ADVANCES IN
Immunology

EDITED BY

FRANK J. DIXON

The Scripps Research Institute
La Jolla, California

ASSOCIATE EDITORS

Frederick Alt
K. Frank Austen
Tadamitsu Kishimoto
Fritz Melchers
Jonathan W. Uhr

VOLUME 66

ACADEMIC PRESS
San Diego London Boston
New York Sydney Tokyo Toronto

This book is printed on acid-free paper. ♾

Academic Press
a division of Harcourt Brace & Company
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
http://www.apnet.com

Academic Press Limited
24-28 Oval Road, London NW1 7DX, UK
http://www.hbuk.co.uk/ap/

International Standard Book Number: 0-12-022466-6

PRINTED IN THE UNITED STATES OF AMERICA
97 98 99 00 01 02 EB 9 8 7 6 5 4 3 2 1

CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions begin.

Lucien A. Aarden (101), Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Laboratory for Experimental and Clinical Immunology, University of Amsterdam, 1066 CX Amsterdam, The Netherlands

Richard Bucala (197), The Picower Institute for Medical Research, Manhasset, New York 11030

C. Erik Hack (101), Department of Internal Medicine, Free University Hospital, 1066 CX Amsterdam, The Netherlands

Berhane Ghebrehiwet (225), Division of Allergy and Clinical Immunology, Department of Medicine, State University of New York, Stony Brook, New York 11794-8161

Juergen Hammer (67), Roche Milano Ricerche, I-20132 Milan, Italy

Kusumam Joseph (225), Division of Allergy and Clinical Immunology, Department of Medicine, State University of New York, Stony Brook, New York 11794-8161

Louis B. Justement (1), Department of Microbiology, Division of Developmental and Clinical Immunology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Allen P. Kaplan (225), Division of Allergy and Clinical Immunology, Department of Medicine, State University of New York, Stony Brook, New York 11794-8161

Christine N. Metz (197), The Picower Institute for Medical Research, Manhasset, New York 11030

Sesha Reddigari (225), Division of Allergy and Clinical Immunology, Department of Medicine, State University of New York, Stony Brook, New York 11794-8161

Yoji Shibayama (225), Division of Allergy and Clinical Immunology, Department of Medicine, State University of New York, Stony Brook, New York 11794-8161

Michael Silverberg (225), Division of Allergy and Clinical Immunology, Department of Medicine, State University of New York, Stony Brook, New York 11794-8161
Francesco Sinigaglia (67), Roche Milano Ricerche, I-20132 Milan, Italy
Tiziana Sturniolo (67), Roche Milano Ricerche, I-20132 Milan, Italy
Lambertus G. Thijz (101), Department of Medical Intensive Care Unit, Free University Hospital, 1066 CX Amsterdam, The Netherlands
Otto O. Yang (273), AIDS Research Center, Massachusetts General Hospital, Boston, Massachusetts 02114
Bruce D. Walker (273), AIDS Research Center, Massachusetts General Hospital, Boston, Massachusetts 02114

ADVANCES IN IMMUNOLOGY, VOL. 66

The Role of CD45 in Signal Transduction

LOUIS B. JUSTEMENT

Department of Microbiology, Division of Developmental and Clinical Immunology, University of Alabama at Birmingham, Birmingham, Alabama 35294

I. Introduction

With the growing awareness of the important role that protein tyrosine kinases (PTKs) play in receptor-dependent signaling processes in cells of the hematopoietic lineage, it has become necessary to characterize the complementary function of protein tyrosine phosphatases (PTPs) in order to develop a comprehensive understanding of the molecular mechanisms whereby inducible tyrosine phosphorylation regulates immune cell development, activation, and differentiation. During the past 20 years the transmembrane receptor-like PTP CD45 has been extensively characterized and is one of the key enzymes responsible for regulation of inducible tyrosine phosphorylation in cells of the hematopoietic lineage (Thomas, 1989; Trowbridge and Thomas; 1994, Alexander, 1997). Although CD45 is expressed by all nucleated cells of the hematopoietic lineage and is involved in regulating the function of several cell types, including monocytes/macrophages, NK cells, mast cells, and basophils, the vast majority of work concerning this PTP has been conducted using T and B lymphocytes and this review will focus predominantly on those studies.

The initial identification of CD45 as a lymphocyte surface antigen was based on studies using monoclonal antibodies (mAbs) that demonstrated that this transmembrane glycoprotein is abundantly expressed by most cells and comprises 5–10% of the surface protein. Subsequent cloning of CD45 at the cDNA and genomic level revealed several interesting characteristics about the primary structure of this molecule (Thomas, 1989). This PTP consists of a large extracellular domain of approximately 400–500 amino acids, a single transmembrane-spanning region, and a cytoplasmic domain of 700 amino acids (Fig. 1). CD45 can be expressed as one of eight potential isoforms that vary in molecular weight from 180 to 220 kDa due to alternative mRNA splicing of up to three exons (exons 4–6) that encode a variable amino-terminal domain rich in O-linked sugars. Therefore, alternative mRNA splicing of CD45 can result in a significant degree of heterogeneity in the extracellular domain due to changes in polypeptide structure as well as differential O-linked glycosylation of the molecule. The expression of CD45 isoforms has been shown to occur in a cell type-specific manner and also varies with the activation or differentia-

0065-2776/97 $25.00

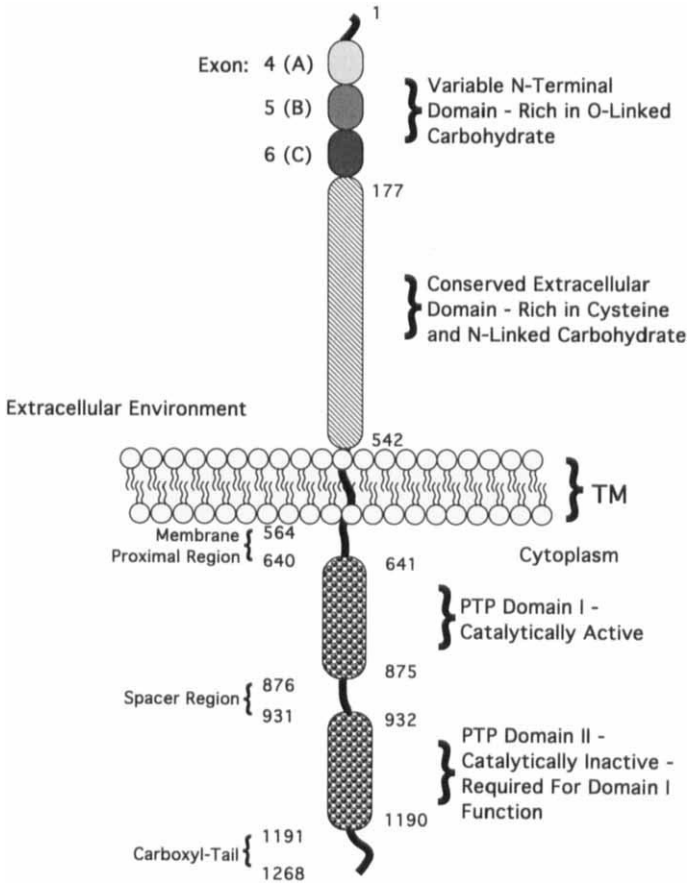


FIG. 1. Schematic representation of the domain structure of murine CD45. The high-molecular-weight isoform of CD45 containing all three alternatively spliced exons is pictured. Exons 4–6 encode amino acids 8–50, 51–99, and 100–146, respectively. All major domain regions are consecutively numbered from amino acid 1 at the amino terminus to amino acid 1268 at the carboxy terminus.

tion state of lymphocytes. Because studies have documented highly regulated patterns of expression for the various isoforms of CD45, a great deal of effort has been directed toward delineating the overall physiological significance of isoform switching. Although evidence is emerging to suggest that specific CD45 isoforms do perform unique functions in the context of lymphocyte development and activation, this continues to be an issue that is of great interest. Because the different isoforms of CD45 possess identical cytoplasmic domains and exhibit equivalent catalytic activity, stud-

ies to elucidate CD45 isoform function have focused predominantly on the role of the extracellular domain in mediating differential interactions with extracellular "ligands" for CD45 (Fig. 2).

Based on the knowledge that CD45 is expressed at high levels in lymphocytes and exhibits unique patterns of isoform-specific expression, it was hypothesized early on that this transmembrane protein serves an important signaling function in lymphocytes. This hypothesis was further strengthened by studies using mAbs demonstrating that cross-linking of CD45 results in significant alterations in lymphocyte activation in response to mitogenic stimuli. That CD45 is indeed an important regulator of signal transduction in lymphocytes was confirmed when its sequence was compared to that of the soluble PTP, PTP1B, demonstrating that the intracellular region of CD45 contains tandem repeats (PTP domain I and PTP domain II) that are 40 and 33% homologous to the catalytic domain of PTP1B, respectively (Charbonneau *et al.*, 1988). Subsequent studies confirmed that CD45 is a functional transmembrane PTP (Tonks *et al.*, 1988), thereby stimulating a renewed interest in characterizing the role that this glycoprotein serves in lymphocyte biology. The results from studies

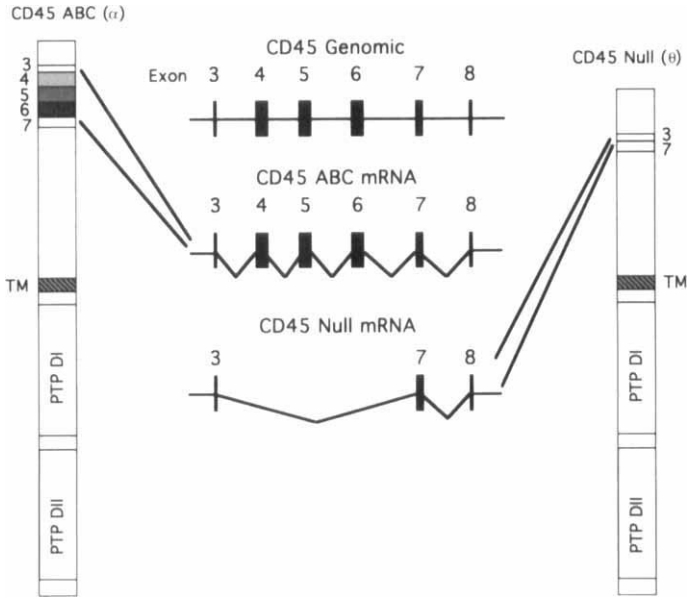


FIG. 2. Generation of CD45 isoforms by alternative mRNA splicing of exons 4–6. Alternative splicing of CD45 results in the formation of distinct isoforms that vary at the amino terminus in terms of their polypeptide and carbohydrate composition.

using mAbs suggested that CD45 acts as a negative regulator of lymphocyte activation in response to antigen receptor (AgR) ligation. However, studies using CD45-deficient T and B cell lines revealed that the expression of CD45 is in fact required for optimal AgR-mediated signal transduction (Trowbridge and Thomas, 1994; Justement *et al.*, 1994). In general, the results obtained from experiments with CD45-deficient cell lines have been supported by CD45 transgenic and CD45 knockout studies that further demonstrate that CD45 expression is crucial not only for lymphocyte activation but also for T cell and to a lesser extent B cell development as well (Kishihara *et al.*, 1993; Byth *et al.*, 1996).

In order to fully delineate the molecular mechanism(s) by which CD45 regulates lymphocyte biology, identification of cellular substrates and elucidation of the effect that reversible tyrosine phosphorylation has on their effector function will be required. Along these lines, studies have convincingly demonstrated that CD45 regulates the tyrosine phosphorylation and activity of the Src family PTKs. Although it is readily apparent that the Src family PTKs are important substrates for CD45, and that inhibition of their activation is likely to be a primary cause of altered lymphocyte responsiveness to AgR ligation in cells lacking CD45, it is likely that there are other intracellular effector proteins whose function is also regulated directly by CD45 as a result of dephosphorylation.

Once CD45 was determined to be a functional PTP, it became clear that delineation of the mechanism(s) by which its catalytic function is regulated would be crucial for understanding the dynamic way in which lymphocyte biology is regulated by this PTP. However, studies have not yet convincingly elucidated the molecular mechanism responsible for regulating the catalytic function of CD45. Although evidence suggests that posttranslational modification of CD45 may be involved, when examined in the context of one another the studies do not support a consensus model. Alternatively, it has been proposed that redistribution of CD45 within the plasma membrane in response to "ligand" binding may result in its physical sequestration, thereby restricting access to substrates within the cell. A related hypothesis proposes that aggregation or redistribution of CD45 within the plasma membrane may lead to alterations in its catalytic activity either through an association with the cytoskeleton or as a result of multimerization with itself. The various mechanisms that have been proposed to play a role in regulation of CD45 catalytic function are not mutually exclusive and it is quite possible that the overall process is complex and multifaceted.

Clearly, a significant amount of progress has been made with regard to the analysis of CD45 structure and function as is evident from the discussion presented in this review. However, there remain several key issues that

relate to the physiological role for CD45 isoforms, including the identity of extracellular ligands, the identification of intracellular substrates, and the mechanism responsible for regulation of CD45 catalytic activity, for which there are no definitive answers. These questions are currently actively being studied using a wide range of technical approaches and cell systems.

II. CD45 Function in Lymphocyte Development and Activation

The discovery that CD45 is a transmembrane PTP has stimulated a significant effort to elucidate its functional role in cells of the hematopoietic lineage. In general, three approaches have been used, including experiments with anti-CD45 mAbs, CD45-deficient cell lines, and, recently, CD45 transgenic and gene knockout mice. The results obtained from these different experimental approaches have not provided an entirely consistent picture of the functional role played by CD45. Nevertheless, most of the experimental discrepancies can be attributed to technical and procedural issues that are inherently associated with the use of a given procedure, and when the data are viewed as a whole, it is possible to begin to develop a general appreciation of the functional relationships that pertain to CD45.

A. Analysis of CD45 Function Using Monoclonal Antibodies

From a historical perspective, mAbs directed against CD45 were used in an attempt to determine whether it is involved in regulation of lymphocyte function long before the identification of this transmembrane protein as a PTP. Several reviews in the literature document studies in which anti-CD45 mAbs have been used to demonstrate a potential role for CD45 in regulation of lymphocyte activation and differentiation, as well as in lymphocyte homotypic aggregation (Thomas, 1989; Trowbridge and Thomas, 1994; Justement *et al.*, 1994; Alexander, 1997). It is important to note that the interpretation of data obtained from studies using mAbs should take into account several issues, including the experimental approach employed, the specificity of the anti-CD45 mAbs used, and the cell population being examined. As mentioned previously, structural and, presumably, functional characteristics of the extracellular domain of CD45 can vary significantly due to alternative mRNA splicing. This results in the production of distinct CD45 isoforms that differ with respect to their polypeptide backbone. Moreover, different isoforms may exhibit selective patterns of glycosylation depending on the cell type in which they are expressed. Due to the high degree of structural plasticity in the extracellular domain, it is formally possible that cells within a given population may express different isoforms and/or glycosylation variants of CD45. It is also

quite possible that individual cells may express more than one structural form of CD45 in certain circumstances (e.g., during activation and/or differentiation). Therefore, the use of anti-CD45 mAbs to study CD45 function may be complicated by the fact that a given mAb may not recognize every cell within a population or may not bind to all CD45 molecules expressed by a given cell. This could potentially lead to selective effects on subpopulations of cells that would not necessarily be apparent based on phenotypic analysis of the population as a whole. Alternatively, the selective engagement of distinct subsets of CD45 molecules on the surface of a cell could elicit qualitatively different responses, depending on the specific intermolecular interactions that might be perturbed. Finally, even though a cell population may express a single structural variant of CD45, selective modulation of CD45 function might be observed depending on the specific epitope recognized by the particular anti-CD45 mAb used. In other words, binding of mAbs to distinct epitopes within the extracellular domain of CD45 could induce selective changes in CD45 that might affect its catalytic activity and/or interactions with other proteins in the cell. As discussed below, numerous studies have demonstrated that these issues are of practical concern.

1. Studies with T Lymphocytes

Despite the numerous studies that have been conducted using anti-CD45 mAbs to study the role of CD45 in T cell biology, questions concerning the physiological significance of the findings obtained still remain. This is in part due to the fact that anti-CD45 mAbs have been used as surrogate ligands for CD45, even though it has not yet been definitively proven that physiological ligands for this PTP exist or, if they do, how they might regulate its function. Therefore, it is difficult to interpret how the effects elicited by treatment of cells with anti-CD45 mAbs relate to physiologic situations. Second, anti-CD45 mAbs have been observed to both potentiate and attenuate T cell activation in response to a number of mitogenic stimuli. These contradictory results are presumably due to one or more of the factors discussed previously relating to differences in experimental approach, CD45 isoform expression, or antibody specificity. Moreover, because very few studies have documented the effects that binding of mAbs have on the conformation of CD45, its interaction with other cellular proteins, or its catalytic activity, it is difficult to delineate the mechanism(s) responsible for the observed alterations in T cell function. Finally, it should be noted that these issues are equally relevant to studies using anti-CD45 mAbs in other cell systems (see below).

Some of the earliest experiments performed examined the effect that anti-CD45 mAbs had on T cell proliferation in response to T cell mitogens

such as PHA. As is the case with many other experimental systems, anti-CD45 was found to potentiate as well as attenuate the response to PHA. Studies in which anti-CD45 was observed to potentiate IL-2 production and proliferation were performed with mAbs that recognize the high-molecular-weight isoform of CD45 (formerly B220 or CD45RA; recently designated CD45α) (Ledbetter *et al.*, 1985; Marvel and Mayer, 1988). In contrast, when cells were incubated in the presence of PHA and anti-CD45 mAbs that recognize determinants common to all isoforms, a significant inhibition of proliferation was observed that correlated with decreased IL-2 receptor expression and IL-2 production (Bernabeu *et al.*, 1987). The contrasting results obtained in these studies could be due, at least in part, to the fact that mAbs specific for CD45α might preferentially recognize a subpopulation of T cells (e.g., unprimed, naive T cells) that responds differently to cross-linking of CD45 when compared with the population to which the pan-specific mAbs bind. It is also possible that binding of mAbs to distinct isoforms of CD45 and/or to distinct epitopes within any given CD45 molecule could result in qualitatively different effects on CD45 function and thus the response to PHA.

Analysis of the effects that anti-CD45 mAbs have on CD2-mediated T cell proliferation indicates that differential responsiveness of T cell subsets to anti-CD45 mAbs does affect whether the net effect on activation is positive or negative. Separation of T cells into CD45α (CD45RA) and CD45θ (CD45RO) expressing subpopulations revealed that those cells expressing the higher molecular weight isoform (presumably naive cells) responded to simultaneous cross-linking of CD2 and CD45 by exhibiting a greater proliferative response (Schraven *et al.*, 1989). In contrast, the proliferative response of CD45θ-positive cells was not appreciably enhanced by binding of anti-CD45. Enhanced proliferation of CD45α-positive cells was observed regardless of whether a pan-specific or a CD45α-restricted anti-CD45 mAb was used (Schraven *et al.*, 1989). Thus, it is possible that either the naive T cell subpopulation responds differently to CD45 ligation or there is something inherently unique about the way in which the high-molecular-weight isoform regulates T cell proliferation in response to mitogenic signals. Findings from other studies in which CD2 was co-cross-linked with CD45 using mAbs that react with all isoforms of CD45 demonstrated inhibition of inducible tyrosine phosphorylation, calcium mobilization, activation of MAP-2K, and proliferation (Nel *et al.*, 1991). These results were interpreted as being indicative of the fact that CD45 plays a negative regulatory role in signal transduction via CD2. Due to the fact that CD45 and CD2 were coligated, it is possible that this experimental procedure causes a nonspecific decrease in the efficiency of CD2 cross-linking, thereby attenuating signal transduction (see below).

However, in one study CD2 and CD45 were independently cross-linked and the same results were obtained, suggesting that binding of mAb to CD45 uncouples CD2 from its signal transduction pathway (Samelson *et al.*, 1990).

The effects of anti-CD45 mAbs on T cell AgR complex-mediated processes have been most extensively studied. As is the case for studies of mitogen and CD2-induced T cell proliferation, there is some degree of variability in terms of the results obtained when T cells are incubated in the presence of reagents that cross-link CD3 and CD45. This is in part due to the fact that the net effect elicited by addition of anti-CD45 mAbs to T cells can vary greatly depending on the specific T cell subpopulation and the specificity of the mAbs used. Studies have documented differences in the involvement of CD45 in AgR signaling when comparing $CD4^+$ versus $CD8^+$ T cell subsets. The simultaneous cross-linking of CD3 with immobilized anti-CD3 mAb in the presence of anti-CD45 mAb resulted in potentiation of IL-2 production and T cell proliferation of $CD4^+$ T cells (Martoreli *et al.*, 1987). Another study demonstrated a significant perturbation of T cell AgR-mediated signaling in $CD4^+$ but not $CD8^+$ T cells following the addition of anti-CD45 (Maroun and Julius, 1994). These results suggest that the involvement of CD45 in coupling of the T cell AgR to second messenger systems is potentially distinct in $CD4^+$ versus $CD8^+$ cells. Differences have also been observed with regard to the effect elicited by anti-CD45 mAb treatment in studies examining naive versus memory T cell subpopulations. Co-cross-linking experiments using CD45 isoform-restricted mAbs revealed that co-cross-linking of CD3 and CD45α (expressed on naive T cells) mediated a significant increase in the proliferative response (Welge *et al.*, 1993). In contrast, a CD45θ-specific mAb that recognizes the isoform expressed by memory T cells was not observed to enhance the proliferative response. The same results were observed when T cells were separated into CD45α-positive and CD45θ-positive subpopulations and a mAb specific for an epitope common to all isoforms was used in conjunction with anti-CD3, indicating that the effect was not due to a particular antibody or the binding of anti-CD45 mAb to a particular epitope (Welge *et al.*, 1993). This finding, however, does not rule out the possibility that anti-CD45 mAbs with unique specificities have the ability to differentially regulate T cell responses in certain circumstances. In one study, T cell proliferation induced by anti-CD2 or anti-CD3 was augmented by the addition of an anti-CD45 mAb that recognizes a specific carbohydrate determinant found predominantly on the 190-kDa form of CD45 expressed by a subpopulation of $CD4^+$ memory T cells (Torimoto *et al.*, 1992). It is apparent from these findings that CD45 expression within the $CD4^+$ subset is heterogeneous and that the addition of a mAb with a unique specificity

results in the selective enhancement of the proliferative response, a result not obtained when pan-specific anti-CD45 mAbs are used.

Although the results from studies examining the role of CD45 in regulation of CD3-dependent T cell activation vary, the general consensus is that binding of anti-CD45 mAbs exerts an inhibitory effect on a wide range of signal transduction events and depresses lymphocyte proliferation. T cell activation in response to cross-linking of the CD3 complex has been extensively studied and involves the early activation of Src family PTKs, which results in phosphorylation of antigen receptor subunits and the recruitment of the PTK ZAP-70 (Chan *et al.*, 1994). Subsequently, PLCγ becomes tyrosine phosphorylated, is inducibly activated, and hydrolyzes phosphatidylinositol to produce the second messengers diacylglycerol and inositol phosphate, which mediate PKC activation and calcium mobilization, respectively. Simultaneous cross-linking of CD3 and CD45 has been observed to abrogate inducible tyrosine phosphorylation of numerous substrates including PLCγ, to inhibit the production of inositol phosphates, and to significantly reduce calcium mobilization (Kiener and Mittler, 1989; Ledbetter *et al.*, 1988, 1991; Turka *et al.*, 1992). The downstream activation of MAP-2K and calmodulin-kinase IV as well as the inducible tyrosine phosphorylation of oncoprotein 18 are also negatively affected by simultaneous cross-linking of CD45 and CD3 (Shivnan *et al.*, 1996).

These data were at first interpreted as evidence that CD45 is a negative regulator of signaling, effectively uncoupling CD3 from its signaling machinery. However, subsequent studies in CD45-deficient T cell lines revealed that optimal signaling via the T cell AgR requires expression of CD45, suggesting that this PTP is in fact a positive regulator of lymphocyte activation (Trowbridge and Thomas, 1994). This indicates that binding of anti-CD45 mAbs does not necessarily lead to the "activation" of a CD45-dependent inhibitory function, but instead has a negative affect on normal PTP function, which is required for maintenance of the AgR in a competent state so that it can respond to ligand. The underlying mechanism(s) responsible for the negative effect elicited by simultaneous cross-linking of CD45 and CD3, and therefore the inherent contradiction between studies using mAbs and those in CD45-deficient cell lines, can perhaps be explained based on an analysis of the intermolecular association between CD45 and CD3 in the plasma membrane. Depending on the experimental protocol used, it is theoretically possible either to facilitate the physical interaction between CD3 and CD45 or to effectively redistribute these molecules in the membrane into mutually exclusive pools. The relative juxtaposition of these molecules in the plasma membrane with respect to one another would in turn have a direct impact on whether CD45 can physically engage substrates associated with the T cell AgR complex.

A consistent finding in the literature is that coligation of CD45 and CD3 using primary IgG isotype mAbs and secondary cross-linking reagents leads to almost complete inhibition of lymphocyte activation and proliferation (Alexander, 1997; Ledbetter *et al.*, 1988; Turka *et al.*, 1992). It has been proposed that the inhibition of T cell responses under these conditions is due to a nonspecific mechanism in which efficient aggregation of the T cell AgR complex is physically inhibited and thus the effects observed are not related to the function of CD45 (Alexander *et al.*, 1992). In studies in which the ratio of CD3 molecules cross-linked to CD45 molecules is maintained at 1 : 1 or 1 : 2 using chemically defined heteroconjugates of Fab fragments against CD45 and CD3, there is no inhibition of CD3-mediated signals (Shivnan *et al.*, 1992). This is in contrast to the results obtained under conditions in which intact IgG isotype anti-CD45 and anti-CD3 mAbs are added in the presence of a secondary cross-linking reagent. Because the number of CD45 molecules expressed on the surface of T cells is 10-fold greater than the number of CD3 molecules, the latter conditions would theoretically favor the co-cross-linking of CD3 to CD45 and would diminish the relative number of dimers, and particularly oligomers, of CD3 that are formed in the membrane. It is well documented that efficient cross-linking of CD3 is required for the productive generation of a signal. Therefore, it is possible that co-cross-linking of CD45 to CD3 results in inhibition of CD3-mediated signaling due to nonspecific interference with CD3 aggregation. If IgM isotype mAbs are used to co-cross-link CD3 and CD45, no inhibition of the CD3-mediated signal is observed presumably because the multimeric nature of the IgM mAb induces sufficient cross-linking of CD3 within the larger heteromolecular aggregates. Further support for the hypothesis that CD3 aggregation is attenuated by co-cross-linking strategies was provided by experiments in which other abundantly expressed surface receptors (e.g., CD5 and LFA-1) were observed to inhibit CD3-mediated signaling in a manner analogous to CD45 when co-cross-linked with CD3 (Shivnan *et al.*, 1992). Not surprisingly, CD2- and CD28-mediated proliferation of T cells is inhibited in experiments in which these molecules are co-cross-linked to CD45 (Ledbetter *et al.*, 1988). In contrast, the CD4-mediated T cell proliferative response is enhanced by co-cross-linking of this molecule with CD45. In this case, the enhanced response is perhaps due to the fact that CD45 is brought into close proximity to the pool of $p56^{lck}$ that associates with CD4. The close aposition of CD45 and $p56^{lck}$ would presumably facilitate dephosphorylation of $p56^{lck}$, causing it to become activated. Under these conditions the activation of CD4-associated $p56^{lck}$ by CD45 could perhaps drive a more efficient signal than that generated by cross-linking of CD4 alone. Co-cross-linking of CD3, CD45, and CD4 has actually been shown to

partially reverse the negative effects on signaling and proliferation that are observed when CD3 and CD45 are co-cross-linked in the absence of CD4 (Ledbetter *et al.*, 1991). The ability of CD4 to partially restore lymphocyte responsiveness may again be due to its association with $p56^{lck}$, which would be recruited into the multireceptor complex where it could be activated by CD45.

Regardless of whether receptor co-cross-linking results in nonspecific inhibition of CD3-mediated signals, other studies in which CD45 and CD3 are cross-linked independently provide evidence to support the hypothesis that CD45 functions as a positive regulator of signal transduction. These studies demonstrate that physical sequestration of CD45 through independent ligation may negatively affect its ability to facilitate signal transduction in response to CD3 ligation. This was most clearly shown in a series of experiments involving the use of reagents that mediate progressively greater degrees of CD45 cross-linking in the plasma membrane (Ledbetter *et al.*, 1991). The addition of anti-CD45 mAb, a conjugate of the mAb, and finally biotinylated anti-CD45 plus avidin was observed to induce partial to complete inhibition of a response initiated by the subsequent addition of anti-CD3 mAb. The degree of inhibition correlated with the degree of CD45 cross-linking indicating that aggregation of CD45 in the membrane is associated with a decrease in signal transduction efficiency. This same result has been obtained by other groups using independent cross-linking protocols in which they demonstrated that anti-CD45 mAbs inhibit a range of CD3-mediated responses from inducible tyrosine phosphorylation to activation of calmodulin-kinase IV (Kiener and Mittler, 1989; Shivnan *et al.*, 1996). In one instance the inhibitory effects of anti-CD45 mAbs were reversed by the addition of vanadate, a nonspecific inhibitor of PTPs (Kiener and Mittler, 1989). Although these results suggest that CD45 exerts a negative effect on signaling, it should be noted that vanadate inhibits most cellular PTPs and also has the ability to activate PTKs directly. Therefore, the net result of vanadate treatment could be enhanced activation of one or more PTKs that effectively override the inhibition of signaling induced by CD45 aggregation. As revealed by confocal microscopy, a likely mechanistic explanation for the inhibition of CD3-mediated signaling is that cross-linking of CD45 causes it to patch and/or cap in the plasma membrane, effectively limiting its ability to engage substrates inside the cell due to its physical sequestration (Shivnan *et al.*, 1996).

A recent series of studies have provided additional insight into the biochemical consequences associated with CD45 cross-linking in the T cell. Cross-linking of CD45 with a cocktail of mAbs, anti-CD45 mAb followed by a secondary antibody, or anti-CD45 mAbs bound to plastic plates results in the activation and redistribution of $p56^{lck}$ within the cell

(Arendt *et al.*, 1995; Guttinger *et al.*, 1992; Cardine *et al.*, 1994). In each of these systems the reagents used would be expected to induce a high degree of CD45 aggregation in the membrane potentially leading to the formation of caps. Immunofluorescence analysis revealed that a significant portion of the total cellular $p56^{lck}$ cocaps with CD45 but not with other abundantly expressed transmembrane receptors (Guttinger *et al.*, 1992). It was also shown that CD45 cross-linking ultimately leads to the translocation of $p56^{lck}$ from the plasma membrane to intracellular compartments of the cell (Cardine *et al.*, 1994). Whether these effects of anti-CD45 are due to a structural–functional change in CD45 itself or are the result of changes in the intermolecular interactions between CD45 and other proteins that associate with $p56^{lck}$ is not clear. Regardless, these data demonstrate that ligand binding to CD45 in the absence of other stimuli has the ability to alter the activation and intracellular localization of $p56^{lck}$, an important effector molecule in T cell activation.

2. *Studies with B Lymphocytes*

Anti-CD45 mAbs have been used to demonstrate that both activation and differentiation of the B cell can be affected by their addition, indicating that CD45 is involved in regulation of signal transduction via the B cell AgR complex. Like the T cell, the results obtained from these studies vary considerably depending on the mAb used and the specific B cell population examined. The use of anti-CD45 mAbs to study B cell activation has raised several interesting questions about the potential role for CD45 ligands and has suggested that the signal transduction pathway activated following cross-linking of the AgR is differentially regulated by CD45 depending on the B cell's inherent state of activation.

B cell activation is both negatively and positively modulated in the presence of anti-CD45 mAbs and the difference in the effect on cellular proliferation in response to AgR cross-linking is predominantly a function of the specificity of the anti-CD45 mAbs used. As discussed previously, the specific experimental protocol used can potentially have a significant impact on the results obtained; however, most studies in the B cell have employed the method in which there is simultaneous cross-linking of CD45 and the AgR in an independent manner. For the most part, experiments demonstrate that the simultaneous addition of anti-CD45 mAbs abrogates B cell AgR-mediated signal transduction as well as proliferation (Justement *et al.*, 1994). Several events associated with B cell activation, including calcium mobilization, phosphoinositide hydrolysis, and increased gene transcription, are inhibited by the simultaneous cross-linking of CD45 and the AgR (Gruber *et al.*, 1989; Mittler *et al.*, 1987; Deane *et al.*, 1991; Alsinet *et al.*, 1990; Smeland *et al.*, 1990). It was further demonstrated that small

resting B cells are the most susceptible to the negative effects elicited by cross-linking of CD45. Isolation of B cells based on size or density gradient centrifugation revealed that as the cells transit from a resting G_0 population to one that contains a higher number of cells that have entered G_1 and exhibit increased RNA synthesis, they become more resistant to the inhibitory effect elicited by cross-linking CD45 (Mittler *et al.*, 1987; Gruber *et al.*, 1989). Although proliferation induced by the addition of anti-IgM mAb is suppressed in all B cell populations by simultaneous addition of anti-CD45 mAb, the degree of inhibition was much less in cells that appear to be partially activated than that observed for resting B cells. This suggests that the functional role of CD45 in regulating signal transduction via the B cell AgR changes with progressive activation such that the involvement of CD45 in AgR signaling does not appear to be as crucial in cells that have received initial activating signals. The inhibitory effect of anti-CD45 mAbs can be reversed by simultaneous stimulation of B cell with anti-Ig and anti-CD40 mAbs (Gruber *et al.*, 1989) but not by the addition of anti-Ig in conjunction with several cytokines, including IL-1, -2, and -4 (Mittler *et al.*, 1987). Clearly, it is possible to override the negative effect of CD45 ligation by providing other accessory signals to the B cell. This observation suggests that differential sensitivity of B cell populations to the effects of anti-CD45 mAbs may be explained by the fact that once B cells have received appropriate accessory signals, the functional relationship between CD45 and the AgR is qualitatively altered.

In certain instances the addition of anti-CD45 mAbs has been observed to enhance B cell proliferation. In particular, incubation of B cells with mAbs that recognize the high-molecular-weight isoform of CD45 (CD45α) in many instances appears to potentiate the proliferative response following stimulation with anti-Ig (Alsinet *et al.*, 1990; Deane *et al.*, 1991). Additionally, mAbs that exhibit specificity for carbohydrate determinants on CD45 appear to potentiate proliferation in response to AgR cross-linking. These results are in many ways similar to those obtained from studies examining T cell activation and proliferation, and they indicate that binding of mAbs to discrete epitopes in the extracellular domain of CD45 may elicit qualitatively different results in terms of the effect that they have on CD45 function. Such findings, if true, are potentially significant because they provide evidence to suggest that structural heterogeneity in the extracellular domain is indeed relevant from a physiological perspective because it facilitates the generation of unique ligand : receptor interactions that could differentially regulate CD45 function.

Studies have utilized anti-CD45 mAbs that recognize a B cell-restricted epitope to analyze the role of this PTP in regulating B cell activation *in vivo*. Administration of anti-CD45 mAb was found to abrogate a T cell-

dependent antibody response against the hapten fluorescein isothiocyanate (FITC) (Domiati-Saad *et al.*, 1993). In these studies both the plaque-forming cell response and serum antibody production were measured and were significantly inhibited by a single dose of anti-CD45 mAb. The effect of antibody administration was transient in that once the mAb had been cleared from the system, anti-FITC responses could be detected. A kinetic analysis of mAb administration provided data to suggest that the anti-CD45 mAb was exerting an effect on B cell activation, thereby preventing cells from responding to antigen and undergoing subsequent differentiation. Administration of anti-CD45 mAb did not affect the secondary response following rechallenge with antigen (Domiati-Saad *et al.*, 1993). Therefore, it is possible that memory B cells have lost the specific epitope recognized by the anti-CD45 mAb used in this study due to isoform switching. Alternatively, the response to antigen by B cells in the memory pool may involve a qualitatively distinct AgR-mediated signal transduction process that is not regulated by CD45 in a manner analogous to that for resting naive B cells. Finally, based on *in vitro* studies it is possible that memory B cells receive accessory signals that override the negative effects elicited by ligation of CD45 (Gruber *et al.*, 1989).

Besides their well-documented effects on B cell activation in response to AgR cross-linking, anti-CD45 mAbs have been shown to affect other postactivation events associated with B cell differentiation and immunoglobulin class switching. Anti-CD45 mAbs have been used to demonstrate that CD45 is involved in regulation of signaling via IL-4 receptors and MHC class II molecules, suggesting that this PTP serves an important function well after the initial activation response (Hasegawa *et al.*, 1990; Lane *et al.*, 1991; Polla *et al.*, 1988). Additional studies have demonstrated that addition of anti-CD45 mAbs to LPS-stimulated cultures *in vitro* abrogates class switching to secondary immunoglobulin isotypes but does not affect the production of IgM (Yakura *et al.*, 1988, 1986). Maximal effects required that the mAbs be added to cultures within 48 hr and it was determined by limiting dilution analysis that the inhibitory effect of anti-CD45 mAbs was related to a decrease in the precursor frequency of IgG-secreting cells (Yakura *et al.*, 1988). In contrast, anti-CD45 mAbs were observed to inhibit both IgM and IgG production *in vitro* in response to stimulation of B cells with *Staphylococcus aureus* Cowan (SAC) (Morikawa *et al.*, 1991). This result was presumably due to abrogation of B cell differentiation as opposed to cellular activation because the anti-CD45 mAbs were added 48 hr after stimulation with SAC. Quite distinct results were obtained when immunoglobulin isotype switching was assayed using T cell-dependent limiting dilution analysis in the presence of anti-CD45 mAbs. In these studies, the addition of anti-CD45 mAb to B cells in the

presence of dendritic cells and alloreactive T cells was observed to induce a three- or fourfold increase in the number of cells that underwent class switching to secondary Ig isotypes (George *et al.*, 1994). The reason for the discrepancy between this study and others using LPS or anti-Ig is not clear; however, it does not appear as though the difference is due to binding of mAbs to either the T cells or dendritic cells in the culture system. In the T cell-dependent isotype switching system, cross-linking of CD45 with anti-CD45 mAb and secondary anti-rat Ig mAbs was observed to enhance secondary isotype switching (George *et al.*, 1994). As previously discussed, the physical localization of CD45 within a discrete cap in the plasma membrane could prevent it from interacting with one or more substrates in the cell, thereby altering their state of tyrosine phosphorylation and presumably their function. Alternatively, because CD45 extends from the plasma membrane as a rod-like structure that is approximately 28–51 nm in length depending on the number of exons expressed, it is one of the largest molecules on the lymphocyte surface (Thomas, 1989). Therefore, another consequence of CD45 cross-linking and capping could be the enhanced interaction between B cells, T cells, and dendritic cells via surface molecules involved in antigen recognition due to reorganization of CD45 within the membrane.

3. *Studies of Lymphocyte Adhesion*

Within the past 3 years a number of studies have documented the involvement of CD45 in lymphocyte adhesion processes through the use of anti-CD45 mAbs. One of the first studies demonstrated that incubation of peripheral blood mononuclear cells with mAbs specific for several different epitopes in the extracellular domain, including CD45RA, CD45RO, and common determinants found in all isoforms, induced the rapid formation of large cell clusters (Lorenz *et al.*, 1993). Cellular aggregation in response to anti-CD45 was blocked by the addition of EDTA, cytochalasin B, or incubation at 4°C, all characteristics of adhesion mediated by integrins. The involvement of LFA-1 and ICAM-1 was confirmed by antibody blocking experiments. Subsequent studies revealed that treatment of highly purified T cells with anti-CD45 mAbs could induce aggregation of the treated cells with untreated monocytes. However, purified T cells treated with anti-CD45 did not undergo aggregation in the absence of monocytes and treatment of monocytes with anti-CD45 mAb did not induce heterotypic aggregation with T cells (Lorenz *et al.*, 1994). Thus, treatment of resting T cells was associated with activation of an integrin-dependent heterotypic aggregation response that was 5- to 10-fold more sensitive to inhibitors of cAMP- and cGMP-dependent protein kinases than the aggregation response induced by PMA. Conversely, the anti-CD45 aggre-

gation response was not affected by PKC or PTK inhibitors (Lorenz *et al.*, 1994).

Whereas anti-CD45-mediated heterotypic aggregation between T cells and monocytes does not require prior activation of the T cell, homotypic aggregation of T cells in response to anti-CD45 treatment occurs only if the cells have been activated by cross-linking of the AgR complex (Spertini *et al.*, 1994). Anti-CD45 mAbs directed against CD45RO and common determinants found on all isoforms, but not CD45RA, were observed to induce CD11a/CD18 (LFA-1)-dependent as well as CD11a/CD18-independent homotypic aggregation of activated but not resting peripheral T cells. Depending on the specific epitope recognized by the different anti-CD45 mAbs, the aggregation response could be inhibited by the addition of PKC and/or PTK inhibitors (Spertini *et al.*, 1994). The results from these studies indicate that in addition to regulating signal transduction via the T cell AgR complex, ligand binding to CD45 induces a signal that leads to enhanced adhesiveness of activated T cells. A final variation concerning the role of CD45 with respect to T cell adhesion was demonstrated by studies in which mAbs reacting with CD45 common epitopes, or the restricted CD45RO determinant, elicited a rapid and potent homotypic aggregation response in all human thymocytes and T cell lines with an immature phenotype (Bernard *et al.*, 1994). In agreement with observations from other groups, anti-CD45 mAbs were not observed to induce homotypic aggregation of mature T cells. Additionally, the aggregation response was at least partially dependent on the interaction between LFA-1 and ICAM-3. However, because mAbs directed against these integrins mediated only partial inhibition of thymocyte aggregation, it is likely that another adhesion process is involved (Bernard *et al.*, 1994).

Recently, it has been shown that CD45 is physically and functionally associated with the semaphorin CD100 (Herold *et al.*, 1996). Coprecipitation studies demonstrated a physical interaction between CD45 and CD100 isolated from resting T cells. The ability to coimmunoprecipitate these molecules was enhanced by T cell activation. Additionally, the interaction between CD100 and CD45 involves isoforms of CD45 in the 190- to 205-kDa range corresponding to all CD45 molecules that contain either one or two of the three variably spliced exons. Ligation of CD45 using mAbs leads to a transient decrease in the level of CD100 expression as well as the secretion of a soluble form of this molecule (Herold *et al.*, 1996). In contrast to findings from other groups, a specific anti-CD45 mAb (4.14) induced LFA-1-independent homotypic aggregation in resting T cells that was potentiated by the simultaneous cross-linking of CD100. These experiments reveal a bidirectional relationship between CD45 and the semaphorin CD100 that may play an important role in terms of regulat-

ing the trafficking of T cells as well as their interaction with other cells of the immune system.

Homotypic aggregation in the B cell is also affected by the addition of anti-CD45 mAbs as demonstrated by studies in which three novel mAbs (Lai1/8, 11, and 15) were observed to induce intercellular adhesion in the absence of other stimuli (Zapata *et al.*, 1995). These mAbs recognize determinants contained within the A exon-encoded region of CD45 and bind to a peptide epitope that can be selectively masked by the addition of carbohydrate. It is worthy to note that epitopes recognized by these mAbs are selectively expressed by peripheral blood and tonsilar B cells, perhaps as a result of differential glycosylation (Zapata *et al.*, 1995). The adhesion response triggered by these mAbs involves the binding of LFA-1 to ICAM-1 and/or ICAM-3 based on the ability to block cell : cell interactions with mAbs against these integrins. Additional experiments revealed that CD45 cross-linking leads to the colocalization of both CD45 and LFA-1 in the membrane. The use of PTK and PTP inhibitors suggests that the phosphatase activity of CD45 may be involved in upregulation of integrin-mediated homotypic aggregation (Zapata *et al.*, 1995). The use of another anti-CD45 mAb (HAB-1) that recognizes a determinant common to all isoforms revealed that ligand binding to CD45 can inhibit homotypic aggregation induced through MHC class I and class II, CD19, CD21, CD40, and Leu-13 surface molecules (Wegner *et al.*, 1993). In contrast, this anti-CD45 mAb did not affect homotypic aggregation induced by PMA. The analysis of 13 additional anti-CD45 mAbs resulted in the identification of only one other mAb (138-3) with similar inhibitory properties. Due to the fact that only one other anti-CD45 mAb exhibited similar properties, it is unlikely that the inhibitory effect on homotypic aggregation was caused by a steric hindrance phenomenon. A final point of interest concerning the functional characteristics of these mAbs relates to the fact that they both potentiate the T cell response to CD2 ligation (Wegner *et al.*, 1993).

B. Analysis of CD45 Function Using CD45-Deficient Cell Lines

The development of CD45-deficient cell lines has provided a valuable experimental tool for elucidation of the role that CD45 plays in regulation of lymphocyte activation. In general, these experimental systems have been developed using previously characterized lymphoid cell lines from which spontaneously arising or mutagenized CD45-deficient cells were isolated after negative selection with anti-CD45 antibodies and complement. Recently, homologous recombination has been used in the DT40 chicken B cell line to create cells that possess a specific genetic defect resulting in the loss of CD45 expression (Yanagi *et al.*, 1996). Although there are

certain caveats that must be considered when using cell lines in general, and particularly those that have been subjected to nonspecific mutagenic procedures, the results obtained using CD45-deficient cells have nevertheless provided relatively consistent information indicating that CD45 expression is important for optimal signal transduction via either the T or B cell AgR complex (Alexander, 1997; Trowbridge and Thomas, 1994).

1. CD45-Deficient T Cell Lines

That CD45 expression is required in order to effectively couple the T cell AgR to its intracellular signal transduction pathway has been clearly documented in studies of murine $CD4^+CD45^-$ and $CD8^+CD45^-$ T cell lines generated by chemical mutagenesis (Pingel and Thomas, 1989; Weaver *et al.*, 1991). In both the $CD4^+$ and $CD8^+$ cell lines, the response to either antigen or anti-CD3 was significantly impaired as evidenced by the failure to proliferate and to produce IL-2 or IFN-γ, respectively. Additionally, the CD45-deficient $CD8^+$ T cell lines exhibited a severe impairment in their ability to lyse either syngeneic or allogeneic target cells (Weaver *et al.*, 1991). Supplementation of cultures with exogenous IL-2 was observed to restore the proliferative response of both $CD4^+$ and $CD8^+$ CD45-deficient T cells indicating that the IL-2 receptor mediates a signal that is not affected by the loss of CD45, possibly because the IL-2 signal converges with the AgR signaling pathway at a point that is downstream of the block caused by the loss in CD45 expression. In both studies, CD45-positive spontaneous revertants were isolated and characterized demonstrating that the reexpression of this PTP was sufficient to restore normal signal transduction via the T cell AgR (Pingel and Thomas, 1989; Weaver *et al.*, 1991). Subsequent studies with the CD45-deficient T cell lines showed that they are unable to proliferate in response to mitogenic stimulation with anti-Thy-1 mAb. In contrast, the response to the T cell mitogens Con A and PHA was not affected by the loss of CD45 expression (Pingel *et al.*, 1994). This result was somewhat surprising because anti-Thy-1 and Con A both require T cell AgR expression in order to function and therefore presumably utilize one or more components of the AgR complex for transduction of a signal. Moreover, neither of these mitogenic stimuli were observed to induce tyrosine phosphorylation in CD45-negative T cells, nor did they induce activation of $p56^{lck}$ as is normally observed (Pingel *et al.*, 1994). Thus, it is apparent that Con A has the ability to bypass the block in AgR signaling by binding to distinct coreceptors on the cell and/or as a result of activating a distinct intracellular effector pathway that is not inhibited by the loss of CD45 expression.

In addition to the use of murine T cell lines, a large number of studies have been conducted with CD45-deficient T lymphoma (BW5147, NZB.1,

and YAC-1) and leukemic (Jurkat, HPB-ALL, and CB1) cells (Koretzky *et al.*, 1990, 1991; Deans *et al.*, 1992; Peyron *et al.*, 1991; Volarevic *et al.*, 1992; Shiroo *et al.*, 1992; Biffen *et al.*, 1993). In general, the results obtained with these cell lines are in agreement with those described previously and have provided a significant amount of information concerning the specific nature of the block in signal transduction associated with the loss of CD45 expression. Analysis of AgR-mediated signaling in CD45-deficient cells reveals little or no induction of tyrosine phosphorylation, inositol phosphate production, calcium mobilization, protein kinase C activation, and, in certain cell types such as Jurkat, no IL-2 production (Koretzky *et al.*, 1990, 1992). In the case of the CD45-deficient Jurkat and HPB-ALL cell lines, reconstitution of CD45 expression following transfection with cDNA encoding the CD45RO or CD45RAB isoforms, respectively, was observed to restore normal signaling via the T cell AgR (Koretzky *et al.*, 1992; Biffen *et al.*, 1993; Shiroo *et al.*, 1992). Besides playing an important role in T cell AgR-mediated signaling, CD45 has also been observed to be required for optimal signaling via several other receptors including CD2 (Koretzky *et al.*, 1991), MHC class I (Skov *et al.*, 1995), and FcεRI (Adamcewski *et al.*, 1995) as demonstrated by studies using Jurkat cells that had been transfected with cDNA encoding this receptor.

It is clear that the loss of CD45 expression and its associated PTP activity results in a profound block in the AgR signal transduction pathway at a point that is proximal to the receptor complex itself. The lack of inducible tyrosine phosphorylation, which is one of the earliest events triggered by AgR ligation (Chan *et al.*, 1994), suggests that CD45 expression is important for proper regulation of PTK function in the cell. Because CD45-deficient T cell lines produce cytokines and proliferate normally in response to PMA and ionomycin, it is clear that downstream activation of these events is sufficient to restore the normal cellular response (Peyron *et al.*, 1991). This in turn indicates that CD45 regulates the function of effector proteins (e.g., $p56^{lck}$ and $p59^{fyn}$) that are membrane proximal in relationship to calcium mobilization and PKC activation. Indirect evidence indicating that the loss of CD45 leads to a block in signaling at the level of Src family PTKs has been provided by studies in the HPB-ALL cell line demonstrating that co-cross-linking of CD3 and either CD4 or CD8 leads to the induction of early signal transduction events including the activation of $p56^{lck}$, tyrosine phosphorylation, and calcium mobilization (Deans *et al.*, 1992, Shiroo *et al.*, 1992). These observations are in agreement with those demonstrating that co-cross-linking of CD3 and CD4 reverses the negative effect elicited by treatment of T cells with anti-CD45 mAbs (Ledbetter *et al.*, 1991). In these experiments, reconstitution of signaling may be related to enhanced recruitment of $p56^{lck}$ to the AgR complex by CD4 or CD8, effectively

compensating for decreased activation of $p56^{lck}$, and perhaps that of $p59^{fyn}$ as well. Several studies have documented aberrant activation of both $p56^{lck}$ and $p59^{fyn}$ in cells that lack CD45 and this is thought to be primarily responsible for the abrogation of downstream signal transduction processes (Shiroo *et al.*, 1992; Biffen *et al.*, 1993; Ostergaard *et al.*, 1989; McFarland *et al.*, 1993; Hurley *et al.*, 1993). Recently, evidence has been obtained indicating that the block in T cell AgR-mediated signal transduction is in fact due to the loss of $p56^{lck}$ activation. Reconstitution of CD45-deficient cells with a chimeric protein composed of the extracellular/transmembrane domain of the EGF receptor fused to a constitutively active form of $p56^{lck}$ (Tyr505 to Phe505) was observed to restore AgR-mediated signaling (Duplay *et al.*, 1996). These findings provide evidence indicating that the block in signaling can be overcome by supplying a pool of active $p56^{lck}$ to the cell. In contrast, neither wild-type nor catalytically deficient forms of $p56^{lck}$ were able to restore signaling in the absence of CD45 expression (Duplay *et al.*, 1996).

In contrast to the consensus viewpoint that can be deduced from the studies described previously, two CD45-deficient cell lines have been analyzed that provide qualitatively different results in terms of their response to AgR ligation. In a series of experiments using a particular clone of CD45-deficient Jurkat cells that was obtained using fluorescence-activated cell sorting, anti-CD3 mAb was observed to induce significant tyrosine phosphorylation and calcium mobilization, albeit to a lesser extent than in parental cells (Peyron *et al.*, 1991). Although early effector responses were clearly detectable in these cells, the downstream production of cytokines was almost completely abrogated. This finding is interesting for two reasons. First, it suggests that the loss of CD45 expression in these cells may have been compensated for by the increased expression/function of another PTP that has the ability to regulate $p56^{lck}$ and $p59^{fyn}$. Analysis of membrane-associated PTP activity in these CD45-deficient cells revealed a significant decrease in the total level of activity, indicating that a compensatory PTP might restore signaling through increased catalytic function as opposed to being overexpressed (Peyron *et al.*, 1991). Alternatively, it is possible that $p56^{lck}$ and/or $p59^{fyn}$ are themselves overexpressed or perhaps aberrantly activated, thus compensating for the loss of CD45. However, an analysis of their relative level of expression and activity in CD45-positive (J.D) versus CD45-negative (JS-7) cells was not performed. Recently, an analysis of the J.D Jurkat cell line and its CD45-deficient variant JS-7 revealed that AgR cross-linking stimulates tyrosine phosphorylation of the ζ chain and the association with $p72^{syk}$, regardless of CD45 expression (Chu *et al.*, 1996). A similar response was not observed in the J45.01 CD45-negative Jurkat cell line that has previously been shown to be unresponsive

to AgR cross-linking (Koretzky *et al.*, 1991). These findings suggest that $p72^{syk}$ expression, and presumably activation, is able to compensate for the loss of CD45. This hypothesis was confirmed by experiments in which $p72^{syk}$ was transfected into J45.01 cells and was observed to restore responsiveness to AgR cross-linking (Chu *et al.*, 1996). Second, based on the fact that AgR cross-linking leads to tyrosine phosphorylation and calcium mobilization, but not production of cytokines, it is possible that CD45 regulates effector molecules that lie downstream of $p56^{lck}$ and $p59^{fyn}$. A caveat to this hypothesis is that the levels of second messengers produced in response to AgR cross-linking might be too low to propagate downstream signals leading to cytokine production, a possibility supported by the fact that these cells produced normal levels of cytokines in response to PMA and ionomycin (Peyron *et al.*, 1991).

Experiments with a CD45-deficient mutant of the retrovirally transformed YAC-1 T cell line reveal a very distinct pattern of signaling in response to CD3 cross-linking (Volarevic *et al.*, 1992). To begin with, the CD45-negative YAC-1 cells are unique from the standpoint that numerous intracellular proteins are constitutively hyperphosphorylated on tyrosine when compared to wild-type cells. This is in contrast to the results obtained with most CD45-deficient cells in which the level of basal tyrosine phosphorylation is not greatly changed compared to parental cells. Thus, the loss of CD45 in the YAC-1 cells results in a significant alteration of the basal homeostasis for tyrosine phosphorylation. This finding suggests that one or more PTKs may be constitutively hyperactivated in these cells in the absence of AgR ligation. In support of this hypothesis, the PTK $p70^{zap}$ is constitutively hyperphosphorylated on tyrosine and coprecipitates with the ζ chain, which is also hyperphosphorylated in CD45-negative YAC-1 cells (Mustelin *et al.*, 1995). In normal T cells $p70^{zap}$ is observed to colocalize with CD45 and tyrosine-phosphorylated $p70^{zap}$ is dephosphorylated by CD45 *in vitro*. These data suggest that CD45 may regulate the activity of $p70^{zap}$, but unlike the Src family kinases, $p70^{zap}$ function may be negatively regulated by CD45.

In agreement with results from other CD45-deficient cells, stimulation of YAC-1 mutant cells with anti-CD3 did not induce an increase in tyrosine phosphorylation above the constitutively elevated background (Volarevic *et al.*, 1992). Similarly, stimulation with anti-CD3 mAb did not induce detectable phosphoinositide hydrolysis and resulted in only a small, delayed calcium flux within the first 7 min. However, CD45-deficient YAC-1 cells did exhibit a delayed and gradual increase in the mean intracellular concentration of Ca^{2+} after stimulation with anti-CD3 mAb, whereas parental cells did not. The delayed increase in the concentration of intracellular Ca^{2+} was due to extracellular influx and suggests that the loss of CD45

may lead to the dysregulation of calcium channels in the plasma membrane of the cell. In both of the studies described previously, CD45-positive revertants were isolated in which AgR-mediated signaling had reverted back to normal when compared to parental cells (Peyron *et al.*, 1991, Volarevic *et al.*, 1992). Therefore, it can be concluded from all the studies with CD45-deficient T cells that expression of this PTP is intimately involved in regulating the quantitative, and perhaps the qualitative, nature of the signal transduced following ligand binding to the AgR complex.

2. *CD45-Deficient B Cell Lines*

Whereas the requirement for CD45 expression is tightly correlated with successful initiation of signal transduction following T cell AgR cross-linking, the expression of this PTP is not absolutely required in order to trigger certain early activation events following B cell AgR ligation (Justement *et al.*, 1994). Moreover, there is a greater heterogeneity in terms of the qualitative and/or quantitative nature of the signals that are transduced when comparisons are made between different CD45-deficient B cell lines. Nevertheless, it is still apparent that this PTP performs an important regulatory function with regard to signal transduction via the B cell AgR.

To date, the most extensive series of studies concerning the role of CD45 in B cell AgR-mediated signal transduction has been performed with the B cell plasmacytoma J558μm3. This cell line expresses membrane IgM specific for the hapten nitrophenyl but lacks CD45 due to normal downregulation of its expression associated with terminal differentiation. Initial experiments were performed with these cells demonstrating that signal transduction in response to either antigen or anti-Ig was abnormal as indicated by the absence of a detectable calcium mobilization response (Justement *et al.*, 1991). The J558μm3 cells were then transfected with a cDNA encoding the high-molecular-weight isoform of CD45 and it was found that expression of this PTP reconstituted the ability to mobilize calcium in response to AgR cross-linking. This observation provided the first indication that AgR signaling in the B cell may be dependent on the expression of CD45. However, unlike T cells, cross-linking of the AgR expressed by CD45-deficient J558μm3 cells leads to the inducible tyrosine phosphorylation of numerous substrates (Kim *et al.*, 1993; Pao and Cambier, 1997; Pao *et al.*, 1997). It should be noted that these cells exhibit both qualitative and quantitative differences in their basal as well as stimulated tyrosine phosphorylation pattern. Based on these findings, and those in other CD45-deficient cell lines, it is apparent that CD45 is not absolutely required for coupling of the AgR to its signal transduction pathway. Moreover, these results suggest that even though CD45 may regulate the activity of one or more Src family PTKs in the B cell, there must be additional PTKs

that do not require CD45 expression for functional activation. Although inducible tyrosine phosphorylation can be detected in the CD45-deficient J558μm3 plasmacytoma, several downstream abnormalities have been observed. First, as expected, recruitment and activation of the Src family kinases p53/56^{lyn}, p59^{fyn}, and p55^{blk} appears to be attenuated in the absence of CD45 (Pao *et al.*, 1997; Pao and Cambier, 1997). However, the PTK p72^{syk} appears to be inducibly activated and recruited to the AgR upon ligand binding (Katagiri *et al.*, 1995; Yanagi *et al.*, 1996; Pao and Cambier, 1997). Not surprisingly, the tyrosine phosphorylation of PLCγ is normal, presumably due to the activation of p72^{syk}. However, despite this, PLCγ activation is significantly decreased in the absence of CD45 and there is a greatly reduced level of inositol trisphosphate production in J558μm3 cells (Kim *et al.*, 1993; Pao *et al.*, 1997). The lack of inositol trisphosphate production apparently leads to attenuation of calcium mobilization in these cells. Activation of the downstream effector proteins MAP kinase and p21^{Ras} is also inhibited in the absence of CD45 (Kim *et al.*, 1993; Kawauchi *et al.*, 1994).

Additional studies with CD45-deficient mutants of the K46-17μmλ B lymphoma cell line support several of the observations previously discussed (L. B. Justement, unpublished observation). AgR cross-linking in these cells mediates inducible tyrosine phosphorylation of several substrates, although the pattern of phosphorylation is again qualitatively and quantitatively different from that observed in cells that express CD45. The activation of p53/56^{lyn} is inhibited in these cells but tyrosine phosphorylation of p72^{syk} is normal, suggesting that this PTK is activated in response to AgR cross-linking. Tyrosine phosphorylation of PLCγ is observed in CD45-deficient K46-17μmλ cells; however, no information has been obtained regarding the production of inositol phospholipid second messengers. Finally, AgR cross-linking in CD45-deficeint K46-17μmλ B cells results in mobilization of calcium from intracellular stores but there is no detectable extracellular influx (L. B. Justement, unpublished observation). Studies with the chicken B cell line DT40, in which CD45 expression has been knocked out using homologous recombination, provide data that support many of the observations described previously (Yanagi *et al.*, 1996). AgR cross-linking induces diminished but detectable tyrosine phosphorylation in these cells. The phosphorylation and activation of p53/56^{lyn} are dysregulated in the absence of CD45, whereas the activation of p72^{syk} is relatively unaffected. The calcium mobilization response in the CD45-deficient DT40 cells is characterized by a delayed and gradual increase in the intracellular concentration of Ca^{2+} (Yanagi *et al.*, 1996). Although this response in not comparable to those in the J558μm3 or K46-17μmλ cell lines, it is characteristic of previous studies in which the abrogation of p53/

56^{lyn} expression was observed to cause a delayed and gradual increase in the concentration of intracellular Ca^{2+} upon AgR cross-linking (Takata *et al.*, 1994).

A somewhat different response pattern was observed in CD45-deficient BAL-17 cells in which AgR ligation induced relatively normal tyrosine phosphorylation and calcium mobilization when compared to either CD45-positive parental cells or revertants (Ogimoto *et al.*, 1995). Although the absence of CD45 expression had only a slight affect on early signal transduction events in these cells, it was shown that AgR-mediated growth arrest was almost completely blocked in cells that were CD45 deficient. Another series of studies involving the immature B cell line WEHI-231 reveal an equally distinct response phenotype when signaling in CD45-deficient mutants is investigated (Ogimoto *et al.*, 1994). The CD45-deficient WEHI-231 cells actually exhibit a phenotype that is very similar to that seen with the CD45-deficient YAC-1 T cell line (Volarevic *et al.*, 1992) in that numerous substrates are constitutively hyperphosphorylated on tyrosine, but cross-linking of the AgR fails to induce any additional phosphorylation. Furthermore, the CD45-deficient WEHI-231 cells exhibit a delayed but prolonged calcium mobilization response that is again very similar to that observed in the YAC-1 cell line.

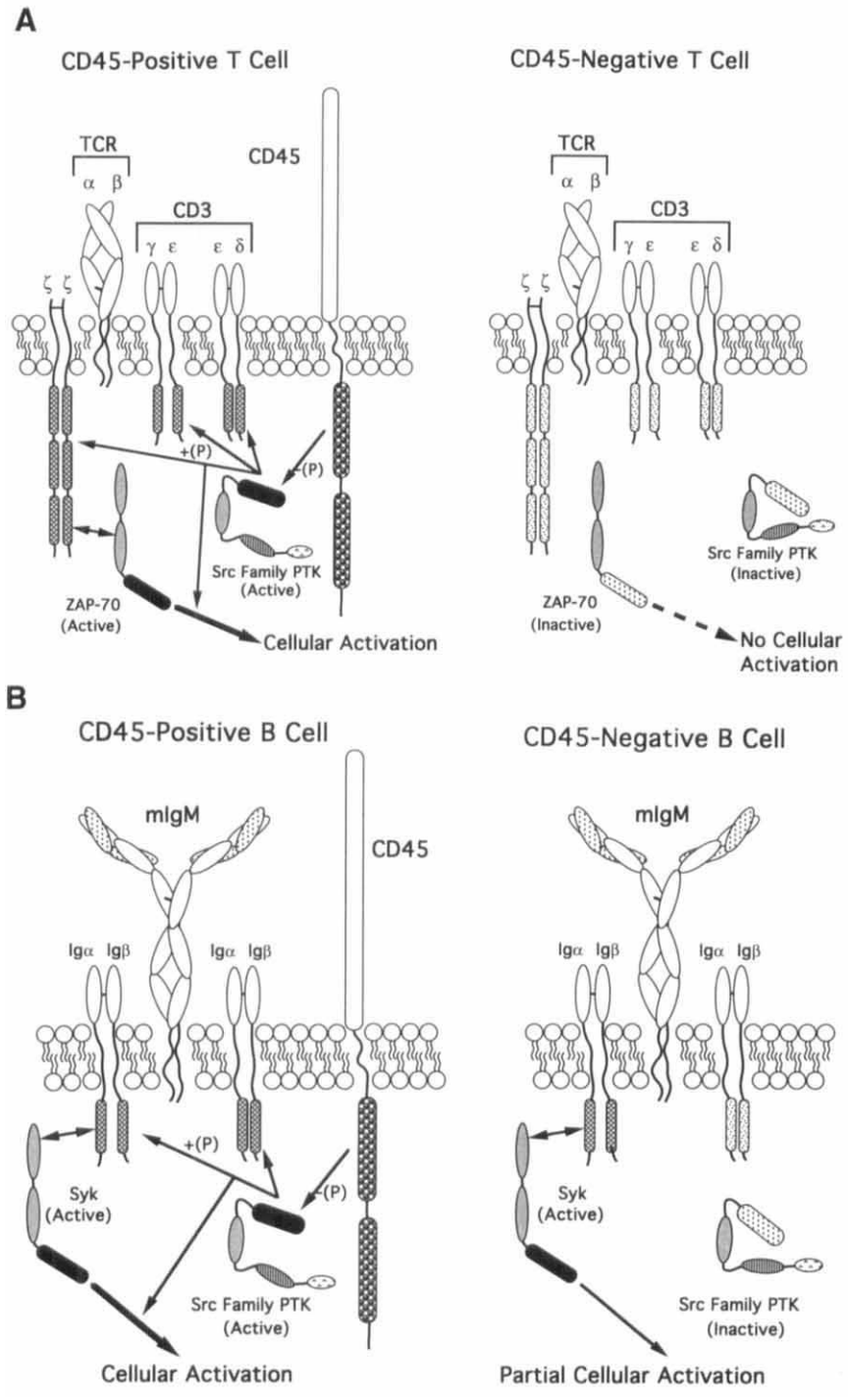
In conclusion, the results from studies of CD45-deficient B cell lines demonstrate that this PTP does indeed regulate signal transduction via the AgR. However, the role for CD45 in B cells is more subtle compared that in to T cells, perhaps because there are other PTKs in the B cell whose function is not dependent on the expression of CD45 (e.g., $p72^{syk}$). This is in contrast to CD45-deficient T cells in which the block in signal transduction does not appear to be effectively circumvented through the activation of alternative PTKs (Fig. 3). Support for this hypothesis has been provided by a recent study demonstrating that $p72^{syk}$, but not $p70^{zap}$, can function independently of either CD45 or $p56^{lck}$ (Chu *et al.*, 1996).

C. Analysis of CD45 Function Using Gene-Targeted Mice

The recent generation of CD45-deficient mice through homologous recombination has confirmed the important role that CD45 serves in regu-

---

FIG. 3. Regulation of T cell and B cell activation by CD45. (A) Schematic representation of signaling via the T cell AgR in the presence or absence of CD45. (B) Schematic representation of B cell AgR signaling in the presence or absence of CD45. Differences exist in terms of the effect that loss of CD45 expression has on AgR-mediated signaling in T cells versus B cells. Whereas T cell activation is essentially abrogated by the loss of CD45 expression, B cell AgR ligation still initiates certain early activation responses. This may be due to the fact that $p72^{syk}$ is able to function in the absence of CD45, whereas $p70^{zap}$ is not.


A
CD45-Positive T Cell
TCR
α β
CD45
CD3
γ ε ε δ
ζ ζ
+(P)
-(P)
Src Family PTK
(Active)
ZAP-70
(Active)
Cellular Activation
CD45-Negative T Cell
TCR
α β
CD3
γ ε ε δ
ζ ζ
Src Family PTK
(Inactive)
ZAP-70
(Inactive)
No Cellular
Activation
B
CD45-Positive B Cell
mIgM
CD45
Igα Igβ
Igα Igβ
+(P)
-(P)
Syk
(Active)
Src Family PTK
(Active)
Cellular Activation
CD45-Negative B Cell
mIgM
Igα Igβ
Igα Igβ
Syk
(Active)
Src Family PTK
(Inactive)
Partial Cellular Activation

lation of lymphocyte development and activation. Initial studies were reported with mice carrying a mutation in CD45 variable exon 6 resulting in a complete loss of CD45 expression by B cells and a significant reduction in its expression on peripheral T cells (Kishihara *et al.*, 1993). Expression of CD45 in the thymus was restricted to mature $CD4^+$ or $CD8^+$ cells and was undetectable on double-negative or double-positive thymocytes. Analysis of the peripheral lymphoid compartment revealed a comparable number of surface IgM^+ cells in the slpeen and bone marrow of both CD45–exon$6^{+/+}$ and CD45–exon$6^{-/-}$ mice. In contrast, the total number of T cells was significantly reduced in all peripheral lymphoid organs examined with a selective skewing toward $CD4^+$ T cells due to a greater reduction in the number of $CD8^+$ T cells. Despite the reduction in the number of T cells, the total number of lymphocytes in the blood, lymph nodes, and bone marrow was unchanged and there was a twofold increase in the number of cells in the spleen. Defects in thymocyte development were apparent based on altered numbers of immature versus mature thymocytes in CD45–exon$6^{-/-}$ mice (Kishihara *et al.*, 1993). A slight increase in double-negative cells and a significant decrease in the number of single-positive thymocytes was observed. In contrast, the number of double-positive cells was comparable to that in CD45–exon$6^{+/+}$ mice. These results indicate that the loss of CD45 expression correlates with a block in thymocyte development at the transition between double-positive immature and single-positive mature cells. A second minor block in the transition from double-negative to double-positive thymocytes was also suggested by the slight increase in $CD4^-CD8^-$ cells. It was also noted that the loss of CD45 expression negatively affects the intrathymic development of Vγ3 T cells and that the block in γ/δ thymocyte maturation is distinct from the block in the α/β lineage (Kawai *et al.*, 1995). Clonal deletion of T cells expressing superantigen-reactive AgRs was analyzed in CD45–exon$6^{+/+}$ and CD45–exon$6^{-/-}$ mice and was observed to occur normally in both, indicating that CD45 expression is not required for negative selection of superantigen-reactive cells (Kishihara *et al.*, 1993; Penninger *et al.*, 1996). Nevertheless, T cells from CD45–exon$6^{-/-}$ mice were unable to mount a proliferative response when stimulated with anti-CD3, anti-TCR$\alpha\beta$, Con A or PMA, and ionomycin. Surprisingly, the lack of responsiveness was observed for CD45-negative as well as CD45-positive T cells isolated from the CD45–exon$6^{-/-}$ mice. Moreover, the defect in proliferation was not overcome by the addition of pharmacologic agents (i.e., PMA and ionomycin) that act well downstream of the putative block in Src family PTK activation associated with the loss of CD45 expression. Finally, the ability to generate an antiviral cytotoxic T cell response was evaluated in CD45–exon$6^{-/-}$ mice based on footpad swelling after local infection with LCMV. Not

surprisingly, mice lacking CD45 exhibited a complete absence of the swelling reaction characteristically mediated by $CD8^+$ cytotoxic T cells. Moreover, secondary restimulation *in vitro* failed to reveal a detectable CTL response against LCMV (Kishihara *et al.*, 1993).

Examination of the B cell compartment in CD45–exon6$^{-/-}$ mice revealed little or no effect on colony formation of bone marrow-derived myeloid or B cell progenitors. Nor was there any difference in the number of colony-forming B cells isolated from spleen, bone marrow, or peritoneum (Kishihara *et al.*, 1993). Although the number of B cells was not decreased in CD45–exon6$^{-/-}$ mice, they did exhibit certain phenotypic alterations suggesting that development within the B cell compartment may be affected by the loss of CD45 expression. Both the high- and low-density splenic B cell populations exhibited a significant decline in the frequency of IgD^{hi},IgM^{lo} cells in addition to a significant decline in the number of B cells expressing CD23 and MHC class II (Benatar *et al.*, 1996). The phenotype of bone marrow B cells was essentially normal. These alterations in B cell phenotype suggest that the loss of CD45 may cause a developmental arrest at the transition from immature to mature B cells. Analysis of the proliferative response following AgR cross-linking demonstrated that CD45-deficient B cells are unable to proliferate when stimulated with either polyclonal or monoclonal anti-IgM, whereas the response to LPS is not affected (Kishihara *et al.*, 1993; Benatar *et al.*, 1996). A more detailed study of AgR-mediated signal transduction with B cells isolated from CD45–exon6$^{-/-}$ mice provided findings that are in agreement with those from CD45-deficient cell lines. AgR cross-linking was observed to mediate induction of tyrosine phosphorylation and in particular it was noted that Igα and PLCγ2 exhibited increased phosphorylation comparable to that in B cells from CD45–exon6$^{+/+}$ animals (Benatar *et al.*, 1996). However, the calcium mobilization response was not normal and was characterized by a rapid and transient increase in the concentration of intracellular Ca^{2+}. The transient rise in Ca^{2+} was determined to be due to the release of Ca^{2+} from intracellular stores (Benatar *et al.*, 1996). These results suggest that CD45 may regulate the movement of Ca^{2+} into the B cell, possibly by controlling the function of Ca^{2+} channels in the plasma membrane.

Experiments to assess the antibody response in CD45–exon6$^{-/-}$ mice were performed and revealed that the T-independent antibody response is only marginally decreased, whereas the T-dependent response is decreased by a factor of 50 (Kishihara *et al.*, 1993). A more detailed examination of the role for CD45 in B cell responses against T-independent and T-dependent antigens was performed following adoptive transfer of $CD4^+$ T cells from wild-type mice into the CD45–exon6$^{-/-}$ animals (Kong *et al.*, 1995). In the presence of CD45-positive T cells, CD45-deficient B cells

were able to respond to both T-independent and T-dependent antigens and were able to undergo class switching to secondary isotypes. These data suggest that CD45 is not required for B cell activation and differentiation per se (Kong *et al.*, 1995). However, a detailed analysis has not been performed to determine whether the elimination of CD45 expression results in an adaptive developmental response leading to the preferential selection of B cells that have effectively compensated for the loss of CD45 expression through increased expression and/or function of PTKs or PTPs.

The role of CD45 in regulation of B cell positive and negative selection was assessed by crossing CD45–exon6$^{-/-}$ mice with mice carrying immunoglobulin transgenes specific for hen egg lysozyme (HEL) (Cyster *et al.*, 1996). B cells isolated from these animals were examined for their ability to respond to soluble HEL *in vitro*. Activation of the ERK/RSK/EGR1 signaling cascade was significantly diminished in CD45-deficient B cells, as was the Ca^{2+} mobilization response when compared to control B cells. Antigenic stimulation of CD45-deficient B cells resulted in decreased expression of CD86 and little or no increase in the expression of CD54. In contrast to previous studies with CD45–exon6$^{-/-}$ B cells, stimulation with polyclonal anti-IgM or PMA was observed to induce comparable activation of ERK2 and calcium mobilization in B cells regardless of whether they expressed CD45 (Cyster *et al.*, 1996). The essential conclusion from these experiments is that expression of CD45, although not absolutely required for initiation and propagation of signaling via the AgR, does play an important role in regulating the intensity of the signal and thus the threshold of sensitivity of the AgR for antigen. It was further shown that alterations in AgR sensitivity have practical consequences during the selection of high-affinity self-reactive B cells. Whereas the presence of circulating HEL autoantigen normally mediates negative selection of autoreactive CD45-positive B cells, loss of CD45 expression leading to a decrease in AgR-mediated signal intensity actually potentiated the positive selection of autoreactive B cells (Cyster *et al.*, 1996). Thus, it is apparent that CD45 is directly involved in regulating the AgR signal threshold and therefore determines whether B cells undergo negative or positive selection.

Recently, CD45-null mice have been generated through the targeted disruption of exon 9 within the CD45 locus (Byth *et al.*, 1996). These animals exhibit a complete loss of CD45 expression on both T cells and B cells, in contrast to the CD45–exon6$^{-/-}$ knockout mice. The spleens from CD45–exon9$^{-/-}$ mice contained twice the number of B cells and only one-fifth as many T cells when compared to wild-type animals. The increased number of B cells in the spleen was due to expansion of two B cell subpopulations that express high levels of IgM. The response to anti-CD40 and IL-4 was normal based on upregulation of MHC class II and

CD40 expression, indicating that CD45 is not involved in regulating signal transduction via these receptors. Mature T cell expression in the periphery of CD45-null mice was dramatically reduced and there was a 10-fold reduction in $CD3^+CD4^+$ cells and at least a 3- or 4-fold decrease in $CD3^+CD8^+$ T cells. In contrast to the CD45–exon$6^{-/-}$ mice, the CD45–exon$9^{-/-}$ animals exhibit a distinct block in both negative and positive thymocyte selection that is probably accounted for by the complete loss of CD45 expression (Byth *et al.*, 1996). Ontogenetic analysis of thymocyte development further suggests that CD45 expression is not required for the development of thymic stem cells nor the entry of prothymocytes into the thymic rudiment. Investigation of the development of thymocytes from CD45–exon$9^{-/-}$ mice in fetal thymic organ culture (FTOC) revealed that there is a significant increase in the basal apoptotic rate of $CD4^+CD8^+$ thymocytes resulting in a general decrease in the total number of thymocytes recovered after 7 days. As a result, CD45-null thymuses contain an aberrantly high percentage of double-negative cells. These results confirm that CD45 expression is important for enhancing the efficiency of positive selection by promoting the transition from the double-positive to single-positive state (Byth *et al.*, 1996). FTOC was also used to demonstrate that CD45 expression is required for negative selection following AgR ligation. Treatment of $CD45^{+/+}$ but not $CD45^{-/-}$ FTOC with anti-CD3 mAb was observed to induce significant levels of apoptosis and the yield of thymocytes from $CD45^{+/+}$ thymuses was reduced by 38%, whereas the number of thymocytes recovered from $CD45^{-/-}$ thymuses was not affected. In contrast, the apoptotic response of $CD45^{-/-}$ double-positive thymocytes was comparable to that of $CD45^{+/+}$ cells following the addition of ionomycin, dexamethasone, or anti-Fas mAb. Therefore, the defect in negative selection observed in $CD45^{-/-}$ cells is specific to the signal delivered by AgR ligation (Alexander, 1997; Byth *et al.*, 1996). Finally, in the absence of CD45 expression, negative selection of TCR-Vβ8 double-positive thymocytes was found to be defective in response to the superantigen *S. aureus* enterotoxin B (Conroy *et al.*, 1997). The abrogation of negative selection in the CD45–exon$9^{-/-}$ mice was in contradiction to results obtained in the CD45–exon$6^{-/-}$ system, demonstrating that negative selection occurs normally. It is possible that the difference in results between these two studies is a function of the low level of CD45 expression that is maintained in the CD45–exon$6^{-/-}$ animals, which is perhaps sufficient to enable the T cell AgR to drive a signal in response to a strong stimulus such as a superantigen.

As in the CD45–exon$6^{-/-}$ mice, proliferation of B cells isolated from CD45–exon$9^{-/-}$ mice in response to AgR cross-linking was absolutely dependent on the expression of CD45 (Byth *et al.*, 1996). Neither polyclonal

anti-IgM nor anti-IgD antibody were observed to induce cellular proliferation of B cells, whereas the response to anti-CD40 mAb was relatively unaffected by the loss of CD45 expression. The functional response to anti-CD40 was not unexpected because anti-CD40 had previously been shown to restore the proliferative response of B cells that had been treated with anti-Ig and anti-CD45 mAbs simultaneously (Gruber *et al.*, 1989). Finally, proliferation of B cells stimulated with anti-CD38 mAb was analyzed, revealing a significant impairment in their responsiveness associated with the loss of CD45 expression. Because signal transduction via CD38 requires the expression of a functional B cell AgR complex (Lund *et al.*, 1996), and may in fact utilize components of the AgR complex, it is not entirely surprising to find that CD38 is nonfunctional in CD45-deficient cells. A more detailed analysis of antibody responses and intracellular signaling by B cells from CD45–exon9$^{-/-}$ mice has not yet been reported, so it is not known how these parameters will compare to data obtained from CD45–exon6$^{-/-}$ mice or cell lines studies; however, such information will be most informative.

In conclusion, CD45 has been shown to play an essential role in T cell development and is required for the productive transmission of a signal from the T cell AgR. In contrast, CD45 serves a more subtle role in the B cell compartment where it appears to be involved in development, although it is not absolutely required. Additionally, signal transduction via the B cell AgR is not completely impaired by a lack of CD45 expression. Many early signaling events are observed but may be noticeably altered in terms of quantitative and qualitative aspects. Finally, a consistent finding is that B cell proliferation *in vitro* is completely abrogated by the loss of CD45, but it is possible to stimulate relatively normal antibody production by CD45$^{-/-}$B cells *in vivo* as long as appropriate T cell help is provided. This suggests that the B cell is able to respond to antigen, proliferate, and differentiate into an antibody-secreting cell. There must therefore be one or more mechanisms that enable the B cell to bypass the block imposed on it by the loss of CD45 expression. It is likely that, in the presence of CD45$^{+/+}$ T cells, antigen-stimulated CD45$^{-/-}$ B cells receive and respond to costimulatory signals that support proliferation and differentiation *in vivo*, despite the fact that they lack CD45.

III. Molecular Mechanisms Involved in CD45 Function

In order to understand the way in which CD45 regulates lymphocyte development and activation at the molecular level, it is essential to characterize the intracellular substrates that are targets for this PTP and to delineate the mechanism(s) responsible for regulation of its catalytic activ-

ity. Elucidation of these issues will facilitate the interpretation of functional data derived from studies with CD45 mAbs, CD45-deficient cell lines, and CD45-deficient mice.

A. Identification of Substrates for CD45

Identification of substrates for CD45 is a major area of emphasis due to the fact that this is one of the most important questions related to the development of a molecular model to explain its function in lymphocytes. Several issues must be taken into consideration when attempting to identify substrates for CD45. First, the subcellular localization of CD45 has to be considered in order to identify likely targets for this PTP. Because CD45 is a transmembrane PTP, it is logical to propose that relevant substrates would be almost exclusively localized to the inner face of the plasma membrane in either a constitutive or inducible manner. Although CD45 is indeed expressed predominantly in the plasma membrane, studies have documented the presence of intracellular pools of CD45 that actually redistribute within the cell in response to activation signals (Minami *et al.*, 1991). Additionally, it is possible that CD45, expressed on the surface of the cell, might undergo capping and be internalized following ligand binding. Thus, there are situations in which CD45 is found within intracellular compartments and thus the possibility cannot be completely ruled out that this PTP may dephosphorylate intracellular substrates under specific conditions. A more subtle aspect of CD45 localization within the cell relates to the potential for selective interactions between CD45 and other transmembrane proteins as a function of isoform expression. Thus, an analysis of intermolecular interactions between CD45 and other surface proteins may yield significantly different results depending on the cell type studied and its relative state of development or activation. The selective interaction of CD45 with other transmembrane proteins could in turn have a significant effect on the complement of intracellular substrates that are accessible to CD45. Second, it is not always possible to interpret changes in the tyrosine phosphorylation status of a protein as definitive proof that that protein is a direct substrate for CD45. Due to the relatively complex and extensive regulatory networks that exist between PTKs and PTPs in most cells, it is possible that changes in the phosphorylation of any given protein could result from alterations in the function of a PTK or a distinct PTP that is in turn regulated by the expression of CD45 or by modulation of its function.

1. Analysis of Intermolecular Associations Involving CD45

The rationale for performing experiments to identify cellular proteins that physically interact with CD45 is twofold. First, the physical association

between CD45 and another protein, particularly a tyrosine phosphoprotein, may be indicative of the fact that that protein is a substrate for CD45. Second, it is possible that protein : protein interactions play a role in regulating the catalytic function of CD45. The interaction between CD45 and other proteins may be involved in the targeting of substrates to CD45, effectively regulating its ability to dephosphorylate selected phosphoproteins within the cell. Alternatively, the catalytic activity of CD45 may be controlled through its interaction with one or more regulatory proteins that are not necessarily substrates.

Not surprisingly, a number of studies demonstrating intermolecular interactions between CD45 and other transmembrane proteins in T and B lymphocytes have been reported. Molecules that interact with CD45 based on chemical cross-linking, coprecipitation, or cocapping/modulation include the ζ chain of the CD3 complex (Furukawa *et al.*, 1994), CD4 and CD8 (Mittler *et al.*, 1991), CD7 (Lazarovits *et al.*, 1994), CD26 (Torimoto *et al.*, 1991), CD2 (Schraven *et al.*, 1990; Altevogt *et al.*, 1989), CD100 (Herold *et al.*, 1996), and Thy-1 (Lynes *et al.*, 1993; Volarevic *et al.*, 1990) on T cells and membrane immunoglobulin (mIg) (Brown *et al.*, 1994), and LFA-1 (Zapata *et al.*, 1995) on B cells. In many instances, the possibility that these interactions are physiologically relevant has been supported through the use of anti-CD45 mAbs or CD45-deficient cell lines.

a. Association of CD45 with Transmembrane Proteins in the T Cell. Many of the transmembrane proteins listed previously interact not only with CD45 but also with the T cell AgR complex as well. Therefore, it is formally possible that large multisubunit complexes exist in the plasma membrane consisting of the T cell AgR, CD45, and one or more coreceptors. If this is in fact true, then it could complicate the interpretation of results obtained from studies of protein:protein interactions between CD45 and specific T cell coreceptors because it may not be possible to accurately discriminate between direct as opposed to indirect associations. Another caveat that must be kept in mind is that CD45 is an extremely abundant cell surface protein. Thus, it is quite possible that artificial interactions may be seen due to nonspecific trapping of other transmembrane proteins as a result of chemical cross-linking and/or immunoprecipitation of CD45.

As previously mentioned, anti-CD45 mAbs have been observed to potentiate T cell responses when added simultaneously with anti-CD2 mAbs (Schraven *et al.*, 1989, 1990). Additional studies using chemical cross-linking suggest that these proteins are intimately associated with one another in the plasma membrane in an activation-independent manner (Schraven *et al.*, 1990). The interaction between CD45 and CD2 was shown to be relatively specific based on the fact that CD45 did not coprecip-

itate with CD27 or CD58 after chemical cross-linking, although the CD45:CD2 complex did contain MHC class I. Similar findings were reported in a study demonstrating that CD45 coprecipitates with CD2 isolated from mouse thymocytes, splenic T cells, Con A blasts, and T cell lines in the absence of chemical cross-linking (Altevogt *et al.*, 1989). Whereas CD2 appeared to be constitutively associated with CD45 in the membrane, there was no detectable interaction with CD3, again suggesting that the interaction with CD2 is specific. However, modulation of CD2 from the cell surface did not lead to comodulation of either CD45 or CD3. The data suggest that CD2 and CD45 preferentially interact with one another in the membrane and that engagement of the extracellular domain of CD45 by ligand may modulate the signal transduced via CD2 by altering the physical relationship between these proteins in the membrane.

Signal transduction via Thy-1, a glycosyl phosphatidylinositol-linked receptor, requires the expression of both the T cell AgR complex (Ashwell and Klausner, 1990) and CD45 (Pingel *et al.*, 1994), suggesting that these transmembrane polypeptides interact with one another in the plasma membrane. In support of this hypothesis, immunoprecipitation of Thy-1 from chemically cross-linked T cells resulted in the coprecipitation of CD45, MHC class I, and β_2-microglobulin. A similar pattern of coprecipitating proteins was observed when the T cell AgR was isolated from chemically treated T cells. Further analysis revealed that a significant proportion of either Thy-1 or the T cell AgR could be isolated in association with CD45, demonstrating that they are intimately associated with this PTP (Volarevic *et al.*, 1990). In contrast, attempts to detect a direct interaction between Thy-1 and the T cell AgR were unsuccessful. Thus, an analysis of the interaction between CD45, Thy-1, and the T cell AgR provides evidence for the potential existence of a multisubunit complex in which CD45 functions as an intermediate between Thy-1 and the T cell AgR complex. Alternatively, CD45 might interact one on one with either Thy-1 or the AgR and thus regulate the function of these receptors independently. Nevertheless, the previous findings, as well as others (Furukawa *et al.*, 1994), suggest that CD45 and the ζ chain of the CD3 complex physically associate with one another in the plasma membrane. Additional proof for the physical interaction between these polypeptides was provided by studies using purified glutathione *S*-transferase–CD45 fusion proteins to isolate high-affinity tyrosine-phosphorylated CD45 substrates from T cell lysates. CD45 fusion proteins were found to bind to CD3 ζ chain, whereas leukocyte common antigen-related (LAR) PTP fusion proteins or CD45–LAR hybrid fusion proteins did not (Furukawa *et al.*, 1994). Moreover, phosphorylated CD3 ζ chain was preferentially dephosphorylated by CD45 under conditions that did not support the dephosphorylation of other

phosphoproteins. The finding that CD3 ζ chain is a potential substrate for CD45 suggests that this PTP may be involved in terminating signal transduction via the T cell AgR or at the least is involved in regulating the basal homeostatic level of CD3 ζ chain phosphorylation. Because CD3 ζ phosphorylation is associated with AgR- mediated activation of $p59^{fyn}$ and/or $p56^{lck}$, one would not expect to observe its hyperphosphorylation in CD45-deficient cells because these PTKs are essentially inactive. However, studies with the YAC-1 lymphoma (Volarevic *et al.*, 1992), which is characterized by elevated basal tyrosine phosphorylation of numerous substrates including CD3 ζ, suggests that this subunit of the T cell AgR complex may in fact be a substrate for CD45. Therefore, it is formally possible that this PTP regulates both the initiation and the termination of signal transduction via the AgR.

Characterization of another multisubunit complex in the membrane of T cells revealed an interaction between CD7, a functional homolog of Thy-1, CD45, and the T cell AgR complex (Lazarovits *et al.*, 1994). Cross-linking of CD7 was observed to induce tyrosine phosphorylation of numerous substrates in the cell, including CD45. Immunoprecipitation and Western blotting analysis demonstrating that CD7 exists in a complex with CD45 and CD3 was confirmed using fluorescence resonance energy transfer (FRET) to obviate potential artifacts caused by incomplete solubilization of proteins in detergent lysates. FRET analysis demonstrated that CD7 is in close apposition to both CD45 and CD3 in the membrane, supporting the existence of an oligomeric complex containing all three polypeptides (Lazarovits *et al.*, 1994). Although cross-linking of CD7 stimulates T cell proliferation and integrin-mediated adhesion, it is not known whether it does so by regulating the function of CD45 or whether CD45 is required in order for CD7 to deliver a signal to the cell. Therefore, the physiological significance of this complex, as well as that of others that contain CD45, is currently unknown.

Not all interactions between CD45 and other transmembrane proteins occur constitutively, as demonstrated by studies of the association between CD45 and CD4 or CD8. Activation of human peripheral blood leukocytes with anti-CD3 mAb or during an MLR response induces the CD3-dependent formation of CD45:CD4 or CD45:CD8 complexes (Mittler *et al.*, 1991). Maximal formation of these heterodimeric complexes does not occur until 72–96 hr after the initial stimulation of cells with anti-CD3, whereas the maximal association during an MLR response did not occur until Day 6 (Mittler *et al.*, 1991). Presumably, the interaction between CD45 and either CD4 or CD8 late during T cell activation is required to deliver additional signals to the cell that promote proliferation once it has received the requisite activating signals. It is likely that a primary target

of CD45 is the Src family kinase $p56^{lck}$ that associates with CD4 and CD8. Antibody-mediated coclustering of CD4 or CD8 results in increased tyrosine phosphorylation and kinase activity of $p56^{lck}$. In contrast, co-cross-linking of either CD4 or CD8 with CD45 inhibits the normal induction of $p56^{lck}$ tyrosine phosphorylation and activation, providing support for the concept that this PTK is a substrate for CD45 (Ostergaard and Trowbridge, 1990).

As previously discussed, the fact that CD45 undergoes isoform switching in response to cellular activation and differentiation suggests that the extracellular domain is involved in regulating its function through specific interactions with other transmembrane proteins and their associated intracellular effector molecules. Such isoform-dependent interactions would effectively target CD45 to selected intracellular substrates and thereby regulate its function by controlling the relative accessibility of substrates. Studies of naive and memory T cells that differ with respect to the isoform of CD45 that they express reveal distinct isoform-specific interactions between CD45, CD4, and the T cell AgR. On naive T cells that express a high-molecular-weight isoform of CD45 (CD45RB), these receptors migrate independently of one another and therefore do not appear to be physically associated (Dianzani *et al.*, 1990). On the other hand, examination of T cells that express the low-molecular-weight isoform of CD45 (CD45RO) and are therefore considered to be memory cells, revealed that CD45, CD4, and the T cell AgR complex all migrate together in the plasma membrane and therefore function as an integrated protein complex. The analysis of isoform-specific interactions was extended in subsequent studies using a panel of anti-CD45 mAbs specific for epitopes encoded by the different alternatively spliced exons A, B, and C of CD45. These experiments demonstrated that the T cell accessory molecule CD2 cocaps with CD45RO and the adhesion molecule LFA-1 interacts with CD45RA (Dianzani *et al.*, 1992). The results from these studies suggest that the interactions between these proteins and CD45 involve low-molecular-weight, single-exon isoforms as opposed to the higher-molecular-weight isoforms of CD45 that contain multiple exons.

b. Association of CD45 with Transmembrane Proteins in the B Cell. To date, the investigation of intermolecular associations between CD45 and other transmembrane proteins in the B cell has been relatively limited in scope. Nevertheless, it has been shown that CD45 interacts with both mIgM and mIgD isolated from resting splenic B cells based on the reciprocal coprecipitation of these proteins (Brown *et al.*, 1994). Furthermore, immunoprecipitation of CD45 from detergent lysates prepared from B cells followed by *in vitro* radiolabeling of immune complex proteins revealed

the presence of tyrosine phosphorylated proteins with molecular weights corresponding to the Igα and Igβ subunits of the mIg-associated heterodimer. The presence of the Igα/Igβ heterodimer in the CD45 immune complex was confirmed by secondary immunoprecipitation with antibodies specific for these subunits. Although this study did not directly address the specific nature of the interaction between CD45 and the B cell AgR complex, it provides evidence to support the conclusion that CD45 interacts with the AgR complex in an activation-independent manner. The close physical association between the AgR complex and CD45 raises the possibility that the basal tyrosine phosphorylation state of the Igα/Igβ heterodimer may be regulated by CD45 in a manner similar to that proposed for the ζ chain in T cells (Furukawa *et al.*, 1994). Support for the hypothesis that CD45 is involved in regulating the phosphorylation state of the B cell AgR complex has been provided by studies demonstrating that physical sequestration of CD45 within the plasma membrane leads to increased phosphorylation of Igα and Igβ (Lin *et al.*, 1992). Incubation of splenic B cells with anti-CD45 mAb alone did not induce CD45 to associate with the cytoskeleton or to form caps within the plasma membrane. Under these conditions little or no change in the phosphorylation of Igα or Igβ was observed. However, incubation of B cells with anti-CD45 mAb and a secondary cross-linking reagent induced the rapid association of CD45 with the cytoskeleton and caused CD45 to form a cap within the plasma membrane. These events were associated with a significant increase in the tyrosine phosphorylation of Igα and Igβ. Kinetic analysis of heterodimer phosphorylation revealed a correlation between this event and the formation of an intermolecular association between CD45 and the cytoskeleton (Lin *et al.*, 1992). These results suggest that the activity of CD45 may be regulated by its interaction with cytoskeletal elements and/or physical redistribution within the membrane. Further support for the hypothesis that Igα and Igβ are substrates for CD45 comes from studies of the CD45-deficient B cell plasmacytoma J558μm3 and a CD45-positive transfectant. Analysis of basal as well as inducible phosphorylation of Igα and Igβ revealed that the heterodimer exhibits elevated basal tyrosine phosphorylation in CD45-deficient cells compared to CD45-positive transfectants (Pao and Cambier, 1997). Moreover, antigen stimulation resulted in hyperphosphorylation of Igα and Igβ in CD45-deficient cells, indicating that CD45 may actually regulate basal as well as inducible phosphorylation of these polypeptides. In contrast to these observations, B cells isolated from CD45–exon6$^{-/-}$ mice exhibited basal and inducible phosphorylation of the heterodimer that was comparable to B cells isolated from CD45–exon6$^{+/+}$ control mice (Benatar *et al.*, 1996).

c. Association of CD45 with Intracellular Substrates. Without question, the largest body of information documenting the physical association between CD45 and intracellular proteins relates to its interaction with the Src family PTKs $p56^{lck}$ (Schraven *et al.*, 1991; Koretzky *et al.*, 1993; Ng *et al.*, 1996) and $p59^{fyn}$ (Mustelin *et al.*, 1992) in the T cell and $p53/56^{lyn}$ (Brown *et al.*, 1994; Burg *et al.*, 1994) in the B cell. Additionally, the physical interaction between CD45 and these kinases has been extensively documented to be of physiological importance in terms of their phosphorylation and activation (Trowbridge and Thomas, 1994; Alexander, 1997). The Src family PTK $p59^{fyn}$ physically associates with the T cell AgR complex and is intimately involved in signal transduction mediated by cross-linking of this receptor complex (Weiss and Littman, 1994). The importance of this PTK in regulating AgR signaling and the early defect in signal transduction associated with the loss of CD45 expression suggested that this kinase might be a potential target for CD45. Studies employing antibody-mediated capping of CD45 and subsequent immunofluorescence analysis demonstrated an interaction between CD45 and $p59^{fyn}$ based on the specific colocalization of a significant proportion of these proteins (Mustelin *et al.*, 1992). However, the specific nature of the interaction has yet to be elucidated. Thus, it is quite possible that the colocalization of CD45 and $p59^{fyn}$ is in fact due to the association between elements of the CD3 complex and CD45 as opposed to a direct association between CD45 and $p59^{fyn}$ itself.

In contrast to the circumstantial evidence for an association between CD45 and $p59^{fyn}$, a number of studies have provided evidence supporting a direct physical interaction between CD45 and $p56^{lck}$. Initially, *in vitro* kinase assays were performed demonstrating that CD45 coprecipitates with a PTK that phosphorylates an immune complex substrate with a molecular weight of 32 kDa (Schraven *et al.*, 1991). Western blot analysis of CD45 immune complexes demonstrated that $p56^{lck}$ was responsible for the CD45-associated *in vitro* kinase activity. These data indicated that CD45 forms a functional complex with $p56^{lck}$ and a 32-kDa phosphoprotein. Subsequent evaluation of the interaction between CD45, $p56^{lck}$, and the low-molecular-weight phosphoprotein pp32 were performed demonstrating that the *in vitro* kinase activity associated with CD45 requires the expression of $p56^{lck}$, suggesting that CD45 and $p59^{fyn}$ do not interact with one another directly. Additionally, the association between CD45 and $p56^{lck}$ was not dependent on expression of the T cell AgR or the transduction of a signal via the AgR. Finally, it was shown that $p56^{lck}$ and pp32 interact with CD45 independently of one another (Koretzky *et al.*, 1993). Recently, recombinant purified $p56^{lck}$ was found to specifically associate with a purified recombinant CD45 cytoplasmic domain protein with a stoichiometry of approximately 1 : 1 but not with the cytoplasmic domain of RPTPα (Ng

et al., 1996). Analysis of the $p56^{lck}$ domains involved in binding to CD45 demonstrated that both the unique N-terminal and SH2, but not the SH3, domains were able to block the binding of $p56^{lck}$ to recombinant CD45. Of the two, however, the SH2 domain bound with higher affinity. Finally, the interaction of the $p56^{lck}$ SH2 domain with CD45 occurred in the absence of CD45 tyrosine phosphorylation, indicating that this domain participates in a nonconventional interaction (Ng *et al.*, 1996). This last result is in contrast to previous findings showing that tyrosine phosphorylation of CD45 by $p50^{csk}$ potentiates the interaction with $p56^{lck}$ (Autero *et al.*, 1994) and that CD45, isolated from detergent lysates of a pervanadate-treated T cell hybridoma, coprecipitates with $p56^{lck}$ (Lee *et al.*, 1996). In the latter study analysis of deletion mutants of $p56^{lck}$ indicated that the interaction between this kinase and CD45 was not absolutely dependent on either the SH2 or SH3 domains, again suggesting that the association may be mediated in part by the unique N-terminal region of $p56^{lck}$ (Lee *et al.*, 1996).

In the B cell, evidence has accumulated indicating that there is a physical association between CD45 and the PTK $p53/56^{lyn}$ (Brown *et al.*, 1994; Burg *et al.*, 1994) but not other Src family PTKs such as $p59^{fyn}$ or $p55^{blk}$. Isolation of CD45 immune complex material from detergent lysates of resting splenic B cells followed by radiolabeling of proteins using an *in vitro* kinase assay revealed that CD4 is constitutively associated with one or more PTKs (Brown *et al.*, 1994). Subsequent analysis of CD45-associated PTK activity with a panel of anti-Src family kinase antibodies indicated that $p53/56^{lyn}$ coprecipitates with CD45 in a selective manner. Further analysis demonstrated that a GST : Lyn fusion protein could be used to immunoprecipitate CD45 from thymocyte lysates indicating that $p53/56^{lyn}$ does not require components of the B cell AgR complex in order to associate with CD45. Because $p53/56^{lyn}$ was constitutively associated with CD45 in lysates prepared from resting B cells or from unstimulated thymocytes, it is apparent that tyrosine phosphorylation of CD45 is not required for this interaction (Brown *et al.*, 1994). However, the results from another study demonstrate the rapid induction of $p53/56^{lyn}$ binding to CD45 in response to B cell AgR cross-linking (Burg *et al.*, 1994). Thus, it is possible that $p53/56^{lyn}$ may associate with CD45 via more than one mechanism and the association between these proteins may be potentiated by tyrosine phosphorylation of CD45.

B. Regulation of Src Family Kinases by CD45

As previously discussed, the loss of CD45 expression leads to a profound block in signal transduction via the T cell AgR and to a lesser extent the B cell AgR (Alexander, 1997; Trowbridge and Thomas, 1994; Justement

et al., 1994). In both cell types the apparent lesion in the signal transduction cascade occurs at a point that is proximal to the AgR itself and in T cells this leads to an abrogation of inducible tyrosine phosphorylation. Thus, it was logical to hypothesize that CD45 expression may be required for regulation of PTK function. It has been well documented that the T cell and B cell AgRs are coupled to Src family PTKs and that activation of these kinases is one of the earliest detectable events following AgR cross-linking (Weiss and Littman, 1994). Evidence that CD45 is involved in regulating Src family kinase phosphorylation and activation was first provided by studies of paired lymphoma cell lines that differed with regard to their level of CD45 expression. An analysis of these well-characterized cell lines revealed the constitutive hyperphosphorylation of $p56^{lck}$ in cells that were CD45 deficient. Phosphoamino acid analysis confirmed that $p56^{lck}$ isolated from CD45-deficient cells was hyperphosphorylated on tyrosine residue 505, the carboxyl-terminal negative regulatory phosphorylation site (Ostergaard *et al.*, 1989). Moreover, activation of $p56^{lck}$ was undetectable in one of the above cell lines lacking CD45, whereas activation was readily detectable in its CD45-positive counterpart (Mustelin *et al.*, 1989). Incubation of purified CD45 with $p56^{lck}$ *in vitro* increased the catalytic activity of the latter more than 2-fold. These findings indicate that expression of CD45 is required to regulate tyrosine phosphorylation of the carboxyl-terminal regulatory tyrosine residue of $p56^{lck}$ in order to maintain the kinase in an active state. Subsequent analysis of CD45 involvement in regulation of Src family kinase function in a CD45-negative $CD8^+$ T cell clone demonstrated increased reactivity of both $p56^{lck}$ and $p59^{fyn}$ with anti-phosphotyrosine antibody and a concomitant decrease in their enzymatic activity (McFarland *et al.*, 1993). Cyanogen bromide digestion and examination of peptide maps revealed that the carboxy-terminal tyrosine 505 of $p56^{lck}$ exhibited an 8-fold increase in tyrosine phosphorylation, whereas tyrosine 531 of $p59^{fyn}$ was increased 2-fold in CD45-negative cells. Similarly, tyrosine phosphorylation of $p56^{lck}$ on the inhibitory Tyr505 was increased by 2-, 6-, or 8-fold in three different CD45-deficient cell lines. In contrast, phosphorylation of $p59^{fyn}$ on the equivalent residue (Tyr531) was observed to be increased by only 2.5-fold in two cell lines and not at all in a third (Hurley *et al.*, 1993). The phosphorylation of $p60^{c\text{-}src}$ at the homologous tyrosine was not increased by the absence of CD45 in one cell line examined. Although expression of CD45 correlates with a reduction in the tyrosine phosphorylation of Src family PTKs and therefore suggests that these kinases are direct substrates for CD45, it is formally possible that CD45 expression actually potentiates the function of another PTP that dephosphorylates the inhibitory tyrosine residues (Hurley *et al.*, 1993). Alternatively, it is possible that CD45 inhibits the function of another PTK

that is responsible for phosphorylating the inhibitory tyrosine residue. However, the fact that CD45 has been shown to dephosphorylate $p56^{lck}$ and $p59^{fyn}$ *in vitro* (Mustelin *et al.*, 1992; Ostergaard and Trowbridge, 1990) and the fact that these PTKs physically interact with CD45 (Mustelin *et al.*, 1992; Ng *et al.*, 1996) argue in favor of the hypothesis that they are indeed direct substrates for CD45. Another point raised from the previous results relates to the differences in the degree of hyperphosphorylation observed between $p56^{lck}$, $p59^{fyn}$, and $p60^{c\text{-}src}$ in the absence of CD45 expression. Such differences can perhaps be explained by the fact that CD45 exhibits a relatively high degree of substrate specificity such that $p56^{lck}$ is preferentially dephosphorylated when compared to $p59^{fyn}$. However, the various members of the Src family exhibit a very high degree of similarity in their carboxyl-terminal sequences flanking the respective inhibitory tyrosine residues, which makes this unlikely. An alternative explanation for the relative differences observed could be that members of the Src family are not equally accessible to the catalytic domain of CD45. Thus, it is conceivable that those kinases that are more readily accessed by CD45 would exhibit the greatest differential in their tyrosine phosphorylation. A final point raised by these studies is that a large percentage of the $p56^{lck}$ isolated from CD45-positive cells is dephosphorylated at the 505 position. This finding suggests that CD45 does not need to become activated by AgR cross-linking in order to perform its function. Rather, it appears as though CD45 is responsible for maintaining a significant proportion of cellular $p56^{lck}$ in a dephosphorylated state in the absence of stimulus, indicating that signaling via the AgR involves the mobilization of a pool of preactivated kinase (Hurley *et. al*, 1993).

It has been proposed that phosphorylation of the carboxy-terminal tyrosine residue of Src family kinases leads to conformational changes that inhibit their catalytic function (Weiss and Littman, 1994). In essence, phosphorylation of the conserved carboxy-terminal tyrosine residue of Src kinases results in the formation of a phosphotyrosine motif that mediates the formation of an intramolecular association with the SH2 domain of the kinase. This intramolecular bond causes the kinase to assume a catalytically inactive conformation in which the interaction between substrate and the catalytic domain is sterically inhibited. Based on this model, it has been proposed that dephosphorylation of the carboxy-terminal inhibitory tyrosine residue enables the kinase to open up and assume an active conformational state (Weiss and Litman, 1994). Studies indicating that CD45 does indeed modulate the conformational state of $p56^{lck}$ in a manner consistent with the intramolecular model of regulation of Src kinase function have been reported (Sieh *et al.*, 1993). Experiments demonstrate that CD45 regulates the binding of $p56^{lck}$ to an 11-amino acid tyrosine phosphopeptide

derived from the carboxy terminus of $p56^{lck}$. Kinase that did bind the phosphopeptide was dephosphorylated at Tyr505 and comprised only 5–10% of the total cellular pool of $p56^{lck}$. The finding that only a small percentage of $p56^{lck}$ bound phosphopeptide in CD45-positive cells could be due to the fact that access of CD45 to $p56^{lck}$ is regulated by the subcellular location of the kinase with respect to CD45. For example, cytosolic pools of $p56^{lck}$ might not be available to CD45, would not be dephosphorylated, and therefore could not interact with the $p56^{lck}$ phosphopeptide. A more likely explanation, based on the observation that a large percentage of the total pool of $p56^{lck}$ exists in a dephosphorylated state in CD45-positive cells, is that the kinase is already associated with endogenous cellular proteins, effectively precluding interaction with the phosphopeptide. Cross-linking of the T cell AgR or CD4 induced a significant increase in the amount of $p56^{lck}$ that bound to the phosphopeptide, suggesting that cellular activation causes $p56^{lck}$ that has been dephosphorylated on Tyr505 to dissociate from endogenous cellular proteins and bind the phosphopeptide. This is consistent with the hypothesis that CD45 functions by maintaining $p56^{lck}$ in a preactivated state and that AgR cross-linking mediates signal transduction by mobilizing this pool of preactivated kinase (Sieh *et al.*, 1993). In conclusion, these studies support a role for CD45 in the intramolecular model of negative regulation and suggest that this PTP maintains $p56^{lck}$ in a dephosphorylated, preactivated state. In the absence of CD45, $p56^{lck}$ is hyperphosphorylated on tyrosine 505, causing it to assume an inactive conformation either through the formation of an intramolecular interaction or possibly as a result of intermolecular associations between $p56^{lck}$ molecules or between $p56^{lck}$ and other cellular components. (Fig. 4).

Despite the large body of evidence indicating that CD45 is required for maintenance of Src family kinases in a dephosphorylated, and thus preactivated state, data from recent studies indicate that the role of this PTP may in fact be more complex. Examination of the tyrosine phosphorylation status of $p56^{lck}$ and $p59^{fyn}$ in murine and human CD45-deficient T cell lines revealed that, despite the fact that these kinases were spontaneously hyperphosphorylated, their catalytic activity was increased when compared to that of kinases from CD45-positive cells (Burns *et al.*, 1994). Exposure of hyperphosphorylated $p56^{lck}$ or $p59^{fyn}$ to CD45 *in vitro* was paradoxically observed to decrease their activity to basal levels. In agreement with previous studies, $p56^{lck}$ isolated from CD45-deficient cells exhibited increased phosphorylation on Tyr505, as well as on the autophosphorylation site Tyr394 (Burns *et al.*, 1994). Subsequent work confirmed that increased phosphorylation of Src family kinases in the absence of CD45 is restricted to tyrosine residues. Thus, experiments were performed to determine the relative effect that tyrosine phosphorylation of specific residues has on the

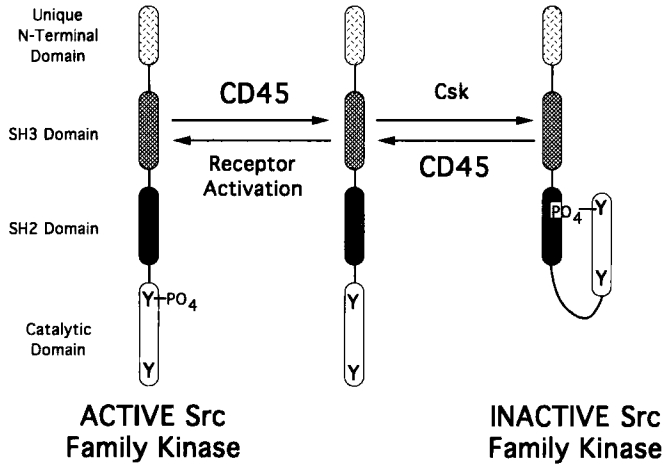


FIG. 4. Regulation of Src family kinase function by CD45. Several studies have demonstrated that CD45 targets the carboxy-terminal negative regulatory tyrosine of Src family kinases. Dephosphorylation of this tyrosine by CD45 causes the kinase to assume an active conformation. In conjunction with receptor activation, Src family kinases are phosphorylated on a second stimulatory tyrosine that promotes maximal catalytic function. Evidence suggests that this tyrosine residue may also be a potential substrate for CD45. Thus, CD45 may be involved in both positive and negative regulation of Src family kinases.

function of $p56^{lck}$ (D'Oro *et al.*, 1996). CD45-deficient YAC-1 cells were transfected with different forms of $p56^{lck}$ in which specific tyrosine residues had been mutated either singly or in combination. Mutation of Tyr192 to phenylalanine had little or no effect on catalytic activity of $p56^{lck}$, whereas mutation of Tyr394 was observed to cause a significant decrease in enzyme function. Interestingly, catalytic activity of $p56^{lck}$ was not restored by the double mutation of Tyr394 and Tyr505. Phosphopeptide analysis of $p56^{lck}$ isolated from CD45-negative cells demonstrated that hyperphosphorylation occurs at Tyr505 and to a lesser extent on Tyr394. Incubation of purified CD45 with hyperphosphorylated kinase resulted in dephosphorylation of the Tyr394 residue *in vitro* (D'Oro *et al.*, 1996). Thus, it is apparent that both Tyr394 and Tyr505 are dephosphorylated by CD45 and that in the absence of CD45 increased phosphorylation of Tyr394 has the ability to potentiate kinase function despite hyperphosphorylation at the inhibitory Tyr505 residue. It seems that the relative activity of $p56^{lck}$, and presumably that of other Src family kinases, is regulated by the balance of phosphorylation on activating versus inhibitory tyrosine residues and that CD45 is in some way involved in regulating this balance.

Regulation of $p59^{fyn}$ phosphorylation and function by CD45 has been documented in a number of studies. Experiments with human mononuclear

leukocytes demonstrated selective colocalization of $p59^{fyn}$ with CD45 but not LFA-1 (Mustelin *et al.*, 1992). Further evidence for a functional interaction between CD45 and $p59^{fyn}$ was provided by experiments showing that this PTK is a substrate for CD45 *in vitro* and that incubation with this PTP results in the rapid dephosphorylation of Tyr531. Dephosphorylation of $p59^{fyn}$ was observed to increase its catalytic activity based on autophosphorylation as well as phosphorylation of an exogenous substrate. Finally, experiments were performed suggesting that CD45 maintains $p59^{fyn}$ in a dephosphorylated state in the absence of cellular activation (Mustelin *et al.*, 1992). Similar findings were reported in studies of CD45-negative HPB-ALL cells and a CD45-positive transfectant derived from these that expresses the $CD45RA^+B^+C^+$ isoform (Shiroo *et al.*, 1992). In the absence of CD45 expression the activity of $p59^{fyn}$ was decreased by 65%, whereas $p56^{lck}$ function was not appreciably affected. The fact that $p59^{fyn}$ function was affected to a greater extent in these cells compared to that in previous studies (McFarland *et al.*, 1993; Hurley *et al.*, 1993; Sieh *et al.*, 1993) suggests that differential regulation of Src family kinases by CD45 may occur and be a function of the specific cell type and/or the relative expression of particular kinases. Nevertheless, AgR-mediated signal transduction in the HPB-ALL cells was directly correlated with $p59^{fyn}$ activity and this was in turn dependent on the expression of CD45 (Shiroo *et al.*, 1992).

The CB-1 T-ALL cell line has been used to demonstrate that the ability of CD45 to regulate Src kinase function is indeed affected by the relative subcellular localization of these molecules (Biffen *et al.*, 1994). Fluorescence cell sorting was used to isolate spontaneously arising populations of CB-1 cells that were either positive or negative for CD45 expression. Stimulation of the paired cell lines by cross-linking the T cell AgR alone or after coligation with CD4 or CD8 failed to drive a signal in CD45-negative cells. However, analysis of total cellular $p56^{lck}$ or $p59^{fyn}$ activity did not reveal a significant difference in the function of these PTKs when CD45-negative and CD45-positive cells were compared. This confounding observation was resolved by measuring the catalytic activity of kinases specifically associated with transmembrane receptors as opposed to the total kinase pool in the cell. This revealed that $p56^{lck}$ associated with CD4 exhibited a 78% reduction in function when isolated from CD45-negative cells. Additionally, T cell AgR-associated PTK activity was decreased by 84% (Biffen *et al.*, 1994). Isolation of T cell AgR immune complexes from CD45-negative cells followed by reconstitution with recombinant $p59^{fyn}$ restored normal phosphorylation of ζ and γ subunits, suggesting that endogenous kinase within the immune complex was in an inactive state. These studies provide evidence demonstrating that tyrosine phosphorylation of

Src family PTKs, and thus their functional status, is regulated by subcellular localization of CD45 and/or kinase.

Until recently, very little direct evidence was available documenting regulation of Src family kinases by CD45 in B cells; however, several studies have now provided data in support of this hypothesis. Examination of kinase function in CD45-negative WEHI-231 cells demonstrated a selective effect on the phosphorylation state and activity of $p53/56^{lyn}$ but not $p56^{lck}$ or $p72^{syk}$ (Katagiri *et al.*, 1995). It was shown that $p53/56^{lyn}$ is hyperphosphorylated in unstimulated CD45-negative cells and does not undergo any further inducible phosphorylation in response to AgR cross-linking. However, it was found that $p53/56^{lyn}$ isolated from CD45-negative cells exhibits enhanced basal as well as inducible kinase activity (Katigiri *et al.*, 1995). This suggests that in the absence of CD45, $p53/56^{lyn}$ is hyperphosphorylated on the tyrosine homolog of $p56^{lck}$ Tyr394 and is thus activated. Although, phosphopeptide analysis of hyperphosphorylated $p53/56^{lyn}$ was not performed in this study so it is not known whether this is the explanation for the observed enhancement in kinase function. The relative specificity of the alteration in $p53/56^{lyn}$ function as opposed to that of $p56^{lck}$ in the WEHI-231 cells is in agreement with previous observations suggesting that a selective physical interaction between CD45 and $p53/56^{lyn}$, but not other Src family kinases, exists (Brown *et al.*, 1994). However, it is equally possible that this result is a function of the developmental state of WEHI-231 cells or some other cell type-specific condition that effectively regulates the interaction between CD45 and $p56^{lck}$.

Studies of Src family kinase function in the CD45-negative J558μm3 plasmacytoma and a reconstituted transfectant clone demonstrate that cross-linking of the AgR leads to increased tyrosine phosphorylation of CD79a (Igα) and CD79b (Igβ) as well as the recruitment and activation of $p72^{syk}$ in cells lacking CD45 (Pao and Cambier, 1997). In contrast, $p53/56^{lyn}$ from CD45-negative cells was characterized as being hyperphosphorylated in the absence of stimulus and did not exhibit increased tyrosine phosphorylation above this basal level in response to AgR ligation. Moreover, $p53/56^{lyn}$ was not recruited to the B cell AgR complex and did not exhibit appreciable catalytic activity. Nor were phosphopeptides derived from CD79a observed to bind to $p53/56^{lyn}$ isolated from CD45-negative cells, suggesting that the kinase was in an inactive conformation. This was supported by phosphopeptide analysis of $p53/56^{lyn}$ isolated from CD45-deficient cells that revealed increased phosphorylation of inhibitory tyrosine residue 508 (Pao and Cambier, 1997). Perhaps the most surprising finding from these studies was that CD79a and CD79b are inducibly phosphorylated and recruit $p72^{syk}$, despite the fact that $p53/56^{lyn}$, $p59^{fyn}$, and $p55^{blk}$ appear to be catalytically inactive in the absence of CD45 (Pao *et al.*,

1997; Pao and Cambier, 1997). Thus, it is possible that $p72^{syk}$ is able to phosphorylate the AgR complex following receptor ligation in the absence of Src family kinase activation. Alternatively, another PTK may exist in the B cell that is inducibly activated and phosphorylates CD79a and CD79b in the absence of CD45 expression. The most recent study to document a role for CD45 in regulation of Src kinase function involves the chicken B cell line DT40 in which the gene for CD45 has been disrupted by gene targeting to create a CD45 knockout cell line (Yanagi *et al.*, 1996). Analysis of $p53/56^{lyn}$ from these cells revealed that both autophosphorylation and carboxy-terminal inhibitory tyrosines are hyperphosphorylated in the absence of CD45. Additionally, the activation of $p53/56^{lyn}$ was observed to be profoundly inhibited in the CD45 knockout B cells. The finding that the autophosphorylation tyrosine is hyperphosphorylated, but the activity of $p53/56^{lyn}$ is inhibited, is in contrast to studies in the T cell demonstrating that phosphorylation of the autophosphorylation residue of $p56^{lck}$ is dominant (D'Oro *et al.*, 1996). In agreement with previous studies, $p72^{syk}$ phosphorylation and activation do not appear to be significantly affected by the loss of CD45 expression (Yanagi *et al.*, 1996).

IV. Regulation of CD45 Function

Determination of the molecular mechanism(s) responsible for regulation of CD45 function continues to be an issue of great interest. Due to the high level of CD45 expression in hematopoietic cells and its important role in lymphocyte biology, it is almost certain that the activity of this PTP is tightly regulated in the cell. Logically, the functional activity of CD45 could be regulated through mechanisms that affect its inherent catalytic activity and/or its ability to access relevant substrates in the cell. As previously mentioned, several hypothetical mechanisms have been proposed and in many instances data have been obtained in their support (Trowbridge and Thomas, 1989; Alexander, 1997). Thus, it is quite possible that regulation of CD45 function is complex and involves more than one process depending on the cell type and its state of development, activation, or differentiation.

A. Structure–Function Analysis of CD45

The cytoplasmic tandem repeat domains of CD45 exhibit a significant degree of homology with PTPase IB (Charbonneau *et al.*, 1988) and possess intrinsic PTP activity (Tonks *et al.*, 1988). This realization provided the impetus for a series of studies to elucidate structure–function principles that might provide clues to the molecular mechanism underlying regulation of CD45 function. Toward this objective, extensive mutational analysis of

the cytoplasmic domain of CD45 has identified a number of critical regulatory elements that are required for optimal catalytic function based on *in vitro* dephosphorylation of exogenous substrates (Streuli *et al.*, 1990; Johnson *et al.*, 1992; Trowbridge, 1991). Mutation of a key cysteine residue (Cys817 in mouse and Cys828 in human) within cytoplasmic domain I caused the complete loss of enzymatic activity, whereas alteration of the homologous cysteine in the second cytoplasmic domain of CD45 had little or no effect on substrate dephosphorylation. These findings indicate that only domain I is catalytically active and that the difference in function between this domain and domain II is related to the amino acids flanking the critical cysteine residues in each. Comparison of the amino acid sequence flanking cysteine 1132 in the second domain of CD45 with the consensus sequence found in active PTPs reveals that this region in the second domain differs at five positions. Substitution of three of these different amino acids into the consensus sequence in cytoplasmic domain I effectively abrogates PTP function (Johnson *et al.*, 1992), suggesting that domain II lacks the proper structural characteristics required for a functional PTP.

Nevertheless, one study has shown that limited proteolysis of CD45 increases its activity significantly and that the proteolytic fragment liberated contains a portion of catalytic domain I plus most of the second domain (Tan *et al.*, 1993). Site-directed mutagenesis was then used to remove 109 residues from the first catalytic domain of CD45 and the recombinant protein was purified from cell lysates using immunoaffinity chromatography. The purified protein was then shown to possess PTP activity. Thus, it is still questionable as to whether one or both domains of CD45 are able to function in a catalytic sense *in vivo*. Expression of EGFR:CD45 chimeric molecules containing the entire cytoplasmic domain of CD45 in which cysteines 828 and 1144 have been mutated to serine either singly or in combination has been used to partially address this question (Desai *et al.*, 1994). Site-directed mutagenesis of individual cysteine residues within domains I and II demonstrated that catalytic activity of domain I is both necessary and sufficient to restore T cell AgR-mediated signaling in a CD45-deficient cell line. Mutation of the catalytic cysteine in domain I was observed to abrogate all functionality of the chimeric molecule both *in vitro* and *in vivo*, whereas mutation of the catalytic site in domain II resulted in the generation of a chimeric molecule that exhibited properties similar to those of the wild-type receptor (Desai *et al.*, 1994). These data suggest that the second domain of CD45 is not catalytically active either *in vitro* or *in vivo*, nor is it inducibly activated in response to ligand binding as has been previously suggested (Tan *et al.*, 1993). Although the second domain of CD45 does not appear to exhibit catalytic activity, mutational

analysis has demonstrated that it plays an important role in regulating the catalytic activity of domain I and potentially its substrate specificity as well (Streuli *et al.*, 1990; Johnson *et al.*, 1992). Additional mutational analysis of CD45 has revealed that the membrane-proximal region of 77 amino acids is required for catalytic function (Johnson *et al.*, 1992), whereas deletion of the 78 residue carboxy-terminal tail of CD45 has little or no effect on its activity (Streuli *et al.*, 1990; Johnson *et al.*, 1992). Further deletion of 13 residues at the carboxy-terminal end of domain II, however, was observed to abrogate function, and deletion of a 21-amino acid insert within domain II caused up to a fourfold decrease in activity. Finally, of the three conserved tyrosine residues contained within domain I, only the mutation of Tyr729 was observed to significantly affect the catalytic activity of CD45 (Johnson *et al.*, 1992).

Several studies in the literature utilizing at least three different experimental approaches have examined the relative importance of the extracellular domain of CD45 for functional reconstitution of signal transduction. In two studies, chimeric molecules, each containing the cytoplasmic domain of CD45 coupled either to the extracellular and transmembrane regions of the EGF receptor (Desai *et al.*, 1993, 1994) or to the extracellular and transmembrane regions of MHC class I (Hovis *et al.*, 1993), have been utilized to demonstrate that expression of the intracellular domain of CD45 is sufficient to reconstitute T cell AgR-mediated signal transduction in CD45-deficient cells. Except for a slight decrease in the magnitude of signaling observed in cells reconstituted with the MHC class I chimera, it appears as though the extracellular/transmembrane domain of CD45 is not required for the function of this PTP. A third study expressed the cytoplasmic domain of CD45 in the absence of any transmembrane or extracellular domain by coupling the cytoplasmic region of CD45 to a short amino-terminal sequence from $p60^{c\text{-}src}$ (Volarevic *et al.*, 1993). This effectively resulted in the generation of a chimeric protein that is targeted to the inner face of the plasma membrane by virtue of the fact that the sequence derived from $p60^{c\text{-}src}$ is myristylated. Reconstitution of T cell AgR-mediated signal transduction in cells expressing this chimera provides the most compelling evidence in support of the fact that the cytoplasmic domain is sufficient to couple the T cell AgR complex to intracellular signaling pathways (Volarevic *et al.*, 1993). Subsequent mutational analysis of the $p60^{c\text{-}src}$:CD45 chimeric protein confirmed previous findings that catalytic function of membrane-proximal cytoplasmic domain I is required in order for the chimeric molecule to restore signal transduction (Niklinska *et al.*, 1994). Another mutation involving a single amino acid substitution within the myristylation motif of the chimera was used to destroy this site, effectively abrogating the ability of the CD45 chimeric protein to localize

to the inner face of the plasma membrane. Despite high-level expression of the chimera and normal enzymatic activity, mutation of the myristylation site was observed to inhibit restoration of signal transduction in CD45-negative cells (Niklinska *et al.*, 1994). This finding provides direct support for the conclusion that CD45 must be in close proximity to membrane-associated substrates in order to function. Additional evidence that plasma membrane-associated PTP activity is required for T cell AgR-mediated signaling has been provided by experiments demonstrating that the expression of a nonreceptor PTP from yeast, in association with the extracellular and transmembrane regions of MHC class I, is sufficient to restore proximal and distal signaling events in CD45-negative cells (Motto *et al.*, 1994).

Although it is evident that the extracellular domain of CD45 is not required for its ability to reconstitute signaling, this does not exclude the possibility that the extracellular domain plays a role in regulation of function. Indeed, the very fact that CD45 undergoes highly regulated isoform switching and exhibits cell type-specific isoform expression indicates that the extracellular domain serves an important regulatory role (Thomas, 1989; Trowbridge and Thomas, 1994). The most likely function of isoform switching is the modification of the amino-terminal extracellular domain to facilitate selective interactions with other proteins. Selective interactions between individual CD45 isoforms and transmembrane proteins have been demonstrated in the T cell as previously mentioned (Dianzani *et al.*, 1990, 1992). Reconstitution of CD45-negative cell lines with specific isoforms of CD45 has provided additional evidence to support a role for the extracellular domain in regulation of CD45 function. In one study, a T cell line expressing an AgR of known specificity in the presence or absence of CD4 was supertransfected with different CD45 isoforms including CD45 ABC, CD45 BC, CD45 C, and CD45 null (Novak *et al.*, 1994). Experiments performed with these CD45 transfectants provided information indicating that signaling via the T cell AgR following stimulation with either antigen–MHC class II complexes or allogeneic MHC class II was significantly enhanced by the expression of CD45 null and single-exon isoforms as opposed to the higher-molecular-weight isoforms. This finding was in agreement with previous observations indicating that increased expression of the CD45 null isoform on $CD4^+$ memory T cells correlates with increased responsiveness to antigenic stimulation (Horgan *et al.*, 1990). Subsequent studies were conducted in order to confirm that isoform-specific differences in CD45 are indeed responsible for regulating protein : protein interactions on the T cell surface, thus affecting signal transduction via the T cell AgR. Analysis of the molecular interaction between CD4 and CD45 was examined by transfecting cells with GPI-linked isoforms of CD45 in order to obviate any involvement of the intracellular domain (Leitenberg *et al.*,

1996). It was observed that the CD45-null isoform preferentially interacts with CD4 when compared to CD45 ABC based on the colocalization of these molecules in the membrane. Moreover, there was a direct correlation between the size of the variable extracellular domain of CD45 and association with CD4 (Leitenberg *et al.*, 1996). These data suggest that the enhanced interaction between the CD45 null isoform and CD4 may facilitate access to CD4-associated $p56^{lck}$ thus increasing the efficiency of signaling. Other studies have documented isoform-related differences in the production of IL-2 and in the regulation of effector molecule phosphorylation (McKenney *et al.*, 1995; Onodera *et al.*, 1996). Investigation of Jurkat cells transfected with CD45 null and CD45 ABC isoforms revealed that cells expressing the high-molecular-weight isoform exhibit increased phosphorylation of p95 Vav and potentiation of its interaction with SLP-76 when compared to cells that express CD45 null. IL-2 production, on the other hand, is potentiated in cells that express CD45 null (McKenney *et al.*, 1995). It is noteworthy that the effects observed in the studies discussed previously are primarily a function of the overall size of the extracellular variable domain of CD45. Because these studies do not indicate that any one alternatively spliced exon is responsible for mediating the effects observed, it remains to be determined whether individual variable exon-encoded polypeptide sequences and/or their glycosylation selectively regulate interactions between CD45 and other proteins.

B. Regulation of CD45–Substrate Interaction

Conceptually there are two mechanisms by which the function of CD45 could possibly be regulated. The first encompasses processes that control the ability of CD45 to interact with substrates in the cell. This overall concept can be further dissected based on the fact that CD45–substrate interactions can be regulated either by restricting the location of CD45 in the plasma membrane with respect to a particular substrate or by restricting the subcellular localization of any given substrate with respect to CD45. Alternatively, the function of CD45 could be regulated by processes that have a direct affect on its catalytic activity such as posttranslational modification or association with intracellular inhibitors. In this regard, evidence exists to suggest that posttranslational modification of CD45 occurs; however, it is not clear at this time whether alterations in the catalytic activity of CD45 play a major role in regulation of its function.

It is clear that intermolecular interactions between CD45 and other transmembrane proteins occur. Such interactions can occur either in *trans*, when CD45 on one cell binds to a transmembrane protein on another, or in cis, when CD45 interacts with another transmembrane protein on the same cell. As discussed in Section III,A, a number of interactions involving

CD45 have been demonstrated to occur in *cis*. In most instances it has been hypothesized that such interactions are involved in targeting of CD45 to specific intracellular substrates. However, it has been difficult to obtain definitive proof that this is indeed the case. Circumstantial evidence suggesting that localization of CD45 within the plasma membrane is involved in regulating its function, and that such interactions are dependent on the specific isoform of CD45 expressed, has been provided by studies with mAbs (Ledbetter *et al.*, 1991; Lin *et al.*, 1992; Shivnan *et al.*, 1996) and reconstitution of CD45-deficient cell lines with specific CD45 isoforms (Novak *et al.*, 1994; Leitenberg *et al.*, 1996) or chimeric receptors (Niklinska *et al.*, 1994).

To date, information concerning protein–protein interactions that involve CD45 and occur in *trans* is limited to studies on the association between CD45 and CD22. CD22 is a B cell-restricted transmembrane glycoprotein that has been shown to play a role in mediating cell–cell adhesion (Clark, 1993). Several studies have documented that CD22 binds to CD45 expressed on both T and B cells and does so in an isoform-independent manner (Stamenkovic *et al.*, 1991; Arrufo *et al.*, 1992; Law *et al.*, 1995). Because CD22 is a sialic acid-binding lectin that recognizes α2,6-linked sialic acids, it binds to a number of proteins expressed by T and B cells in addition to CD45. Therefore, its interaction with CD45 does not represent a selective receptor–ligand pair. Moreover, the interaction between CD45 and CD22 probably occurs both in *trans*, potentially mediating homotypic aggregation between B cells or heterotypic interactions involving B and T cells, as well as in *cis*. The physiological significance of these interactions is not currently known. However, experiments have demonstrated that cross-linking of the T cell AgR to ligands bound by a soluble CD22 fusion protein blocks the CD3-mediated increase in tyrosine phosphorylation of PLCγ as well as intracellular calcium mobilization (Aruffo *et al.*, 1992). Subsequent studies confirmed that the ability of CD22 fusion proteins to modulate T cell AgR-mediated signaling depends on the expression of CD45 (Sgroi *et al.*, 1995). These findings suggest that the interaction between CD22 and CD45 might play a role in regulating cellular function by altering the distribution of CD45 within the plasma membrane or by disrupting existing protein–protein interactions between CD45 and surface receptors on the cell. Nevertheless, the studies described previously employ an experimental design that utilizes extensive artificial cross-linking of CD3 and CD45 to elicit an effect on signal transduction. Thus, it is possible that nonspecific alterations in the degree of CD3 aggregation are responsible for the effects observed (Alexander *et al.*, 1992).

Support for the concept that ligand binding to CD45 may regulate its function via an allosteric mechanism involving dimerization and functional

inactivation has been provided by reconstitution studies using chimeric CD45 constructs that are composed of the extracellular/transmembrane region of the EGF receptor fused to the cytoplasmic domain of CD45. Transfection of this chimeric molecule into CD45-negative cells restores signal transduction via the T cell AgR (Desai *et al.*, 1993, 1994). However, the addition of EGF to cells expressing the EGF–CD45 chimeric molecule effectively inhibits signal transduction in response to cross-linking of the AgR, suggesting that ligand binding downregulates CD45 function. The inhibitory effect of EGF could be reversed by cotransfecting cells with a truncated form of the EGF receptor that lacks a cytoplasmic domain. Increasing levels of truncated EGF receptor expression were observed to reverse the ability of EGF to inhibit signal transduction. These results indicate that ligand-mediated dimerization of the EGF–CD45 chimera is required in order to elicit a negative effect on signaling. Cotransfection of cells with wild-type CD45 was also observed to restore signal transduction, even though the EGF–CD45 chimeric molecules undergo dimerization, presumably because native CD45 is unaffected by the addition of EGF and therefore does not undergo dimerization in the membrane (Desai *et al.*, 1993). These results provide convincing evidence that ligand binding to CD45 negatively regulates its function via a process that is dependent on dimerization of the cytoplasmic domain of the molecule. It is possible that ligand-mediated dimerization of CD45 plays an important role in regulating access of substrate to the catalytic site. Support for such a mechanism has been provided by analyzing the crystal structure of the membrane-proximal catalytic domain of murine RPTPα (Bilwes *et al.*, 1996). Examination of a dimeric form of this domain indicates that the amino-terminal segment of each monomer forms a helix–turn–helix structural wedge that fits into the active site of the opposing monomer. Therefore, based on the primary sequence conservation of the dimer interface between CD45 and RPTPα (Biswel *et al.*, 1996), the results with the EGF–CD45 chimera study (Desai *et al.*, 1993), and the fact that CD45 can be recovered from cells as a homodimer (Takeda *et al.*, 1992), it is quite possible that ligand-mediated dimerization of CD45 restricts its interaction with substrate.

Clearly, interactions that occur in *trans* between CD45 and its potential ligands may be extremely important in terms of regulating the function of this PTP. Nevertheless, the identification of a physiologically relevant ligand for CD45 has yet to be reported. An alternative possibility is that intermolecular associations that occur in *cis* play an important role in regulating the function of CD45. In this regard, CD45 has been shown to interact with a 116-kDa transmembrane glycoprotein of unknown function that is ubiquitously expressed in hematopoietic cells (Arendt and Ostergaard,

1995). A series of recent studies have characterized another transmembrane protein, referred to as lymphocyte phosphatase-associated phosphoprotein (LPAP), that forms a noncovalent association with CD45 in *cis*. Initial identification of LPAP was reported after immunoprecipitation of CD45 from human T cells or continuously proliferating T lymphoma cell lines, followed by radiolabeling of immune complex proteins using an *in vitro* kinase assay (Schraven *et al.*, 1991). It was consistently found that CD45 coprecipitates with $p56^{lck}$ as well as a tyrosine phosphorylated protein of 32 kDa (Schraven *et al.*, 1991, 1992). Further analysis of pp32 revealed distinct species in resting and activated T cells of 29/32 kDa and 30/31 kDa, respectively (Schraven *et al.*, 1992). Phosphorylated pp32 was effectively dephosphorylated by CD45 *in vitro*, suggesting that CD45 exists in a complex consisting of $p56^{lck}$ and pp29–32. The association between CD45 and a potential murine homolog of LPAP, called CD45-associated protein (CD45-AP), has been shown (Takeda *et al.*, 1992). The cDNA and deduced amino acid sequences for LPAP and CD45-AP have recently been reported and these molecules exhibit 66% identity on the nucleotide level and 64% identity on the amino acid level, suggesting that they are indeed homologs of one another (Schraven *et al.*, 1994; Takeda *et al.*, 1994). The expression of LPAP/CD45-AP is restricted to cells of the lymphoid lineage and is not detected in monocytes or neutrophils even though they express CD45. It appears that the interaction between CD45 and LPAP/CD45-AP is required for stabilization of this protein because expression of LPAP/CD45-AP is not detected in CD45-deficient T or B cells due to rapid turnover as a result of proteolytic degradation. Several groups have mapped the sites of interaction between CD45 and LPAP/CD45-AP demonstrating that they associate via their transmembrane regions (McFarland *et al.*, 1995; Kitamura *et al.*, 1995; Bruyns *et al.*, 1996). It is interesting to note that the transmembrane regions of both LPAP/CD45-AP and CD45 are highly conserved across species (McFarland *et al.*, 1995). Although one group reported that the membrane-proximal cytoplasmic domain of LPAP may also be required for the interaction with CD45 (Bruyns *et al.*, 1996), there is little or no evidence to suggest that either the extracellular or carboxy-terminal cytoplasmic regions of LPAP/CD45-AP are involved in the interaction with CD45. Not surprisingly, the interaction between LPAP/CD45-AP and CD45 is not dependent of $p56^{lck}$ expression (Koretzky *et al.*, 1993; Kitamura *et al.*, 1995; Bruyns *et al.*, 1996). Similarly, the interaction between $p56^{lck}$ and CD45 is not mediated by LPAP/CD45-AP (Ng *et al.*, 1996) and there is little or no evidence to suggest that these proteins interact with one another (McFarland *et al.*, 1995). The functional role of LPAP/CD45-AP is currently unknown; however, this protein has been hypothesized to be an adapter molecule that

targets substrates or regulatory molecules to CD45. It is intriguing to note that CD45 associated with CD45-AP appears to be less active than is free CD45 (Kitamura *et al.*, 1995). Whether this is an indication that CD45-AP recruits an inhibitor protein remains to be seen. Several studies have demonstrated that chimeric CD45 molecules that lack the transmembrane region are able to reconstitute signaling via the T cell AgR complex (Desai *et al.*, 1993; Hovis *et al.*, 1993; Volarevic *et al.*, 1993), despite the fact that these chimeric receptors almost certainly do not associate with LPAP/CD45-AP. Because the interaction between LPAP/CD45-AP and CD45 is not required for its ability to regulate Src family kinases, or other early activation events mediated by AgR cross-linking, it is possible that this interaction may be involved in novel regulatory processes that have yet to be defined.

C. Posttranslational Modification of CD45

Posttranslational modification of CD45 has been documented both *in vitro* and *in vivo* in a number of studies. However, a consistent effect of either serine/threonine or tyrosine phosphorylation on the catalytic activity of CD45 has yet to be observed. Studies *in vitro* have demonstrated that CD45 is indeed phosphorylated to a high stoichiometry by casein kinase II, glycogen synthase kinase, and protein kinase C (PKC), with little or no effect on its catalytic activity against RCM-lysozyme or myelin basic protein (Tonks *et al.*, 1990). In contrast, the results from another study report that the catalytic activity of CD45 is potentiated by sequential phosphorylation on tyrosine, followed by serine/threonine phosphorylation mediated by casein kinase II (Stover and Walsh, 1994). The enhanced activity of CD45 under these phosphorylation conditions was directed against LCM-lysozyme, but not myelin basic protein. Moreover, enhancement of CD45 activity was observed in response to tyrosine phosphorylation by any one of four different PTKs including v-abl, $p56^{lck}$, $p60^{c\text{-}src}$, and the EGF receptor. The activation of CD45 is dependent on the order of phosphorylation as it is enhanced when phosphorylated on tyrosine first followed by serine, but not when phosphorylated in the reverse order (Stover and Walsh, 1994).

In agreement with *in vitro* data, CD45 has been shown to be phosphorylated on both tyrosine (Stover *et al.*, 1991; Autero *et al.*, 1994) and serine residues (Yamada *et al.*, 1990; Valentine *et al.*, 1991; Autero and Gahmberg, 1987; Ostergaard and Trowbridge, 1991) *in vivo*. Studies of serine phosphorylation provide evidence suggesting that both PKC-dependent and PKC-independent phosphorylation of CD45 occurs (Autero and Gahmberg, 1987; Yamada *et al.*, 1990). In the case of PKC-mediated phosphorylation, alterations in CD45 isoform expression were observed that most likely were the result of cellular activation, not serine phosphorylation (Yamada

et al., 1990). Analysis of phosphorylated CD45 revealed a 50% decrease in its activity against the synthetic tyrosine phosphopeptide Raytide (Yamada *et al.*, 1990). Treatment of T cells with IL-2 was observed to induce serine phosphorylation of CD45 that was qualitatively different from that mediated by PKC (Valentine *et al.*, 1991). In contrast to stimulation with PMA, IL-2-induced phosphorylation of CD45 was not observed to alter its surface expression or catalytic activity. These findings suggest that serine phosphorylation of CD45 may have very different outcomes as far as its catalytic function is concerned. This is further indicated by the finding that ionomycin treatment of thymocytes and murine T cell lines causes a concurrent decrease in catalytic activity and serine phosphorylation. Thus, in certain circumstances, it is apparent that serine phosphorylation of CD45 potentiates its activity (Ostergaard and Trowbridge, 1991).

Analysis of CD45 tyrosine phosphorylation *in vivo* is somewhat problematic due to the potential for autodephosphorylation or *trans*-dephosphorylation. Nevertheless, through the use of PTP inhibitors, it has been possible to demonstrate transient tyrosine phosphorylation of CD45 following stimulation of T cells with anti-CD3 mAb (Stover *et al.*, 1991). In a more detailed analysis of the potential role that tyrosine phosphorylation plays in regulation of CD45 function, studies have demonstrated that addition of PTP inhibitors leads to *in vivo* phosphorylation of CD45 on tyrosine 1193 (Autero *et al.*, 1994). Cotransfection of CD45 and $p50^{csk}$ into COS-1 cells demonstrated that tyrosine phosphorylation of CD45 is mediated by this PTK as opposed to $p56^{lck}$ or $p59^{fyn}$. It was further demonstrated that tyrosine phosphorylation of CD45 leads to increased binding of $p56^{lck}$ via its SH2 domain and increased catalytic activity of CD45. These data indicate that posttranslational modification of CD45 can affect both its inherent catalytic activity and its physical association with substrates (Autero *et al.*, 1994).

The interaction of CD45 with regulatory molecules in the cell constitutes another mechanism by which its catalytic activity may be controlled. As alluded to previously, the interaction between CD45 and CD45-AP appears to decrease the catalytic activity of CD45 (Bruyns *et al.*, 1996). Alternatively, it has been well documented that cross-linking of CD45 causes it to become associated with elements of the cytoskeleton (Bourguignon *et al.*, 1978, 1985). Isolation of CD45 from cells following induction of receptor rearrangement reveals that CD45 is tightly associated with the integral membrane protein fodrin in a 1 : 1 molar ratio (Bourguignon *et al.*, 1985). Subsequent analysis of the interaction between CD45 and the cytoskeletal proteins fodrin and spectrin demonstrates that these proteins bind to one another directly in a saturable manner (Lokeshwar and Bourguignon, 1992). Most significant, binding of either fodrin or spectrin to CD45 was

observed to increase its activity by 7.5- and 3.2-fold, respectively. These results strongly suggest that the physical interaction between CD45 and elements of the cytoskeleton plays an important role in regulating the activity of CD45. Mapping of the fodrin-binding domain on CD45 revealed that this region lies between amino acids 930 and 939, and that this sequence is involved in the fodrin-mediated upregulation of CD45 activity (Iida *et al.*, 1994).

V. Conclusion

Almost 20 years after the initial characterization of CD45 as an abundantly expressed protein on the surface of lymphocytes our understanding of the role that this PTP performs in cells of the immune system has just begun to take shape. Through the use of a wide range of molecular techniques, including gene targeting, it has been possible to determine that the expression of CD45 is important for lymphocyte development and activation. However, subtleties have been observed in terms of the role that CD45 plays in T lymphocytes as opposed to B lymphocytes. Whereas the expression of CD45 is absolutely required for proper development and antigen responsiveness in the T cell compartment, such is not the case for B cells. Thus, it is apparent that further analysis of the molecular mechanisms underlying CD45 function is required in order to fully appreciate its role in the complex process of tyrosine phosphorylation-dependent signal transduction. In this regard, a significant amount of work has yet to be done in order to characterize substrates for CD45 and to elucidate the mechanisms that are responsible for controlling its catalytic activity in the cell. Additionally, a number of questions remain unresolved concerning the importance of the extracellular domain of CD45. The regulation of isoform switching and the importance of differential isoform expression, as well as the identification of ligands for CD45, are issues that still engender great interest. Finally, as ongoing studies progress, novel functions for CD45 may be uncovered that are not currently appreciated. Such an area of potential development concerns the role of CD45 in regulation of cellular communication and trafficking. Evidence is only now being presented to suggest that the interaction of CD45 with integrins, semaphorins, and components of the cytoskeleton may be of significance in regulating lymphocyte biology. Through a combination of molecular genetics, cell biology, and biochemistry it should be possible to unravel many of the remaining questions pertaining to CD45 in the years to come.

Acknowledgments

The author thanks the following individuals for providing reprints, preprints, and helpful comments during the preparation of this review: Drs. M. L. Thomas, H. L. Ostergaard,

D. R. Alexander, G. A. Koretzky, A. Weiss, J. C. Cambier, J. Penninger, J. Marth, and P. Johnson. This work was supported by grant NIH GM-46524.

REFERENCES

Adamczewski, M., Numerof, R. P., Koretzky, G. A., and Kinet, J. P. (1995). Regulation by CD45 of the tyrosine phosphorylation of high-affinity Ig receptor beta-chains and gamma-chains. *J. Immunol.* **154,** 3047–3055.

Alexander, D., Shiroo, M., Robinson, A., Biffen, M., and Shivnan, E. (1992). The role of CD45 in T-cell activation-resolving the paradoxes? *Immunol. Today* **13,** 477–481.

Alexander, D. R. (1997). The role of the CD45 phosphotyrosine phosphatase in lymphocyte signalling. *In* "Lymphocyte Signalling Mechanisms, Subversion and Manipulation" (M. M. Harnett and K. P. Righley, Eds.), in press. Wiley, New York.

Alsinet, E., Ingles-Esteve, J., Vilella, R., Lozano, F., Mila, J., Rojo, I., Martorell, J., Vives, J., and Gaya, A. (1990). Differential effects of anti-CD45 monoclonal antibody on human B cell proliferation: A monoclonal antibody recognizing a neuraminidase-sensitive epitope of the T200 molecule enhances anti-immunoglobulin-induced proliferation. *Eur. J. Immunol.* **20,** 2801–2804.

Altevogt, P., Schreck, J., Schraven, B., Meuer, S., Schirrmacher, V., and Mitsh, A. (1989). Association of CD2 and T200 (CD45) in mouse T lymphocytes. *Int. Immunol.* **2,** 353–360.

Arendt, C. W., and Ostergaard, H. L. (1995). CD45 protein-tyrosine phosphatase is specifically associated with a 116-kDa tyrosine-phosphorylated glycoprotein. *J. Biol. Chem.* **270,** 2313–2319.

Arendt, C. W., Hsi, G., and Ostergaard, H. L. (1995). Immobilized antibodies to CD45 induce rapid morphologic changes and increased tyrosine phosphorylation of $p56^{lck}$-associated proteins in T cells. *J. Immunol.* **155,** 5095–5103.

Aruffo, A., Kanner, S. B., Sgroi, D., and Ledbetter, J. A. (1992). CD22-mediated stimulation of T cells regulates T-cell receptor/CD3-induced signaling. *Proc. Natl. Acad. Sci. USA* **89,** 10242–10246.

Ashwell, J. D., and Klausner, R. D. (1990). Genetic and mutational analysis of the T-cell antigen receptor. *Annu. Rev. Immunol.* **8,** 139–167.

Autero, M., and Gahmberg, C. G. (1987). Phorbol diesters increase the phosphorylation of the leukocyte common antigen CD45 in human T cell. *Eur. J. Immunol.* **17,** 1503–1506.

Autero, M., Saharinen, J., Pessa-Morikawa, T., Soula-Rothhut, M., Oetken, C., Gassmann, M., Bergman, M., Alitalo, K., Burn, P., Gahmberg, C. G., and Mustelin, T. (1994). Tyrosine phosphorylation of CD45 phosphotyrosine phosphatase by $p50^{csk}$ kinase creates a binding site for $p56^{lck}$ tyrosine kinase and activates the phosphatase. *Mol. Cell. Biol.* **14,** 1308–1321.

Benatar, T., Carsetti, R., Furlonger, C., Kamalia, N., Mak, T., and Paige, C. J. (1996). Immunoglobulin-mediated signal transduction in B cells from CD45-deficient mice. *J. Exp. Med.* **183,** 329–334.

Bernabeu, C., Carrera, A. C., De Landazuri, M. O., and Sanchez-Madrid, F. (1987). Interaction between the CD45 antigen and phytohemagglutinin. Inhibitory effect on the lectin-induced T cell proliferation by anti-CD45 monoclonal antibody. *Eur. J. Immunol.* **17,** 1461–1466.

Bernard, G., Zoccola, D., Ticchioni, M., Breittmayer, J. P., Aussel, C., and Bernard, A. (1994). Engagement of the CD45 molecule induces homotypic adhesion of human thymocytes through a LFA-1/ICAM-3-dependent pathway. *J. Immunol.* **152,** 5161–5170.

Biffen, M., Shiroo, M., and Alexander, D. (1993). Selective coupling of the T cell antigen receptor to phosphoinositide-derived diacylglycerol production in HPB-ALL T cells correlates with CD45-regulated p^{59fyn} activity. *Eur. J. Immunol.* **23,** 2980–2987.

Biffen, M., McMichael-Phillips, D., Larson, T., Venkitaraman, A., and Alexander, D. (1994). The CD45 tyrosine phosphatase regulates specific pools of antigen receptor-associated p^{59fyn} and CD4-associated p^{56lck} tyrosine kinases in human T-cells. *EMBO J.* **13,** 1920–1929.

Bilwes, A. M., den Hertog, J., Hunter, T., and Noel, J. P. (1996). Structural basis for inhibition of receptor protein-tyrosine phosphatase-α by dimerization. *Nature* **382,** 555–559.

Bourguignon, L. Y. W., Hyman, R., Trowbridge, I., and Singer, S. J. (1978). Participation of histocompatibility antigens in capping of molecularly independent cell suface components by their specific antibodies. *Proc. Natl. Acad. Sci. USA* **75,** 2406–2410.

Bourguignon, L. Y. W., Suchard, S. J., Nagpal, M., and Glenney, J. R., Jr. (1985). A T-lymphoma transmembrane glycoprotein (gp 180) is linked to the cytoskeletal protein, Fodrin. *J. Cell Biol.* **101,** 477–487.

Brown, V., Ogle, E., Burkhardt, A., Rowley, R., Bolen, J., and Justement, L. (1994). Multiple components of the B cell antigen receptor complex associate with the protein tyrosine phosphatase CD45. *J. Biol. Chem.* **269,** 17238–17244.

Bruyns, E., Hendricks-Taylor, L. R., Meuer, S., Koretzky, G. A., and Schraven, B. (1996). Identification of the sites of interaction between lymphocyte phosphatase-associated phosphorylation (LPAP) and CD45. *J. Biol. Chem.* **270,** 31372–31376.

Burg, D., Furlong, M., Harrison, M., and Geahlen, R. (1994). Interactions of Lyn with the antigen receptor during B cell activation. *J. Biol. Chem.* **269,** 28136–28142.

Burns, C., Sakaguchi, K., Appella, E., and Ashwell, J. (1994). CD45 regulation of tyrosine phosphorylation and enzyme activity of *src* family kinases. *J. Biol. Chem.* **269,** 13594–13600.

Byth, K. F., Conroy, L. A., Howlett, S., Smith, A. J. H., May, J., Alexander, D. R., and Holmes, N. (1996). CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of CD^+CD8^+ thymocytes, and in B cell maturation. *J. Exp. Med.* **183,** 1701–1718.

Cardine, A. M., Maridonneau-Parini, I., and Fischer, S. (1994). Activation and internalization of $p56^{lck}$ upon CD45 triggering of Jurkat cells. *Eur. J. Immunol.* **24,** 1255–1261.

Chan, A. C., Desai, D. M., and Weiss, A. (1994). The role of protein-tyrosine kinases and protein-tyrosine phosphatases in T-cell antigen receptor signal transduction. *Annu. Rev. Immunol.* **12,** 555–592.

Charbonneau, H., Tonks, N. K., Walsh, K. A., and Fischer, E. H. (1988). The leukocyte common antigen (CD45): A putative receptor-linked protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA* **85,** 7182–7186.

Chu, D. H., Spits, H., Peyron, J. F., Rowley, R. B., Bolen, J. B., and Weiss, A. (1996). The Syk protein tyrosine kinase can function independently of CD45 or Lck in T cell antigen receptor signaling. *EMBO J.* **15,** 6251–6261.

Clark, E. A. (1993). CD22, a B cell-specific receptor, mediates adhesion and signal transduction. *J. Immunol.* **150,** 4715–4718.

Conroy, L. A., Byth, K. F., Howlett, S., Holmes, N., and Alexander, D. R. (1997). Defective depletion of CD45-null thymocytes by the *Staphylococcus aureus* enterotoxin B superantigen. *Immunol. Lett.* in press.

Cyster, J. G., Healy, J. I., Kishihara, K., Mak, T. W., Thomas, M. L., and Goodnow, C. C. (1996). Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45. *Nature* **381,** 325–328.

Deane, D. L., Harvey, E., and Steel, C. M. (1991). Differential effects of CD45 CD45R and CD45RO monoclonal antibodies in modulating human B cell activation. *Clin. Exp. Immunol.* **83,** 175–181.

Deans, J. P., Kanner, S. B., Torres, R. M., and Ledbetter, J. A. (1992). Interaction of CD4:lck with the T cell receptor/CD3 complex induces early signaling events in the absence of CD45 tyrosine phosphatase. *Eur. J. Immunol.* **22,** 661–668.

Desai, D. M., Sap, J., Schlessinger, J., and Weiss, A. (1993). Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell* **43,** 541–554.

Desai, D. M., Sap, J., Silvennoinen, O., Schlessinger, J., and Weiss, A. (1994). The catalytic activity of the CD45 membrane-proximal phosphatase domain is required for TCR signaling and regulation. *EMBO J.* **13,** 4002–4010.

Dianzani, U., Luqman, M., Rojo, J., Yagi, J., Baron, J., Woods, A., Janeway, C. A., Jr., and Bottomly, K. (1990). Molecular associations on the T cell surface correlate with immunological memory. *Eur. J. Immunol.* **20,** 2249–2257.

Dianzani, U., Redoglia, V., Malavasi, F., Bragardo, M., Pileri, A., Janeway, C. A., Jr., and Bottomly, K. (1992). Isoform-specific associations of CD45 with accessory molecules in human T lymphocytes. *Eur. J. Immunol.* **22,** 365–371.

Domiati-Saad, R., Ogle, E. W., and Justement, L. B. (1993). Administration of anti-CD45 mAb specific for a B cell-restricted epitope abrogates the B cell response to a T-dependent antigen in vivo. *J. Immunol.* **151,** 5936–5947.

D'Oro, U., Sakaguchi, K., Apella, E., and Ashwell, J. D. (1996). Mutational analysis of Lck in CD45-negative T cells: Dominant role of tyrosine 394 phosphorylation in kinase activity. *Mol. Cell. Biol.* **16,** 4996–5003.

Duplay, P., Alcover, A., Fargeas, C., Sekaly, R. P., and Branton, P. E. (1996). An activated epidermal growth factor receptor/Lck chimera restores early T cell receptor-mediated calcium response in a CD45-deficient T cell line. *J. Biol. Chem.* **271,** 17896–17902.

Furukawa, T., Itoh, M., Krugger, N. X., Streuli, M., and Saito, H. (1994). Specific interaction of the CD45 protein-tyrosine phosphatase with tyrosine-phosphorylated CD3 ζ chain. *Proc. Natl. Acad. Sci. USA* **91,** 10928–10932.

George, A., Rath, S., Shroff, K. E., Wang, M., and Durdik, J. M. (1994). Ligation of CD45 on B cells can facilitate production of secondary Ig isotypes. *J. Immunol.* **152,** 1014–1021.

Gruber, M. F., Bjorndahl, J. M., Nakamura, S., and Fu, S. M. (1989). Anti-CD45 inhibition of human B cell proliferation depends on the nature of activation signals and the state of B cell activation. *J. Immunol.* **142,** 4144–4152.

Guttinger, M., Gassmann, M., Amrein, K. E., and Burn, P. (1992). CD45 phosphotyrosine phosphatase and $p56^{lck}$ protein tyrosine kinase: A functional complex crucial in T cell signal transduction. *Int. Immunol.* **4,** 1325–1330.

Hasegawa, K., Nishimura, H., Ogawa, S., Hirose, S., Sato, H., and Shirai, T. (1990). Monoclonal antibodies to epitope of CD45R (B220) inhibit interleukin 4-mediated B cell proliferation and differentiation. *Int. Immunol.* **2,** 367–375.

Herold, C., Elhabazi, A., Bismuth, G., Bensussan, A., and Boumsell, L. (1996). CD100 is associated with CD45 at the surface of human T lymphocytes. *J. Immunol.* **157,** 5262–5268.

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.

Hovis, R. R., Donovan, J. A., Musci, M. A., Motto, D. G., Goldman, F. D., Ross, S. E., and Koretzky, G. A. (1993). Rescue of signaling by a chimeric protein containing the cytoplasmic domain of CD45. *Science* **260,** 544–546.

Hurley, T., Hyman, R., and Sefton, B. (1993). Differential effects of expression of the CD45 tyrosine protein phosphatase on the tyrosine phosphorylation of the *lck, fyn,* and c-*src* tyrosin protein kinases. *Mol. Cell. Biol.* **13,** 1651–1656.

Iida, N., Lokeshwar, V. B., and Bourguignon, L. Y. W. (1994). Mapping the fodrin binding domain in CD45, a leukocyte membrane-associated tyrosine phosphatase. *J. Biol. Chem.* **269,** 28576–28583.

Johnson, P., Ostergaard, H. L., Wasden, C., and Trowbridge, I. S. (1992). Mutational analysis of CD45. *J. Biol. Chem.* **267,** 8035–8041.

Justement, L. B., Campbell, K. S., Chien, N. C., and Cambier, J. C. (1991). Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science* **252,** 1839–1842.

Justement, L. B., Brown, V., and Lin, J. (1994). Regulation of B-cell activation by CD45: A question of mechanism. *Immunol. Today* **15,** 399–406.

Katagiri, T., Ogimoto, M., Hasegawa, K., Mizuno, K., and Yakura, H. (1995). Selective regulation of Lyn tyrosine kinase by CD45 in immature B cells. *J. Biol. Chem.* **270,** 27987–27990.

Kawai, K., Kishihara, K., Molina, T. J., Wallace, V. A., Mak, T. W., and Ohashi, P. S. (1995). Impaired development of Vγ3 dendritic epidermal T cells in $p56^{lck}$ protein tyrosine kinase-deficient and CD45 protein tyrosine phosphatase-deficient mice. *J. Exp. Med.* **181,** 345–349.

Kawauchi, K., Lazarus, A. H., Rapoport, M. J., Harwood, A., Cambier, J. C., and Delovitch, T. L. (1994). Tyrosine kinase and CD45 tyrosine phosphatase activity mediate $p21^{ras}$ activation in B cells stimulated through the antigen receptor. *J. Immunol.* **152,** 3306–3316.

Kiener, P. A., and Mittler, R. S. (1989). CD45-protein tyrosine phosphatase cross-linking inhibits T cell receptor CD3-mediated activation in human T cells. *J. Immunol.* **143,** 23–28.

Kim, K. M., Alber, G., and Reth, M. (1993). Signaling function of the B-cell antigen receptors. *Immunol. Rev.* **132,** 124–146.

Kishihara, K., Penninger, J., Wallace, V. A., Kundig, T. M., Kawai, K., Wakeham, A., Timms, E., Pferrer, K., Ohashi, P. S., Thomas, M. L., Furlonger, C., Paige, C. J., and Mak, T. W. (1993). Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice. *Cell* **74,** 143–156.

Kitamura, K., Maiti, A., Ng, D. H. W., Johnson, P., Maizel, A. L., and Takeda, A. (1995). Characterization of the interaction between CD45 and CD45-AP. *J. Biol. Chem.* **270,** 21151–21157.

Kong, Y., Kishihara, K., Sumichika, H., Nakamura, T., Kaneko, M., and Nomoto, K. (1995). Differential requirements of CD45 for lymphocyte development and function. *Eur. J. Immunol.* **25,** 3431–3436.

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* **346,** 66–68.

Koretzky, G. A., Picus, J., Shultz, T., and Weiss, A. (1991). Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. *Biochemistry* **88,** 2037–2041.

Koretzky, G. A., Kohmetscher, M. A., Kadleck, T., and Weiss, A. (1992). Restoration of T cell receptor-mediated signal transduction by transfection of CD45 cDNA into a CD45-deficient variant of the jurkat T cell line. *J. Immunol.* **149,** 1138–1142.

Koretzky, G. A., Kohmetscher, M., and Ross, S. (1993). CD45-associated kinase activity requires lck but not T cell receptor expression in the jurkat T cell line. *J. Biol. Chem.* **268,** 8958–8964.

Lane, P. J., Ledbetter, J. A., McConnell, F. M., Draves, K., Schieven, G. L., and Clark, E. A. (1991). The role of tyrosine phosphorylation in signal transduction through surface Ig in human B cells. Inhibition of tyrosine phosphorylation prevents intracellular calcium release. *J. Immunol.* **146,** 715–722.

Law, C. L., Aruffo, A., Chandran, K. A., Doty, R. T., and Clark, E. A. (1995). Ig domains 1 and 2 of murine CD22 constitute the ligand-binding domain and bind multiple sialylated ligands expressed on B and T cells. *J. Immunol.* **155,** 3368–3376.

Lazarovits, A. I., Osman, N., LeFeuvre, C. E., Ley, S., and Crumpton, M. J. (1994). CD7 is associated with CD3 and CD45 on human T cells. *J. Immunol.* **153,** 3956–3966.

Ledbetter, J. A., Rose, L. M., Spooner, C. E., Beatty, P. G., Martin, P. J., and Clark, E. A. (1985). Antibodies to common leukocyte antigen p220 influence human T cell proliferation by modifying IL 2 receptor expression. *J. Immunol.* **135,** 1819–1825.

Ledbetter, J. A., Tonks, N. K., Fischer, E. H., and Clark, E. A. (1988). CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T and B cells. *Proc. Natl. Acad. Sci. USA* **85,** 8628–8632.

Ledbetter, J. A., Schieven, G. L., Uckun, F. M., and Imboden, J. B. (1991). CD45 cross-linking regulates phospholipase C activation and tyrosine phosphorylation of specific substrates in CD3/Ti-stimulated T cells. *J. Immunol.* **146,** 1577–1583.

Lee, J. M., Fournel, M., Veillette, A., and Branton, P. E. (1996). Association of CD45 with Lck and components of the Ras signaling pathway in pervanadate-treated mouse T-cell lines. *Oncogene* **12,** 253–262.

Leitenberg, D., Novak, T. J., Farber, D., Smith, B. R., and Bottomly, K. (1996). The extracellular domain of CD45 controls association with the CD4–T cell receptor complex and the response to antigen-specific stimulation. *J. Exp. Med.* **183,** 249–259.

Lin, J., Brown, V. K., and Justement, L. B. (1992). Regulation of basal tyrosine phosphorylation of the B cell antigen receptor complex by the protein tyrosine phosphatase CD45. *J. Immunol.* **149,** 3182–3190.

Lokeshwar, V. B., and Bourguignon, L. Y. W. (1992). Tyrosine phosphatase activity of lymphoma CD45 (GP180) is regulated by a direct interaction with the cytoskeleton. *J. Biol. Chem.* **267,** 21551–21557.

Lorenz, H. M., Harrer, T., Lagoo, A. S., Baur, A., Eger, G., and Kalden, J. R. (1993). CD45 mAb induces cell adhesion in peripheral blood mononuclear cells via lymphocyte function-associated antigen (LFA-1) and intercellular cell adhesion molecule 1(ICAM-1). *Cell. Immunol.* **147,** 110–128.

Lorenz, H. M., Lagoo, A. S., and Hardy, K. J. (1994). The cell and molecular basis of leukocyte common antigen (CD45)-triggered, lymphocyte function-associated antigen-1-/intercellular adhesion molecule-1-dependent, leukocyte adhesion. *Blood* **83,** 1862–1870.

Lund, F. E., Yu, N., Kim, K. M., Reth, M., and Howard, M. C. (1996). Signaling through CD38 augments B cell antigen receptor (BCR) responses and is dependent on BCR expression. *J. Immunol.* **157,** 1455–1467.

Lynes, M. A., Tibbetts, D. J., Swenson, L. M., and Sidman, C. L. (1993). Dynamic associations of CD45 and Thy-1 on plasma membranes of C3H-gld/gld and C3H-lpr/lpr. *Cell. Immunol.* **151,** 65–79.

Maroun, C. R., and Julius, M. (1994). Distinct involvement of CD45 in antigen receptor signalling in $CD4^+$ and $CD8^+$ primary T cells. *Eur. J. Immunol.* **24,** 967–973.

Martoreli, J., Vilella, R., Borche, L., Rojo, I., and Vives, J. (1987). A second signal for T cell mitogenesis provided by monoclonal antibodies CD45 (T200). *Eur. J. Immunol.* **17,** 1447–1451.

Marvel, J., and Mayer, A. (1988). CD45R gives immunofluorescence and transduces signals on mouse T cells. *Eur. J. Immunol.* **18,** 825–828.

McFarland, E. D. C., and Thomas, M. L. (1995). CD45 protein-tyrosine phosphatase associates with the WW domain-containing protein, CD45AP, through the transmembrane region. *J. Biol. Chem.* **270,** 28103–28107.

McFarland, E. D. C., Hurley, T. R., Pingel, J. T., Sefton, B. M., Shaw, A., and Thomas, M. L. (1993). Correlation between Src family member regulation by the protein-tyrosine-phosphatase CD45 and transmembrane signaling through the T-cell receptor. *Proc. Natl. Acad. Sci. USA* **90,** 1402–1406.

McKenney, D. W., Onodera, H., Gorman, L., Mimura, T., and Rothstein, D. M. (1995). Distinct isoforms of the CD45 protein-tyrosine phosphatase differentially regulate interleukin 2 secretion and activation signal pathways involving Vav in T cells. *J. Biol. Chem.* **270,** 24949–24954.

Minami, Y., Stafford, F. J., Lippincott-Schwartz, J., Yuan, L. C., and Klausner, R. D. (1991). Novel redistribution of an intracellular pool of CD45 accompanies T cell activation. *J. Biol. Chem.* **266,** 9222–9230.

Mittler, R. S., Greenfield, R. S., Schacter, B. Z., Richard, N. F., and Hoffmann, M. K. (1987). Antibodies to the common leukocyte antigen (T200) inhibit an early phase in the activation of resting human B cells. *J. Immunol.* **138,** 3159–3166.

Mittler, R. S., Rankin, B. M., and Kiener, P. A. (1991). Physical associations between CD45 and CD4 or CD8 occur as late activation events in antigen receptor-stimulated human T cells. *J. Immunol.* **147,** 3434–3440.

Morikawa, K., Oseko, F., and Morikawa, S. (1991). The role of CD45 in the activation, proliferation and differentiation of human B lymphocytes. *Int. J. Hematol.* **54,** 495–504.

Motto, D. G., Musci, M. A., and Koretzky, G. A. (1994). Surface expression of a heterologous phosphatase complements CD45 deficiency in a T cell clone. *J. Exp. Med.* **180,** 1359–1366.

Mustelin, T., Coggeshall, K. M., and Altman, A. (1989). Rapid activation of the T-cell tyrosine protein kinase $pp56^{lck}$ by the CD45 phosphotyrosine phosphatase. *Proc. Natl. Acad. Sci. USA* **86,** 6302–6306.

Mustelin, T., Pessa-Morikawa, T., Autero, M., Gassmann, M., Anderson, L. C., Gahmberg, C. G., and Burn, P. (1992). Regulation of the $p59^{fyn}$ protein tyrosine kinase by the CD45 phosphotyrosine phosphatase. *Eur. J. Immunol.* **22,** 1173–1178.

Mustelin, T., Williams, S., Tailor, P., Couture, C., Zenner, G., Burn, P., Ashwell, J. D., and Altman, A. (1995). Regulation of the $p70^{zap}$ tyrosine protine kinase in T cells by the CD45 phophotyrosine phosphatase. *Eur. J. Immunol.* **25,** 942–946.

Nel, A. E., Ledbetter, J. A., Williams, K., Ho, P., Akerley, B., Franklin, K., and Katz, R. (1991). Activation of MAP-2 kinase activity by the CD2 receptor in jurkat T cells can be reversed by CD45 phosphatase. *Immunology* **73,** 129–133.

Ng, D. H., Watts, J. D., Aebersold, R., and Johnson, P. (1996). Demonstration of a direct interaction between $p56^{lck}$ and the cytoplasmic domain of CD45 *in vitro*. *J. Biol. Chem.* **271,** 1295–1300.

Niklinska, B. B., Hou, D., June, C., Weissman, A. M., and Ashwell, J. D. (1994). CD45 tyrosine phosphatase activity and membrane anchoring are required for T-cell antigen receptor signaling. *Mol. Cell. Biol.* **14,** 8078–8084.

Novak, T. J., Farber, D., Leitenberg, D., Hong, S., Johnson, P., and Bottomly, K. (1994). Isoforms of the transmembrane tyrosine phosphatase CD45 differentially affect T cell recognition. *Immunity* **1,** 109–119.

Ogimoto, M., Katagiri, T., Mashima, K., Hasegawa, K., Mizuno, K., and Yakura, H. (1994). Negative regulation of apoptotic death in immature B cells by CD45. *Int. Immunol.* **6,** 647–654.

Ogimoto, M., Katagiri, T., Mashima, K., Hasegawa, K., Mizuno, K., and Yakura, H. (1995). Antigen receptor-initiated growth inhibition is blocked in CD45-loss variants of a mature B lymphoma, with limited effects on apoptosis. *Eur. J. Immunol.* **25,** 2265–2271.

Onodera, H., Motto, D. G., Koretzky, G. A., and Rothstein, D. M. (1996). Differential regulation of activation-induced tyrosine phosphorylation and recruitment of SLP-76

to Vav by distinct isoforms of the CD45 protein-tyrosine phosphatase. *J. Biol. Chem.* **271,** 22225–22230.

Ostergaard, H. L., and Trowbridge, I. S. (1990). Coclustering CD45 with CD4 or CD8 alters the phosphorylation and kinase activity of $p56^{lck}$. *J. Exp. Med.* **172,** 347–350.

Ostergaard, H. L., and Trowbridge, I. S. (1991). Negative regulation of CD45 protein tyrosine phosphatase activity by inomycin in T cells. *Science* **252,** 1423–1425.

Ostergaard, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Sefton, B. M., and Trowbridge, I. S. (1989). Expression of CD45 alters phosphorylation of the *lck*-encoded tyrosine protein kinase in murine lymphoma T-cell lines. *Proc. Natl. Acad. Sci. USA* **86,** 8959–8963.

Pao, L. I., and Cambier, J. C. (1997). Syk but not Lyn recruitment to BCR and activation following stimulation of $CD45^-$ B cells. *J. Immunol.,* **158,** 2663–2669.

Pao, L. I., Bedzyk, W. D., Persin, C., and Cambier, J. C. (1997). Molecular targets of CD45 in B cell antigen receptor signal transduction. *J. Immunol.* **158,** 1116–1124.

Penninger, J. M., Wallace, V. A., Molina, T., and Mak, T. W. (1996). Lineage-specific control of superantigen-induced cell death by the protein tyrosine kinase $p56^{lck}$. *J. Immunol.* **157,** 5359–5366.

Peyron, J. F., Verma, S., de Waal Malefyt, R., Sancho, J., Terhorst, C., and Spits, H. (1991). The CD45 protein tyrosine phosphatase is required for the completion of the activation program leading to lymphokine production in the Jurkat human T cell line. *Int. Immunol.* **3,** 1357–1366.

Pingel, J. T., and Thomas, M. L. (1989). Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell* **58,** 1055–1065.

Pingel, J. T., McFarland, E. D. C., and Thomas, M. L. (1994). Activation of CD45-deficient T cell clones by lectin mitogens but not anti-Thy-1. *Int. Immunol.* **6,** 169–178.

Polla, B. S., Ohara, J., Paul, W. E., Nabavi, N., Myer, A., Liou, H., Shen, F., Gillis, S., Bonventre, J. V., and Glimcher, L. H. (1988). Differential induction of class II gene expression in murine pre-B-cell lines by B-cell stimulatory factor-1 and by antibodies to B-cell surface antigens. *J. Mol. Cell. Immunol.* **3,** 363–373.

Samelson, L. E., Fletcher, M. C., Ledbetter, J. A., and June, C. H. (1990). Activation of tyrosine phosphorylation in human T cells via the CD2 pathway. *J. Immunol.* **145,** 2448–2454.

Schraven, B., Roux, M., Hutmacher, B., and Meuer, S. C. (1989). Triggering of the alternative pathway of human T cell activation involves members of the T 200 family of glycoproteins. *Eur. J. Immunol.* **19,** 397–403.

Schraven, B., Samstag, Y., Altevogt, P., and Meuer, S. C. (1990). Association of CD2 and CD45 on human T lymphocytes. *Nature* **345,** 71–74.

Schraven, B., Kirchgessner, H., Gaber, B., Samstag, Y., and Meuer, S. (1991). A functional complex is formed in human T lymphocytes between the protein tyrosine phosphatase CD45, the protein tyrosine kinase $p56^{lck}$ and pp32, a possible common substrate. *Eur. J. Immunol.* **21,** 2469–2477.

Schraven, B., Schirren, A., Kirchgessner, H., Siebert, B., and Meuer, S. C. (1992). Four CD45/$P56^{lck}$-associated phosphorproteins (pp29–pp32) undergo alterations in human T cell activation. *Eur. J. Immunol.* **22,** 1857–1863.

Schraven, B., Schoenhaut, D., Bruyns, E., Koretzky, G., Eckerskorn, C., Wallich, R., Kirchgessner, H., Sakorafas, P., Labkovsky, B., Ratnofsky, S., and Meuer, S. (1994). LPAP, a novel 32-kda phosphoprotein that interacts with CD45 in human lymphocytes. *J. Biol. Chem.* **269,** 29102–29111.

Sgroi, D., Koretzky, G. A., and Stamenkovic, I. (1995). Regulation of CD45 engagement by the B-cell receptor CD22. *Proc. Natl. Acad. Sci. USA* **92,** 4026–4030.

Shiroo, M., Goff, L., Biffen, M., Shivnan, E., and Alexander, D. (1992). CD45 tyrosine phosphatase-activated $p59^{fyn}$ couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx. *EMBO J.* **11,** 4887–4897.

Shivnan, E., Biffen, M., Shiroo, M., Pratt, E., Glennie, M., and Alexander, D. (1992). Does coaggregation of the CD45 and CD3 antigens inhibit T cell antigen receptor complex-mediated activation of phospholipase C and protein kinase C? *Eur. J. Immunol.* **22,** 1055–1062.

Shivnan, E., Clayton, L., Alldridge, L., Keating, K. E., Gullberg, M., and Alexander, D. R. (1996). CD45 monoclonal antibodies inhibit TCR-mediated calcium signals, calmodulin-kinase IV/Gr activation, and oncoprotein 18 phosphorylation. *J. Immunol.* **157,** 101–109.

Sieh, M., Bolen, J. B., and Weiss, A. (1993). CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck. *EMBO J.* **12,** 315–321.

Skov, S., Odum, N., and Claesson, M. H. (1995). MHC class-I signaling in T-cells leads to tyrosine-kinase activity and PLC-gamma-1 phosphorylation. *J. Immunol.* **154,** 1167–1176.

Smeland, E. B., Holte, H., Blomhoff, H. K., Asheim, H. C., Stokke, T., Torjesen, P., and Funderud, S. (1990). Inhibition of polyphosphoinositide breakdown and *c-myc* induction accompanying inhibition of human B-cell activation by two monoclonal antibodies against the leucocyte common antigen (CD45). *Scand. J. Immunol.* **31,** 583–591.

Spertini, F., Wang, A. V. T., Chatila, T., and Geha, R. S. (1994). Engagement of the common leukocyte antigen CD45 induces homotypic adhesion of activated human T cells. *J. Immunol.* **153,** 1593–1602.

Stamenkovic, I., Sgroi, D., Aruffo, A., Sy, M. S., and Anderson, T. (1991). The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T cells and α2-6 sialyltransferase, CD75, on B cells. *Cell* **66,** 1133–1144.

Stover, D. R., and Walsh, K. A. (1994). Protein-tyrosine phosphatase activity of CD45 is activated by sequential phosphorylation by two kinases. *Mol. Cell. Biol.* **14,** 5523–5532.

Stover, D. R., Charbonneau, H., Tonks, N. K., and Walsh, K. A. (1991). Protein-tyrosine-phosphatase CD45 is phosphorylated transiently on tyrosine upon activation of Jurkat T cells. *Proc. Natl. Acad. Sci. USA* **88,** 7704–7707.

Streuli, M., Krueger, N., Thai, T., Tang, M., and Saito, H. (1990). Distinct functional roles of the two intracellular phosphatase like domains of the receptor-linked protein tyrosine phosphatases LCA and LAR. *EMBO J.* **9,** 2399–2407.

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukuda, T., Yamamura, H., and Kurosaki, T. (1994). Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled $Ca2^+$ mobilization through distinct pathways. *EMBO J.* **13,** 1341–1349.

Takeda, A., Wu, J. J., and Maizel, A. L. (1992). Evidence for monomeric and dimeric forms of CD45 associated with a 30-kDa phosphorylated protein. *J. Biol. Chem.* **267,** 16651–16659.

Takeda, A., Maizel, A. L., Kitamura, K., Ohta, T., and Kimura, S. (1994). Molecular cloning of the CD45-associated 30-kDa protein. *J. Biol. Chem.* **269,** 2357–2360.

Tan, X., Stover, D. R., and Walsh, K. A. (1993). Demonstration of protein tyrosine phosphatase activity in the second of two homologous domains of CD45. *J. Biol. Chem.* **268,** 6835–6838.

Thomas, M. L. (1989). The leukocyte common antigen family. *Annu. Rev. Immunol.* **7,** 339–369.

Tonks, N. K., Charbonneau, H., Diltz, C. D., Fischer, E. H., and Walsh, K. A. (1988). Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase. *Biochemistry* **27,** 8695–8701.

Tonks, N. K., Diltz, C. D., and Fischer, E. H. (1990). CD45, an integral membrane protein tyrosine phosphatase. *J. Biol. Chem.* **265,** 10674–10680.

Torimoto, Y., Dang, N. H., Vivier, E., Tanaka, T., Schlossman, S. F., and Morimoto, C. (1991). Coassociation of CD26 (dipeptidyl peptidase IV) with CD45 on the surface of human T lymphocytes. *J. Immunol.* **147,** 2514–2517.

Torimoto, Y., Dang, N. H., Streuli, M., Rothstein, D. M., Saito, H., Schlossman, S. F., and Morimoto, C. (1992). Activation of T cells through a T cell-specific epitope of CD45. *Cell. Immunol.* **145,** 111–129.

Trowbridge, I. S. (1991). CD45: A prototype for transmembrane protein tyrosine phosphatases. *J. Biol. Chem.* **266,** 23517–23520.

Trowbridge, I. S., and Thomas, M. L. (1994). CD45: An emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu. Rev. Immunol.* **12,** 85–116.

Turka, L. A., Kanner, S. B., Schieven, G. L., Thompson, C. B., and Ledbetter, J. A. (1992). CD45 modulates T cell receptor/CD3-induced activation of human thymocytes via regulation of tyrosine phosphorylation. *Eur. J. Immunol.* **22,** 551–557.

Valentine, M. A., Widmer, M. B., Ledbetter, J. A., Pinault, F., Voice, R., Clark, E. A., Gallis, B., and Brautigan, D. L. (1991). Interleukin 2 stimulates serine phosphorylation of CD45 in CTLL-2.4 cells. *Eur. J. Immunol.* **21,** 913–919.

Volarevic, S., Burns, C. M., Sussman, J. J., and Ashwell, J. D. (1990). Intimate association of Thy-1 and the T-cell antigen receptor with the CD45 tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA* **87,** 7085–7089.

Volarevic, S., Niklinska, B. B., Burns, C. M., Yamada, H., June, C. H., Dumont, F. J., and Ashwell, J.D. (1992). The CD45 tyrosine phosphatase regulates phosphotyrosine homeostasis and its loss reveals a novel pattern of late T cell receptor-induced Ca^{2+} oscillations. *J. Exp. Med.* **176,** 835–844.

Volarevic, S., Niklinska, B. B., Burns, C. M., June, C. H., Weissman, A. M., and Ashwell, J. D. (1993). Regulation of TCR signaling by CD45 lacking transmembrane and extracellular domains. *Science* **260,** 541–546.

Weaver, C. T., Pingel, J. T., Nelson, J. O., and Thomas, M. L. (1991). $CD8^+$ T-cell clones deficient in the expression of the CD45 protein tyrosine phosphatase have impaired responses to T-cell receptor stimuli. *Mol. Cell. Biol.* **11,** 4415–4422.

Wegner, N., Engel, P., and Tedder, T. F. (1993). Regulation of the tyrosine kinase-dependent adhesion pathway in human lymphocytes through CD45. *J. Immunol.* **150,** 4887–4899.

Weiss, A., and Littman, D. R. (1994). Signal transduction by lymphocyte antigen receptors. *Cell* **76,** 263–274.

Welge, T., Wolf, M., Jaggle, C., and Luckenbach, G. A. (1993). Human naive T cells are preferentially stimulated by crosslinking of CD3 and CD45RA with monoclonal antibodies. *Cell. Immunol.* **148,** 218–225.

Yakura, H., Kawabata, I., Shen, F., and Katagiri, M. (1986). Selective inhibition of lipopolysaccharide-induced polyclonal IgG response by monoclonal Ly-5 antibody. *J. Immunol.* **136,** 2729–2733.

Yakura, H., Kawabata, I., Ashida, T., and Katagiri, M. (1988). Differential regulation by Ly-5 and Lyb-2 of IgG production induced by lipopolysaccharide and B cell stimulatory factor-1 {IL-4}. *J. Immunol.* **141,** 875–880.

Yamada, A., Streuli, M., Saito, H., Rothstein, D. M., Schlossman, S. F., and Morimoto, C. (1990). Effect of activation of protein kinase C on CD45 isoform expression and CD45 protein tyrosine phosphatase activity in T cells. *Eur. J. Immunol.* **20,** 1655–1660.

Yanagi, S., Sugawara, H., Kurosaki, M., Sabe, H., Yamamura, H., and Kurosaki, T. (1996). CD45 modulates phosphorylation of both autophosphorylation and negative regulatory tyrosines of Lyn in B cells. *J. Biol. Chem.* **271,** 30487–30492.

Zapata, J. M., Campanero, M. R., Marazuela, M., Sanchez-Madrid, F., and O. de Landazuri, M. (1995). B-cell homotypic adhesion through exon-A restricted epitopes of CD45 involves LFA-1/ICAM-1, ICAM-3 interactions, and induces coclustering of CD45 and LFA-1. *Blood* **86,** 1861–1872.

This article was accepted for publication on February 20, 1997.

ADVANCES IN IMMUNOLOGY, VOL. 66

HLA Class II Peptide Binding Specificity and Autoimmunity

JUERGEN HAMMER, TIZIANA STURNIOLO, and FRANCESCO SINIGAGLIA

Roche Milano Ricerche, I-20132 Milan, Italy

I. Introduction

The genes for the human leukocyte antigen (HLA) class II molecules lie within the major histocompatibility complex (MHC) on chromosome 6. The HLA class II loci are clustered into three regions, known as DR, DQ, and DP. Each of these regions contains at least one α gene and one β gene. The gene products form an $\alpha\beta$ heterodimer that is expressed as a membrane-bound protein on the cell surface. HLA class II proteins play a central role in T cell selection and activation. They bind peptide fragments derived from protein antigens and display them on the cell surface for interaction with the antigen-specific receptors of T lymphocytes.

HLA class II loci are extremely polymorphic. Allelic variation between HLA class II molecules of different individuals accounts for the functional differences revealed by HLA typing specificities, allograft reactivity, and, most important, differential ability to bind and display antigenic peptides. Allelic variations of HLA class II genes also seem to play a major role in autoimmunity. HLA typing of large groups of patients with various autoimmune diseases revealed that some HLA alleles occurred with a higher frequency in these patients than in the general population. Among the diseases strongly associated with HLA class II are, for example, rheumatoid arthritis, insulin-dependent diabetes mellitus, and multiple sclerosis.

The purpose of this review is to integrate two different areas of extensive HLA research: (i) the association between peptides and HLA class II molecules and (ii) the linkage of HLA class II alleles with autoimmune disease susceptibility. In Section II, we summarize recent developments that have led to a clearer understanding of the association between peptides and HLA class II molecules. We describe how recent breakthroughs and technological advances have resulted in a nearly global and quantitative coverage of HLA-DR peptide-binding specificity. Section II also examines newly emerging bioinformatic tools that enable the computational identification of T cell epitopes, such as autoantigenic peptides, from large sequence databases. In Section III, we focus on disease-linked HLA class II molecules. We discuss how the detailed knowledge of class II peptide interaction, summarized in Section II, can be applied to investigate whether disease-associated HLA class II molecules have unique peptide-binding

0065-2776/97 $25.00

characteristics and, consequently, the capacity to present unique autoantigenic peptides. We also examine how comparative class II peptide-binding studies can be implemented to resolve the effects of polymorphic disease-linked class II residues on peptide binding. Finally, we document some of the known autoantigenic peptides and demonstrate their possible identification by bioinformatic tools. These findings are further examined in Section IV, in which we discuss possible prospects for immune intervention.

II. Toward a Global Coverage of HLA Class II Ligand Specificity

A. The Architecture of the Class II-Binding Groove

Class II molecules are $\alpha\beta$ dimers of similar size, both of which are membrane-inserted chains. The three-dimensional structures of several HLA class II molecules have been determined by X-ray crystallography (Brown *et al.*, 1993; Stern *et al.*, 1994; Ghosh *et al.*, 1995). The class II peptide binding site is formed by the membrane-distal domains of both class II chains, each contributing one α-helix and four β-strands. The β sheet floors and helical walls define a groove of suitable dimensions for occupancy by 9 peptide residues. The class II cleft is open at both ends. As a consequence, the class II cleft allows peptides to extend out and enables the binding of a broad range of peptide lengths, typically 12–24 residues (Rudensky *et al.*, 1991; Hunt *et al.*, 1992; Chicz *et al.*, 1993).

One of the most important features of MHC molecules is their ability to form stable complexes with thousands or millions of different peptide sequences. This enormous binding capacity arises from the interaction between conserved MHC residues and the peptide main chain, thus providing sequence-independent affinity for peptide ligands (Stern *et al.*, 1994; Madden *et al.*, 1991; Matsumura *et al.*, 1992). For MHC class II molecules, the location and the identity of the conserved residues interacting with the peptide main chain are distributed along the binding groove. As a consequence, the peptide main chain is kept in close contact with the MHC cleft, resulting in a relatively flat conformation of class II-bound peptides.

The sequence-independent network of hydrogen bonds between conserved MHC residues and the peptide main chain gives rise to a broad but not unlimited peptide-binding capacity. Indeed, most natural peptide sequences lack the characteristics necessary to bind to MHC molecules. This is because peptide main chain interactions are not the only mode of MHC binding. Some of the peptide side chains contact residues within the MHC cleft and increase the overall binding affinity and specificity of the associated peptides (anchor residues) (Sette *et al.*, 1987; Jardetzky *et al.*, 1990; Falk *et al.*, 1991; Hammer *et al.*, 1993); others interfere

with residues of the MHC cleft and reduce binding (inhibitory residues) (Boehncke *et al.*, 1993; Sette *et al.*, 1993; Hammer *et al.*, 1994b). These sequence-dependent interactions are due to the irregular surface of the MHC cleft. MHC side chains protrude into the cleft and form pockets or ridges, resulting in strong preferences for interaction with particular amino acid side chains.

Interestingly, most pockets in the MHC groove are shaped by clusters of polymorphic MHC residues and are thus of distinct chemical and size characteristics in different MHC alleles. For example, a negatively charged side chain in one MHC molecule may preferentially interact with positively charged peptide residues, whereas a positively charged side chain in another MHC molecule may only bind to negatively charged peptide residues. MHC alleles can therefore be characterized either topographically, i.e., by differences in the precise nature and position of the polymorphic pockets, or functionally, i.e., by the resulting allele-specific differences in their interaction with peptides.

B. Different Approaches to Defining HLA Class II-Binding Motifs

1. Identification of General Class II-Binding Motifs Using Large Peptide Repertoires

The interaction of peptide side chains with pockets of the MHC cleft imposes sequence requirements on the peptides that can bind. These requirements are summarized in peptide-binding "motifs" (Sette *et al.*, 1987; Rothbard and Gefter, 1991). A breakthrough for the analysis of MHC-binding motifs was the characterization of large, MHC-selected peptide pools that allowed a generalization of rules for peptide binding to MHC molecules (Falk *et al.*, 1991; Hammer *et al.*, 1992).

For class II HLA-DR molecules, motifs were identified by the analysis of large peptide pools selected from M13 bacteriophage peptide display libraries (Hammer *et al.*, 1992, 1993, 1994a). This powerful technique is based on the ability of filamentous bacteriophage to display peptides on their outer surface and involves the screening and enrichment of bacteriophage-displaying peptides that bind to a particular protein (Scott and Smith, 1990; Devlin *et al.*, 1990; Cwirla *et al.*, 1990). By inserting oligonucleotide-encoding peptides known to bind to DRB1*0101 into the protein III-encoding gene of the bacteriophage M13, we could demonstrate that bacteriophage displaying the appropriate class II ligand can bind specifically to the DR groove (Hammer *et al.*, 1992). Based on this observation, we selected large DRB1*0101 binding peptide repertoires from a M13 peptide display library consisting of millions of random peptides.

Sequence analysis of the DNA encoding the DRB1*0101-selected peptides led to the identification of peptide positions in which amino acids with similar side chains occurred with increased frequency (anchor residues), thus resulting in a DRB1*0101 peptide-binding motif (Hammer *et al.*, 1992). The motif consists of four anchors at relative positions 1, 4, 6, and 9 that are at fixed distances from one another; it thus reflects the architecture of the DRB1*0101 groove in that both the spacing and the chemical characteristics of anchor residues correspond to the major pockets 1, 4, 6, and 9 of the HLA-DR cleft (Stern *et al.*, 1994).

The screening of bacteriophage libraries has also been applied to the identification of other HLA-DR-binding motifs, such as DRB1*0401 and DRB1*1101 (Hammer *et al.*, 1993). An important finding was the identification of conserved anchor residues, i.e., anchors found in each of the HLA-DR-selected peptide pools, as well as allele-specific anchor residues. For example, most of the DRB1*0101-, DRB1*0401-, and DRB1*1101-selected peptide pools were found to have aromatic and aliphatic amino acid residues at anchor position 1 and 4, respectively, whereas strong allele-specific amino acid preferences were identified at position 6: Ala and Gly for DRB1*0101, Ser and Thr for DRB1*0401, and Arg and Lys for DRB1*1101. These results provided a molecular basis for both the promiscuity and the specificity of peptide recognition by HLA-DR molecules. Thus, peptides with conserved anchor residues at positions 1 and 4, and small Ala residues at position 6 should bind, at least intermediately, to several HLA-DR alleles. Alternatively, DRB1*1101 ligands with a conserved anchor at position 1 and a large, positively charged Arg at position 6 will not bind to DRB1*0101 and DRB1*0401 because Arg will not be accommodated by the pocket 6 of both molecules (Hammer *et al.*, 1993).

Increasingly complex HLA class II motifs were identified by varying the conditions used to elute bacteriophage from the class II cleft (Hammer *et al.*, 1994a). For example, a "low-pH" wash step prior to bacteriophage elution increased the stringency of peptide selection and resulted in the identification of secondary anchors at positions 2, 3, and 7. Based on these findings we could design short peptides in which six of the seven residues were anchors. These peptides bound tightly to DR molecules and provided the basis for the design of nonpeptidic MHC blockers (Hanson *et al.*, 1996).

General HLA class II motifs were also identified by characterizing large endogenous bound peptide pools. With the pooled peptide sequencing technique, a technique originally developed for the definition of class I motifs (Falk *et al.*, 1991; Falk and Rötzschke, 1993), endogenous class II-bound peptide pools were eluted and subsequently analyzed by Edman

sequencing (Falk *et al.*, 1994; Verreck *et al.*, 1994). Because the class II-binding cleft is open at both ends and endogenous peptides are not aligned due to the variable length of class II ligands, pool sequencing approaches with class II-eluted peptides failed to reveal patterns as clear as those of class I ligands. However, pool sequencing combined with the alignment of natural ligands and the consideration of predicted pocket structure resulted in class II motifs similar to the ones obtained by the bacteriophage display technology (Friede *et al.*, 1996). In conclusion, it should be emphasized that motifs derived from both M13 display library screening and pooled peptide sequencing are the result of studies of MHC-binding to large peptide repertoires. They indicated, therefore, a general mode of peptide binding and provided the rational basis for a more quantitative characterization of class II-binding specificity (Section II,B,3).

2. *Identification of Specific Class II-Binding Motifs by Single-Substitution Experiments on Naturally Processed Peptides*

Another approach for studying human class II molecule–peptide interaction has been to characterize the effects of single-residue substitutions in naturally MHC-bound peptides, which reveals residues critical to the interaction of these peptides with HLA class II molecules. In the case of DRB1*0101, the importance of an aromatic residue at relative position 1 was initially found by Ala substitutions of the influenza hemagglutinin (HA) epitope 307–319 (Jardetzky *et al.*, 1990). More extensive truncation and single-residue substitution studies on HA 307–319 or tetanus toxoid 830–843 revealed specific class II-binding motifs for DRB1*0401, DRB1*1101, and DRB1*0701, in addition to DRB1*0101 (Sette *et al.*, 1993; O'Sullivan *et al.*, 1991; Krieger *et al.*, 1991). Single-residue substitution studies were also performed on the mycobacterial heat shock protein 65 peptide 3–13 (Geluk *et al.*, 1992; Sidney *et al.*, 1992) and on naturally associated ligands (Malcherek *et al.*, 1993), disclosing a prominent position 4 anchor (Asp) for the DRB1*0301 allele. Finally, substitution experiments on the myelin basic protein peptide 84–102 revealed differential binding frames for DRB1*1501 and DRB5*0101 (Vogt *et al.*, 1994; Wucherpfennig *et al.*, 1994).

Initially, most single-substitution results could not easily be generalized on the basis of only a few modified peptides. However, the growing number of single-substitution experiments and the comparison of these results with other methods, such as the usage of M13-displayed peptide repertoires, confirmed the generality of motifs derived from single-substitution experiments. Moreover, only single-substitution experiments on synthetic peptides could reveal the presence of peptide side chains that interfere

with peptide binding. These inhibitory residues are of similar importance for association with class II molecules as the presence of anchor residues (Boehncke *et al.*, 1993; Sette *et al.*, 1993; Hammer *et al.*, 1994b). Single-substitution experiments were thus an important factor in the recent identification of quantitative matrix-based class II motifs.

3. *Class II Motifs Based on Quantitative Matrices*

X-ray crystallographic studies indicated that different peptides bind with a similar conformation to HLA-DR molecules (Stern *et al.*, 1994; Ghosh *et al.*, 1995). The analyses of large HLA-DR ligand repertoires selected from M13 bacteriophage display libraries supported this hypothesis (Hammer *et al.*, 1993). Although most of the HLA-DR-selected peptides differed significantly in their primary sequence, they all shared perfectly spaced anchor residues, thus suggesting an overall similar conformation of HLA-DR ligands. A ligand conformation that is only marginally influenced by its primary sequence, if at all, implies that no significant contacts are made among the side chains of the ligand. Single-substitution experiments on T cell epitopes and designer peptides provided direct evidence for this hypothesis. Peptide side chain effects (anchor, inhibitory, or neutral effects) seemed to depend on the position within a particular peptide frame rather than on neighboring amino acids (Hammer *et al.*, 1994b).

Taken together, these observations led to the approximation that each amino acid in a peptide sequence contributes to the affinity of the peptide independently of the neighboring amino acids (Hammer *et al.*, 1994a,b, 1995; Parker *et al.*, 1994; Reay *et al.*, 1994; Marshall *et al.*, 1995). The combination of multiple-peptide synthesis technology with high-throughput *in vitro* MHC–peptide-binding assays allowed this approximation to be tested. The determination of the effects of each amino acid at all peptide positions resulted in matrices that define quantitatively MHC class II ligands specificity (Hammer *et al.*, 1994b; Reay *et al.*, 1994; Marshall *et al.*, 1995).

The determination of a quantitative matrix, however, does not automatically lead to an effective motif. For the substituted peptide (basis peptide), the probability of frameshifting needed to be reduced and the affinity optimized to guarantee the accurate measurement of side chain effects over a wide range of binding affinities. For DRB1*0401 molecules, we have designed a strategy for determining quantitative matrices that is widely applicable to many other human class II DR molecules (Hammer *et al.*, 1994b). First, the binding frame of Ala-based designer peptides was fixed by the obligatory position 1 anchor. Next, only 9- or 10-residue-long peptides were used for scanning. The reduced length amplified both the positive effect of the anchors and the negative effect of the inhibitory

residues due to the reduced main chain interaction (Hammer *et al.*, 1994b) and decreased the probability of shifting within the position 1 peptide frame. Finally, the affinity of the basis peptide was adjusted by incorporating either additional conserved anchor residues or by increasing the main chain interaction through an N-terminal Gly or 6-aminocaproic acid. Side chain scanning with optimized basis peptides resulted in a sensitive analysis of the effects of all peptide side chains on DRB1*0401 binding. As for DRB1*0401, a similar approach was used for the determination of quantitative matrices for other HLA class II molecules including, for example, most rheumatoid arthritis-associated HLA-DR1 and DR4 alleles and subtypes (Hammer *et al.*, 1995). A DRB1*0401 matrix was also determined by using less sensitive, 13-residue-long designer peptides (Marshall *et al.*, 1995). This approach, however, failed to identify several known DRB1*0401-specific anchor residues (Hammer *et al.*, 1993), due to the lack of affinity optimization of the basis peptide.

A comparison of quantitative matrices provided detailed information about the effects of polymorphic HLA class II residues on peptide binding (Section III,B). Another benefit of matrix-based motifs is their great predictive power (Section II,C). New algorithms based on quantitative side chain scanning data were developed, resulting in computational identification of HLA class II ligands (Sections II,C,2 and II,C,4). Finally, quantitative matrices are considered "fingerprints" of class II clefts because they reflect, as a sum of all peptide side chain values, the architecture of the polymorphic DR groove. We expect that, in the near future, this will allow a reclassification of HLA class II polymorphism on the basis of the resulting peptide-binding specificity rather than on the basis of primary sequences.

4. Global Coverage of HLA-DR Ligand Specificity by Using a "Pocket-Specificity Database" for the Generation of Quantitative Matrices

HLA-DR molecules account for more than 90% of the HLA class II isotypes expressed on antigen presenting cells. Although the HLA-DRA locus is monomorphic, more than 100 alleles have been described for the HLA-DRB1 locus (Marsh and Bodmer, 1995). The determination of quantitative matrices for each of these HLA-DR alleles would therefore require hundreds of thousands of peptide-binding assays. A global coverage of HLA-DR-binding specificity may, however, not necessarily require an individual analysis of quantitative matrices. The relative independence of peptide side chains from one another (Section II,B,3) allows matrix-based motifs to be subdivided into individual pocket-specificity profiles, representing the sum of all quantitative side chain values for a given peptide

position and its corresponding pocket or cleft area (Fig. 1). The comparison of pocket-specificity profiles analyzed on different class II alleles revealed that these profiles are not only independent of neighboring peptide side chains but also independent of the remaining class II-binding cleft (T. Sturniolo *et al.*, manuscript in preparation). For example, the polymorphic residues constituting pocket 9 in DRB1*0401 and DRB1*1101 are identical; consequently, peptide side chain scanning on both pockets resulted in overall similar pocket-specificity profiles (Fig. 1). In contrast, different pocket 9 residues in DRB1*0801 and DRB1*1101 led to different pocket-specificity profiles (Fig. 1). Comparison studies on pocket 1 and pocket 6 (Hammer *et al.*, 1994b, 1995) (Fig. 1) provided further evidence that supports an overall allele independence of pocket-specificity profiles. Thus, pocket-specificity profiles need only to be determined once and can subsequently be used in matrix-based motifs of several HLA class II molecules (Fig. 2).

Most HLA-DR subtypes and allotypes share pockets along the class II groove. For example, DRB1*1101 shares pockets 1, 2, 3, and 9 with DRB1*0401 and pocket 6 with DRB1*0801. As the peptide side chains

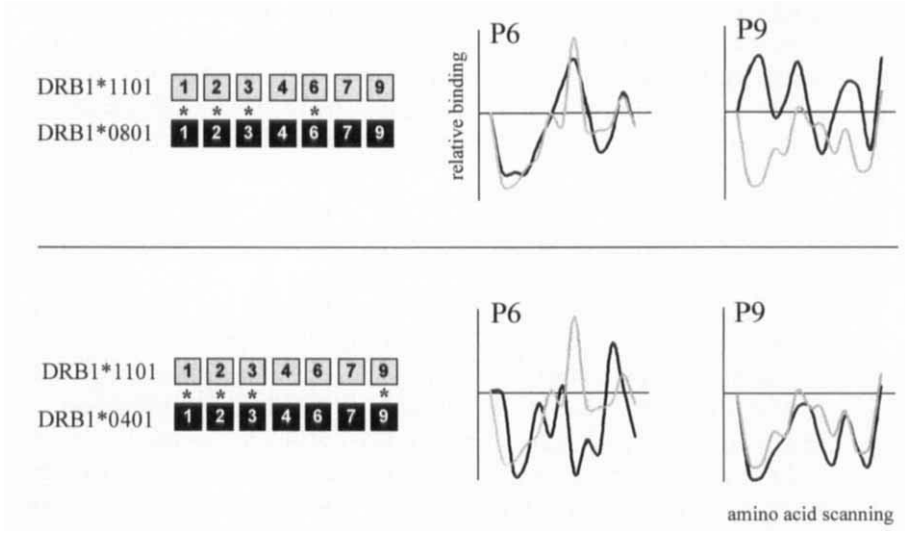


FIG. 1. Pocket specificity profiles are HLA class II allele independent. Polymorphic residues constituting pocket 6 (P6) in DRB1*1101 and DRB1*0801 (top) and pocket 9 (P9) in DRB1*1101 and DRB1*0401 are identical (marked by *), resulting in overall similar pocket specificity profiles.

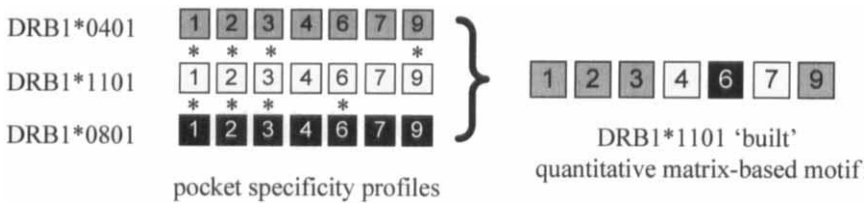


FIG. 2. Generation of quantitative matrices by the combination of pocket specificity profiles. A DRB1*1101 matrix-based quantitative motif is "built" by determining the pocket specificity profiles for the two DRB1*1101 pockets 4 and 7 (white) and by combining them with profiles derived from DRB1*0401 (gray) and DRB1*0801 (black).

of peptide positions 5 and 8 are directed toward the T cell receptor, they have only minor effects on binding and can therefore be neglected (Stern *et al.*, 1994). Thus, in this example, a DRB1*1101 matrix-based quantitative motif could easily be generated by determining the pocket-specificity profiles for the two DRB1*1101 pockets 4 and 7 and, subsequently, by combining them with pocket-specificity profiles derived from DRB1*0401 and DRB1*0801 matrices (Fig. 2).

In conclusion, relatively small databases of pocket-specificity profiles are required to generate quantitative matrices for many HLA class II alleles. The usage of pocket-specificity profiles as "building blocks" for quantitative matrices will ultimately lead to a global coverage of human class II-binding specificity. For example, we have recently illustrated that the creation of a small pocket-specificity database led to a complete assembly of 25 matrix-based HLA-DR motifs and to the near completion of the building of a further 40 motifs (T. Sturniolo *et al.*, manuscript in preparation). The 25 fully built motifs allow the determination of already a large proportion of the HLA-DR class II-binding specificity, which, for example, covers more than 90% of the North American Caucasian population (Tsuji *et al.*, 1992).

5. *HLA-DP and -DQ*

Of all class II isotypes, HLA-DR is the best characterized structurally and functionally. For instance, all available structural information on HLA class II molecules was derived from X-ray analyses of HLA-DR molecules (Section II,A); likewise, most information on HLA class II peptide interaction was gained from HLA-DR molecules (Sections II,B,1–II,B,4). This explains why relatively little is known about peptide-binding characteristics of HLA-DQ and -DP molecules in comparison to HLA-DR molecules. For HLA-DP molecules, for example, only simple binding motifs have been suggested based on the pooled peptide sequencing technique (Ram-

mensee *et al.*, 1995); this, however, needs confirmation because no reliable peptide-DP-binding assays are currently available. For HLA-DQ molecules, information on ligand specificity has only recently been available. Single-substitution experiments defined a simple motif for DQA1*0301/DQB1*0301 that was quite different from the motifs recognized by DR molecules (Sidney *et al.*, 1994). Its prominent feature is the requirement of two small and/or hydrophobic residues spaced at positions i + 2 and i + 4. However, because these features can basically be found in almost every natural peptide frame, this motif is not suitable for predicting HLA-DQ ligands (Section II,C,2). Simple motifs have also been described for the autoimmune disease-linked HLA molecules DQA1*0501/DQB1*0201 (Johansen *et al.*, 1995; Vartdal *et al.*, 1996; Van de Wal *et al.*, 1996) and DQA1*0301/DQB1*0302 (Kwok *et al.*, 1996a,b). As for DQA1*0301/DQB1*0301, these motifs were different from the ones recognized by DR molecules. For example, no prominent position 1 anchors were found, as indicated by Ala-substitution experiments. Both motifs consisted mainly of inhibitory residues, with the exception of a negatively charged anchor residue at position 9. The tendency of HLA-DQ ligands to be less dependent on the interaction of peptide side chains with the class II cleft than HLA-DR ligands has recently been confirmed by the determination of a quantitative matrix-based motif for DQA1*0501/DQB1*0301 (Raddrizzani *et al.*, 1997). This motif revealed the capability of DQA1*0501/DQB1*0301 molecules to bind peptide structures without the involvement of large peptide side chains. Based on this finding, we have succeeded in modifying DR-selected peptide repertoires such that they lost the binding capacity for HLA-DR molecules and bound exclusively to DQA1*0501/DQB1*0301, thus demonstrating, at least in part, a complementary function of HLA-DR and -DQ isotypes in antigen presentation. Together, the initial studies on HLA-DQ peptide interaction indicate differences between the binding mode of HLA-DR and -DQ molecules that may consequently maximize the diversity of peptide repertoires available for T cell recognition.

C. Computational Identification of HLA Class II Ligands

1. Prediction of HLA Class II Ligands as a Means to Identify T Cell Epitopes

The goal of T cell epitope prediction is the ability to accurately identify peptide sequences within a given protein that will elicit desired T cell responses in the context of a defined MHC molecule. Initially, T cell epitope prediction was based solely on the analysis of known T cell antigenic sites, mainly on the identification of amphipathic helices (DeLisi and Ber-

zofsky, 1985; Rothbard and Taylor, 1988). Such methods were superseded, however, when evidence emerged for the existence of epitopes bound in extended conformation by HLA class II molecules (Section II,A). Other approaches that analyzed the primary structure rather than the secondary structure, identified common patterns of amino acids in immunogenic peptides and utilized them for prediction. All these approaches, however, ignored the key requirements for MHC specificity.

Recently, advances in the field of antigen presentation led to a general consensus that epitope prediction ought to be based on a molecular understanding of the events that determine antigen presentation; that is, antigen processing and HLA class II binding. The ultimate goal is the identification of rules underlying these events and their application for the precise prediction of T cell epitopes. Inherent structural properties of protein antigens, however, exclude simple rules for the determination of antigen processing in that, for example, the structure of a given antigen could influence its susceptibility to proteolysis. Additional parameters that add to the complexity of antigen processing are, for example, the specificity of the proteolytic enzymes involved or the stability of the generated peptides. In short, antigen processing is a multistep process that, at least for now, can neither be described by simple rules nor utilized for T cell epitope prediction.

MHC peptide binding constitutes the second "bottleneck" in the natural selection of T cell epitopes and appears to be less complex than antigen processing. Most natural peptide frames lack the capacity to interact with HLA class II molecules (Hammer *et al.*, 1994b). HLA class II peptide-binding rules can therefore be used to "filter out" potential T cell epitopes, thus minimizing the number of peptides to be synthesized and assayed. T cell epitope prediction based on HLA class II-binding rules also facilitates the identification of epitopes with certain characteristics, such as promiscuous or allele-specific epitopes (Section II,B,4). Finally, increasingly available sequence and expression pattern databases provide new opportunities for MHC-based epitope prediction in sectors in which traditional approaches, such as the synthesis and testing of overlapping peptide sequences (Sinigaglia *et al.*, 1992), would fail: the simultaneous epitope scanning on large numbers of gene sequences that are, for example, specifically expressed or upregulated in tumor cells or in tissues implicated in autoimmune diseases.

The prospect of utilizing MHC binding as a means to predict T cell epitopes spurred the rapid identification of HLA class II motifs. As a result, different binding motifs (Sections II,B,1–II,B,5) with variable capabilities to predict class II ligands were identified. In parallel, different bioinformatic approaches were developed utilizing these binding motifs and/or

large class II ligand databases for the prediction of peptide binding to HLA class II molecules.

2. *Bioinformatic Tools to Predict T Cell Epitopes*

Motifs analyzed by the M13 bacteriophage display technology (Section II,B,1), pooled peptide sequencing technique (Section II,B,1), or single-substitution experiments (Section II,B,2) often encode only the most prominent characteristics of peptide–MHC interaction (simple motifs). Although these peptide characteristics correspond to known or modeled class II structures, they are insufficient for a comprehensive prediction of class II ligands. For example, the alignment of DRB1*0101-selected, M13 displayed peptide repertoires led to the identification of four anchor positions (Section II,B,1). Accordingly, most selected peptide sequences (Fig. 3) or natural peptides (Hammer *et al.*, 1994a) with three or four anchors in frame bound with high affinity to DRB1*0101. However, a large proportion of peptides bound to DRB1*0101 even, if they had only two anchors in frame (Fig. 3). Therefore, all or nothing algorithms selecting peptides with three or more anchors ("all") but omitting peptides with less than three anchors ("nothing") would miss large proportions of class II ligands; on the other hand, algorithms predicting peptides with only two anchors in frame would pick an unacceptable number of false positives because two anchors can be found in most natural or random peptides (Fig. 3). In conclusion, simple motifs and all or nothing algorithms (Hammer *et al.*, 1994a; Sidney *et al.*, 1994) do not account for the complexity of class II peptide interaction and have therefore only a limited capacity to predict potential T cell epitopes.

Matrix-based motifs (Sections II,B,3 and II,B,4) are refined binding motifs that allow for more sophisticated prediction algorithms. In contrast

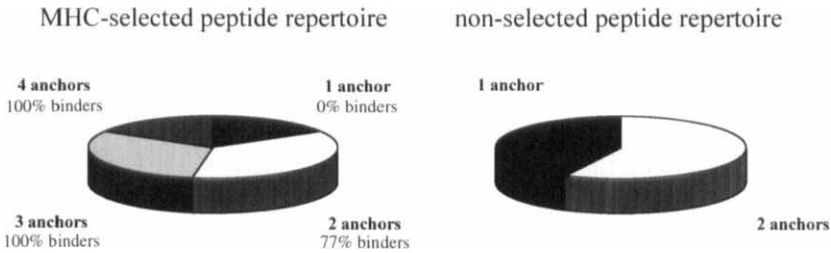
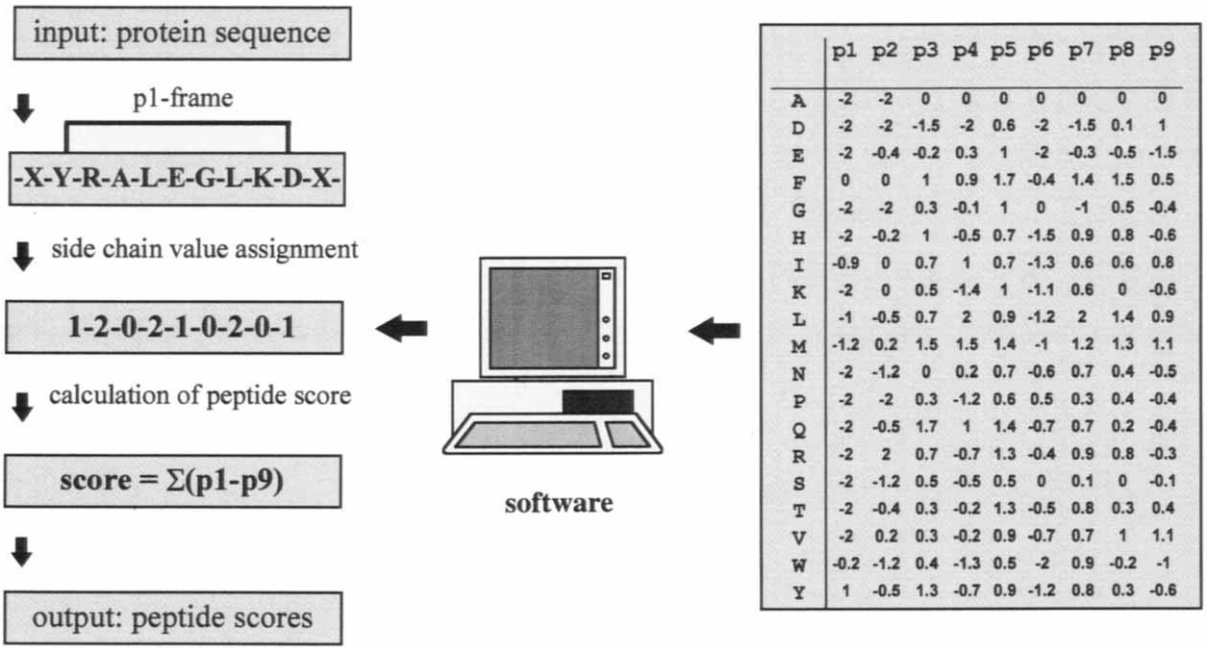


FIG. 3. "All or nothing" algorithms have only limited capacity to predict HLA class II ligands. A large proportion of peptides selected by DRB1*0101 (Hammer *et al.*, 1993; L. Raddrizzani *et al.*, manuscript in preparation) have only two anchors in frame (left). Algorithms predicting peptides with only two anchors pick, however, more than 50% of all random peptides (right), thus lacking the capacity to efficiently predict class II binders.

to simple all or nothing rules, epitope prediction relies on mathematical processing of individual peptide side chain data that are derived either from quantitative matrices (Hammer *et al.*, 1994b; Reay *et al.*, 1994; Marshall *et al.*, 1995) or from amino acid frequency matrices (Davenport *et al.*, 1995). In principle, matrix-based epitope prediction can be divided into four steps (Fig. 4): First, all possible peptide frames are extracted from a given protein sequence. Second, the corresponding position- and amino acid-specific matrix values are assigned to each residue of a given peptide frame. Next, the side chain values of each peptide are added or multiplied, resulting in the peptide "score." Lastly, peptides are selected based on their peptide score. Thus, instead of simply counting anchor residues, matrix-based algorithms take into account the relative importance of every amino acid residue in a peptide sequence, as charged by their effect on binding (quantitative matrix) or frequency within an aligned peptide pool (frequency matrix).

The capacity to predict HLA class II ligands using quantitative matrix-based algorithms was first demonstrated for DRB1*0401 molecules (Hammer *et al.*, 1994b; Marshall *et al.*, 1995). These algorithms ranked naturally processed peptides and T cell epitopes in the top 2–4% of all possible peptide frames of given antigens even if they owned only one or two anchor residues, as charged by simple motifs. More important, however, a correlation between the peptide score and the binding affinity was demonstrated (Fig. 5) (Hammer *et al.*, 1994b) that therefore supports the underlying approximation that a given residue contributes to binding independently of its neighboring amino acid residues (Section II,B,3). Recently, many more quantitative matrix-based algorithms were established, including algorithms for DRB1*0101, DRB1*1501, DRB1*1101, DRB1*0701, and DRB1*0801 molecules, to name a few. The predictive power of some of these algorithms was validated by computer simulating the screening of M13 peptide display libraries (Fig. 5): Quantitative matrix-based algorithms were used instead of purified HLA–class II molecules to enrich for large class II-binding peptide repertoires.

Algorithms based on frequency matrices are less common for HLA class II molecules (Davenport *et al.*, 1995). The underlying assumption is that amino acid residues are more important for class II peptide interaction if they occur with higher frequencies in aligned peptide pools. Although this criterium turned out to be useful for the identification of anchor residues (amino acids occuring with high frequency) (Section II,B,1), there is considerable doubt about the possibility of accurately differentiating among amino acids occurring with lower frequency. For example, an aligned DRB1*0101-selected peptide pool revealed more than 80% aromatic residues at relative position 1 (Hammer *et al.*, 1992); these results are in full



	p1	p2	p3	p4	p5	p6	p7	p8	p9
A	-2	-2	0	0	0	0	0	0	0
D	-2	-2	-1.5	-2	0.6	-2	-1.5	0.1	1
E	-2	-0.4	-0.2	0.3	1	-2	-0.3	-0.5	-1.5
F	0	0	1	0.9	1.7	-0.4	1.4	1.5	0.5
G	-2	-2	0.3	-0.1	1	0	-1	0.5	-0.4
H	-2	-0.2	1	-0.5	0.7	-1.5	0.9	0.8	-0.6
I	-0.9	0	0.7	1	0.7	-1.3	0.6	0.6	0.8
K	-2	0	0.5	-1.4	1	-1.1	0.6	0	-0.6
L	-1	-0.5	0.7	2	0.9	-1.2	2	1.4	0.9
M	-1.2	0.2	1.5	1.5	1.4	-1	1.2	1.3	1.1
N	-2	-1.2	0	0.2	0.7	-0.6	0.7	0.4	-0.5
P	-2	-2	0.3	-1.2	0.6	0.5	0.3	0.4	-0.4
Q	-2	-0.5	1.7	1	1.4	-0.7	0.7	0.2	-0.4
R	-2	2	0.7	-0.7	1.3	-0.4	0.9	0.8	-0.3
S	-2	-1.2	0.5	-0.5	0.5	0	0.1	0	-0.1
T	-2	-0.4	0.3	-0.2	1.3	-0.5	0.8	0.3	0.4
V	-2	0.2	0.3	-0.2	0.9	-0.7	0.7	1	1.1
W	-0.2	-1.2	0.4	-1.3	0.5	-2	0.9	-0.2	-1
Y	1	-0.5	1.3	-0.7	0.9	-1.2	0.8	0.3	-0.6

FIG. 4. Principle of a matrix-based epitope prediction. The position- and amino acid-specific matrix values, e.g., binding data or amino acid frequencies, are assigned to each residue of a given peptide frame. Subsequently, values are mathematically processed, resulting in a peptide "score." The correlation between peptide score and binding affinity is the prerequisite for an effective HLA class II ligand prediction (see Fig. 5).

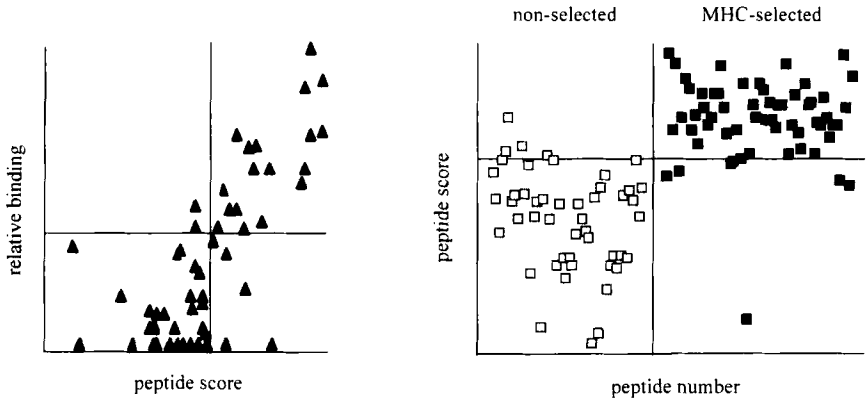


FIG. 5. The peptide score as a means to select HLA class II ligands. The peptide score correlates with the relative binding affinity (left) and differentiates between class II-selected and nonselected peptides displayed on M13 bacteriophage (right).

agreement with *in vitro* binding data (Jardetzky *et al.*, 1990; O'Sullivan *et al.*, 1991). The same peptide pool also showed approximately 2% Asp, 0% Met, and 3% Ile at position 1; however, no correlation was found between these low frequencies and *in vitro* binding data. Met and Ile are weak position 1 anchor residues, whereas Asp is not accepted at position 1 (Hammer *et al.*, 1994a). Thus, the data/noise differentiation may prove difficult for algorithms based on frequency matrices, especially, if matrices were derived from improperly aligned peptide pools (Hammer *et al.*, 1994b; Davenport *et al.*, 1995).

Although algorithms based on quantitative matrices predict large subsets of HLA class II ligands reasonably well, they cannot deal with nonlinearity within data. Double-substitution experiments on DQA1*0501/DQB1*0301 ligands, for example, revealed that the acceptance of peptide side chains at relative position "i" depends on the type of residue at position "i + 2" and vice versa (Raddrizzani *et al.*, 1997). Although these findings are rather exceptional for class II peptide interaction (Section II,B,3), bioinformatic tools, such as artificial neural networks (ANN), can deal with nonlinearity (Beale and Jackson, 1990; Weiss and Kulikowski, 1990). When trained on a large amount of input data, ANNs can extract and retain generalized patterns present in the training data set and subsequently recognize these patterns in a new, previously unseen input. ANNs have been successfully used for class I prediction (Brusic *et al.*, 1994). The amount of required data and, especially, the variable length of class II ligands (Section II,A) prevented, however, a broader application of ANNs for class II prediction. Recently, an effective bioinformatic approach termed PERUN was devel-

oped for the prediction of HLA class II ligands (Brusic *et al.*, 1997). PERUN solves the problem of variable peptide length by using evolutionary algorithms (Forrest, 1993) to derive alignment matrices. These matrices align peptides before they are used to train ANNs. The PERUN approach was tested on DRB1*0401 prediction. A comparative study revealed that PERUN predictions were marginally better than prediction using quantitative matrices and significantly better than simple motif-based prediction.

A major advantage of PERUN is the capacity to automatically refine itself as new data become available. In terms of a global coverage of HLA class II-binding specificity, however, PERUN has several disadvantages: (i) the training of ANNs requires large amounts of data—even though different sources of data can be used to train ANNs, these data are not available for most class II alleles; and (ii) an ANN is data driven, i.e., it can only utilize data derived from the class II allele for which the ANN is established. This is in strong contrast to algorithms based on quantitative matrices that can be built by combining pocket-specificity profiles derived from several class II alleles (Section II,B,4). Thus, quantitative matrices provide, at least for now, the only basis for a comprehensive T cell epitope prediction. More important, the capacity to predict promiscuous or selectively binding HLA class II ligands is not yet possible by other approaches.

TEPITOPE is a recently developed Windows95 application (J. Hammer *et al.*, manuscript in preparation) that enables the computational identification of (i) class II ligands binding in a promiscuous or allele-specific mode and (ii) the effects of polymorphic residues on class II ligand specificity. Twenty-five quantitative matrix-based HLA-DR motifs (Section II,B,4) covering a large part of human class II ligand specificity were incorporated into the first version of TEPITOPE and provide the basis for various algorithms included into the TEPITOPE software package. One of them, the prediction algorithm, permits the prediction and parallel display of ligands for each of the 25 DR alleles starting from any protein sequence. Due to the compact design of the user interface, class II ligands are immediately identified as promiscuous or allele-specific binders, allowing a rapid selection of peptide sequences for further evaluation in biological assays (Fig. 6).

TEPITOPE also enables the calculation of score distribution curves for each class II allele based on any natural protein database. As a consequence, prediction thresholds are expressed as "percentage of natural peptide frames." This compensates allelic differences in absolute peptide scores (Section II,C,2) and improves the prediction of promiscuous or allele-specific class II binders. Another algorithm incorporated in the TEPITOPE software package allows for the possibility to computationally improve

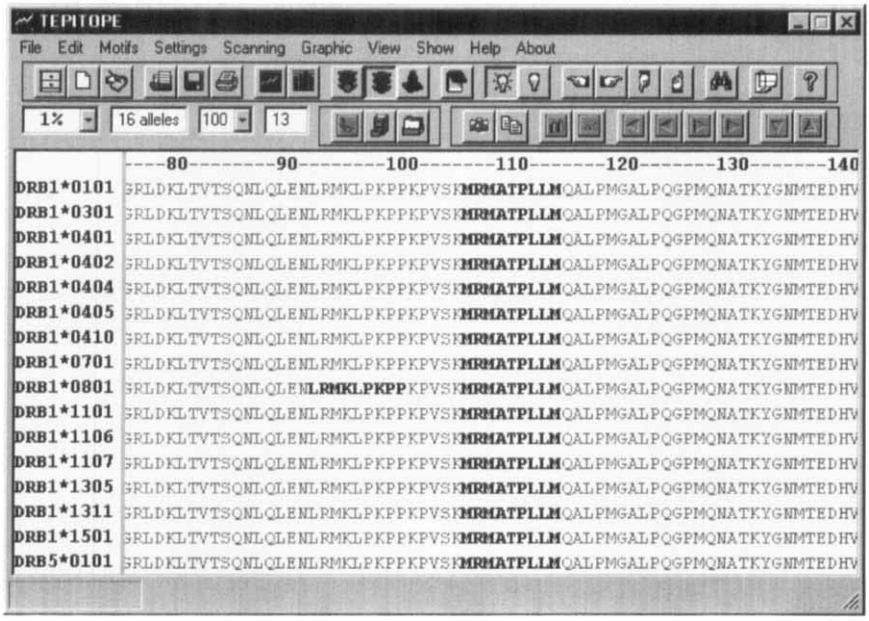


FIG. 6. Prediction of the core sequence of the class II-associated invariant chain peptide (CLIP) with TEPITOPE. The user interface design of TEPITOPE enables the rapid identification of promiscuous ligands, here demonstrated for CLIP (Cresswell, 1994; Romagnoli and Germain, 1994) of the invariant chain. The prediction thresholds were set to 1%, thus only peptide frames were predicted with a score similar to or higher than the 1% best-scoring natural peptide frames.

quantitative matrix-based motifs by expert rules, resulting in "expert matrices." Individual pocket thresholds can be set and pocket weights can be modified; both tools can be applied to compensate for nonlinear data, such as dominant negative effects. The expert rule-setting unit is directly linked to a huge database consisting of thousands of HLA-DR peptide-binding data. The predictive power of the generated expert matrices can therefore immediately be tested, providing a useful feedback loop. Thus, as for ANNs, a data-driven optimization of quantitative matrices will lead to future improvements of ligand prediction, at least for those HLA class II alleles for which *in vitro* binding assays are available.

Finally, TEPITOPE enables the rapid comparison of pocket-specificity profiles (Section II,B,4), revealing in many cases the effects of polymorphic residues on class II ligand specificity. As demonstrated in Section III, this feature proves very useful in displaying the effects of disease-linked class II residues on peptide binding.

III. Peptide-Binding Specificity of Autoimmune Disease-Associated HLA Class II Molecules

A. HLA and Disease Associations

Autoimmune diseases appear to occur when a specific immune response is mounted against itself. Although what triggers the autoimmune response is not known, both environmental and genetic factors are important. Genes in the HLA complex appear to account for the strongest genetic predisposition. For example, a genomewide scan for diabetes susceptibility genes has recently demonstrated that several genes contribute to the disease process, but that genes of the HLA complex are the most important (Davies *et al.*, 1994).

The HLA complex consists of more than 100 different HLA genes (Campbell and Trowsdale, 1994). Serological or RFLP analyses, haplotype comparisons, and the analysis of individual HLA genes suggested that most HLA-linked diseases are primarily associated with genes encoding the peptide presenting HLA molecules, among them several HLA-DR and -DQ alleles. Moreover, sequencing of these HLA genes indicated that disease-associated class II molecules often share unique amino acid residues in the peptide-binding cleft (reviewed by Todd *et al.*, 1988; Nepom and Erlich, 1991; Thorsby, 1995). These residues could affect predisposition to autoimmune disease by several mechanisms that are not mutually exclusive, including for example, shaping of the T cell receptor repertoire and altering the specificity of the peptide binding site (Roy *et al.*, 1989; Kappler *et al.*, 1987; Teh *et al.*, 1988).

The effect of disease-associated class II polymorphic residues in determining the peptide-binding specificity has been addressed by detailed HLA class II peptide-binding studies. Although these studies alone cannot definitely establish the role of MHC class II alleles in determining resistance or susceptibility to autoimmune disease, they provide important information toward a clearer understanding of the autoimmune disease process. The identification of a correlation between binding specificity and disease association, for example, strongly supports the hypothesis that selective binding of autoantigenic peptides is the major mechanism underlying HLA disease association (Section III,B). Furthermore, the knowledge of HLA class II peptide-binding rules could lead to the identification of autoantigenic peptides selectively presented by the disease-associated HLA class II molecules (Sections II,C and III,B).

B. Rheumatoid Arthritis

Susceptibility to rheumatoid arthritis (RA) is specifically associated with the HLA-DR locus (Tiwari and Terasaki, 1985). More than 80% of Cauca-

sian RA patients express DRB1*0401, DRB1*0404, DRB1*0408, or DRB1*0101 (Winchester, 1994). In Japanese patients, the frequency of another DR4 subtype, encoded by DRB1*0405, is increased (Otha *et al.*, 1982). Interestingly, the RA-associated DR molecules all carry a short stretch of amino acids at DRβ 67–74 ("shared epitope") that is highly conserved, even though DRβ 67–71 covers an otherwise very polymorphic region of the DR cleft (Gregersen *et al.*, 1986; Nepom *et al.*, 1989; Wordsworth *et al.*, 1989). The fact that DRB1*0402, a closely related molecule not associated with RA, differs from some RA-linked molecules only in this shared epitope region suggests that this part of the molecule plays a critical role in disease association.

In an attempt to analyze the role of DRβ 67–74 on HLA-DR peptide-binding specificity, we compared the pocket-specificity profiles (Section II,B,4) of RA-associated DR4 subtypes with profiles of nonassociated DR molecules (Hammer *et al.*, 1995). Striking differences, especially in the specificity of pocket 4, were identified between DR4 subtypes that are associated with RA and those that are not (Fig. 7*a*). For example, peptides with negatively charged residues at position 4 bound to RA-associated DRB1*0401 or DRB1*0404 molecules but not to the nonassociated DRB1*0402 molecule; the reverse was true for peptides with positively charged residues at position 4. Site-directed mutagenesis demonstrated that positively (DRB1*0401) or negatively (DRB1*0402) charged residues at DRβ71 were responsible for most of these effects (Hammer *et al.*, 1995). Similar conclusions were also reached by the pooled peptide sequencing technique (Friede *et al.*, 1996) and by selected single-substitution experiments (Woulfe *et al.*, 1995). Altogether, these results demonstrated a striking correlation between binding specificity and disease association, thus supporting the hypothesis that selective binding of autoantigenic peptides is the mechanism underlying HLA association in RA.

Although the target self-antigen that initiates the autoimmune process is unknown, a number of candidate autoantigens have been implicated in the pathogenesis of RA. Some were derived from normal self-proteins, such as type II collagen or other joint-associated proteins (Holmdal *et al.*, 1990; Mikecz *et al.*, 1987), and others from microorganisms (Gaston *et al.*, 1990). Recently, a panel of DRB1*0401-restricted mouse T cell hybridomas specific for bovine type II collagen were generated from DRB1*0401 transgenic mice (Fugger, 1996). The vast majority recognized a single determinant corresponding to the conserved residues 390–402 in human, which was predicted using a quantitative matrix-based DRB1*0401 motif (Marshall *et al.*, 1995). Further analysis using TEPITOPE (Section II,C,2) indicated that this determinant was predicted to bind selectively to RA-

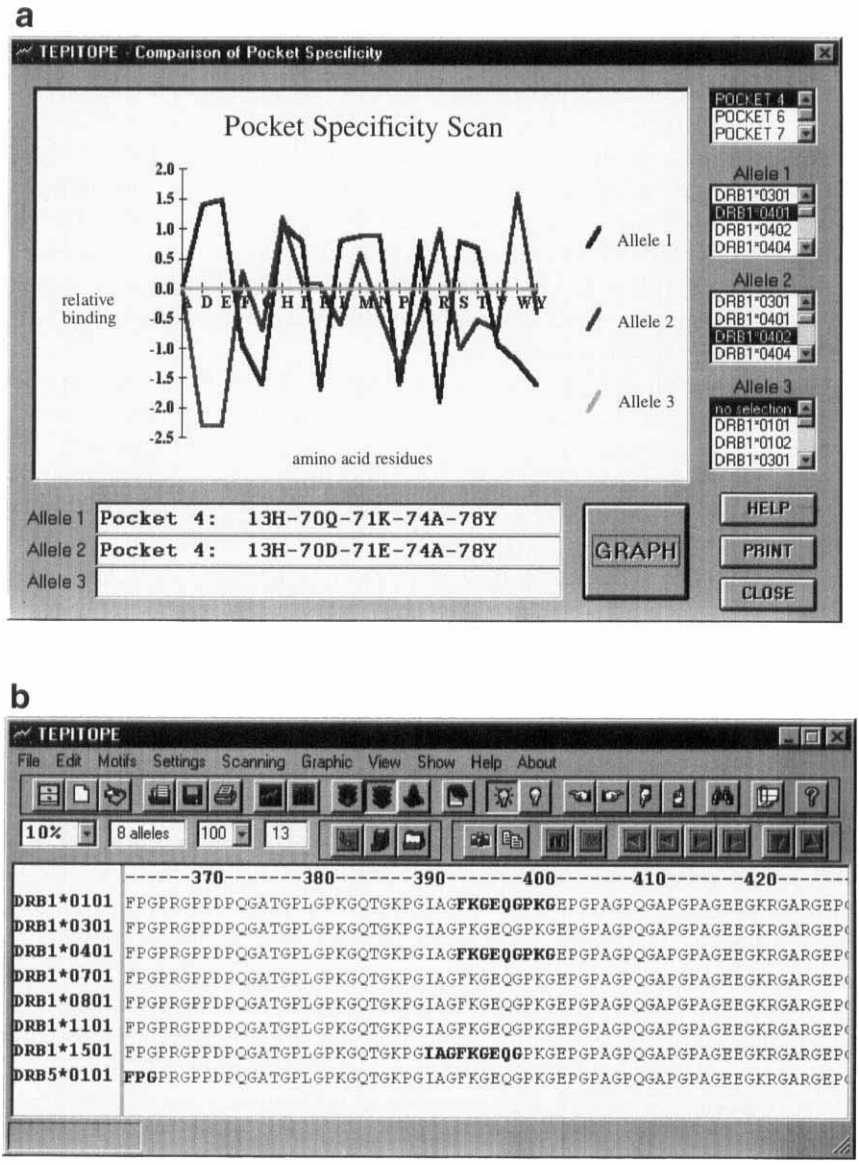


FIG. 7. (a) Comparison of pocket 4 specificity profiles of DRB1*0401 and DRB1*0402 with TEPITOPE. The selection of the pocket and HLA class II alleles (right) results in the display of the corresponding pocket specificity profiles (center) and the different polymorphic residues that constitute the pocket (bottom). (b) *Prediction of a selective peptide in human type II collagen with TEPITOPE.* The predicted region corresponds to a dominant T cell epitope in bovine collagen identified in DRB1*0401 transgenic mice (Fugger *et al.*, 1996). The prediction thresholds were set to 10% (see Fig. 6). Major class II alleles of the Caucasian population were selected.

associated HLA-DR molecules (Fig. 7*b*), thus supporting the value of this approach for the identification of potential autoantigens.

C. Pemphigus Vulgaris

Pemphigus vulgaris (PV) is an autoimmune disease of the skin in which autoantibody production to an epidermal cell adhesion molecule, desmoglein 3, is believed to result in a loss of keratinocyte adhesion and subsequent severe blister formation (Amagai *et al.*, 1991). PV is associated in several ethnic groups with the DRB1*0402 allele (Ahmed *et al.*, 1990, 1991). As discussed under Section III,B, DRB1*0402 differs only at DRβ 67–74 from some RA-associated DR4 subtypes. The pocket 4 of the DRB1*0402 cleft has a negatively charged residue (Glu) at position DRβ71. Correspondingly, peptides with positively charged residues at position 4 can bind to DRB1*0402, whereas peptides with negatively charged residues at this position cannot bind due to a "charge clash" (Fig. 7*a*). The detailed analysis of pocket-specificity profiles using TEPITOPE revealed, however, that PV-associated and nonassociated DR4 subtypes do not differ only in their acceptance of charged position 4 residues; Trp, for example, is an anchor residue for DRB1*0402 but an inhibitory residue for DRB1*0401. Thus, efforts to predict selectively presented T cell epitopes (Wucherpfennig *et al.*, 1995) also need to include binding characteristics that are less obvious than charge–charge interactions or clashes.

A recent study demonstrated that T cells from PV patients responded to a desmoglein 3 peptide 190–204 (Wucherpfennig *et al.*, 1995). This peptide is presented by the disease-associated DRB1*0402 molecule but not by other DR4 subtypes. Site-directed mutagenesis of DRB1*0402 could indeed demonstrate that selective presentation was due to the negatively charged residue at DRβ71 of pocket 4.

D. Methimazole-Induced Insulin Autoimmune Syndrome

The P4 pocket may also be important for the allele-specific binding and presentation of an insulin peptide by the DRB1*0406 molecule that is associated with susceptibility to a drug-induced insulin autoimmune syndrome (IAS) (Uchigata *et al.*, 1992). The disease, which is reported to be a frequent cause of hypoglycemia among Japanese, is characterized by the presence of high titers of anti-insulin antibodies and by the fact that a large number of patients had been taking reducing agents, such as methimazole or glutathione, prior to the onset of the disease.

A simple DRB1*0406 motif has recently been identified by single-substitution experiments (Matsushita *et al.*, 1994). This motif allowed the identification of an insulin peptide (TSICSLYQLE) that bound selectively to the IAS-associated DRB1*0406 but not to the nonassociated

DRB1*0405 molecule. Interestingly, this peptide fragment may not be available under normal physiological conditions for MHC binding due to a disulfide bond with a flanking Cys residue. It is hypothesized that a reducing compound such as methimazole may cleave the disulfide bond *in vivo*, thus allowing the binding of the insulin peptide to the DRB1*0406 molecules, which could in turn lead to the activation of self-insulin-specific T cells.

E. Multiple Sclerosis

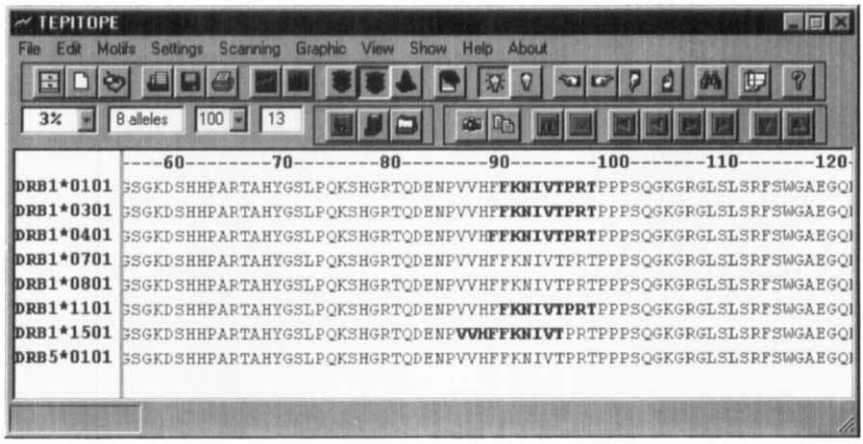
Multiple sclerosis (MS) is a chronic inflammatory disease of the human central nervous system (CNS) characterized by demyelination and by focal infiltrates of macrophages, plasma cells, and T cells in the CNS. Susceptibility to MS is specifically associated with the HLA-DR locus (Tiwari and Terasaki, 1985). Approximately 50–70% of MS patients carry the DRB1*1501 allele versus 20–30% of normal individuals (Tiwari and Terasaki, 1985). Besides DRB1*1501, other HLA class II alleles are overrepresented in certain ethnic groups, such as DRB1*0401 in Southern Italians and Arabs (Marrosu *et al.*, 1988; Yacub and Daif, 1988).

Myelin basic protein (MBP) and proteolipid protein (PLP) are putative autoantigens involved in the pathogenesis of MS and can induce experimental autoimmune encephalitis (EAE) in mice and rats (Pettinelli *et al.*, 1982; Fritz *et al.*, 1983; Enddoh *et al.*, 1986). In humans, MBP- or PLP-reactive HLA-DR-restricted T cells have been isolated from both MS patients and healthy controls (Martin *et al.*, 1990; Ota *et al.*, 1990; Pette *et al.*, 1990). MBP-specific T cells in MS patients were activated, whereas those from controls were in a resting state (Zhang *et al.*, 1994). Activated MBP- and PLP-reactive T cells might therefore be involved in the pathogenesis of MS.

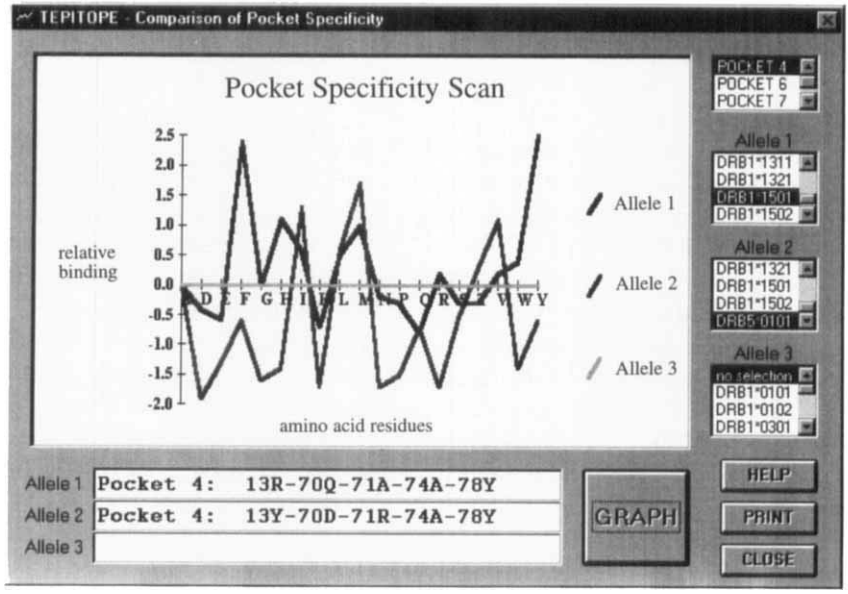
An epitope scan of MBP with TEPITOPE identified a promiscuous MBP region 87–99, whereby DRB1*1501, in contrast to most other DR molecules, is predicted to bind in a slightly different binding frame (Fig. 8*a*). The pocket-specificity comparison function of TEPITOPE (Fig. 8*b*)

Fig. 8. (a) Prediction of a promiscuous peptide in MBP with TEPITOPE. MBP 87–99 represents a immunodominant region of MBP (Wucherpfennig *et al.*, 1994; Ota *et al.*, 1990). The prediction thresholds were set to 3% (see Fig. 6). (b) *Comparison of pocket 4 specificity profiles of DRB1*1501 and DRB5*0101 with TEPITOPE.* The pocket comparison function of TEPITOPE reveals DRB1*1501-specific anchor residues if compared to DRB5*0101 or other HLA-DR alleles (not shown). (c) *Prediction of an immunodominant peptide in PLP with TEPITOPE.* PLP 175–192 is an immunodominant epitope in HLA-DR4 individuals (Markovic-Plese *et al.*, 1995). The prediction thresholds were set to 1% (see Fig. 6). All DR4 subtypes included in TEPITOPE were selected.

a


TEPITOPE
File Edit Motifs Settings Scanning Graphic View Show Help About
3%
8 alleles
100
13
----60--------70--------80--------90--------100-------110-------120-
DRB1*0101 GSGKDSHHPARTAHYGSLPQKSHGRTQDENPVVHFFKNIVTPRTPPPSQGKGRGLSLSRFSWGAEGQ
DRB1*0301 GSGKDSHHPARTAHYGSLPQKSHGRTQDENPVVHFFKNIVTPRTPPPSQGKGRGLSLSRFSWGAEGQ
DRB1*0401 GSGKDSHHPARTAHYGSLPQKSHGRTQDENPVVHFFKNIVTPRTPPPSQGKGRGLSLSRFSWGAEGQ
DRB1*0701 GSGKDSHHPARTAHYGSLPQKSHGRTQDENPVVHFFKNIVTPRTPPPSQGKGRGLSLSRFSWGAEGQ
DRB1*0801 GSGKDSHHPARTAHYGSLPQKSHGRTQDENPVVHFFKNIVTPRTPPPSQGKGRGLSLSRFSWGAEGQ
DRB1*1101 GSGKDSHHPARTAHYGSLPQKSHGRTQDENPVVHFFKNIVTPRTPPPSQGKGRGLSLSRFSWGAEGQ
DRB1*1501 GSGKDSHHPARTAHYGSLPQKSHGRTQDENPVVHFFKNIVTPRTPPPSQGKGRGLSLSRFSWGAEGQ
DRB5*0101 GSGKDSHHPARTAHYGSLPQKSHGRTQDENPVVHFFKNIVTPRTPPPSQGKGRGLSLSRFSWGAEGQ

b


TEPITOPE - Comparison of Pocket Specificity
Pocket Specificity Scan
relative binding
amino acid residues
Allele 1
Allele 2
Allele 3
POCKET 4
POCKET 6
POCKET 7
Allele 1
DRB1*1311
DRB1*1321
DRB1*1501
DRB1*1502
Allele 2
DRB1*1321
DRB1*1501
DRB1*1502
DRB5*0101
Allele 3
no selection
DRB1*0101
DRB1*0102
DRB1*0301
Allele 1 Pocket 4: 13R-70Q-71A-74A-78Y
Allele 2 Pocket 4: 13Y-70D-71R-74A-78Y
Allele 3
GRAPH
HELP
PRINT
CLOSE

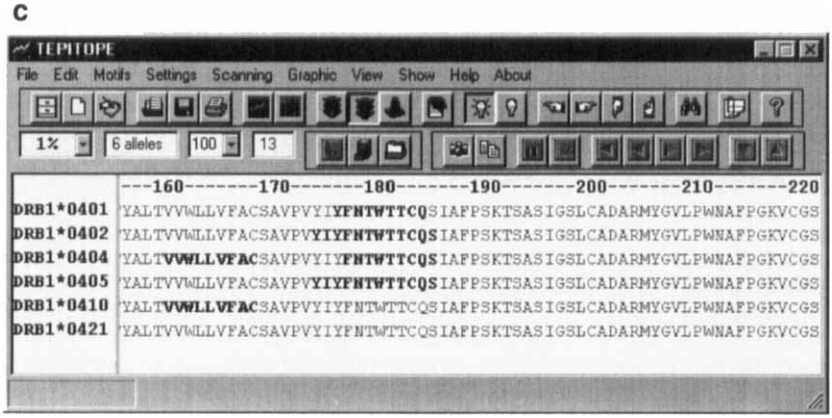


FIG. 8—*Continued*

leads to the identification of DRB1*1501-specific aromatic anchor residues at peptide position 4, which explains, at least in part, the DRB1*1501-specific binding frame. A number of investigators could demonstrate that MBP 87–99 indeed represents the immunodominant region of MBP in the context of the MS-associated DR alleles DRB1*1501 (Wucherpfennig *et al.*, 1994; Ota *et al.*, 1990; Valli *et al.*, 1993), and that DRB1*1501 binds a peptide frame that is different from the one recognized by most other DR alleles (Vogt *et al.*, 1994).

Epitope scanning with overlapping 20-residue-long peptides of PLP led to the identification of an immunodominant epitope (PLP 175–192) for HLA-DR4 individuals (Markovic-Plese *et al.*, 1995). Once again, this region could be predicted by TEPITOPE (Fig. 8*c*). HLA-DR4-IE chimeric class II transgenic mice were immunized with MBP 87–106 and PLP 175–192 to investigate the development of the HLA-DR-associated disease (Ito *et al.*, 1996). PLP 175–192 provoked a strong proliferative response of lymph node T cells and caused inflammatory lesions in the white matter of the CNS and symptoms of experimental allergic encephalomyelitis. Immunization with MBP 87–106 elicited a very weak proliferative T cell response and caused mild EAE. The amino acid sequences of both PLP 175–192 and MBP 87–106 are identical in humans and in mice, thus representing potential autoantigens in both species. Notably, mice lacking the DRB1*0401 transgene did not develop EAE after immunization with either PLP 175–192 or MBP 87–106, indicating that a human MHC class II binding site alone can confer susceptibility to an experimentally induced murine autoimmune disease.

F. Insulin-Dependent Diabetes Mellitus

Susceptibility to insulin-dependent diabetes mellitus (IDDM) is most strongly determined by DQ alleles that encode Ser, Ala, or Val at position β57 (Horn *et al.*, 1988). In contrast, Asp at DQβ57 mediates resistance to IDDM (Morel *et al.*, 1988; Ronningen *et al.*, 1989). Similarly, the diabetes-prone NOD mouse strain has Ser at position 57 in the I-Aβ chain (the murine homolog of DQβ), whereas most other mouse strains, including the closely related nonobese normal, have Asp at this position. These findings have led to the intriguing hypothesis that Asp57 in the DQβ chain protects against IDDM, whereas its absence increases susceptibility.

The structural analysis of HLA-DR molecules revealed that Asp57 of DRβ forms a salt bridge with Arg79 of DRα (Stern *et al.*, 1994). The pocket-specificity comparison function of TEPITOPE reveals a strong effect of residue β57 on HLA-DR peptide interaction (Fig. 9): Negatively charged and aromatic residues at peptide position 9 are not accepted by pocket 9 when β57 is an Asp. Peptide elution and binding studies (Chicz *et al.*, 1994; Kwok *et al.*, 1996a; Nepom *et al.*, 1996) led to the identification of similar effects for HLA-DQ peptide interaction, thus supporting a possi-

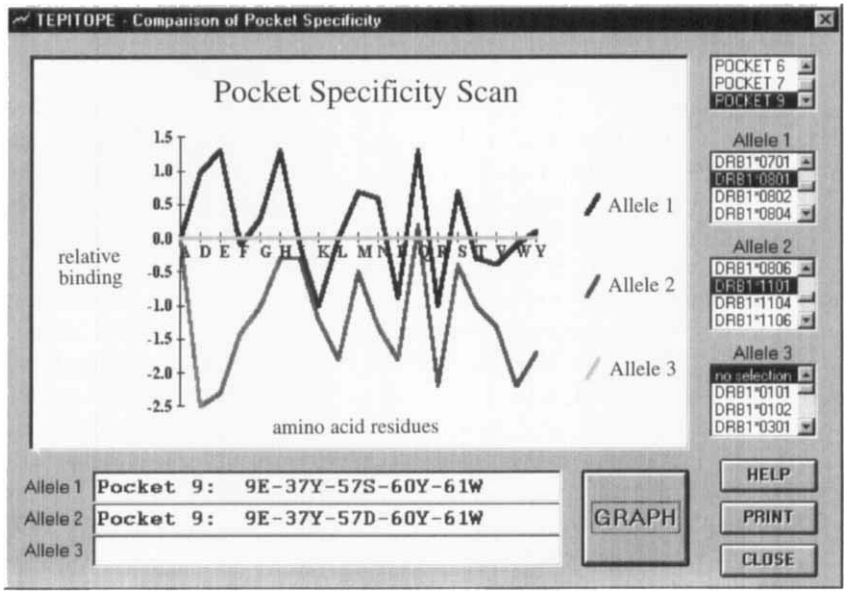


FIG. 9. Comparison of pocket 9 specificity profiles of DRB1*0801 and DRB1*1101 with TEPITOPE. The pocket comparison function of TEPITOPE reveals the effects of DRβ57 on the binding specificity of HLA-DR molecules.

ble correlation between HLA-DQ-binding specificity and IDDM association.

Several observations suggest, however, that the residue at position 57 plays a significant but not exclusive role in IDDM association (Awata *et al.*, 1990; Lund *et al.*, 1990). Thus, both comparative binding studies and more advanced peptide-binding motifs are required for HLA-DQ molecules (Section II,B) to elucidate the role of other HLA-DQ residues on peptide binding and to predict potential autoantigenic peptides.

IV. Prospects for Immune Intervention

Detailed knowledge of HLA class II peptide-binding specificity might have interesting applications for immunointervention in autoimmune diseases. One strategy ought to be directed at the development of MHC-specific antagonists that block the peptide binding site of disease-linked class II MHC molecules. An antagonist is expected to interfere with the presentation of antigenic peptide ligands, including those involved in the activation of autoimmune T cells. Indeed, several groups have used this strategy to prevent induction of T cell-mediated autoimmune diseases such as EAE in mice (Lamont *et al.*, 1990; Gautam *et al.*, 1992) and rats (Wauben *et al.*, 1994), autoimmune myocarditis (Smith and Allen, 1991), and insulin-dependent diabetes mellitus (Hurtenbach *et al.*, 1993). The advantage of this strategy is that it is applicable without specific knowledge of the disease-inducing autoantigen(s). Because different class II molecules exhibit different peptide-binding specificities, the antagonist is expected to be selective, i.e., to bind to the disease-linked class II molecules only. Thus, a considerable part of the host's antigen presenting capacity would remain intact, and consequently patients would not be severely immunocompromised.

Another attractive approach for a specific therapy of autoimmune diseases is the induction of peripheral tolerance to the pathogenic self-antigen. Induction of peripheral tolerance has been demonstrated in several autoimmune models. For example, EAE can be ameliorated by inducing T cell anergy to synthetic peptides corresponding to the immunodominant epitopes of MBP (Gaur *et al.*, 1992). Similarly, tolerance to glutamic acid decarboxylase, a putative autoantigen in IDDM, prevents diabetes development in NOD mice (Kaufman *et al.*, 1993; Tisch *et al.*, 1993). A current limit to this approach is that information on the autoantigens involved in most human autoimmune diseases is still fragmentary and not fully verified. It is hoped that the type of research described here and, in particular, the computational epitope scanning of large numbers of gene sequences implicated in autoimmune diseases (Section II,C) will lead to the rapid identification of new autoantigen candidates.

Finally, specific inhibition of $CD4^+$ (De Magistris *et al.*, 1992; Racioppi *et al.*, 1993) or $CD8^+$ (Jameson *et al.*, 1993) autoreactive T cells can also be induced by antigen analogs acting as T cell receptor (TCR) antagonists. For example, it has recently been demonstrated that TCR antagonist peptides can inhibit EAE induced by a proteolipoprotein epitope in SJL mice (Franco *et al.*, 1994). Thus, if the epitopes inducing pathogenic T cells could be identified, administration of TCR antagonists could prevent, or possibly even treat, autoimmune diseases. Detailed knowledge of HLA class II peptide-binding specificity should prove useful for both the identification of pathogenic T cell-inducing epitopes and the design of a TCR antagonist. Obviously, the antagonist has to be designed in such a way that it will never become an agonist. These requirements are likely to limit considerably the clinical applicability of this approach.

V. Concluding Remarks

A striking characteristic of autoimmune diseases is the increased frequency of certain HLA class II alleles in affected individuals. Although there is much to learn about the pathogenesis of autoimmunity, it is generally believed that disease-associated HLA class II molecules have the capacity to bind and present autoantigenic peptides to T cells. A nearly global coverage of HLA-DR peptide-binding specificity, combined with newly emerging bioinformatic tools, has led to effective ways of studying the role of disease-associated class II residues and to the identification of peptides selective for disease-associated molecules. The detailed knowledge of HLA class II peptide interaction may therefore represent a step forward in the understanding of autoimmune disease processes.

Acknowledgments

This work was supported by Grant BI04-CT95-0263 from the European Community.

References

Ahmed, A. R., Yunis, E. J., Khatri, K., Wagner, R., Notani, G., Awdeh, Z., and Alper, C. A. (1990). Major histocompatibility complex haplotype studies in Ashkenazi Jewish patients with pemphigus vulgaris. *Proc. Natl. Acad. Sci. USA* **87,** 7658–7662.

Ahmed, A. R., Wagner, R., Khatri, K., Notani, G., Awdeh, Z., Alper, C. A., and Yunis, E. J. (1991). Major histocompatibility complex haplotypes and class II genes in non-Jewish patients with pemphigus vulgaris. *Proc. Natl. Acad. Sci. USA* **88,** 5056–5060.

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* **67,** 869–877.

Awata, T., Kuzuya, T., Matsuda, A., Iwamoto, Y., Kanazawa, Y., Okuyama, M., and Juji, T. (1990). High frequency of aspartic acid at position 57 of HLA-DQ beta-chain in Japanese IDDM patients and nondiabetic subjects. *Diabetes* **39,** 266–269.

Beale, R., and Jackson, T. (1990). "Neural Computing: An Introduction." Hilger, Bristol, UK.

Boehncke, W. H., Takeshita, T., Pendleton, C. D., Houghten, R. A., Sadegh, N., Racioppi, S., Berzofsky, J. A., and Germain, R. N. (1993). The importance of dominant negative effects of amino acid side chain substitution in peptide–MHC molecule interactions and T cell recognition. *J. Immunol.* **150,** 331–341.

Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L., and Wiley, D. C. (1993). The three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* **364,** 33–39.

Brusic, V., Rudy, G., and Harrison, L. C. (1994). "Complex Systems: Mechanisms of Adaptation." IOS Press, Amsterdam.

Brusic, V., Rudy, G., Honeyman, M., Hammer, J., and Harrison, L. C. (1997). Prediction of MHC class II-binding peptides using an evolutionary algorithm and artificial neural network. Submitted for publication.

Campbell, R. D., and Trowsdale, J. (1994). Map of the human MHC. *Immunol. Today* **14,** 349–352.

Chicz, R. M., Urban, R. G., Gorga, J. C., Vignali, D. A. A., Lane, W. S., and Strominger, J. L. (1993). Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J. Exp. Med.* **178,** 27–47.

Chicz, R. M., Lane, W. S., Robinson, R. A., Trucco, M., Strominger, J. L., and Gorga, J. C. (1994). Self-peptides bound to the type I diabetes associated class II MHC molecules HLA-DQ1 and HLA-DQ8. *Int. Immunol.* **6,** 1639–1649.

Cresswell, P. (1994). Assembly, transport and function of MHC class II molecules. *Annu. Rev. Immunol.* **12,** 259–293.

Cwirla, S. E., Peters, E. A., Barret, R. W., and Dower, W. J. (1990). Peptides on phage: A vast library of peptides for identifying ligands. *Biochemistry* **87,** 6378–6382.

Davenport, M. P., Ho Shon, I. A., and Hill, A. V. (1995). An empirical method for the prediction of T cell epitopes. *Immunogenetics* **42,** 392–397.

Davies, J. L., Kawaguchi, Y., Bennett, S. T., Copeman, J. B., Cordell, H. J., Pritchard, L. E., Reed, P. W., Gough, S. C. L., Jenkins, S. C., Palmer, S. M., Balfour, K. M., Rowe, B. R., Farrall, M., Barnett, A. H., Bain, S. C., and Todd, J. A. (1994). A genome-wide search for human type 1 diabetes susceptibility genes. *Nature* **371,** 130–136.

DeLisi, C., and Berzofsky, J. A. (1985). T-cell antigenic sites tend to be amphipathic structures. *Proc. Natl. Acad. Sci. USA* **82,** 7048–7052.

De Magistris, T. M., Alexander, J., Coggeshall, M., Altman, A., Gaeta, F. C. A., Grey, H. M., and Sette, A. (1992). Antigen analog–major histocompatibility complexes act as antagonists of the T cell receptor. *Cell* **68,** 625–634.

Devlin, J. J., Paniganiban, L. C., and Devlin, P. E. (1990). Random peptide libraries: A source of specific protein binding molecules. *Science* **249,** 404–406.

Enddoh, M., Tabira, T., Kunishita, T., Sakai, K., Yamamura, T., and Taketomi, T. (1986). A proteolipid apoprotein is an encephalitogen of acute and relapsing autoimmune encephalomyelitis in mice. *J. Immunol.* **137,** 3832–3835.

Falk, K., and Rötzschke, O. (1993). Consensus motifs and peptide ligands of MHC class I molecules. *Semin. Immunol.* **5,** 81–94.

Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H.-G. (1991). Allele specific motifs revealed by sequencing of self peptides eluted from MHC molecules. *Nature* **351,** 290–296.

Falk, K., Roetzschke, O., Stevanovic, S., Jung, G., and Rammensee, H.-G. (1994). Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constraints of processing, and general rules. *Immunogenetics* **39,** 230–242.

Forrest, S. (1993). Genetic algorithms: Principles of natural selection applied to computation. *Science* **261,** 872–878.

Franco, A., Southwood, S., Arrhenius, T., Kucroo, V. K., Grey, H. M., Sette, A., and Ishioka, G. Y. (1994). T cell receptor antagonist peptides are effective inhibitors of experimental allergic encephalomyelitis. *Eur. J. Immunol.* **24,** 940–946.

Friede, T., Gnau, V., Jung, G., Keilholz, W., Stevanovic, S., and Rammensee, H. (1996). Natural ligand motifs of closely related HLA-DR4 molecules predict features of rheumatoid arthritis associated peptides. *Biochim. Biophys. Acta* **1316,** 85–101.

Fritz, R. B., Chou, C. H. J., and McFarlin, D. E. (1983). Relapsing murine experimental allergic encephalomyelitis induced by myelin basic protein. *J. Immunol.* **130,** 1024–1026.

Fugger, L., Rothbard, J., and Sonderstrup-McDevitt, G. (1996). Specificity of an HLA-DRB1°0401 restricted T cell response to type II collagen. *Eur. J. Immunol.* **26,** 928–933.

Gaston, J. S. H., Life, P. F., Jenner, P. J., Colston, M. J., and Bacon, P. A. (1990). Recognition of a mycobacteria-specific epitope in the 65-kD heat-shock protein by synovial fluid-derived T cell clones. *J. Exp. Med.* **171,** 831–841.

Gaur, A., Wiers, B., Liu, A., Rothbard, J., and Fathman, C. G. (1992). Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy. *Science* **258,** 1491–1494.

Gautam, A. M., Pearson, C. I., Sinha, A. A., Smilek, D. E., Steinman, L., and McDevitt, H. O. (1992). Inhibition of experimental autoimmune encephalomyelitis by a nonimmunogenic non-self peptide that binds to I-A^u. *J. Immunol.* **148,** 3049–3054.

Geluk, A., Meijgaarden, K. E., Janson, A. A. M., Drijfhout, J. W., Meloen, R. H., Vries, R. R. P., and Ottenhof, T. H. M. (1992). Functional analysis of DR17(DR3)-restricted mycobacterial T cell epitopes reveals DR17-binding motif and enables the design of allele-specific competitor peptides. *J. Immunol.* **149,** 2864–2871.

Ghosh, P., Amaya, M., Mellins, E., and Wiley, D. C. (1995). The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature* **378,** 457–462.

Gregersen, P. K., Shen, M., Song, Q. L., Merryman, P., Degar, S., Seki, T., Maccari, J., Goldberg, D., Murphy, H., Schwenzer, J., Wang, C. Y., Winchester, R. J., Nepom, G. T., and Silver, J. (1986). Molecular diversity of HLA-DR4 haplotaypes. *Proc. Natl. Acad. Sci. USA* **83,** 2642–2646.

Hammer, J., Takacs, B., and Sinigaglia, F. (1992). Identification of a motif for HLA-DR1 binding peptides using M13 display libraries. *J. Exp. Med.* **176,** 1007–1013.

Hammer, J., Valsasnini, P., Tolba, K., Bolin, D., Higelin, J., Takacs, B., and Sinigaglia, F. (1993). Promiscuous and allele-specific anchors in HLA-DR binding peptides. *Cell* **74,** 197–203.

Hammer, J., Belunis, C., Bolin, D., Papadopoulos, J., Walsky, R., Higelin, J., Danho, W., Sinigaglia, F., and Nagy, Z. A. (1994a). High-affinity binding of short peptides to major histocompatibility complex class II molecules by anchor combinations. *Proc. Natl. Acad. Sci. USA* **91,** 4456–4460.

Hammer, J., Bono, E., Gallazzi, F., Belunis, C., Nagy, Z., and Sinigaglia, F. (1994b). Precise prediction of major histocompatibility complex class II peptide interaction based on peptide side chain scanning. *J. Exp. Med.* **180,** 2353–2358.

Hammer, J., Gallazzi, F., Bono, E., Karr, R. W., Guenot, J., Valsasnini, P., Nagy, Z. A., and Sinigaglia, F. (1995). Peptide binding specificity of HLA-DR4 molecules: Correlation with rheumatoid arthritis association. *J. Exp. Med.* **181,** 1847–1855.

Hanson, G. J., Vuletich, J. L., Bedell, L. J., Bono, C. P., Howard, S. C., Woulfe, S. L., and Zacheis, M. L. (1996). Design of MHC class II (DR4) ligands using conformationally restricted iminoacids at p3 and p5. *Bioorg. Med. Chem. Lett.* **6,** 1931–1936.

Holmdal, R., Andersson, M., Goldschmidt, K., Gustafsson, K., Jansson, L., and Mo, J. A. (1990). Type II collagen autoimmunity in animals and provocations leading to arthritis. *Immunol. Rev.* **118,** 193–232.

Horn, G., Bugawan, T., Long, C., and Erlich, H. (1988). Allelic sequence variation of the HLA DQ loci: Relationship to serology and to IDDM susceptibility. *Proc. Natl. Acad. Sci. USA* **85,** 6012–6016.

Hunt, D. F., Michel, H., Dickinson, T. A., Shabonawitz, J., Cox, A. L., Sakaguchi, K., Apella, E., Grey, H. M., and Sette, A. (1992). Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad. *Science* **256,** 1817–1820.

Hurtenbach, U., Lier, E., Adorini, L., and Nagy, Z. A. (1993). Prevention of autoimmune diabetes in non-obese diabetic mice by treatment with a class II major histocompatibility complex-blocking peptide. *J. Exp. Med.* **177,** 1499–1504.

Ito, K., Bian, H.-J., Molina, M., Han, J., Magram, J., Saar, E., Belunis, C., Bolin, D. R., Arceo, R., Campbell, R., Falcioni, F., Vidovic, D., Hammer, J., and Nagy, Z. A. (1996). HLA-DR4-IE chimeric class II transgenic, murine class II-deficient mice susceptible to experimental allergic encephalomyelitis. *J. Exp. Med.* **183,** 2635–2644.

Jameson, S. C., Carbone, F. R., and Bevan, M. J. (1993). Clone-specific T cell receptor antagonists of major histocompatibility complex class I-restricted cytotoxic T cells. *J. Exp. Med.* **177,** 1541–1550.

Jardetzky, T. S., Gorga, J. C., Busch, R., Rothbard, J. B., Strominger, J. L., and Wiley, D. C. (1990). Peptide binding to HLA-DR1: A peptide with most residues substituted to alanine retains MHC binding. *EMBO J.* **9,** 1797–1804.

Johansen, B. H., Vartdal, F., Eriksen, J. A., Thorsby, E., and Sollid, L. M. (1995). Identification of a putative motif for binding of peptides to HLA-DQ2. *Int. Immunol.* **8,** 177–182.

Kappler, J., Roehm, N., and Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. *Cell* **49,** 273–280.

Kaufman, D. L., Clare-Salzler, M., Tian, J., Forsthuber, T., Ting, G. S. P., Robinson, P., Atkinson, M. A., Sercarz, E. E., Tobin, A. J., and Lehmann, P. V. (1993). Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* **366,** 69–72.

Krieger, J. I., Karr, R. W., Grey, H. M., Yu, W.-Y., O'Sullivan, D., Batovsky, L., Zheng, Z.-L., Colòn, S. M., Gaeta, F. C. A., Sidney, J., Albertson, M., Del Guercio, F. C. A., Chesnut, R. W., and Sette, A. (1991). Single amino acid changes in DR and antigen define residues critical for peptide–MHC binding and T cell recognition. *J. Immunol.* **146,** 2331–2340.

Kwok, W. W., Domeier, M. E., Johnson, M. L., Nepom, G. T., and Koelle, D. M. (1996a). HLA-DQB1 codon 57 is critical for peptide binding and recognition. *J. Exp. Med.* **183,** 1253–1258.

Kwok, W. W., Domeier, M. E., Raymond, F. C., Byers, P., and Nepom, G. T. (1996b). Allele-specific motifs characterize HLA-DQ interaction with a diabetes-associated peptide derived from glutamic acid decarboxylase. *J. Immunol.* **156,** 2171–2177.

Lamont, A. G., Powell, M. F., Colon, S. M., Miles, G., Grey, H. M., and Sette, A. (1990). The use of peptide analogs with improved stability and MHC binding capacity to inhibit antigen presentation in vitro and in vivo. *J. Immunol.* **144,** 2493–2498.

Lund, T., O'Reilly, L., Hutchings, P., Kanagawa, O., Simpson, E., Gravely, R., Chandler, P., Dyson, J., Picard, J. K., Edwards, A., Kioussis, D., and Cooke, A. (1990). Prevention of insulin-dependent diabetes mellitus in non-obese diabetic mice by transgenes encoding modified I-A beta chain or normal I-E alpha-chain. *Nature* **345,** 727–729.

Madden, D. R., Gorga, J. C., Strominger, J. L., and Wiley, D. C. (1991). The structure of HLA-B27 reveals nonamer self-peptides bound in an extended conformation. *Nature* **353,** 321–325.

Malcherek, G., Falk, K., Roetzschke, O., Rammensee, H.-G., Stevanovic, S., Gnau, V., Jung, G., and Melms, A. (1993). Natural peptide ligand motifs of two HLA molecules associated with myasthenia gravis. *Int. Immunol.* **5,** 1229–1237.

Markovic-Plese, S., Fukaura, H., Zhang, J., Al-Sabbagh, A., Southwood, S., Sette, A., Kuchroo, V. K., and Hafler, D. A. (1995). T cell recognition of immunodominant and cryptic proteolipid protein epitopes in humans. *J. Immunol.* **155,** 982–992.

Marrosu, M. G., Muntoni, F., Murru, M. R., Spinicci, G., Pischedda, M. P., Goddi, F., Cassu, P., and Piratsu, M. (1988). Sardinian multiple sclerosis is associated with HLA-DR4: A serologic and molecular analysis. *Neurology* **348,** 1749–1753.

Marsh, S. G. E., and Bodmer, J. G. (1995). HLA class II region nucleotide sequences, 1995. *Tissue Antigens* **45,** 258–280.

Marshall, K. W., Wilson, K. J., Liang, J., Woods, A., Zaller, D., and Rothbard, J. B. (1995). Prediction of peptide affinity to HLA DRB1°0401. *J. Immunol.* **154,** 5927–5933.

Martin, R., Jaraquemada, D., Flerlage, M., Richert, J., Whitaker, J., Long, E. O., McFarlin, D. E., and McFarland, H. F. (1990). Fine specificity and HLA restriction of myelin basic protein-specific cytotoxic T cell lines from multiple sclerosis patients and healthy individuals. *J. Immunol.* **145,** 540–548.

Matsumura, M., Fremont, D. H., Peterson, P. A., and Wilson, I. A. (1992). Emerging principles for the recognition of peptide antigens by MHC class I molecules. *Science* **257,** 927–934.

Matsushita, S., Takahashi, K., Motoki, M., Komoriya, K., Ikagawa, S., and Nishimura, Y. (1994). Allele specificity of structural requirement for peptides bound to HLA-DRB1°0405 and -DRB1°0406 complexes: Implication for the HLA-associated susceptibility to methimazole-induced insulin autoimmune syndrome. *J. Exp. Med.* **180,** 873–883.

Mikecz, K., Glant, T. T., and Poole, A. R. (1987). Immunity to cartilage proteoglycans in BALB/c mice with progressive polyarthritis and nkylosing spondylitis induced by injection of human cartilage proteoglycan. *Arthritis Rheum.* **30,** 306–318.

Morel, P. A., Dorman, J. S., Todd, J. A., McDevitt, H. O., and Trucco, M. (1988). Aspartic acid at position 57 of the HLA-DQ beta chain protects against type I diabetes. A family study. *Proc. Natl. Acad. Sci. USA* **85,** 8111–8115.

Nepom, G. T., and Erlich, H. A. (1991). MHC class II molecules and autoimmunity. *Annu. Rev. Immunol.* **9,** 493–520.

Nepom, G. T., Byers, P., Seyfried, C., Healey, L. A., Wilskie, K. R., Stage, D., and Nepom, B. S. (1989). HLA genes associated with rheumatoid arthritis. Identification of susceptibility alleles using specific oligonucleotides probes. *Arthritis Rheum.* **32,** 15–21.

Nepom, B. S., Nepom, G. T., Coleman, M., and Kwok, W. (1996). Critical contribution of b chain residue 57 in peptide binding ability of both HLA-DR and -DQ molecules. *Proc. Natl. Acad. Sci. USA* **93,** 7202–7206.

O'Sullivan, D., Arrhenius, T., Sidney, J., Del Guercio, M.-F., Albertson, M., Wall, M., Oseroff, C., Southwood, S., Colòn, S. M., Gaeta, F. C. A., and Sette, A. (1991). On the interaction of promiscuous antigenic peptides with different DR alleles. *J. Immunol.* **147,** 2664–2669.

Ota, K., Matsui, M., Milford, E. L., Mackin, G. A., Weiner, H. L., and Hafler, D. A. (1990). T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature* **346,** 183–187.

Otha, N., Nishimura, Y. K., Tanimoto, T., *et al.* (1982). Association between HLA and Japanese patients with rheumatoid arthritis. *Hum. Immunol.* **5,** 123–132.

Parker, K. C., Bednarek, M. A., and Coligan, J. E. (1994). Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide sidechains. *J. Immunol.* **152,** 163–175.

Pette, M., Fujita, K., Wilkinson, D., Altmann, D., Trowsdale, J., Giegerich, G., Hinkkanen, A., Epplen, J., Kappos, L., and Wekerle, H. (1990). Myelin autoreactivity in multiple sclerosis: Recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes of multiple-sclerosis patients and healthy donors. *Proc. Natl. Acad. Sci. USA* **87,** 7968–7972.

Pettinelli, C. B., Fritz, R. B., Chou, C. H. J., and McFarlin, D. E. J. (1982). Encephalitogenic activity of guinea pig myelin basic protein in the SJL mouse. *Immunology* **129,** 1209–1211.

Racioppi, L., Ronchese, F., Matis, L. A., and Germain, R. N. (1993). Peptide–major histocompatibility complex class II complexes with mixed agonist–antagonist properties provide evidence for ligand-related differences in T cell receptor-dependent intracellular signaling. *J. Exp. Med.* **177,** 1047–1060.

Raddrizzani, L., Sturniolo, T., Guenot, J., Bono, E., Gallazzi, F., Nagy, Z., Sinigaglia, F., and Hammer, J. (1997). Different strategies in peptide interaction allow HLA-DQ and DR isotypes to bind complementary peptide repertoires. *J. Immunol.* (in press).

Rammensee, H.-G., Friede, T., and Stevanovic, S. (1995). MHC ligands and peptide motifs: First listing. *Immunogenetics* **41,** 178–228.

Reay, P. A., Kantor, R. M., and Davis, M. M. (1994). Use of global amino acid replacements to define the requirements for MHC binding and T cell recognition of moth cytochrome c (93–103). *J. Immunol.* **152,** 3946–3957.

Romagnoli, P., and Germain, R. N. (1994). The CLIP region of invariant chain plays a critical role in regulating major histocompatibility complex class II folding, transport and peptide occupancy. *J. Exp. Med.* **180,** 1107–1113.

Ronningen, K. S., Iwe, T., Halstensen, T. S., Spurkland, A., and Thorsby, E. (1989). The amino acid at position 57 of the HLA-DQ beta chain and susceptibility to develop insulin-dependent diasbetes mellitus. *Hum. Immunol.* **26,** 215–225.

Rothbard, J. B., and Gefter, M. L. (1991). Interaction between immunogenic peptides and MHC proteins. *Annu. Rev. Immunol.* **9,** 527–565.

Rothbard, J. B., and Taylor, W. R. (1988). A sequence pattern common to T cell epitopes. *EMBO J.* **7,** 93–100.

Roy, S., Scherer, M. T., Briner, T. J., Smith, J. A., and Gefter, M. L. (1989). Murine MHC polymorphism and T cell specificities. *Science* **244,** 572–575.

Rudensky, A., Preston, H. P., Hong, S. C., Barlow, A., and Janeway, C. J. (1991). Sequence analysis of peptides bound to MHC class II molecules. *Nature* **353,** 622–627.

Scott, J. K., and Smith, G. P. (1990). Searching for peptide ligands with an epitope library. *Science* **248,** 386–390.

Sette, A., Buus, S., Colon, S., Smith, J. A., Miles, C., and Grey, H. M. (1987). Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature* **328,** 395–399.

Sette, A., Sidney, J., Oseroff, C., del Guercio, M.-F., Southwood, S., Arrhenius, T., Powell, M. F., Colon, S. M., Gaeta, F. C., and Grey, H. M. (1993). HLA DR4w4 binding motifs illustrate the biochemical basis of degeneracy and specificity in peptide–DR interactions. *J. Immunol.* **151,** 3163–3170.

Sidney, J., Oseroff, C., Southwood, S., Wall, M., Glenn, I., Koning, F., and Sette, A. (1992). DRB1*0301 molecules recognize a structural motif distinct from the one recognized by most DRB1 alleles. *J. Immunol.* **149,** 2634–2640.

Sidney, J., Oseroff, C., del Guercio, M. F., Southwood, S., Ishioka, G. Y., Sakaguchi, K., Appella, E., and Sette, A. (1994). Definition of a DQ3.1 - specific binding motif. *J. Immunol.* **152,** 4516–4525.

Sinigaglia, F., Romagnoli, P., Guttinger, M., Takacs, B., and Pink, J. R. L. (1992). Selection of T-cell epitopes and vaccine engineering. *Methods Enzymol.* **203,** 370–386.

Smith, S. C., and Allen, P. M. (1991). Myosin-induced acute myocarditis is a T cell-mediated disease. *J. Immunol.* **147,** 2141–2147.

Stern, L. J., Brown, J. H., Jardetzky, T. S., Gorga, J. C., Urban, R. G., Strominger, J. L., and Wiley, D. C. (1994). Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature* **368,** 215–221.

Teh, H. S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Blüthmann, H., and von Boehmer, H. (1988). Thymic major histocompatibility complex antigens and the ab T-cell receptor determine the CD4/CD8 phenotype of T cells. *Nature* **335,** 229–233.

Thorsby, E. (1995). HLA-associated disease susceptibility—Which genes are primarily involved? *Immunologist* **3,** 51–58.

Tisch, R., Yang, X.-D., Singer, S. M., Liblau, R. S., Fugger, L., and McDevitt, H. O. (1993). Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. *Nature* **366,** 72–75.

Tiwari, J. L., and Terasaki, P. I. (1985). "HLA and Disease Associations." Springer-Verlag, Berlin/New York.

Todd, J. A., Acha-Orbea, H., Bell, J. I., Chao, N., Fronek, Z., Jacob, C. O., McDermott, M., Sinha, A. A., Timmerman, L., Steinman, L., and McDevitt, H. O. (1988). A molecular basis for MHC class II-associated autoimmunity. *Science* **240,** 1003–1009.

Tsuji, K., Aizawa, M., and Sasazuki, T. (1992). "Proceedings of the Eleventh International Histocompatibility Workshop and Conference." Oxford Science, Oxford.

Uchigata, Y., Omori, Y., Nieda, M., Kuwata, S., Tokunaga, K., and Juji, T. (1992). HLA-DR4 genotype and insulin-processing in insulin autoimmune syndrome. *Lancet* **340,** 1467.

Valli, A., Sette, A., Kappos, L., Oseroff, C., Sidney, J., Miescher, G., Hochberger, M., Albert, E. D., and Adorini, L. (1993). Binding of myelin basic protein peptides to human histocompatibility leukocyte antigen class II molecules and their recognition by T cells from multiple sclerosis patients. *J. Clin. Invest.* **91,** 616–628.

Van de Wal, Y., Kooy, Y. M. C., Drijfhout, J. W., Amons, R., and Koning, F. (1996). Peptide-binding characteristics of the Coeliac disease-associated DQ (A1°0501, B1°0201) molecule. *Immunogenetics* **44,** 246–253.

Vartdal, F., Johansen, B. H., Friede, T., Thorpe, C. J., Stevanovic, S., Eriksen, J. E., Sletten, K., Thorsby, E., Rammensee, H.-G., and Sollid, L. M. (1996). The peptide binding motif of the disease associated HLA-DQ (alfa 1° 0501, beta 1° 0201) molecule. *Eur. J. Immunol.* **26,** 2764–2772.

Verreck, F. A. W., van de Poel, A., Termijtelen, A., Amons, R., Drijfhout, J.-W., and Koning, F. (1994). Identification of an HLA-DQ2 peptide binding motif and HLA-DPw3-bound self-peptide by pool sequencing. *Eur. J. Immunol.* **24,** 375–379.

Vogt, A. B., Kropshofer, H., Kalbacher, H., Kalbus, M., Rammensee, H.-G., Coligan, J. E., and Martin, R. (1994). Ligand motifs of HLA-DRB5°0101 and DRB1°1501 molecules delineated from self-peptides. *J. Immunol.* **153,** 1665–1673.

Wauben, M. H. M., Joosten, I., Schlief, A., van der See, R., Boog, C. J. P., and van Eden, W. (1994). Inhibition of experimental autoimmune encephalomyelitis by MHC class II binding competitor peptides depends on the relative MHC binding affinity of the disease-inducing peptide. *J. Immunol.* **152,** 4211–4220.

Weiss, S. M., and Kulikowski, C. A. (1990). "Computer Systems That Learn." Kaufman, San Mateo, CA.

Winchester, R. (1994). The molecular basis of susceptibility to rheumatoid arthritis. *Adv. Immunol.* **56,** 289–466.

Wordsworth, B. P., Lanchbury, J. S., Sakkas, L. I., Welsh, K. I., Panayi, G. S., and Bell, J. I. (1989). HLA-DR4 subtype frequencies in rheumatoid arthritis indicate that DRB1

is the major susceptibility locus within the HLA class II region. *Proc. Natl. Acad. Sci. USA* **86,** 10049–10053.

Woulfe, S. L., Bono, C. P., Zacheis, D. A., Kirschmann, T. A., Baudino, C. S., Karr, R. W., and Schwartz, B. D. (1995). Negatively charged residues interacting with the p4 pocket confer binding specificity to DRB1°0401. *Arthritis Rheum.* **38,** 1744–1753.

Wucherpfennig, K. W., Sette, A., Southwood, S., Oseroff, C., Matsui, M., Strominger, J. L., and Hafler, D. A. (1994). Structural requirements for binding of an immunodominant myelin basic protein peptide to DR2 isotypes and for its recognition by human T cell clones. *J. Exp. Med.* **179,** 279–290.

Wucherpfennig, K. W., Yu, B., Bhol, K., Monos, D. S., Argyris, E., Karr, R. W., Ahmed, A. R., and Strominger, J. L. (1995). Structural basis for major histocompatibility complex (MHC)-linked susceptibility to autoimmunity: Charged residues of a single MHC binding pocket confer selective presentation of self-peptides in pemphigus vulgaris. *Proc. Natl. Acad. Sci. USA* **92,** 11935–11939.

Yacub, B. A., and Daif, A. K. (1988). Multiple sclerosis in Saudi Arabia. *Neurology* **328,** 621–625.

Zhang, J., Markovic-Plese, S., Lacet, B., Raus, J., Weiner, H. L., and Hafler, D. A. (1994). Increased frequency of interleukin 2-responsive T cell specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J. Exp. Med.* **179,** 973–984.

This article was accepted for publication on February 20, 1997.

ADVANCES IN IMMUNOLOGY, VOL. 66

Role of Cytokines in Sepsis

C. ERIK HACK,*,† LUCIEN A. AARDEN,* and LAMBERTUS G. THIJS‡

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Laboratory for Experimental and Clinical Immunology, University of Amsterdam, 1066 CX Amsterdam, The Netherlands; and †Department of Internal Medicine and ‡Department of Medical Intensive Care Unit, Free University Hospital, 1066 CX Amsterdam, The Netherlands

I. Introduction

The development of sophisticated host defense mechanisms is of vital importance for the human being to survive. These defense mechanisms are mediated by the release and activation of endogenous proteins and cells, called inflammatory mediators, in response to invading microorganisms. Within hours to days these mediators bring about the following: the microorganisms are localized and neutralized, dead and damaged cells are removed, and tissue repair starts. Host defense mechanisms are indispensable: Genetic or acquired deficiencies of these often results in an increased risk for infections. Man also has to deal with a number of diseases resulting from inappropriate or excessive activation of host defense mechanisms. A typical example is sepsis: This disease, which in the United States has an estimated incidence of 70,000–300,000 cases per year with mortality rates of 10–50% (1), results from an excessive and systemic release and activation of endogenous inflammatory mediators (2–4).

Sepsis is defined as the systemic response to infection (1, 5): Patients are said to have sepsis when they fulfill the criteria for systemic inflammatory response syndrome (SIRS) (at least two of the following: fever or hypothermia, tachycardia, tachypnea, or leukocytosis or leukopenia) and have evidence for the presence of an infectious process. Severe sepsis is defined as sepsis associated with organ dysfunction, hypoperfusion, or hypotension; septic shock is defined as sepsis with hypotension despite adequate fluid resuscitation, along with the presence of perfusion abnormalities such as lactic acidosis, oliguria, or altered mentation. Further details of these definitions are given elsewhere (1, 5).

Inflammatory mediators include cytokines; neutrophils, monocytes, macrophages, endothelial cells, platelets, and other cells; plasma protein cascade systems such as the complement, coagulation, fibrinolytic, and contact systems; tissue damaging proteinases; lipid mediators such as eicosanoids and platelet-activating factor; and oxygen and nitrogen radicals. In addition, during host defense reactions a number of agents are released that blunt the inflammatory response, such as anti-inflammatory cytokines, soluble

0065-2776/97 $25.00

cytokine receptors, acute phase proteins including proteinase inhibitors, and stress hormones.

Here we will review the role of cytokines in the pathogenesis of sepsis. Because much of our understanding of the role of inflammatory mediators in sepsis is based on studies in experimental models, we will first discuss some aspects of these models.

II. Models for Sepsis

A. Human Models

The human model for sepsis most frequently studied is experimental human endotoxemia: A low dose of endotoxin (2–4 ng/kg body wt) is given intravenously to healthy human volunteers. Usually, mild general constitutional and systemic responses, including a rise in core temperature, often initiated with chills and rigors (1–2 hr after the challenge), and varying degrees of headache, myalgias, arthralgias, and nausea, develop (6). Although hypotensive reactions are usually minimal, the cardiovascular responses following administration of endotoxin in part mimick those of sepsis (7). The endotoxin-induced clinical symptoms resolve within 24 hr and are reduced by specific endotoxin-neutralizing agents such as recombinant bactericidal/permeability-increasing protein (8). A model resembling human experimental endotoxemia, with respect to clinical signs, dose of endotoxin used, the release of cytokines, and the activation patterns of coagulation, fibrinolysis, and neutrophils, was developed in chimpanzees (9). This primate model provides an alternative for studies in man, in particular when investigating the effect of specific interventions. The course of circulating cytokines following a challenge with a low dose of endotoxin is reproducible and characteristic: Tumor necrosis factor-α (TNFα) is the first cytokine to appear in plasma after the challenge (typically after 45–60 min with peak levels at 90 min), followed by interleukin-1β (IL1), which reaches peak levels at 2 or 3 hr, and finally by IL6, IL8, IL10, IL12, monocyte chemotactic protein-1 (MCP-1), leukemia inhibitory factor (LIF), and granulocyte colony-stimulating factor (G-CSF).

B. Animal Models

Various animal models for sepsis have been described. It is beyond the scope of this review to discuss in detail these models, this has been done elsewhere (10). In general, sepsis is induced in these models by intravenous administration of live or dead microorganisms, mostly *Escherichia coli,* or endotoxin and usually evolves relatively rapidly (compared to human sepsis) and mortality or recovery follows within 1 or 2 days. Hence, these models provide no or only limited information about long-standing (days to weeks)

septic processes, which frequently occur in clinical practice. Furthermore, the challenge is usually given over a short period (up to 2 hr) and the inflammatory mediators are activated and released in a more or less reproducible sequence. In clinical sepsis, however, exposure to the challenge occurs in a less standardized way because the entrance of microorganisms or endotoxin often is more chronic and repetitive. This may allow synergisms and antagonisms between mediators to take place that are not apparent in the animal models. Other aspects of the animal models include the size and the nature of the animal and its sensitivity to endotoxin. Small animals, in particular mice, are suitable to determine the LD100, whereas larger animals (dogs and pigs) are more suitable for hemodynamic studies. Primates allow an extensive analysis of the course of inflammatory parameters in blood because assays developed for measurements in humans often can be used in primates. Notably, a challenge with high doses of endotoxin or large numbers of gram-negative bacteria in animals relatively insensitive to endotoxin, such as baboons, may induce phenomena [for example, substantial activation of complement due to direct interaction with endotoxin (11)] that do not occur in animals that are more sensitive to endotoxin. Finally, the intravenous administration of endotoxin or live bacteria bypasses local defense mechanisms. Hence, interventions that potentially impair these local defense mechanisms (such as anti-TNF) should be evaluated in more appropriate models, for example, that of cecal ligation and puncture.

III. Cytokines

Cytokines mediate communication between cells of the body. They resemble classical hormones in that they exert their effects at picomolar–femtomolar concentrations and affect the function of target cells by binding to specific cellular receptors. Binding of cytokines to their receptors often transduces signals into the target cell, leading to an altered functional state of these cells [see for review Refs. (12)-(15)]. Cytokines usually have an auto- or paracrine mode of action, although in some situations, in particular in sepsis, they exert effects at a distance from their site of production. Unlike classical hormones, most cytokines have overlapping functions, can be produced by many cells in the body, and also have multiple target cells. Consequently, their biologic effects are pleiotropic. Cytokines may be grouped based on their main biological effects: those with main effects on activation and proliferation of lymphocytes, those with effects on hematopoiesis, those with pro- and anti-inflammatory properties, and those that orchestrate cell growth and differentiation during tissue repair. A breakthrough regarding the role of cytokines in sepsis was the observation by

Cerami and co-workers (16) that neutralizing antibodies against endogenous TNF could protect mice against the lethal effects of endotoxin. This observation not only for the first time demonstrated a pathogenic role of cytokines in sepsis but also illustrated that this disease results from an overreacting host defense rather than from the release of bacterial toxins (17, 18). Subsequent studies have provided compelling evidence that in particular IL1, IL6, chemokines such as IL8 and MCP-1, IL10, IL12, IL1 receptor antagonist (IL1-ra), macrophage migration inhibition factor (MIF), LIF, G-CSF, interferon-γ (IFNγ), and TNF are involved in the pathogenesis of sepsis and septic shock.

The notion that cytokines are involved in the pathogenesis of sepsis is based on several lines of evidence: (i) Intravenous administration of cytokines [TNF, IL1, and IL2 (19, 20)] induces a septic shock-like syndrome in animals or humans; (ii) inhibition of the effects of some cytokines [TNF, IL1; IFNγ; IL6 (inconsistently), IL12, LIF, and MIF) by administration of neutralizing antibodies, soluble cytokine receptors or receptor antagonists attenuates sepsis in animal models; (iii) administration of anti-inflammatory cytokines, such as IL10, mitigates severe sepsis in animals; and (iv) plasma levels of some cytokines (TNF, IL1, IL6, IL8, IL10, IL12, IFNγ; MCP-1, G-CSF, LIF, and MIF) increase in experimental models for sepsis in animals as well as in humans and may be elevated in septic patients. Therefore, many investigators consider cytokines as pivotal mediators involved in the pathogenesis of sepsis (17, 21, 22).

IV. Measurement of Circulating Cytokines

Although animal models have greatly contributed to our understanding of the role of cytokines in sepsis, most of our knowledge of their role in human sepsis is based on studies of circulating levels in patients. Circulating levels of cytokines are, however, difficult to interpret. In general, cytokines have a rather short half-life time of clearance, on the order of minutes. The results of studies on circulating levels thus will be greatly influenced by timing of blood sampling and by the strength and duration of the stimulus for synthesis. Circulating levels may, therefore, not correctly reflect the synthesis of cytokines. This notion is substantiated by the observation that mice deficient for the 75-kDa TNF receptor have higher levels of circulating TNF upon a challenge with endotoxin than normal mice (23) indicating the importance of receptors for the clearance of cytokines. Furthermore, a study in septic patients treated with a TNF receptor–Fc fusion protein, which prevents TNF from binding to its cellular receptors and hence induces accumulation of TNF in the circulation, demonstrated that in only 4% of the 141 patients TNF was detected in the circulation

before treatment, whereas TNF was detected in 40% after treatment (24). Apparently, TNF was synthesized in considerably more patients then was evident from circulating levels before treatment. Similarly, circulating IL6 has been shown to increase in patients with multiple myeloma following administration of a monoclonal antibody that prevents binding of IL6 to its receptor. It is also not known to what extent complications of sepsis, such as diminished function of kidneys and other organs, may influence synthetic and clearance rates of cytokines. Finally, it is to be noted that the immunoassays used to detect circulating cytokines do not perform similarly. For example, TNF bound to its soluble receptor is not detected in immunoassays in which monoclonal antibodies against the receptor binding site of TNF are used (25). It is, therefore, not surprising that significant differences in TNF results were found when five different assays for TNF were evaluated in patients with adult respiratory distress syndrome (26). Thus, circulating levels of cytokines should be interpreted with caution.

V. Tumor Necrosis Factor and Interleukin-1

The cytokines TNF and IL1 are major endogenous mediators of sepsis. Although they bind to different cellular receptors and differ in their three-dimensional structure, both cytokines have multiple overlapping and synergistic activities (15, 27, 28). For example, either cytokine is able to induce a septic shock-like state in animals upon intravenous administration; inhibition of either cytokine in animal models for sepsis may attenuate the severity of the disease and protect the animals against the lethal consequences of a bacterial challenge. Because their activities largely overlap, TNF and IL1 are discussed together.

A. Biochemistry of TNF and IL1

The history of TNF started at the end of the last century when an American surgeon, William Coley, explored the possibility to treat cancer by administering broths of cultures of Streptococcus and Serratia microorganisms to patients (18). The active principle of the broths was discovered to be endotoxin, which itself did not kill tumor cells but induced the release of an endogenous "tumor necrosis factor" into the circulation. In 1985 this factor was isolated and characterized (29). Independently, Cerami and co-workers (30) in 1985 cloned the gene of "cachectin," a protein mediating wasting of chronic disease. TNF and cachectin turned out to be the same protein (31). TNF shares 28% amino acid sequence homology with lymphotoxin (32). Both cytokines bind to the same receptors, but their synthesis is differentially regulated (33). Because of closely overlapping activities

with TNF, lymphotoxin is sometimes called TNFβ, whereas TNF is indicated with the suffix "α." Because there is no evidence for its involvement in the pathogenesis of septic shock, we will not discuss lymphotoxin further. The term TNF will be used here to denote TNF-α.

Biologically active TNF consists of a trimer of three identical polypeptide chains with a molecular mass of 17 kDa each (34, 35). The 17-kDa subunit originates from a 26-kDa precursor protein, which exists as a membrane-associated protein (36). Membrane-associated 26-kDaTNF is believed to constitute a killing mechanism of activated macrophages (37). The proteinase cleaving the precursor protein into the 17-kDa mature form, TNF convertase, has been identified as a metalloproteinase (38–40). Very recently, it has been cloned (41, 42). Inhibition of TNF convertase *in vivo* reduces circulating TNF levels following a challenge with endotoxin and reduces mortality (38), indicating that conversion of membrane-bound TNF into its soluble form is essential for the development of lethal endotoxin shock. Whether metalloproteinase(s) is the only TNF convertase is debated (43): Proteinase 3, a serine proteinase expressed by phagocytic cells, may also function as such (43, 44). The TNF gene is located on the short arm of chromosome 6 in man, near to that of lymphotoxin (45), indicating that both genes presumably have originated from tandem duplication of an ancestral gene. In addition, the TNF gene is located near the major histocompatibility complex (MHC) genes, which may explain the linkage between some MHC genotypes and the potency to generate TNF.

The cytokine IL1 has been studied for many years under various names including endogenous pyrogen and lymphocyte-activating factor (27, 46). Purification and cloning of complementary DNA coding for these factors revealed that these activities were mediated by two structurally related cytokines, termed IL1α and IL1β (47–52). Both IL1 species are synthesized as 31-kDa precursor molecules, which lack a hydrophobic signal peptide characteristic for secretory proteins (50, 51, 53). IL1α and IL1β share 26% amino acid sequence homology (51). The mature forms of IL1 have a molecular mass of 17 kDa. In contrast to IL1α, IL1β requires cleavage of the precursor for optimal activity (54). It has been speculated that IL1α functions mainly as an intracellular messenger (54). In addition, cell injury will result in the release of intracellular IL1α, which then will act as an extracellular mediator of tissue damage. In contrast to IL1α, IL1β can be detected in the circulation and appears to function primarily as an extracellular mediator consistent with the notion that its biological activity is mediated by the 17-kDa form only. How IL1β is secreted by cells is not clear (55). IL1β precursor is proteolytically processed by IL1β converting enzyme (ICE) (56–58), a cysteine proteinase (59–61) also involved in the execution phase of the apoptotic machinery (62–64). Specific

inhibitors of ICE have been developed and indeed are able to prevent the formation of mature IL1β *in vivo,* while having no effect on the release of IL1α (65).

A third member of the IL1 family showing 26 and 19% amino acid sequence homology to human IL1β and IL1α, respectively (66, 67), has been identified. This cytokine differs from IL1α and IL1β in at least two aspects: (i) Its sequence contains a hydrophobic signal peptide, a feature of secretory proteins; and (ii) though it binds to IL1 receptors (IL1-R) and competes with IL1α and IL1β for this binding, it does not transduce signals into the cell interior and, hence, to activate the cell. Thus, this third member of the IL1 family behaves as a receptor-antagonist and inhibits the function of IL1α and IL1β, and, hence, has been called IL1-ra (15, 68). Additional forms of IL1-ra lacking the signal peptide sequence have been identified (69), although their precise function is not clear.

B. Receptors for TNF and IL1

Both TNF and IL1 exert their effects by binding to specific cellular receptors. TNF-receptors (TNF-R) originally were identified by investigators looking for TNF inhibitors in biological fluids such as human urine. The inhibitors found turned out to be soluble TNF-R shed from cells. Two different TNF-R have been cloned and characterized: TNF-R with a molecular mass of 55 kDa (TNF-R55) and TNF-R of 75 kDa (TNF-R75), respectively (70–75). These receptors are homologous to a number of other receptors, which all together constitute the TNF receptor protein family (75). Most cells and tissues express both types of receptors. Like many receptors, TNF-Rs have an extracellular, a single hydrophobic transmembrane, and an intracellular domain. The sequence of the intracellular domains of either TNF-R is different and does not provide any clue regarding the mechanism of signal transduction via these receptors (76). The extracellular parts show amino acid sequence identity of approximately 25%. A TNF trimer has three binding sites for TNF-R, each located at the interface between two subunits. Thus, one TNF trimer molecule can cross-link up to three TNF-Rs. This aggregation of TNF-Rs presumably is sufficient for signal transduction. *In vitro* studies have indicated that the cytotoxic and most inflammatory effects of TNF are mediated via TNF-R55, whereas proliferative effects are triggered via TNF-R75 (77–80). Furthermore, the transmembrane form of TNF preferentially triggers TNF-R75 (81). Attempts to map the binding sites for TNF on TNF-Rs have indicated that different domains of TNF-R55 and TNF-R75, and, hence, likely different domains on TNF, are involved (82). Indeed, TNF mutants that selectively bind to either TNF-R55 or TNF-R75, have been developed. Studies with these mutants have confirmed that the effects of

TNF *in vivo* on the induction of shock and on the release and activation of other inflammatory mediators, such as IL1, IL6, IL8, coagulation, and so on, are mediated via TNF-R55, except for the release of secretory phospholipase A_2, which mainly occurs via triggering of TNF-R75 (83, 84).

IL1-Rs were initially identified using radiolabeled IL1 as a probe. Two different IL1Rs have been cloned and characterized: IL1-R with a molecular mass of 80 kDa (IL1-R type I) and IL1-R of 68 kDa (IL1-R type II), respectively (85–87). IL1-R type I occurs on many cells in the body, including T cells, endothelial cells, fibroblasts, synovial lining cells, chondrocytes, keratinocytes, and hepatocytes. The distribution of IL1-R type II is more restricted: This IL1-R is found only on B cell lineages, neutrophils, and bone marrow cells (88). Under physiological conditions cells express only a few hundred IL1-Rs, but under inflammatory conditions up to 20,000 per cell. It is now clear that IL1-R type I mediates signaling of cells by IL1, whereas IL1-R type II does not (52, 89). Rather, this latter receptor antagonizes IL1 action by taking away IL1 from IL1-R type I. Thus, in addition to IL1-ra, IL1-R type II also inhibits IL1 by serving as a decoy to lessen the likelihood of a productive interaction of IL1 with IL1-R type I (90). Apparently, the effects of IL1 may be so toxic that the activity of this cytokine needs to be fine-tuned by two different mechanisms.

C. Regulation of TNF and IL1 Activity

TNF and IL1 are toxic cytokines, which nevertheless are necessary for some defense mechanisms. Presumably, because of this toxicity, the activities of TNF and IL1 *in vivo* are tightly controlled to prevent excessive activities of these potentially detrimental molecules. Control occurs at several levels. Transcription of the TNF and IL1 genes is regulated by nuclear factors such as NF-κB and is suppressed by short-lived repressors (91). mRNA transcripts of the TNF gene have a relatively short half-life time due to the presence of UA-rich sequences in the 3′ untranslated region (92), that also occur in mRNA transcripts of other genes encoding for inflammatory cytokines. This posttranscriptional control prevents too abundant synthesis of TNF and IL1 once their genes have been transcribed. Differences in the regulatory sequences of the TNF gene potentially may influence the response of TNF following a microbial challenge. In patients with severe sepsis a genomic polymorphism within the TNF locus has been found to be associated with outcome and the TNF response (93).

The activity of TNF in biological fluids is controlled at several levels. Proteinases released by activated neutrophils can inactivate TNF (94). Similarly, IL1 can be degraded by matrix metalloproteinases (95). It is unknown whether proteolytic inactivation is a regulatory mechanism of IL1 and TNF *in vivo*. The control exerted by soluble forms of TNF

receptors (sTNF-Rs) has been claimed to be more important for regulation of TNF activity. Originally, these sTNF-Rs have been purified from urine and characterized as proteins that bind and inactivate TNF (72). Later on these TNF-binding proteins were found to represent soluble forms of the two TNF-Rs, more precisely their extracellular parts (75, 96, 97). *In vitro* studies suggest that TNF-R75 is mainly released by blood mononuclear cells, whereas TNF-R55 is shed by endothelial cells (98). The precise mechanism of how TNF-Rs are released by cells is not known, although it likely involves proteolysis. Release of TNF-R75 from neutrophils may be mediated by elastase as well as other proteinases (99). Recent observations suggest a metalloproteinase, possibly identical to TNF convertase, to be responsible for cleavage of either TNF-R (100, 101). Assuming a K_D of 10^{-10} M (76), one can calculate that at normal concentrations of sTNF-R [25×10^{-12} M for sTNF-R55 and 100×10^{-12} M for sTNF-R75 (76, 102)] about 50% of TNF is bound by circulating sTNF-Rs. Similarly, at 10-fold increased concentrations, which may occur during sepsis, approximately 90% of TNF will be bound to sTNF-Rs. Enhanced affinity has been shown for constructs consisting of Fc and hinge region of IgG and sTNF-Rs (103), which, therefore, are potent inhibitors of the activity of TNF. In particular sTNF-R55–IgG constructs seem to be suitable inhibitors, whereas sTNF-R75–IgG constructs are less powerful because TNF dissociates relatively easily from this receptor (104). Although sTNF-Rs significantly attenuate TNF activity, it is unlikely that this inhibition is sufficient to neutralize toxic activities of TNF in sepsis, because even at the highest concentrations of sTNF-Rs, 10% of TNF is still active. Observations in patients have shown that the increase of sTNF-Rs during sepsis is not sufficient to counteract TNF activity (105, 106). Moreover, at lower concentrations sTNF-Rs also may stabilize TNF activity, presumably by preventing the TNF trimer to dissociate into inactive monomers (107). In our view the most likely role for sTNF-Rs is due to their effects on the half-life of TNF: Interaction of TNF with its soluble receptors greatly enhances this half-life by serving as a reservoir that slowly releases bioactive TNF.

The activity of IL1 in biological fluids is also regulated by soluble receptors; for example, a soluble IL1-R type II has been identified (108). This receptor preferentially binds (and neutralizes) IL1β but not IL1-ra (109) and also inhibits processing of IL1β precursor protein into active IL1β (109). IL1-R is, among others, released from the surface of neutrophils in response to TNF or lipopolysaccharide (LPS) (110). Circulating levels of IL1-R II are increased by approximately 40-fold in sepsis (89). The activity of IL1 is also regulated in another unique way, i.e., via the production and release of the IL1-ra. The synthesis and release of this cytokine is differentially regulated compared with that of IL1 (55, 111–114). *In vitro*

studies indicate that IL1-ra should be present at 100-fold excess over IL1α or IL1β to achieve 50% inhibition of the effects of these cytokines (115). As explained previously, membrane-bound IL1-R type II serves as a decoy. Therefore, the activity of IL1 is also regulated by the ratio of type I and II receptors on cells.

The synthesis and release of TNF and IL1 are influenced by many other factors. Norepinephrine, PAF, granulocyte/macrophage colony-stimulating factor (GM-CSF), the complement activation product C5a, the acute phase protein C-reactive protein (CRP), engagement of CD11b/CD18, and nitric oxide may enhance the synthesis of TNF and IL1, whereas glucocorticosteroids, epinephrine, eicosanoids, and the cytokines IL4, IL10, IL13, and transforming growth factor-β (TGFβ) decrease the synthesis of IL1 and TNF by mononuclear cells stimulated by endotoxin (116–142). *In vivo* some of these agents also are important (127). For example, adrenalectomy enhances susceptibility for TNF and IL1 (143). In contrast, nitric oxide (NO) does not seem to have an influence on TNF and IL1 release *in vivo* upon a challenge with endotoxin because mice deficient for inducible NO synthase have comparable levels to wild-type mice (144). The synthesis of TNF and IL1 by peripheral blood mononuclear cells is depressed in patients with sepsis (145, 146), but whether this is due to the action of one or more of the agents just listed remains to be established.

D. *In Vitro* Effects of TNF and IL1

TNF and IL1 have considerably overlapping actions and synergize in many effects (147). In addition, IFNγ synergizes at least in some of the TNF/IL1-induced effects, possibly because it enhances transcription of the genes of either cytokine (91). Binding of TNF and IL1 to their cellular receptors induces activation and generation of a number of second messengers such as G proteins, adenyl cyclase, phospholipases A_2 and C, and oxygen free radicals. In addition, a number of genes are transcribed, including those for the adhesion molecules intercellular adhesion molecule-1 (ICAM-1), endothelial–leukocyte adhesion molecule (ELAM); those for the clotting and fibrinolytic proteins tissue factor, urokinase-type plasminogen activator and plasminogen activator inhibitor-1; those for the proinflammatory cytokines IL1, IL6, IL8, and TNFα and the anti-inflammatory cytokines IL4, IL10, and IL1-ra; that for secretory phospholipase A_2; that for inducible nitric oxide synthase; and that for cycloxygenase [reviewed in Refs. (12), (15), and (88)]. The actual effects of these processes strongly depend on the cell type stimulated. In addition, in susceptible cells, binding of TNF may induce death via apoptosis or even necrosis, depending on the metabolic state (12, 148).

Both TNF and IL1 have multiple cell-specific effects (27, 88, 120, 149, 150). Only those relevant for the pathogenesis of sepsis are discussed here. Interaction of TNF and IL1 with endothelial cells is presumably of major importance for inflammatory reactions. These interactions induce the following effects: enhancement of permeability (151); expression of tissue factor and downregulation of thrombomodulin, which reduces the anticoagulant properties and enhances the procoagulant activity of the endothelial surface (151–156); expression of adhesion molecules such as ICAM-1, ELAM, and vascular cell adhesion molecule (VCAM)-1 (27, 157–162); production of hematopoietic growth factors, IL1, IL6, probably LIF, IL8, platelet-derived growth factor, MCP-1, RANTES (regulated on activation normal T expressed secreted chemokine), PAF, and prostaglandins PGE_2 and PGI_2 (27, 149, 163–173); generation of nitric oxide and related compounds via the induction of NO synthase and cat-2, the transporter of L-arginine (174–177); generation of the vasoconstrictor peptide endothelin-1 (178); and the release of urokinase-type plasminogen activator as well as the synthesis of plasminogen activator inhibitor-1 (179–184). Part of the effects of IL1 and TNF are due to a decrease of cyclic adenosine monophosphate (cAMP) and an increase of cyclic guanosine monophosphate (cGMP) in the endothelial cells (151, 185). The increase of cGMP does not only result from the induction of nitric oxide but also occurs through a novel nitric oxide-independent pathway (185). Notably, some effects of TNF and IL1 on the endothelium (induction of VCAM-1 expression and release of IL6 and IL8) are counteracted by nitric oxide (186), which therefore may constitute a negative feedback for IL1/TNF-induced effects.

Interaction of TNF with neutrophils induces an increased expression of surface adherence glycoproteins, C3bi, receptors, and L-selectin; augmentation of superoxide production, phagocytosis, and enzyme release, and aggregation of the cells (27, 149, 187–191). The effects of IL1 on neutrophil function are less clear (55, 88). Some evidence suggests that IL1 can prime neutrophils for other agonists (88), although this effect is probably weak compared to that of other cytokines such as TNF, GM-CSF, or IL8 (192). TNF as well as IL1 induce leukocytosis by stimulating the release of neutrophils from the bone marrow (27, 150). Interaction with macrophages increases the cytotoxic capacity and the production of oxygen radicals and induces the release of other cytokines such as IL1, IL6, and IL8, and PAF and prostaglandin E_2 (149, 150). TNF and IL1 induce the production of many other proteins by mononuclear and other cells. Among these are cycloxygenase-2 (193, 194) and the protein TSG-6, which has the interesting feature that it enhances the inhibition of plasmin by inter-α-trypsin inhibitor. Presumably via this effect TSG-6 is able to attenuate the inflam-

matory potential of locally administered IL1 (195). Thus, besides stimulating proteins that enhance inflammation, TNF and IL1 also induce the synthesis of proteins that limit their proinflammatory effects.

E. *In vivo* Effects of TNF and IL1

A key observation suggesting a central role for TNF and IL1 in the pathogenesis of sepsis is that the intravenous administration of these cytokines to animals induces hemodynamic alterations that closely resemble those seen in experimental endotoxemia and sepsis (196–202). Moreover, both cytokines potentiate each other *in vivo* (147, 201). The mechanism of the hypotension induced by TNF and IL1 is not completely clear. It may be related to the direct effects of either cytokine on endothelial cells including enhancement of permeability and cytotoxic effects (148, 203–205). However, we favor the hypothesis that the hypotension induced by either cytokine is at least in part due to activation of other mediator systems: Hypotensive effects of TNF and IL1 are dependent on the production of NO (206) and prostaglandins (201, 207). The nature of the agonists inducing NO is not clear. Either TNF and IL1 directly stimulate the synthesis of the inducable and calcium-independent form of NO synthase or they induce the generation of agonists for the constitutive NO synthase, such as bradykinin or thrombin. In agreement with their supposed interactions with the endothelium, TNF or IL1 can induce an increase in circulating soluble intercellular adhesion molecule following intravenous injection into mice (208).

Histological studies in animals that have received either TNF or IL1 strongly suggest involvement of activated neutrophils and fibrin formation in the pathogenesis of the toxic effects of either cytokine (28, 195, 200, 201, 209–213). Indeed, locally injected TNF or IL1 induces infiltration of neutrophils (214). Interestingly, infiltration of neutrophils at sites injected with TNF or IL1 is enhanced by the presence of thrombin (215) but not by IL8 or C5a. Apparently, the active site of thrombin is required for this effect on TNF-induced neutrophil infiltration. In addition to TNF and IL1, other cytokines, in particular IL8, are involved in the recruitment and activation of neutrophils in sepsis (216). The role of neutrophils in the detrimental effects of TNF and IL1 is illustrated by observations showing that the permeability changes of the endothelium following administration of TNF or IL1 are more pronounced in the presence of neutrophils (217–219). The dependency of TNF and IL1 on neutrophils to mediate toxicity may explain why oxygen radical scavengers enhance tolerance for TNF in mice (220) and also why α1 acid glycoprotein, which may interfere with the adherence of neutrophils to the endothelium (221), reduces the lethality of TNF (222). Also in humans TNF is able to activate and degranu-

late neutrophils *in vivo* (223). When occurring intravascularly, this may cause neutropenia (due to adherence to the endothelium with subsequent damage to the latter) as well as impaired responses to chemoattractant agents locally produced resulting in less intense neutrophil infiltration in the tissues (224). Low doses of IL1β (3 ng/kg) injected intravenously in patients with metastatic cancer do not induce degranulation of neutrophils, although they cause leukocytosis (225). Whether degranulation of neutrophils in humans may occur at higher doses of IL1 remains to be established. In baboons, however, much higher concentrations of IL1α did not induce degranulation of neutrophils as assessed by measuring circulating elastase (226).

TNF has several effects on other inflammatory mediators *in vivo* including activation of coagulation and fibrinolysis. Typically, coagulation proceeds for hours, whereas fibrinolysis is only shortly (approximately 60 min) activated to become inhibited thereafter, resulting in a so-called procoagulant state (227–230). Remarkably, in baboons a dose of 100 μg of TNF per kilogram of body weight did not induce the formation of thrombin–antithrombin III complexes unless a monoclonal antibody that blocks the anticoagulant protein C was coadministered (231). One may therefore speculate that the responsiveness of the clotting system to TNF may differ in various animal species. In humans, a procoagulant state can also be induced by a low dose of IL1β (3 ng/kg body wt) (225). Notably, both TNF and IL1 are able to induce the release of tissue-type plasminogen activator (tPA) into the circulation, which is in contrast with observations *in vitro* that either cytokine decreases the synthesis of tPA by endothelial cells. A possible explanation is that TNF and IL1 induce the release of vasopressin (232), which in turn causes the release of tPA (233). Furthermore, TNF is able to induce the release of IL1, GM-CSF, macrophage colony-stimulating factor (163), IL6 (223), IL8 (234), IL10 (235), and IL1-ra (236)—effects shared with IL1 (225, 237). In addition to these effects, observations in patients indicate that recombinant TNF is also able to activate the complement and contact systems (238). Such an activation does not occur following administration of a low dose of endotoxin (239–242) ruling out the possibility that the observed activation is due to contaminating endotoxin and indicating that the amount of TNF released during experimental endotoxemia is insufficient to induce complement activation. Complement activation also occurs in patients receiving high doses of interleukin-2 (243–245), which therapy induces the release of TNF (246, 247). Some studies indicate that complement activation by TNF contributes to the tissue injury induced by this cytokine (248–250). Whether IL1 can similarly induce activation of complement system is unknown.

TNF and IL1 have remarkable effects on the heart. They exert a reversible negative inotropic effect on adult cardiomyocytes, presumably by interfering with intracellular calcium homeostasis resulting in reduced peak intracellular calcium levels during contraction (251). In addition, TNF, IL1, and IFNγ may induce myocyte death by stimulating inducable nitric oxide synthase, which is inhibited by TGFβ (252). The molecular mechanisms via which nitric oxide exerts its negative effects on cardiomyocytes are not precisely known but may involve reversible inhibition of mitochondrial activity (253). These myocardial effects also occur *in vivo:* Administration of TNF to animals induces myocardial depression (254, 255), whereas neutralizing anti-TNF antibody largely prevents myocardial suppression during endotoxemia (256). The negative inotropic effect by TNF is partially independent (251, 255, 257) and partially dependent on the generation of nitric oxide (252, 258, 259). TNF (and IL1) may, therefore, represent a "cardiac depressant factor" that circulates during sepsis and is held responsible for the myocardial dysfunction that occurs in sepsis.

TNF is well known for its property to induce cells to go into apoptosis *in vitro.* Also, during endotoxemia this cytokine induces apoptosis in organs and, therefore, may be involved in the pathogenesis of multiple organ failure, which often complicates sepsis (260). Furthermore, TNF and IL1 not only mediate inflammatory reactions but also have metabolic and neuroendocrine effects *in vivo.* Although they likely play a role in sepsis, these effects are beyond the scope of this review and will not be discussed here [for review, see Refs. (120), and (261)].

Finally, TNF and IL1 can substitute for the priming dose of endotoxin in the Shwartzman reaction, in particular when both cytokines are given simultaneously (28).

F. Role of TNF and IL1 in Experimental Endotoxemia

Michie and co-workers (262) were the first to demonstrate that the intravenous injection of a low dose of endotoxin elicits the release of TNF in human volunteers with peak levels at 90 min following the challenge. This observation has been confirmed by a number of studies in humans (102, 138, 239, 263–269) as well as in chimpanzees (9, 242). Levels are reduced upon pretreatment with epinephrine (138). Much higher levels than those than occur during experimental endotoxemia have been observed following (self)administration of a toxic amount (1 mg) of Salmonella endotoxin in man (270). The precise role of TNF in mediating clinical signs and symptoms during experimental endotoxemia is not clear. The magnitude of the TNF release correlates with the extent of clinical signs, the degree of neutropenia, and the release of ACTH, suggesting TNF as the main substance mediating these events (262). Ibuprofen pretreatment

consistently has been found to blunt the clinical response to a low dose of endotoxin, but the effect of this drug on the release of TNF is variable: It had no effect in one (262) and enhanced this release in others (102, 263, 267). These results suggest not only that most of the clinical symptoms of experimental endotoxemia are due to the generation of prostaglandins but also that the formation of these lipid mediators forms a negative feedback loop for the synthesis of TNF. Remarkably, in a more severe model for sepsis, i.e., pigs challenged with live *Pseudomonas aeruginosa*, ibuprofen not only attenuated the septic syndrome but also reduced (rather than increased) circulating TNF (271). The phosphodiesterase inhibitor pentoxifylline is able to inhibit TNF synthesis *in vitro*. Administration of pentoxifylline to healthy volunteers induces hyporesponsiveness of blood mononuclear cells to produce TNF upon stimulation with endotoxin (272). Intravenous administration of this drug also reduces the release of TNF during experimental endotoxemia in human volunteers. However, pentoxyfylline does not abrogate or reduce the clinical responses of experimental endotoxemia (269), suggesting that these responses at least in part are not mediated by TNF. Similarly, clinical symptoms were also not affected by pentoxyfylline in a chimpanzee model for experimental endotoxemia, although this drug attenuated the activation of neutrophils, the release of TNF and IL6 but not that of IL8 (242), and the release of tissue-type plasminogen activator (273). Thus, although pentoxyfylline is able to reduce the release and activation of a number of inflammatory mediators including TNF, in experimental endotoxemia it has no effect on the clinical symptoms suggesting that these symptoms are not induced by these mediators. In agreement herewith, administration of anti-TNF mAb in the same model also had no effect on the clinical symptoms (273, 274). In contrast to pentoxyfylline, anti-TNF also markedly reduced the release of IL8, suggesting that IL8 is induced by TNF during experimental endotoxemia. Furthermore, in humans the administration of a TNF-R75–IgG construct did not affect clinical symptoms of experimental endotoxemia, although it did reduce the release of IL1β, IL8, IL1-ra, and G-CSF (275). Finally, in the chimpanzee model inhibition of PAF reduced the release of TNF (276). Together these data indicate that during experimental endotoxemia TNF mediates the release of other cytokines, the degranulation of neutrophils, and the activation of fibrinolysis (but not that of coagulation), but also that this cytokine is not the main mediator inducing clinical symptoms of experimental endotoxemia. These symptoms are likely due to direct interaction of endotoxin with cells because they are attenuated by agents that specifically neutralize endotoxin activity (8).

Not only TNF but also its soluble receptors are released during experimental endotoxemia in humans and in chimpanzees, reaching peak values

at 2 or 3 hr after the challenge (102, 106, 277–279). This behavior of soluble TNF-Rs has been proposed to explain why some inflammatory mediators are released and activated in a biphasic pattern during experimental endotoxemia (279) because decreasing concentrations of sTNF-R favor the release of bioactive TNF from these receptors. Several observations suggest that TNF itself is not involved in the release of its receptors: In the chimpanzee model the release of sTNF-R was hardly influenced by pentoxifylline (277), although this drug significantly reduced TNF release (242). Similarly, ibuprofen caused a minimal increase of sTNF-R75 and had no effect on the course of circulating sTNF-R55 during experimental endotoxemia, whereas it induced a nearly twofold increase of the peak levels of TNF (102). Finally, in mice injected with endotoxin the release of sTNF-R was not affected by neutralizing anti-TNF antibodies (280). Conversely, in the chimpanzee endotoxin model a PAF inhibitor was able to reduce the increase of sTNF-R (276) suggesting a role for PAF in the endotoxin-induced increase of sTNF-R. The significance of the increase of sTNF-R during endotoxemia is not clear, although one may speculate that it contributes to the blunting of the clinical and inflammatory responses.

An increase of circulating IL1 during human experimental endotoxemia has been found inconsistently: In some studies an increase of circulating IL1 was not detected (237, 239, 262, 263, 265, 267), whereas in others IL1β was reported to increase at 2 or 3 hr following the endotoxin challenge (264, 266, 281, 282). We have compared the effects of intravenous administration of TNFa or IL1β on other inflammatory mediators in healthy volunteers (TNF; 223, 227, 228) or patients with cancer (IL1β; 225): Except for degranulation of neutrophils, which was not observed following the administration of IL1β, the effects of TNF at a dose of approximately 1 μg/kg were more or less comparable with those of IL1β given at a dose of 3 ng/kg. Thus, on a weight basis IL1β is more toxic than TNFα, at least in man. Consequently, one reason for the discrepant results regarding IL1 measurements in humans during experimental endotoxemia and in sepsis is that IL1β, because of its potential toxicity, circulates at rather low levels, even in these conditions. These levels may simply not be detected by most immunoassays.

The role of IL1 in mediating the release and activation of other inflammatory parameters or the development of the clinical symptoms of experimental endotoxemia is not clear. In one study (282) coadministration of IL1-ra did not change the clinical symptoms, including fever, and did not modify the release of the cytokines IL1, TNF, IL6, IL8, and G-CSF, whereas it had only a mild effect on the neutrophilia. In another study IL1-ra also had no effect on objective clinical signs but had a minor effect

on the severity of the subjective symptoms of experimental endotoxemia (279). Also in this study IL1-ra had no significant effect on the release and activation of a number of inflammatory mediators (279). Experimental endotoxemia induces the release of not only IL1 but also L1-ra (138, 237, 276, 281). The increase of this antagonist occurs 30–60 min after IL1 levels start to rise (281). Moreover, levels of IL1-ra remain elevated for a longer period (up to 24 hr) than those of IL1 (return to baseline between 6 and 12 hr). In the chimpanzee model the release of IL1-ra depends in part on PAF (276), indicating that the inflammatory response induced by experimental endotoxemia contains a negative feedback loop at the level of IL1.

Although drugs such as ibuprofen mitigate the clinical signs of experimental endotoxemia, anti-TNF and anti-IL1 reagents have surprisingly little effects. This is puzzling because the latter agents reduce mortality in lethal models. A tentative explanation may be that during experimental endotoxemia the ratio between TNF and IL1 on the one side and sTNF-R, sIL1-R, and IL1-ra on the other is still in balance and that, consequently, the contribution of either cytokine to the clinical response is modest, whereas during lethal challenges the amount of TNF and IL1 produced exceeds by far the amount that can be handled by these cytokine inhibitors. Indeed, during experimental endotoxemia circulating levels of IL1-ra and sTNF-R are within the same range as those found in critically ill patients (106, 237). Furthermore, an imbalance between TNF and its soluble receptors has been claimed to occur in severe meningococcemia (105).

G. TNF and IL1 in Animal Models for Sepsis

TNF and IL1 are also released into the circulation following a (sub)lethal challenge with endotoxin or (live) bacteria (38–40, 65, 114, 127, 141, 209, 248, 264, 280, 283–307). Peak levels of TNF occur in these models approximately 1 or 2 hr after the challenge, whereas IL1 reaches its summit 1 hr later. In animal models for T cell-mediated lethal shock induced by superantigens such as staphylococcal enterotoxin B TNF is released into the circulation, reaching peak values 30–60 min after the intravenous injection of the superantigen (288, 308). Furthermore, circulating levels of TNF also increase following a challenge with gram-positive bacteria (309). It is often assumed that the release of IL1β is more important than that of IL1α during endotoxemia. However, some studies report that not only IL1β but also IL1α increase following a challenge with endotoxin, with the highest levels occurring at 1.5–3 hr after the challenge (65, 301, 304). This raises the possibility that IL1α is also involved in the pathogenesis of (sub)lethal sepsis. Indeed, mice made deficient for IL1β by targeted gene disruption had similar levels of IL1α, IL6, and TNF

following a challenge with a low or a high dose of endotoxin (301, 310). Comparable results, i.e., reduced levels of IL1β and no effect on IL1α, IL6, or TNF, were found in other inflammatory models with inhibitors of ICE (65). These results not only indicate that IL1α in addition to IL1β is produced during endotoxemia and other inflammatory conditions but also that IL1α apparently is sufficient to substitute for IL1β in the inflammatory response induced by endotoxin.

The release of TNF into the circulation in endotoxin models in mice is not directly correlated to survival (305). In addition, the production of IL1 and/or TNF during animal sepsis/endotoxemia can be modulated by many agents. For example, estrogen agonists increase serum TNF levels (303), whereas pentoxifylline reduces TNF release as shown in a cecal ligation and puncture model in rats (311). In the latter situation, pentoxifylline also diminished IL6 response and improved mortality. The role of endogenous PAF in the release of TNF following (sub)lethal challenge with endotoxin is not clear: One study in mice has indicated a role for PAF in this release (299), but this was not confirmed by another (300).

The site of production of TNF and IL1 *in vivo* following the administration of endotoxin is not precisely known. Most studies addressing this issue have been done with TNF. Hepatic venous levels of this cytokine are higher than arterial levels suggesting significant contribution of the splanchnic tissues to the systemic TNF levels (265). Immunohistochemical studies identified Kupffer cells as major sources of TNF and IL1 (127). In another study measuring TNF mRNA produced by various tissues, the liver and the spleen were the major sources (312). In contrast, in a study using transgenic mice bearing a reporter gene flanked by the mouse TNF promoter 5' untranslated region and the 3' untranslated region to assess the tissue distribution of TNF biosynthesis, kidney, heart, islets of Langerhans, spleen, lung, fallopian tubes, and uterus but not other organs, including the liver, were found to be the major sites of TNF production during endotoxemia (313). In addition, circulating leukocytes as well as leukocytes sequestered in the lungs have also been found to produce TNF following a challenge with endotoxin (294). Neutrophils can indeed produce a number of cytokines, in particular in response to endotoxin (314), which may explain why in a lethal mice model a neutralizing antibody against the integrin CD18 reduced the release of TNF (and that of IL8) and diminished mortality (141, 315). Similarly, administration of anti-CD18 monoclonal antibody or a monoclonal antibody against its ligand, intercellular adhesion molecule-1, to rabbits challenged with endotoxin prevented hypotension and largely abrogated the TNF response and reduced TNF-induced hypotension (315).

Following a (sub)lethal challenge with endotoxin not only TNF but also its soluble receptors are released (316–319). Shedding of receptors probably occurs via two separately regulated pathways that, as in experimental endotoxemia, are not influenced by TNF itself (317): One pathway is involved in the early appearance of sTNF-R (i.e., during the first 4 hr) and another in the relatively late appearance (i.e., approximately 8 hr after the challenge) (317). Other studies claim, however, that endogenous TNF released into the circulation also contributes to the release of sTNF-R55 (306). Some observations suggest that the release of sTNF-R is dependent on the release of IL1, at least in severe sepsis (319): Administration of IL1α at a dose of 10 or 100 μg/kg body wt caused an increase of sTNF-R55 and sTNF-R75; treatment of septic baboons with IL1-ra significantly reduced the increase of sTNF-R55; and, finally, administration of IL1-ra to septic patients resulted in a significant decrease of circulating levels of sTNF-R (319). The source of sTNF-R during sepsis is unknown, although one study suggested neutrophils as a major source, at least for sTNF-R75 (320).

A milestone regarding the pathogenic role of cytokines in sepsis in general and that of TNF in particular was the observation by Beutler, Milsark, and Cerami (16) that neutralization of endogenous TNF by administration of neutralizing antibodies could reduce mortality in a lethal model for sepsis. This definitely established that endogenous TNF is released during a lethal septic shock and plays a pivotal role in the development of the detrimental complications of this condition. The observation by Beutler *et al.* (16) has been confirmed in various models for sepsis, including mice or rabbits challenged with endotoxin, baboons challenged with live *E. coli*, *P. aeruginosa*, *S. aureus*, or streptococci, and mice challenged with *P. acnes* and endotoxin (141, 284, 287, 290, 296–298, 309, 321–328), as well as in models for toxic shock syndrome (288, 308, 309). Similar results were observed when TNF was neutralized by recombinant sTNF-R (106, 289, 302, 329–331) or the release of soluble TNF from membrane-bound TNF was prevented by inhibition of TNF convertase (38). In addition, mice expressing a transgene coding for sTNF-R55 were resistant to endotoxin-induced shock (332). The administration of monoclonal antibodies that blocked the function of membrane-bound TNF-R55 also afforded protection against a lethal dose of endotoxin (333). Together these studies convincingly show that endogenous TNF is released in response to infecting microorganisms or their products, and, at least in some animal models, mediates the lethal complications of sepsis by binding to TNF-R55. Consistent herewith, mice lacking TNF-R55 are resistant to TNF-mediated toxicity and to lethality induced by relatively low doses of endotoxin (334, 335). Surprisingly, however, mice deficient for TNF-R75 also have a decreased

sensitivity for TNF but a normal sensitivity for endotoxin and normal T cell development (23). These results fit with the "ligand passing" model for the interaction of TNF with its receptors, that is, that TNF-R75 helps to recruit TNF for interaction with TNF-R55, thereby lowering the concentration of TNF required for signal transduction via TNF-R55 (336, 337).

In general, the protection afforded by anti-TNF agents is optimal when these agents are given before the challenge, although some studies found a protective effect when given 30 min after the challenge (287, 298, 323). In two studies with recombinant sTNF-R, a beneficial effect was observed when these agents were given as late as 3 hr after the (intravenous) challenge (289, 302). This is remarkable considering that peak plasma levels of TNF are found 1 or 2 hr after the challenge, although at least in human experimental endotoxemia there is evidence for a second phase of TNF activity approximately 6 hr after the challenge (279). Notably, anti-TNF agents do not provide protection in all models for sepsis. For example, beneficial effects were not found in a mouse model for peritonitis induced by sublethal cecal ligation and puncture (325, 328, 338–342), a rabbit model for *E. coli* septic peritonitis (343), and even in an endotoxin model (338) or in mice receiving intravenously *S. aureus* (344) or *Klebsiella pneumoniae*, though in the latter model protection was achieved when the animals were challenged with *E. coli* (296). These contrasting effects are difficult to explain. One study indicates that efficacy of anti-TNF is among others dependent on the isotype of the antibody (297), the isotypes that easily activate complement and/or bind to Fc receptors being more efficacious. Furthermore, TNF (as well as IL1) may also have beneficial effects in a lethal endotoxin-induced shock: When given approximately 24 hr before the challenge, recombinant TNF reduces the mortality of this condition (345, 346). The mechanism of this protecting effect is not clear, although in the case of IL1 (which also may afford protection), downregulated production of IL1α, TNF, and IL6, increased production of IL1-ra, and decreased production of cellular TNF-R55 (and hence decreased responsiveness of cells to TNF) have been claimed to explain the protective effects of IL1 (114). Finally, in some of the local models (for example, cecal ligation and puncture) the amount of endotoxin released is not well controlled. Therefore, the lack of efficacy of anti-TNF agents in these models may be due to the fact that the endotoxin challenge is supralethal because protection by anti-TNF agents is lost upon challenge with endotoxin at concentrations exceeding LD100 (302). In the cecal ligation and puncture model mast cells appear to afford protection because in mice deficient for these cells mortality of this model is higher than that in normal mice (341). This protective effect of mast cells is abrogated by anti-TNF

antibody and, hence, protection may be mediated by mast cell-derived TNF (341).

Analysis of the effects of anti-TNF antibody or recombinant sTNF-R treatment on the release and activation of other inflammatory mediators in animals lethally challenged with endotoxin or bacteria suggests that the beneficial effects of these agents are in part mediated by inhibiting the inflammatory response; that is, the synthesis and release of other cytokines, such as IL1, IL6, LIF, RANTES, macrophage inflammatory protein-1α and IL-8 (but not that of IL10); the activation of coagulation; the release of phospholipase A_2 and that of endothelin (106, 172, 284, 287, 290, 295, 347–352). Furthermore, structural deterioration and dysfunction of the endothelium, i.e., diminished response to acethylcholine, was also prevented by administration of sTNF-R in a rat model of cecal ligation and puncture (353). Remarkably, although the adherence of neutrophils to the endothelium is inhibited by anti-TNF (354), the effects on the degranulation of neutrophils are inconsistent: In one study anti-TNF treatment did not modify the release of elastase (287), whereas in another it attenuated this release (295).

The most commonly used agent to block the activity of IL1 *in vivo* is IL1-ra, although antibodies against IL1-R type I also attenuate the host inflammatory response (355, 356). IL1-ra reduced local inflammatory conditions (356, 357). In animal models for lethal sepsis IL1-ra afforded protection against mortality, at least when given before or shortly (20 min) after the challenge (291–293, 358, 359), whereas it hardly had an effect on the host responses following a sublethal challenge (279, 282, 292). In one study a beneficial effect of IL1-ra was observed when this anti-IL1 agent was given as late as 2 hr after the challenge (302). The beneficial effect of IL1-ra in the lethal models is in part due to an improvement of hemodynamic performance and to inhibition of adherence of neutrophils to the endothelium (291–293). Notably, blockade of IL-1R is not always beneficial: In murine listeriosis a negative effect was observed (360). Although *in vitro* studies have suggested that IL1 may induce the synthesis of TNF, the process of which can be blocked by IL1-ra (361, 362), the effects of IL1-ra in the lethal sepsis models are likely not due to inhibition of IL1-induced release of TNF because, except for one study (293), IL1-ra treatment did not affect circulating TNF (291, 292, 318, 359). The beneficial effects of IL1-ra are thus more likely due to inhibition of other mediators, such as activation of coagulation and neutrophils (226), and the release of cytokines such as IL6 (292).

H. TNF AND IL1 IN CLINICAL SEPSIS

Waage *et al.* (363), using the WEHI bioassay, were the first to demonstrate increased circulating levels of TNF in 30% of the patients with

meningococcal disease, these levels correlated with clinical outcome. These results were confirmed by Girardin *et al.* (364), who observed increased plasma levels of TNF, measured with a radioimmunoassay, in the majority of children with meningococcal sepsis and purpuric lesions; these levels correlated with mortality. Girardin and co-workers also measured levels of IL1β in their patients using a radioimmunoassay and found these levels to be elevated in 21% of the children (364). Increased levels of IL1β also correlated with a poor outcome. These initial studies suggested that circulating levels of TNF and IL1 are increased in patients with sepsis (TNF more frequently than IL1) and correlate with the clinical outcome. Later studies only in part confirmed these findings: Some studies report that circulating TNF and, to a lesser extent, IL1 [notably, IL1 is not always found to be increased in patients with sepsis (365)] may be increased in septic patients and correlate with mortality or severity of disease (105, 366–375), whereas others have failed to find such an association (266, 365, 370, 376–383). Several explanations exist for these conflicting data. First, in the animal models for lethal septic shock, levels of TNF and IL1 reach their maximal values quickly (i.e., within 1–3 hr) after the challenge and are no longer detected during the later stages. Therefore, differences in time between the onset of sepsis and the time of entry into the study may greatly influence the results, as has been documented in children with meningococcal septic shock (374). Second, plasma levels may not properly reflect the synthesis of cytokines by cells. For example, patients with sepsis may have detectable monocyte-associated TNF in the presence of undetectable plasma levels (380). Third, it is now clear that the plasma concentrations of TNF and in particular IL1 are relatively low, i.e., in the 10–100 ng/liter range for TNF and even somewhat lower for IL1 (in line with their relative toxicity). The initial immunoassays to detect TNF and IL1 in plasma were relatively insensitive with lower limits of detection of approximately 100 ng/liter or even higher. Therefore, levels measured in most patients by these "first-generation" assays were around the detection limit and consequently the reproducibility of these measurements was poor. Fourth, the various assays may not measure all species of TNF or IL1 with equal sensitivity. Cytokines in plasma may be bound to carrier proteins such as soluble receptors (see above) and a2-macroglobulin (384). Bioassays measure only the active fraction of a given cytokine, whereas immunoassays may measure both active and inactive species depending on the antibodies used. If they recognize binding sites on cytokines for other proteins, anti-TNF antibodies may not detect the cytokine fraction bound to these proteins. Similarly, it is also not clear which IL1β species (inactive pro-IL1β, active IL1β, or proteolytically degraded IL1β) are detected by the assays for IL1β currently used (385). Thus, although initial

studies suggested that high circulating levels of TNF or IL1 in sepsis were correlated with a poor outcome, later studies suggest that plasma levels of TNF or IL1 in sepsis at the time of diagnosis at best only moderately correlate with outcome. In a study of 97 patients with the sepsis syndrome, TNF and IL1 levels were measured with carefully validated and sensitive (detection limit, 20 ng/liter) assays (377). Fifty-four percent of the patients had increased plasma levels of TNF at the time they were first identified as having the sepsis syndrome, whereas 37% had increased IL1. Levels of either cytokine were not different between surviving and nonsurviving patients, although patients with increased levels tended to have a higher mortality. The cytokine best correlating with mortality in these patients was IL6. Because of the poor correlation of individual cytokines with outcome, these authors proposed a cytokine–endotoxin score (a sum of TNFα, IL1β, IL6, and endotoxin scores) to predict mortality in patients with sepsis. In another study investigating the effect of TNF-R–Fc fusion protein in 141 patients with septic shock, only the minority had detectable IL1 or TNF (approximately 12 and 4%, respectively), whereas IL6 was increased in most patients (24). However, TNF was synthesized in considerably more patients because after administration of the TNF inhibitor TNF levels were detectable in 40% of them (24). Finally, in a large series of 146 patients with the sepsis syndrome, circulating TNF and IL1 were detected in 14 and 29% of the patients, respectively (386). Neither cytokine was correlated with mortality, although IL6 was (386).

Some studies comment on the course of TNF and IL1 in sepsis patients. Usually, TNF levels, when detectable, are higher at the time of diagnosis and decrease in time in survivors, whereas they remain constant in nonsurviving patients (365, 366, 378, 387–389). Similar data have been published for IL1 (372). In a limited number of patients [=13] with severe septic shock, however, levels of TNF and IL1 decreased toward normal independently from outcome (373). Apparently, the course of TNF and IL1 in clinical sepsis needs further studies, particularly because this may have important implications for therapy with cytokine antagonists.

It is not known to what extent circulating TNF and IL1 contribute to the inflammatory reactions occurring in the organs during sepsis. Circulating TNF levels were associated with the development of complications such as shock and adult respiratory distress syndrome (ARDS) in one study (366), but this was not observed in other studies (26, 376, 390). Furthermore, levels of TNF in patients with SIRS correlate with decreased expression of L-selectin on neutrophils (191), suggesting that in clinical sepsis TNFα is involved in the activation of neutrophils. On the other hand, there is growing evidence that cytokines locally produced in organs contribute to the organ dysfunction observed in sepsis. For example, pa-

tients with ARDS have substantially higher levels of TNF and IL1 in bronchoalveolar lavage (BAL) fluid than in plasma (391–393), suggesting local production. In agreement herewith, alveolar macrophages of patients with ARDS contain mRNA for IL1 and TNF and synthesize these cytokines (394, 395).

Correct interpretation of TNF and IL1 levels is only possible when information about their inhibitors is also available. Levels of either type of sTNF-R are increased in patients with sepsis (396) and correlate with outcome (374, 383, 386, 397). This increase of sTNF-R is accompanied by a decrease of TNF-R on monocytes (397). Increased levels of sTNF-R may identify surgical patients at risk for developing sepsis (398). Similarly, IL1-ra is also increased in patients with sepsis although circulating levels inconsistently correlate with severity or mortality (373, 386, 399). Finally, levels of sIL1-R type II are increased in patients with sepsis, whereas those of sIL1-R type I are decreased (399).

I. Treatment with Anti-TNF or IL1-ra

The compelling evidence for a detrimental role of TNF and IL1 in the pathogenesis of animal sepsis has prompted the evaluation of the effects of anti-TNF antibodies or sTNF-R and IL1-ra to prevent the harmful events and to reduce the mortality of clinical sepsis.

As discussed previously, in experimental endotoxemia pentoxifylline is able to reduce TNF concentrations. There is only limited experience with this drug in patients with sepsis. In 8 patients with septic shock pentoxifylline reduced circulating TNF within 24 hr after start of the treatment but had no effect on levels of IL6 and IL8, nor did it affect hemodynamics or oxygenation variables compared to patients receiving placebo (400). Initial studies with an anti-TNF murine monoclonal antibody in patients with sepsis have not yielded unequivocally favorable results (401, 402). Although anti-TNF treatment was accompanied by an increase in mean arterial blood pressure, 11 of the 14 patients participating in a phase I study did not survive for 28 days (401). In a study of 80 patients who received various doses of the same anti-TNF monoclonal antibody, no survival benefit for the total study population was found, although the highest dose of anti-TNF (10 mg/kg) appeared to reduce the mortality in patients with increased TNF levels (>50 pg/ml): Only 1 of the 7 patients in this subgroup died, whereas the mortality in the other subgroups was at least 50% (379). Furthermore, a beneficial effect of anti-TNF treatment on myocardial performance has been postulated (403), consistent with observations in animal models (256, 307). However, a beneficial effect of anti-TNF antibodies was neither observed in 42 patients with septic shock (402) nor in a large double-blind placebo-controlled multicenter trial in

patients with sepsis (404). In the latter trial a trend toward reduction of mortality was noted in the subgroup of patients with septic shock (404). The results of a trial with a soluble recombinant fusion protein of human sTNF-R75 covalently linked to the Fc portion of IgG1 were recently published (24). Unfortunately, a negative effect of this TNF inhibitor was observed: In the patient groups receiving the higher doses an increase of mortality was observed compared to placebo-treated patients (53 vs 30% mortality, respectively) (24). This negative effect, in particular observed in patients with gram-positive infections, may be related to the fact that TNF is necessary for the host defense (345, 346), especially in local infections.

The impressive effects of IL1-ra in animal models for lethal sepsis have raised expectations regarding the application of this cytokine antagonist in clinical sepsis. The initial results in 99 patients treated in an open-label placebo-controlled phase II multicenter clinical trial using three different doses of human recombinant IL1-ra fulfilled these expectations: A dose-dependent reduction of mortality was observed: 44% in the placebo group versus 16% in the group that received the highest dose of IL1-ra (405). However, these hopeful results were not confirmed by a subsequent randomized, double-blind, placebo-controlled, multicenter clinical trial in which 893 patients with the sepsis syndrome were treated with placebo or a low dose or a high dose of IL1-ra. A significant increase in survival time was not observed with recombinant IL1-ra treatment in the total group or in the group with shock at study entry (713 patients). However, secondary and retrospective analyses of efficacy suggested that there was a significant dose-related increase in survival time in patients with organ dysfunction and/or a predicted risk of mortality $\geq$24% (406, 407). In a second multicenter trial these beneficial effects of IL1-ra in some subgroups of patients with sepsis could not be confirmed, and clinical studies of the effect of IL1-ra as a treatment for sepsis have now been stopped. In a subgroup of the first large multicenter trial that consisted of 26 patients, the effect of IL1-ra treatment on the course of a number of inflammatory parameters was analyzed (408). The results indicated that in clinical sepsis IL1-ra significantly reduced in a dose-dependent manner the release and activation of a number of inflammatory parameters, including IL6, neutrophil degranulation, phospholipase A_2, complement, and fibrinolysis, as was similarly observed in baboons challenged with a lethal dose of *E. coli* (226). In a similar study, IL1-ra reduced circulating IL6 and various eicosanoids in septic patients (409). Thus, it can concluded that IL1 receptor blockade via the administration of IL1-ra modulates the systemic inflammatory response in sepsis. The lack of efficacy of IL1-ra treatment in patients with sepsis, therefore, may have been due to a

too short treatment period (the influence of IL1-ra on the inflammatory response was lost 2 days after cessation of the therapy). It should, however, also be noted that similar to TNF, IL1 also has been shown to possess protective effects in lethal models for sepsis, at least when given before the challenge (410, 411). Hence, treatment with antagonists of IL1 may not be beneficial for all patients with sepsis.

The usefullness of therapies aiming at neutralization of endogenous TNF and IL1 activity in the clinical treatment of sepsis still needs to be established. It should, however, be noted that in most animal models circulating levels of TNF and IL1 reach maximal values 2 or 3 hr after the challenge and decrease rapidly thereafter. Anti-TNF or anti-IL1 treatment consequently affords protection only when instituted shortly after, and preferably prior to, the challenge. Whether this implicates that these interventions are only efficacious in the very early stages of clinical sepsis needs to be demonstrated.

J. Conclusions about TNF and IL1 in Sepsis

During the past decade a central role of TNF and IL1 in the pathogenesis of experimental sepsis has convincingly been demonstrated. These proinflammatory cytokines are pivotal mediators and inhibition of these cytokines is beneficial in most but not all models for sepsis. In particular, in the models for localized infections the effect of anti-TNF agents is minimal, if not negative. Therefore, TNF and, possibly, IL1 likely have counteracting effects and resemble double-edged swords: TNF and IL1 are neccessary to keep infections localized and to prevent the development of sepsis, but once an infection has become systemic, their effects are detrimental and sepsis ensues. Consequently, administration of an anti-TNF agent will be of no benefit in patients in whom the former effect of TNF predominates because this will impair local defense mechanisms and hence promote entry of the infecting organisms and their products into the circulation. However, patients in whom local defenses have been bypassed and in whom systemic inflammatory responses propagate sepsis will benefit from anti-TNF therapy. The assessment of the contribution of these contrasting effects of TNF and IL1 to the septic process in individual patients will be a challenge for future studies. In addition, it will be important to delineate the role of membrane-bound TNF versus that of soluble TNF in local defense mechanisms. If membrane-bound TNF predominates in local defense mechanisms, inhibitors of TNF convertase may be the anti-TNF agent of choice in sepsis. Finally, although initial studies have shown correlations between circulating TNF or IL1 levels and outcome, subsequent studies did not confirm these results. Hence, determination of circu-

lating TNF and IL1 in patients with sepsis in a general clinical setting has only limited value.

VI. Interleukin-6

In addition to TNF and IL1, the first cytokine recognized to play a role in sepsis was IL6. IL6 was discovered by various investigators, each studying a factor with a different biological effect, such as interferon-β_2, 26-kDa protein, B cell stimulatory factor 2, hybridoma/plasmacytoma growth factor, hepatocyte stimulating factor, monocyte-granulocyte inducer type 2, cytotoxic T cell differentiation factor, and thrombopoietin (412). Molecular cloning of these factors revealed these diverse biological effects were mediated by a single cytokine, which subsequently was called IL6. Thus, IL6 is a multifunctional pleiotropic cytokine. Plasma levels of IL6 can increase enormously in experimental and clinical sepsis, reaching values of up to several hundreds of nanograms per milliliter (normal level, <10 pg/ml). Moreover, compared to other cytokines, circulating levels of IL6 best correlate with outcome in patients with the sepsis syndrome. However, the precise role of IL6 in the pathogenesis of this condition is still not well established.

A. Biochemistry of IL6

Human IL6 is composed of 212 amino acid residues including a N-terminal hydrophobic signal peptide of 28 residues (413). IL6 contains two potential N-glycosylation sites, but its carbohydrate groups are not essential for function, although they cause some molecular weight heterogeneity (412). A motif of four cysteines reveals homology of IL6 with G-CSF, myelomonocytic growth factor, leukemia-inhibitory factor, and oncostatin-M (414, 415). The ternary structure of these cytokines is supposed to consist of four α-helices and interconnecting loops (412). Helix D presumably contains the primary receptor binding site. The gene of human IL6 is located on the short arm of chromosome 7 (416).

IL6 exerts its effects via binding to a specific cellular receptor, IL6-R (417), which is structurally related to receptors for IL2, IL3, IL4, IL5, IL7, G-CSF, GM-CSF, erythropoietin and LIF. Together these constitute a new family of cytokine receptors (14, 418). Analysis of the amino acid sequence of IL6-R predicts a molecular mass of 80 kDa and reveals that IL6-R has only a very short intracytoplasmatic portion, which does not mediate signal transduction. Not unsurprisingly, therefore, IL6-R is associated with a second membrane glycoprotein, termed gp130, which transduces signals (419, 420). It has been proposed that IL6 first binds to IL6-R, which then causes IL6-R to associate with gp130. Binding of IL6 to

IL6-R is of a low affinity, subsequent association of the IL6–IL6-R complex with gp130 creates a high-affinity binding site (14, 412). Presumably, binding of IL6 to the receptor involves the formation of a hexameric complex consisting of two IL6, two IL6-R, and two gp130 molecules leading to dimerization of the latter (421, 422). One interaction site for IL6-R, and two for gp130, have been identified on the IL6 molecule (423, 424). Gp130 is not only involved with signal transduction of IL6 but also serves as a signal transducer for several other cytokines, i.e., oncostatin M, LIF, IL11, and ciliary neurotropic factor (14, 412, 425, 426). This explains why these cytokines have overlapping activities, although each also possesses unique activities (14). As will be discussed later, there is evidence that LIF is also involved in the pathogenesis of sepsis.

There is no evidence for the existence of IL6 antagonists *in vivo*. Soluble IL6-R, which probably is generated from membrane-bound IL-6R by a metalloproteinase (101), does not appear to function as such but rather is able, after having bound IL6, to associate with membrane-bound gp130 and to transduce signals to the cell (427). Soluble gp130, on the other hand, may negatively regulate the IL6 signal (412). *In vitro,* mutagenesis of the different sites involved in binding of IL6 to IL6-R/gp130 has yielded a mutant that behaves like a receptor antagonist, i.e., it binds to the receptor but does not transduce signals (421, 428).

B. *In Vitro* Effects of IL6

Virtually every cell in the body can be induced to synthesize IL6 (412), including monocytes/macrophages, granulocytes, T and B lymphocytes, endothelial cells, smooth muscle cells, fibroblasts, and mast cells. Stimuli for IL6 production are rather diverse: cytokines such as TNF, IL1, platelet-derived growth factor (PDGF), IFNγ and GM-CSF, endotoxin, virusses, prostaglandin E_2, and bradykinin. Monocytes/macrophages and endothelial cells may be the predominant producers of IL6 in sepsis, whereas TNF, IL1, and endotoxin are likely the main inducers (347, 429–431). The production of IL6 by macrophages stimulated with endotoxin can be inhibited by estrogen analogs *in vitro* as well as *in vivo* (303).

IL6 has a wide variety of activities: It stimulates growth of some and inhibits that of other cells, it is a growth and differentiation factor for B lymphocytes, it promotes activation, proliferation, and differentiation of T lymphocytes, synergistically with IL3 it hastens the appearance of multilineage blast cells from human bone marrow progenitors, it has thrombopoietin activity, it has some effects on macrophage differentiation, it stimulates bone resorption presumably via an effect on osteoclastogenesis, it induces PDGF in endothelial cells, it plays a role in the regulation of growth and development trophoblasts or embryonic stem cells during embryonic

development (412, 432), and it represents a link between immune system and the neuroendocrine axis by stimulating the production of corticotropin releasing factor. Actually, some of the functions formerly ascribed to IL1, such as the induction of fever, are in part mediated by IL6 (433, 434). Perhaps the most important function of IL6 *in vivo* is its capability to induce the synthesis of acute phase proteins by hepatocytes (435–439). Observations in mice deficient for IL6 confirm the important role of IL6 as a mediator of the acute phase response, although in particular that induced by endotoxin is not completely abrogated in these mice indicating involvement of related cytokines such as LIF (442–443). *In vivo* studies in man show that IL6 levels correlate well with the extent of acute phase reactions (444, 445).

The role of IL6 in inflammation is not clear. It is far less toxic than TNF or IL1. For example, it is unable to activate neutrophils [although an activating effect has been claimed (446)] and to induce expression of either adhesion molecules or tissue factor by endothelial cells. It is often considered as an anti-inflammatory cytokine because most of the acute phase proteins have anti-inflammatory properties, such as scavenging of oxygen radicals and inhibition of proteinases. Moreover, IL6 itself, as well as some of the acute proteins induced by it in the liver, is able to induce the release of IL1-ra and sTNF-R, inhibitors of IL1 and TNF, respectively (447, 448). Furthermore, IL6 has been found to reduce the endotoxin-induced production of TNF by human monocytes (449, 450), which may explain why IL6 is able to inhibit local inflammatory responses induced by endotoxin (451). IL6 is therefore sometimes considered as an alarm hormone with anti-inflammatory properties that can be produced by virtually any cell in the body under inflammatory stress (that is, cells confronted with high concentrations of TNF and IL1) and that signals the liver to produce anti-inflammatory proteins (439). IL6, in addition to IL1 (46) and TNF (452), is an endogenous pyrogen (453). Thus, fever in septic patients may be due to the release of IL6, which is substantiated by the observation that the increase in body temperature in experimental human endotoxemia occurs when plasma levels of TNF and IL6 are rising (239, 262). Finally, IL6 may have negative inotropic effects on cardiomyocytes; these effects are dependent on nitric oxide (454).

C. *In Vivo* Effects of IL6

To establish the potential role of IL6 in inflammatory conditions, investigators have administered IL6 to animals with or without other cytokines and also to humans, particularly patients with cancer. From these studies IL6 appeared to be a relatively nontoxic cytokine that does not induce a septic shock-like state (455–458): Even doses as high as 1 mg/kg per day

for 9 weeks were well tolerated by nonhuman primates and were not accompanied by the development of shock, vascular leak, organ dysfunction, or other toxic side effects (456). These doses of IL6 did, however, induce a two- or threefold increase of platelet numbers, an increase of immunoglobulins and of soluble IL-2 receptors, and a rise of acute phase proteins (455, 457). Thus, from these observations one may conclude that IL6 is an anti-inflammatory cytokine. In agreement herewith IL6 pretreatment enhanced resistance against infection (459, 460), although this has not been found consistently (461). However, IL6 has proinflammatory properties as well: It is able to induce the synthesis of the enzyme phospholipase A_2, which is involved in the formation of eicosanoids (462), as well as that of CRP, which can promote activation of complement (463). There is some evidence for CRP-dependent activation of complement in a baboon model for sepsis (11) as well as in patients receiving immunotherapy with high doses of IL2 (464), which is a human model for sepsis. Moreover, as will be discussed in the next section, activation of coagulation in a chimpanzee model of low-grade endotoxemia is markedly attenuated by pretreatment with a mAb that neutralizes the activity of IL6 (465), indicating that IL6 stimulates coagulation. In agreement herewith, clotting times were prolonged in baboons receiving recombinant IL6 subcutaneously at a dose of 100 μg/kg body wt (458).

D. Role of IL6 in Models for Sepsis

Soon after the initial reports that TNF was released into the circulation of healthy volunteers following an intravenous injection of a low dose of endotoxin, it was demonstrated that IL6 was released as well (239, 466). Typically, IL6 reaches peak levels at 2 or 3 hr after the administration of endotoxin and returned to normal levels at 5 or 6 hr. Similar observations were made in a chimpanzee model for low-grade endotoxemia (242, 276). In that model the endotoxin-induced release of IL6 was attenuated by pretreatment with a monoclonal antibody against TNF (274), a PAF inhibitor (276), and pentoxifylline (242), but not by a monoclonal antibody that inhibits tissue factor activity (9). Pentoxifylline and the PAF inhibitor each diminished the release of TNF as well; therefore, the effects of these drugs on the endotoxin-induced release of IL6 may well be related to their inhibiting effects on the production of TNF, especially because TNF can induce the release of IL6 *in vivo* (223, 467, 468). Remarkably, despite its effects on the release of IL6 in the chimpanzee model (242), pentoxifylline has no effect on the release of IL6 in human experimental endotoxemia (269). Administration of IL1-ra also has no effect on circulating IL6 (226, 279, 282). Thus, the release of IL6 during experimental endotoxemia is not stimulated by IL1, although this cytokine is able to induce the release

of IL6 *in vivo* (202, 468, 469). For example, in cancer patients a dose of IL1β as low as 3 ng/kg given as an intravenous injection elicits a measurable release of IL6 with peak levels of 50–60 pg/ml (225). Thus, altogether these data indicate that during low-grade endotoxemia, TNF and possibly endotoxin itself, but not IL1, stimulate the synthesis and release of IL6. In addition, glucocorticoid therapy may alter the IL6 response induced by endotoxin either negatively, when given shortly before or simultaneously with endotoxin, or positively, when given 12 hr or more prior to the endotoxin challenge (121). In contrast, epinephrine has only a mild reducing effect, if any, on the release of IL6 in healthy volunteers challenged with a low dose of endotoxin (138), which likely results from a reduced release of TNF. Only one study sought to determine the contribution of IL6 to the release and activation of other mediators during experimental endotoxemia: Chimpanzees were challenged with a low dose of endotoxin after pretreatment with a monoclonal antibody that blocked the activity of IL6. No effect of IL6 on the release of TNF or its soluble receptors, on the release of IL8, the degranulation of neutrophils (465), or on the release of IL10 (470) was observed. Surprisingly, inhibition of IL6 markedly attenuated activation of coagulation (465). Thus, during low-grade endotoxemia IL6 may contribute to coagulation via an unknown mechanism.

Plasma levels of IL6 increase upon a challenge with a (sub)lethal dose of endotoxin, live bacteria (301), or the superantigen staphylococcal enterotoxin B (471). The course of IL6 in sublethally challenged animals is comparable to that during low-grade endotoxemia, i.e., levels are the highest at 3 hr after the challenge and decrease thereafter (285). Following a lethal challenge, this course is different, however: IL6 still increases after 6–8 hr to reach peak levels that are 50- to 100-fold higher than those observed after a sublethal challenge (285, 347, 472). This remarkable difference in the course of IL6 following a sublethal or a lethal challenge may explain why IL6 levels show a good correlation with clinical outcome in patients with sepsis, in particular during the later stages when IL6 after a sublethal challenge decreases but after a lethal challenge still increases. The reason for this different course is not known, but one may speculate that following a sublethal challenge IL6 is mainly produced by monocytes and kupffer cells, and that after a lethal challenge other cells produce IL6 as well. A number of studies have shown that the release of IL6 following a lethal challenge is grossly reduced by inhibition of TNF (106, 287, 290, 311, 347), in agreement with *in vitro* observations that TNF is a potent inducer of IL6 production by various cells (468, 473). Consistent with a supposed role for TNF in the induction and release of IL6 during sepsis is that IL6 levels in patients with sepsis decrease upon treatment with high-dose anti-TNF monoclonal antibody (379). In addition, in an

animal model for sepsis IL1 receptor blockade also reduced the release of IL6 (292) suggesting that after a lethal challenge IL1 also contributes to the release of IL6. In agreement herewith treatment of septic patients with IL1-ra reduces IL6 levels (408, 409) suggesting that in clinical sepsis IL1 also contributes to the synthesis and release of IL6. Surprisingly, treatment of baboons lethally challenged with live *E. coli* with tissue factor pathway inhibitor (TFPI), an inhibitor of extrinsic pathway of coagulation, also reduces the release of IL6 without affecting the TNF response (474, 475). Elucidating the mechanism via which TFPI affects IL6 *in vivo* is important because TFPI reduces mortality in this model. One possibility is that the effect of TFPI on cytokines is related to the formation of thrombin because, at least *in vitro,* TFPI can affect thrombin-induced cytokine IL8 release in whole blood cultures (476). Finally, IL6 response was also reduced in septic pigs receiving anti-C5a indicating that during sepsis complement activation, and in particular C5a, also contributes to the release of IL6 (477).

The role of IL6 in the development of clinical signs and mortality of the lethal sepsis models is not clear. Administration of IL6 does not induce a sepsis syndrome in the host (456, 457), although this does not exclude that IL6 in synergy with another cytokine may do so. Starnes *et al.* (478) were the first to study the effect of passive immunization with monoclonal antibodies that neutralize the activity of IL6 on TNFα or *E. coli*-induced mortality in mice. They claimed that anti-IL6 afforded protection against mortality similarly to anti-TNF antibodies (478). However, part of the results of this study were retracted (H. F. Starnes *et al., J. Immunol.* **148;**1968, 1992). Administration of anti-IL6 antibodies afforded protection in a generalized Shwartzman reaction (479) and in an anti-CD3-induced cytokine syndrome (480), both models for septic shock. This effect of anti-IL6 was mediated without affecting circulating TNF levels. Furthermore, anti-IL6 appeared to be able to reduce mortality in a model for burns-related sepsis, in which mice were subjected to a thermal injury and challenged with *E. coli* (481). A protective effect of anti-IL6 antibodies was, however, not confirmed by others studying various mouse models for septic shock (460, 482), including a model with the superantigen staphylococcal entrotoxin B in galactosamine-sensitized mice (471, 483). In the latter model, and in particular in mice receiving the superantigen without D-galactosamine, levels of TNF and IFNγ were higher in the animals receiving anti-IL6, suggesting involvement in the production of both cytokines (471). Furthermore, pretreatment with IL6 (or IL11) reduced mortality in this model (483). Together with anti-TNF (mimicking septic conditions with limited TNF activity) in the lipopolysaccharide–galactosamine septic shock model, anti-IL6 enhanced whereas IL6 reduced mortality

(460). Experiments with IL6-deficient mice similarly yielded discrepant results (484): On the one hand, no effect of IL6 deficiency was observed in a LD50 endotoxin model, whereas mortality of mice challenged intraperitoneally with live *E. coli* was increased in IL6-deficient mice. The reasons for these discrepant results are not clear. One may speculate that the effect of IL6 neutralizing agents may depend on the sepsis model used: For example, in models in which mortality occurs independently of coagulation or complement activation, no effect of anti-IL6 agents may be expected. On the other hand, for quantitative reasons one should be careful to interpret the anti-IL6 experiments. For example, administration of an antibody with a very high affinity, i.e., a K_D of 10^{-11} M, at a concentration of 0.16 μg/ml (1 μM) in plasma (to achieve this, 6–10 mg/kg body wt should be administered) leaves one IL6 molecule per100,000 free. Under certain septic conditions 5 μg of IL6 per milliliter of plasma may occur. At these concentrations 50 pg of IL6 per milliliter remains free, i.e., active. A monoclonal antibody with a 10-fold lower affity would allow 500 pg of IL6 to be active. *In vitro*, such a level is sufficient for cellular activation. Thus, an amount of 10 mg of antibody per kilogram of body weight may at first glance seem to be sufficient for an intervention study, whereas in reality it is not.

E. IL6 in Clinical Sepsis

Several extremely sensitive bioassays for IL6, including the B9 assay (485), have enabled studies regarding the role of this cytokine in sepsis. In 1989 three groups independently reported on increased plasma levels of IL6 in sepsis: Waage *et al.* (367) described increased levels of bioactive IL6 in 69 of 79 serum samples from patients with severe meningococcal disease, the highest levels being associated with a fatal outcome. Helfgott *et al.* (486) reported on elevated levels of IL6 in patients with various bacterial infections. In a third study, Hack *et al.* (487) described (sometimes very) high levels of plasma IL6 in 86% of the septic patients at the time of admission to the intensive care unit. These levels were related to clinical outcome. In addition, levels significantly correlated with plasma levels of C3a, elastase, and lactate (this latter parameter is supposed to reflect at least in part tissue hypoxia in septic shock). These correlations are consistent with the hypothesis that in sepsis, endothelial cells are injured by oxygen radicals and proteases released by neutrophils, stimulated by agonists such as the complement-derived C5a, and adhere to the endothelial cells under influence of cytokines such as TNF and IL1. As a result, the endothelial cells will swell and become more permeable for proteins and fluid leading to interstitial edema. Therefore, part of the IL6 observed in sepsis may be derived from the injured endothelial cells that secrete this cytokine as

a kind of alarm hormone (439), which induces the synthesis of acute phase proteins in the liver. Among these proteins, for example, is α1-antitrypsin, the main inhibitor in plasma of the proteinase elastase released by neutrophils.

Subsequent studies have confirmed increased production of IL6 in sepsis although the observed concentrations show some variation. In general, 64–100% of the septic patients have elevated circulating levels that correlate with mortality and shock (24, 105, 373, 374, 377, 379, 380, 382, 386, 396, 488–492), although the association with mortality was not always found (365, 493). Only a few studies comment on the course of IL6 in sepsis: In most studies the highest levels of IL6 occurred at the time of diagnosis and decreased thereafter independently from outcome (365, 367, 388, 487, 488, 490, 491). Persistently elevated levels, however, may be associated with mortality (492) or the development of multiple system organ failure (365), though this was not confirmed in a later study (491). Also, in patients with cirrhosis and SIRS a relation between IL6 levels and an organ failure score was found (381). Furthermore, two studies did not observe declining IL6 levels during the course of the disease (380, 489). Finally, plasma IL6 concentrations on admission predict bacteremia and mortality in emergency department patients (494). Bronchoalveolar lavage fluid from patients with ARDS contains high IL6 levels that correlate with parameters reflecting blood–alveolar permeability (393).

Only a few studies in septic patients have addressed the question regarding the stimuli for IL6. In patients treated with a TNF receptor–IgG construct, no effect on IL6 levels was observed (24), whereas in patients treated with an anti-TNF antibody or IL1ra there was a small reduction (402, 408, 409). Similarly, polyclonal sheep antibodies against human TNF were able to mitigate the release of IL6 in humans suffering from Jarisch–Herxheimer reaction following antimicrobial treatment of *Borrelia recurrentis* infection (495). One study has reported on the levels of sIL6-R in septic patients. These levels appeared to be lower than those in normal individuals and inversely correlated with IL6 levels (396).

F. Conclusions about IL6 in Sepsis

IL6 seems to be the cytokine most frequently elevated in clinical sepsis, and circulating levels of this cytokine correlate with outcome in most studies. Determination of these levels either alone or in combination with those of TNF, IL1, and endotoxin to yield a so-called LPS–cytokine score (377) seems to be helpful in predicting survival of patients. It should, however, be realized that IL6 levels are the highest at the time of diagnosis and decrease thereafter in most patients independently of outcome. Despite the association with mortality, the role of IL6 in the pathophysiology

of sepsis is not clear. Animal studies have shown both beneficial and detrimental effects of endogenous IL6. Further studies are needed to solve this issue.

VII. The Chemokine Interleukin-8

A salient feature of acute and chronic inflammation is the infiltration of the affected tissues by polymorphonuclear and mononuclear cells. This recruitment of inflammatory cells is mainly directed by a number of structurally related cytokines, the chemokines (496). Chemokines are composed of at least two superfamilies, the so-called C-C chemokine superfamily, characterized as a juxtaposition of the first two cysteine amino acid residues, and the C-X-C superfamily, in which the first two cysteine amino acid residues are separated by one other amino acid (496). An example of the C–C superfamily is MCP-1 (discussed below), and an example of the C–X–C superfamily is interleukin-8, formerly known as neutrophil-activating peptide 1 (NAP-1), neutrophil activating factor, or monocyte-derived neutrophil chemotactic factor (216, 497, 498).

A. Biochemistry and Biology of IL8

The IL8 precursor protein is synthesized as a single peptide chain consisting of 99 amino acid residues and containing a signal sequence of 20 residues (499). The mature form consists of 79 amino acid residues, which is proteolytically processed at its amino-terminal end to yield various N-terminally processed forms with slightly different biological properties (500, 501). The predominant form of IL8 consists of 72 amino acid residues, which can be generated by conversion of the 77-amino acid form by thrombin (502). Presumably, these two forms account for more than 80% of the IL8 species *in vivo* (498). IL8, in contrast to other chemotactic agents such as C5a, PAF, and leukotrien B4, is remarkably resistant to chemical or proteolytical inactivation, and its biological activity in inflamed tissues may last for several hours (501). The biological effects of IL8 are mediated via binding to specific receptors with subsequent signal transduction. Two different IL8 receptors, IL8-Rα and IL8-Rβ, have been identified (503–506), both belong to the extending family of chemokine receptors.

IL8 is homologous to the other members of the C–X–C chemokine superfamily, i.e., neutrophil-activating peptide-2 (NAP-2), the strongly homologous cytokines GROα, GROβ, and GROγ (three different gene products with only a few differences in amino acid sequence), platelet factor 4, and the interferon-γ-inducible peptide 10. Of these, NAP-2 and the GRO cytokines have the same pattern of activity toward neutrophils as

IL8, although they are less potent (507). This is a reflection of the fact that NAP-2 and the GRO cytokines bind with a high affinity to only one of the two high-affinity receptors for IL8, while having a low affinity for the other (508). Although the GRO cytokines can be synthesized by mononuclear cells triggered with endotoxin, IL1, or TNF (501), and thus likely are formed during sepsis, they as well as the other members of the C–X–C chemokine syperfamily will not be discussed further here because, to our knowledge, there are no reports on their involvement in the pathogenesis of sepsis.

IL8 receptors are members of the superfamily of receptors that are coupled to guanine nucleotide-binding proteins (503, 504). These serpentine-like receptors are characterized by seven transmembrane domains. Due to this structure no soluble receptors are expected to exist. IL8 is produced by a variety of cells including blood monocytes, macrophages, neutrophils, and endothelial cells (216, 496, 498, 501). Agonists that induce the release of IL8 by these cells *in vitro* are TNF, IL1, and endotoxin (216, 496, 498, 501), all of which are involved in the pathogenesis of sepsis. TNF-induced release of IL8 by endothelial cells is dependent on nitric oxide, as was apparent from studies *in vitro* with nitric oxide synthase inhibitors (509). It is likely that thrombin, which can stimulate the formation of nitric oxide by endothelial cells, also induces the release of IL8 by these cells because this clotting enzyme can stimulate the synthesis and the release of other chemokines, such as GROα and MCP-1, by monocytes and endothelial cells via catalytic activation of the thrombin receptor (510, 511). In addition, thrombin can induce monocytes to secrete IL8 and MCP-1 indirectly by activating platelets, which in turn stimulate monocytes via P-selectin and P-selectin glycoprotein ligand-1 (512). Furthermore, ischemia reperfusion may also serve as a trigger to release IL8 by monocytes (513). Also, complement activation products such as the membrane attack complex can stimulate endothelial cells to produce IL8 and MCP-1 (514).

The activity of IL8 is regulated in various ways. *In vivo* IL8 binds to erythrocytes (515, 516), which may contribute to the regulation of its activity. Another mechanism to regulate the activity of IL8 *in vivo* may involve autoantibodies, which seem to be present in about 75% of normal healthy individuals (517). These autoantibodies have been claimed to neutralize IL8 activity. As for anti-IL6 antibodies, autoantibodies against IL8 must have a very high affinity to be able to inhibit this chemokine substantially during sepsis, especially because very high levels may occur in sepsis. IL8 can also bind to glycosaminoglycans, which are supposed to play a role in stabilizing IL8 gradients in the interstitial space, along which neutrophils may invade the tissues.

With regard to its biological effects, IL8 shows remarkable specifity for neutrophils and basophils and hardly has an effect on other cells, in contrast to other chemoattractants such as C5a (501). IL8 has chemoattractant activity (518–522) and is able to induce degranulation, to elicit a respiratory burst, and to activate arachidonate-5-lipoxygenase in neutrophils (498, 518, 523), the processes of which enhance inflammation. In addition, IL8 may promote adherence of neutrophils to endothelium by increasing β2-integrin expression and regulate transendothelial migration of these cells (524, 525). On the other hand, under certain conditions IL8 may also inhibit the adherence of neutrophils to the endothelium (526). Thus, *in vivo* IL8 is presumably an important regulator of neutrophil activation and migration. Intradermal injection of IL8 induces a rapid inflammatory response with massive neutrophil infiltration and accumulation, without participation of monocytes, eosinophils, basophils, and lymphocytes (527). High local concentrations of IL8 may also elicit edema formation due to neutrophil-mediated endothelial damage and subsequent plasma leakage (520, 522). High circulating levels, however, inhibit the migration of neutrophils into the tissues (528). Thus, the role of IL8 may be anti- as well as proinflammatory, depending on its site of production.

Intravascular administration of IL8 does not induce the hemodynamic and metabolic alterations of sepsis although it does induce a transient neutropenia due to sequestration in the lungs, followed by granulocytosis (528, 529). The release of endogenous IL8 is stimulated by the intravenous administration of TNF, IL1, or IL2 (225, 234, 516, 530, 531). Thus, although IL8 alone cannot induce the development of a sepsis-like state, one might speculate that in conjunction with other cytokines it does.

B. IL8 in Experimental Sepsis Models

Local production of IL8 in inflamed tissues is important for recruitment of neutrophils and may contribute to tissue damage. Administration of a neutralizing antibody against IL8 should, therefore, be an effective treatment to prevent local tissue damage by inflammatory reactions, as indeed has been demonstrated in a number of animal models (532). In systemic inflammatory reactions such as those that occur in sepsis, IL8 likely also plays a role because circulating levels of this cytokine increase during experimental endotoxemia (138, 234, 263, 533, 534). In addition, circulating neutralizing autoantibodies against IL8 decrease after an intravenous endotoxin challenge suggesting consumption of these antibodies and, hence, a role in the regulation of active IL8 *in vivo* (534). Typically, the course of endogenous IL8 following an intravenous challenge with a low dose of endotoxin is very similar to that of IL6 with peak levels at 2 hr. Released IL8 may be responsible for the alteration of neutrophil function during

endotoxemia, such as a loss of TNF receptors and L-selectin and a diminished chemotactic activity toward IL8 (533). IL8 also increases in humans following a challenge with a high dose of endotoxin, as was demonstrated in a patient who self-administered endotoxin because of a supposed anticancer effect (270).

A molecule similar to IL8 has not been identified in rats. However, one of the rat molecules closely related to human IL8 is macrophage inflammatory protein-2 (MIP-2), which was shown to be involved in a rat model in which endotoxin is locally instilled in the lungs. In this model MIP-2 appeared to be responsible for the recruitment of neutrophils (535).

The production of IL8 in endotoxin-stimulated human whole blood is biphasic, the initial phase being due to a direct stimulation of leukocytes by endotoxin and the later phase was claimed to be due to stimulation with TNF and IL1 (536, 537). Recently, such a biphasic release of IL8 (and of several other mediators) was also observed during experimental endotoxemia *in vivo*, with the second release of mediators starting at 6 hr after the challenge (M. Boermeester *et al.*, unpublished observations). Similarly, a biphasic release of IL8 has also been found following the administration of TNF (234).

The effect of specific interventions on the course of IL8 in human experimental endotoxemia has been studied only scarcely. In one study treatment with IL1-ra did not influence IL8 levels (282). Furthermore, epinephrine has a marginally depressing effect on the release of IL8 during experimental endotoxemia (138). The effect of interventions on the course of IL8 has been studied more extensively in a chimpanzee model for experimental endotoxemia, in which peak levels of IL8 occur at 2 or 3 hr after the administration of endotoxin (242, 274, 276, 465). In this model the release of IL8 is inhibited by a PAF antagonist and pentoxifylline (242, 276), as well as by anti-TNF (274), but not by anti-IL6 antibodies (465). The observed effects of the PAF antagonist and pentoxifylline on the course of IL8 are presumably indirect because these drugs also attenuate the release of TNF. Preliminary observations in this same model indicate that neutralizing anti-IL8 antibodies do not affect circulating levels of elastase and lactoferrin, which may indicate that endogenous IL8 does not significantly contribute to the degranulation of neutrophils during experimental endotoxemia (T van der Poll *et al.*, unpublished observations).

Circulating levels of IL8 also increase following a challenge with lethal doses of live *E. coli* (472, 530, 538) or endotoxin (141) and, similar to IL6, its course is different in a sublethally challenged animal from that in lethally challenged animals. In the latter, IL8 still rises at 6–8 hr. Furthermore, circulating levels of IL8 strongly correlate with those of MCP-1 (538) suggesting that similar stimuli stimulate the release of either chemokine.

There are no studies identifying the source of IL8 during lethal animal sepsis. Therefore, it remains to be established to what extent other cells besides blood leukocytes and macrophages, such as endothelial cells, contribute to the production of endogenous IL8 in this condition. Treatment of rabbits with a monoclonal antibody that neutralizes IL8 prevents lung reperfusion injury (539), suggesting a critical role of IL8 in this condition. In a lethal mice endotoxin model, pretreatment of the animals with an IL8-neutralizing antibody provided a slightly improved mortality (141).

C. IL8 in Clinical Sepsis

There are only a limited number of studies of the role of IL8 in clinical sepsis. We have observed elevated levels of IL8 in 42 of 47 (89%) patients with sepsis on admission to the intensive care unit (540). Levels were comparable in patients with gram-positive or gram-negative infections. IL8 levels were higher in patients with shock and in patients who died (the largest difference in IL8 between survivors and nonsurvivors was found when only patients with positive bacterial cultures were considered). Moreover, IL8 correlated with lactate levels and inversely with leukocyte and platelet numbers and mean arterial pressure. In addition, IL8 correlated with elastase-a1-antitrypsin complexes, suggesting a role for IL8 in the degranulation of neutrophils, as well as with IL6, supporting that the synthesis of these cytokines in sepsis (and in experimental models for sepsis, see above) is induced by similar stimuli. Serial observations revealed that in most patients IL8, similar to IL6, decreased irrespective of outcome (540). These findings were essentially confirmed in a preliminary communication by Danner *et al.* (541) [although the (weak) association between IL8 and a poor outcome was not observed in that study] and by Endo *et al.* (542). In addition, IL8 levels are higher in patients with sepsis than in those with trauma (492). Increased levels of IL8 have also been observed in serum and cerebrospinal fluid from patients with meningococcal disease, in which serum levels were found to correlate with those of IL6 and of TNF and to be the highest in patient with septic shock (543). In that study 4 of the 5 patients who died had high levels of IL8 (543). Furthermore, circulating IL8 was also increased in 8 of the 18 patients with septic or localized *P. pseudomallei* infection and correlated with outcome (489). Finally, in a study of burn patients circulating IL8 appeared to be elevated shortly after the injury, dependent on the size of the burn, and gradually decrease thereafter in patients without serious infectious complications. Increasing IL8 levels later on were indicative for the development of sepsis (544).

No data exist on the effect of anti-TNF treatment on IL8 levels in septic shock patients. However, in a recent study, polyclonal sheep antibodies

against human TNF reduced the release of IL8 in humans suffering from Jarisch–Herxheimer reaction following antimicrobial treatment of *B. recurrentis* infection (495), showing that in a condition characterized by hypotension and fever, TNF is indeed involved in the production of IL8. In septic shock patients receiving IL1-ra a slight decrease of circulating IL8 was observed (408), indicating that IL1 also contributes in part to the IL8 response in human sepsis.

In a recent study we found that circulating IL8, presumably via its effects on neutrophils, closely correlated with pulmonary microvascular permeability in patients undergoing aortic surgery (545). In contrast, in sepsis patients who have or are developing ARDS, plasma IL8 is similar to that in patients who do not develop this complication (540), suggesting that circulating IL8 is not an important mediator of ARDS in sepsis. On the other hand, in a small number of patients [=13] with ARDS mostly due to sepsis, we have found that IL8 not only correlated with levels of IL6 and elastase-α1-antitrypsin complexes but also with a lung injury score (546), suggesting cytokine-induced activation of neutrophils to be important for the pathogenesis of ARDS. It is not clear whether these somewhat conflicting results are due to differences in patient populations or to other reasons. Nevertheless, IL8 locally produced in the lungs may be a more important mediator in this regard. In 1988 Cohen *et al.* (547) observed increased levels of a peptide, called enzyme-releasing peptide because of its potency to induce the release of enzymes from neutrophils and most likely identical to IL8, in BAL fluid from patients with ARDS (547). Subsequently, it was shown that BAL fluid samples from patients at risk for the development of or already having ARDS contain increased levels of IL8 (548–551). Donnelly *et al.* (548) observed the highest BAL levels of IL8 in patients who progressed to ARDS. Immunocytochemistry suggested alveolar macrophages to be the main sources of IL8 (548). Miller *et al.* (549) reported that levels of IL8 in BAL fluid from patients with ARDS are increased and correlate with neutrophil numbers and with mortality. Moreover, these investigators demonstrated that most of the chemotactic activity of BAL fluid toward neutrophils was absorbed by anti-IL8 antibodies. Chollet-Martin *et al.* (550) found that the ratio of levels in BAL fluid and plasma was significantly greater for IL8 than that for TNF indicating a higher local production of the former. Moreover, levels of IL8 in BAL fluid correlated with mortality, shock, and a general clinical severity index. Torre *et al.* (551) also reported increased levels of IL8 in BAL fluid from patients with ARDS. Notably, although all these studies noted correlations of IL8 in BAL fluid to important clinical parameters, such correlations were not observed for circulating IL8 (548, 550, 551). Moreover, IL8 levels in BAL fluid inversely correlate with circulating levels of soluble L-selectin

(552), suggesting a relation between local release of chemokines in the lung and microvascular endothelial–neutrophil interactions. Thus, taken together these studies demonstrate the importance of local production of IL8 in the development of ARDS. More generally, these studies imply that in sepsis local production of inflammatory mediators may greatly influence the inflammatory processes in the various organs. A challenge for future studies will be to delineate the contributions of local inflammatory reactions to the systemic sepsis process.

D. Conclusions about IL8 in Sepsis

IL8 and related cytokines presumably are important regulators of neutrophil function *in vivo*. Results of studies in animal models for sepsis, in experimental endotoxemia and in clinical sepsis, indicate that IL8 is synthesized and released in significant amounts during sepsis. However, there are no experimental data substantiating that this cytokine is indeed involved in the pathophysiology of sepsis, for example, by activating neutrophils. The precise role of IL8 in the pathogenesis of sepsis, therefore, remains to be established. Few studies measuring circulating IL8 in clinical sepsis show weak correlations between increased levels and fatal outcome and the occurrence of shock. More convincing are studies in patients with ARDS that strongly suggest that the local production of IL8 contributes to the pulmonary dysfunction. We suggest that local production of IL8 may be involved in the development of dysfunction of other organs as well. To what extent IL8-like cytokines such as the GRO molecules also contribute to this dysfunction is as yet unclear.

VIII. The Chemokine Monocyte Chemoattractant Protein-1

MCP-1 is a C–C chemokine. The main function of MCP-1 is to direct monocytes to the inflammatory focus (496). Furthermore, it may contribute to the degranulation of mast cells. These effects may explain that local application of MCP-1 affords protection against local infections (553). MCP-1 can be produced by many cells including macrophages, fibroblasts, epithelial and endothelial cells, and neutrophils, with TNF and IL1 being the main inducers for the synthesis (496). MCP levels in volunteers receiving a low dose of endotoxin increase, reaching peak levels at 3 hr after the challenge (534). Recent observations in septic baboons indicate that substantial amounts of MCP are released following a sublethal challenge with live *E. coli*, with peak levels at 4 hr after the challenge. In lethally challenged animals levels are not only higher but also show a more protracted course (538). Moreover, levels of MCP correlate closely with those of IL8 in this animal model. The majority (57%) of patients with sepsis

have increased circulating levels of MCP-1 (554). Levels of MCP-2 are also increased in the majority (59%) of these patients, but this more often occurs in patients with gram-positive infections, suggesting that the synthesis of MCP-1 and MCP-2 is differentially regulated in sepsis (554). MCP-1 levels weakly correlated with those of MCP-2 and IL8 and were associated with a more severe course. In addition to MCP-1 and -2, another C–C chemokine, RANTES, is also involved in the inflammatory response following endotoxemia. In mice challenged with endotoxin, RANTES mRNA has been found to increase in the lungs, liver, and, slightly, in circulating leukocytes (349). Furthermore, RANTES protein was detected in the lungs and in part was responsible for the influx of macrophages, as was evident from experiments in which endotoxemic mice were treated with anti-RANTES antibodies. Finally, anti-TNF treatment reduced the expression of RANTES mRNA, indicating that the induction of RANTES was dependent on TNF (349). Similarly, expression of other C–C chemokines involved in the recruitment of macrophages and neutrophils in the lungs, such as MIP-1α, is induced in the lungs following endotoxin challenge (352). Inhibition of this chemokine in a mouse endotoxemia model resulted in a decreased influx of neutrophils and macrophages in the lungs, a diminished lung permeability, and slightly improved mortality (352). Together these observations suggest that chemokines orchestrate the migration of leukocytes during endotoxemia and sepsis. To what extent this contributes to the complications of sepsis remains to be established.

IX. Other Cytokines

In addition to TNF, IL1, IL6, and IL8, other cytokines are involved in the pathogenesis of sepsis. A brief discussion of these cytokines and their supposed role in sepsis is given here.

A. The Anti-inflammatory Cytokine Interleukin-10

IL10 is a typical anti-inflammatory cytokine that was initially characterized as a factor produced by T helper subset 2 (Th2) lymphocytes inhibiting the production of cytokines by Th1 cells (555). However, monocytes stimulated with bacterial products can also produce significant amounts of IL10. Its amino acid sequence has been unraveled (556). Also, the structure of one of its receptors is known. This receptor is structurally related to that for interferon (557).

IL10 is a potent inhibitor of the synthesis and release of proinflammatory cytokines TNF, IL1, IL6, IL8, and IFNγ by endotoxin-stimulated monocytes, macrophages, or neutrophils (537, 558–562), probably by inhibiting the transcription of the genes of these cytokines (563). IL10 has different

effects on endothelial cells in that it does not interfere with or even enhances the production of IL6 and MCP-1 by these cells (564). Furthermore, IL10 inhibits endotoxin-stimulated tissue factor expression and induction of procoagulant activity by monocytes (565, 566). The important role of IL10 in inhibiting the release of proinflammatory cytokines under physiological conditions *in vivo* is demonstrated in IL10-deficient mice: These animals develop chronic enterocolitis presumably from uncontrolled immune responses stimulated by enteric antigens (567).

Circulating IL10 levels increase during experimental endotoxemia in human volunteers, reaching peak levels 90–120 min. after the endotoxin gift (138, 235, 470, 561); these levels are not affected by inhibitors of platelet-activating factor or IL6 (470) but are potentiated by intravenous administration of epinephrine (138). Studies with neutralizing anti-TNF antibodies suggest that TNF induces the release of IL10 during experimental enotoxemia, which is confirmed by the observation that the administration of TNF induces the release of IL10 in human volunteers, reaching maximal levels at 45 min after the TNF injection (235). However, in severe sepsis TNF is probably not the main inducer of IL10 because in a lethal *E. coli* model in baboons anti-TNF does not affect the release of IL10 (351).

Neutralization of endogenous IL10 by pretreatment with an anti-IL10 monoclonal antibody in animal models for sepsis results in higher circulating TNF levels and a greater mortality (342, 561), demonstrating that IL10 is an important inhibitor of proinflammatory cytokine synthesis during endotoxemia. Observations in mice deficient for IL10 have confirmed that this cytokine is indeed a natural suppressant of TNF production during systemic and local inflammation (568, 569). Because of its anti-inflammatory properties, IL10 has been evaluated for its efficacy to reduce mortality of animals suffering from sepsis. In mice, the administration of IL10 at a dose of approximately 1 μg per animal reduces the release of proinflammatory cytokines and substantially improves outcome when also given 30 min after the challenge (304, 340, 561, 570, 571). In a cecal ligation puncture model, IL10 decreased mortality even when given 6 hr after induction of sepsis (340). Furthermore, it can reduce mortality of mice challenged with staphylococcal enterotoxin B (572). Similar effects were observed with an IL10/Fc-γ construct (573). Notably, in these experiments IL10 also induced a general state of immunosuppression that may be important for clinical application because it augments susceptibility to repeated infections or continuous invasion of microorganisms (304). Gene therapy with an expression plasmid containing cDNA coding for human IL10 was successfully applied in mice to diminish the inflammatory response induced by endotoxin (574). The results of this novel approach for

the treatment of sepsis were encouraging in that significant protection was afforded and that TNF release was reduced.

Circulating IL10 is increased in most patients with sepsis: In one study levels were increased in 22 of 48 (46%) patients with normotensive sepsis and in 17 of 21 (81%) patients with shock (575). In another study 83% of the patients with septic shock and only 25% of critically ill patients had increased levels of IL10 (576). In the latter study it was further concluded that IL10, because of its correlations with circulating neopterin, TNF, IL6, and IL8, likely is produced by cells of the monocyte–macrophage lineage. In 25 children with fulminant meningococcal septic shock, circulating IL10 was increased in all patients, with the highest levels occurring in patients who died (577). These results were confirmed by others showing that levels of IL10 were increased in all patients with neisseria meningococcal septic shock and were higher than levels in patients without shock (578). Comparable findings were reported by others (374, 579, 580). In patients with septic shock caused by other bacteria besides *Neisseria* species, levels of circulating IL10 were increased and higher than those in patients with non-septic shock (581). In addition, levels correlated with parameters, reflecting severity of shock. In another study in patients with trauma, 40 of 66 patients had elevated IL10 levels, which were associated with hypotension and the development of sepsis (582). Whether endogenous IL10 is able to control the release of proinflammatory cytokines by monocytes–macrophages completely during sepsis is not known. IL10 administration to healthy volunteers suppresses in a dose-dependent fashion the production of IL1 and TNF by endotoxin *ex vivo* (583, 584). Leukocytes from patients with sepsis are indeed unresponsive to endotoxin *in vitro* with regard to the production of TNF and IL1 (145, 146, 585). This state of unresponsiveness may reflect interaction of leukocytes with IL10 (578) and not TGFβ or IL4 (578, 580). Surprisingly, however, IL10 correlates positively, and not inversely, with levels of proinflammatory cytokines, such as TNF, IL6, and IL8, in septic shock (578–581).

IL4 and IL10 have overlapping effects *in vitro* (586–588). However, the administration of IL4 to animals with sepsis increases mortality (589). In agreement herewith, mice deficient for IL4 develop local inflammatory reactions similar to those of wild-type mice (568), indicating that at least in some inflammatory models IL4 is not an important suppressant of endogenous TNF release. The reason for these diverging effects of IL10 and IL4 *in vivo* is unknown, although there is some evidence for dissimilar effects *in vitro* as well. For example, in contrast to IL10 and TGFβ, IL4 is unable to downregulate procoagulant activity and tissue factor expression by human macrophages stimulated with endotoxin (590). Increased levels of active TGFβ have been observed in a baboon model for sepsis (351) as

well as in occasional patients with sepsis or septic shock (578). Furthermore, circulating and bronchoalveolar levels of IL4 may be increased in patients with sepsis (591).

Taken together the data discussed in this section indicate that in most animal models for sepsis the major role of IL10 is to limit the synthesis of TNF and IL1. Whether a similar role is played by endogenous IL10 in human sepsis remains to be established. Furthermore, exogenous IL10 is a promising candidate for therapeutic use, although considering the unresponsiveness of peripheral blood mononuclear cells from patients with sepsis for endotoxin-induced TNF and IL1 release, it may not be efficacious in some patients.

B. Interferon-γ and Interleukin-12

Interferons were among the first cytokines to be discovered and represent a family of (glyco)proteins that can be produced by virtually every cell in the body in response to viral and nonviral stimuli such as bacteria, protozoa, and endotoxin. Interferons are divided into a few subgroups, some of which contain several members each produced by a different gene. For example, at least 14 different types of IFNα are now recognized (592). IFNγ, constituting one of the subgroups of interferon, has a number of effects on and is produced by immune cells and has, therefore, also been called immune interferon. This cytokine was cloned in 1982 (593), is mainly produced by T cells and NK cells, and is able to activate macrophages [for review, see Ref. (592)]: It enhances the transcription of IL1 and TNF by these cells (91) and increases the production of reactive oxygen and nitrogen intermediates. IFNγ can also interact with endothelial cells. For example, it potentiates the ability of these cells to produce reactive nitrogen intermediates in response to TNF, IL1, or endotoxin (594) and thereby affects vascular tone, and it enhances TNF-induced hyperpermeability (218). Similarly, it synergizes with TNF in the induction of cytokines such as RANTES by endothelial cells (173). Subcutaneous injection of IFNγ to patients with cancer induces the release of TNF *in vivo* (595). Because of these and other effects, IFNγ is, in the context of sepsis, a proinflammatory cytokine.

Involvement of IFNγ in the pathogenesis of sepsis is evident from a number of observations. First, circulating levels of this cytokine increase in baboons receiving an infusion with a lethal dose of live *E. coli*, reaching peak levels at 8 hr after the challenge (264, 596), or in D-galactosamine-treated or normal mice challenged with staphylococcal enterotoxin B (471), but not in human volunteers after a challenge with a low dose of endotoxin (264). A similar increase of circulating IFNγ occurs in mice challenged with a lethal dose of endotoxin (597). Second, levels of circulating IFNγ

are increased in some children (19%) with severe infectious purpura and associated with a fatal outcome (364). A small percentage of adults with sepsis also have increased plasma levels of IFNγ (372), although this is not consistently found (367). The third and perhaps most convincing piece of evidence is that passive immunization of animals with antibodies that neutralize the activity of interferon-γ confers significant protection against a lethal endotoxin-induced Shwartzman reaction (598, 599), a lethal challenge with endotoxin (589, 597, 600), and a lethal challenge with live *S. aureus* (344) or *E. coli* (601), even when given as late as 2 hr after the challenge (589, 597). It is not clear whether protection is due to diminished TNF synthesis: In some studies (318, 589, 599) it is and in others it is not associated with reduced circulating levels of TNF (471, 597, 601). Anti-IFNγ partially inhibited endotoxin-induced pulmonary edema in mice (602). Anti-IFNγ, however, did not afford protection against staphylococcal enterotoxin B-induced mortality in galactosamine-sensitized mice (471). On the other hand, the administration of IFNγ to animals receiving a sublethal challenge with endotoxin or live bacteria increases mortality, which is accompanied by increased plasma levels of TNF and IL6 (589, 597, 601). Studies in mice deficient for IFNγ-R challenged with *S. aureus*, however, have revealed that IFNγ may have beneficial effects as well: In the deficient mice bacterial growth and levels of proinflammatory cytokines are higher during the initial stages of sepsis resulting in an increased mortality during the acute stage, whereas in the deficient mice surviving the acute phase, mortality was lower during the later stages of the septic process (603). These data indicate that IFNγ has detrimental (but also some beneficial) effects in sepsis and, in addition, may be an interesting target for immunotherapy in sepsis, particularly because anti-IFNγ therapy seems to be most effective at the later stages. However, one should realize that in situations of impaired host defenses such as in neonates, IFNγ, rather than anti-IFNγ, decreases mortality probably by restoring impaired defenses, as has been shown in a mouse model for sepsis with group B streptococci (604).

The source of IFNγ in sepsis is not clear. Considering that they are potent sources for IFNγ and necessary for the full development of the endotoxin-induced Shwartzman reaction (605), NK cells are probably major sources of IFNγ in sepsis. If so, IL12 may be an important cytokine in sepsis as well because this cytokine can be produced by macrophages stimulated *in vitro* with endotoxin, particularly in the presence of IFNγ (606).

Bioactive IL12 is a heterodimer consisting of two subunits with molecular masses of 40 and 35 kDa, respectively, i.e., the p40 and p35 subunits. This cytokine is known for its potency to regulate the balance between Th1

and Th2 lymphocytes, favoring a shift toward the former (607). In addition, it is a potent inducer for the secretion of IFNγ by NK cells. The receptor for IL12 consists of two subunits (608, 609), both of which are homologous to gp130, the signal transducing peptide chain of the IL6 receptor, and to the receptors of G-CSF and LIF.

IL12 is released into the circulation following a challenge with endotoxin (610, 611). Studies in baboons challenged with a lethal or a sublethal dose of *E. coli* show that similar levels of bioactive IL12 as well as its free 40-kDa chain are released following a lethal or a sublethal dose of *E. coli* despite that IFNγ levels are different in both conditions (264, 596). Thus, IL12 cannot be the only factor required for release of IFNγ during sepsis. It synergizes with TNF in this regard. However, in addition to IL12 and TNF, other cytokines are likely involved in the synthesis of IFNγ because administration of both cytokines to mice was not sufficient to induce IFNγ (612). Cytokines recently identified to fulfill this role in IFNγ production are IL15 (613) and IFNγ-inducing factor (IL18) (614–616). Whether these cytokines are induced during sepsis is unknown.

The important role of IL12 in the pathogenesis of sepsis is illustrated by observations in animal models that administration of neutralizing anti-IL12 antibody reduces the release of IFNγ (611, 612, 617) and affords protection against the development of shock (612, 617). Administration of IL12 to animals, however, does not induce a sepsis-like syndrome (618), indicating that IL12 acts in conjunction with other cytokines. On the other hand, intraperitoneal administration of IL12 in D-galactosamine mice challenged with staphylococcal enterotoxin B conferred protection (619), showing that IL12 may also have beneficial effects in sepsis. Increased production of IL12 also occurs in human sepsis: In a recent study of a group of children with meningococcal sepsis, circulating levels of IL12 p40 were increased in the majority of patients, with the highest levels occurring in those who died (J. Hazelzet *et al.*, submitted for publication).

The role of IFNα in sepsis seems to be opposite to that of IFNγ because this cytokine affords protection against a lethal challenge with endotoxin (599, 620), which is accompanied by a diminished synthesis and release of TNF. Surprisingly, observations in mice suggest that IFNα, as well as IFNβ, may act as a cofactor for IFNγ induction by gram-negative bacteria (621). Levels of IFNα in plasma increase during a lethal endotoxin-induced Shwartzman reaction (599) and in some patients with sepsis (364, 372).

C. Leukemia Inhibitory Factor

LIF is a cytokine with a remarkable number of seemingly unrelated activities involved in metabolism, growth, and differentiation (622). Among these activities are induction of acute phase protein synthesis in the liver

[LIF is identical to hepatocyte stimulatory factor III (440, 441)], differentiation of murine monocytic leukemia cells into macrophages, proliferation of myoblasts, differentiation and proliferation of megakaryocytes, inhibition of melanocyte-derived lipoprotein lipase, activation of osteoclasts, differentiation of cholinergic neurons, proliferation of DA-1 myeloid cells, as well as a role in early embryogenesis [for review, see Ref. (622)]. LIF shares amino acid sequence homology with oncostatin-M and ciliary neurotrophic factor and, to a lesser degree, with IL6 and G-CSF (415, 623, 624). LIF binds to cells initially via a low-affinity receptor (LIF-R), which has been cloned recently and found to be structurally related to gp130 (625), the signal transducing molecule of the IL6-R. Moreover, a high-affinity binding for LIF is induced once LIF-R becomes associated with gp130 (14, 626), as has been found for the interaction between IL6, IL6-R, and gp130 (see above). Considering that IL6 and oncostatin-M also use gp130 for signal transduction, it is not surprising that LIF, IL6, and oncostatin-M have overlapping functions (14).

Although its precise mechanism of action is not known, LIF is involved in the pathogenesis of sepsis: First, LIF enhances host resistance against lethal endotoxemia in mice, at least when administered 2–24 hr before the challenge, and particularly when combined with low doses of IL1 or TNF (627, 628). Whether this effect is dependent on the induction of acute phase proteins (629) is not known. Second, passive immunization of mice against LIF reduces lethality, which is accompanied by a diminished release of TNF, IL1, and IL6 during endotoxemia (630), suggesting that LIF affects the endotoxin-stimulated synthesis of these cytokines. *In vitro* LIF can induce the synthesis of TNF, IL1, IL6, and IL8 by different cell lines (631). Moreover, using a radioreceptor competition assay Waring *et al.* (632) demonstrated increased levels of LIF in some occasional patients with gram-negative sepsis. The same authors reported increased levels of LIF in 13 of 33 patients with meningococcemia; these levels correlated with disease severity and mortality (633). In patients with sepsis due to other organisms besides meningococci, approximately 33% had detectable levels of LIF (634). Furthermore, in septic baboons large quantities of LIF are released into the circulation following a challenge with a lethal or, to a lesser extent, a sublethal dose of *E. coli* (172). The course of LIF in this model is remarkably parallel to that of IL6. Moreover, intravenous injection of TNF induces LIF in baboons, suggesting that, as for IL6, most of LIF produced during sepsis is induced by endogenous TNF (172).

D. Macrophage Migration Inhibition Factor

The precise role of MIF in sepsis is far from clear. During endotoxemia in mice MIF is synthesized by the pituitary gland and possibly also by

other organs, which finally results in increased circulating levels that reach their maximum values 20 hr after the challenge (635). Neutralization of MIFs (using specific rabbit anti-MIF antiserum in mice) that are subsequently challenged with a LD50 of endotoxin confers significant protection against mortality, whereas coadministration with recombinant MIF enhances mortality (635). These data demonstrate that MIF potentiates lethal endotoxemia and may play an important role in the pathogenesis of sepsis. Recently, low concentrations of glucocorticoids were found to induce rather than to inhibit MIF production from macrophages, raising the intriguing possibility that this cytokine overrides glycocorticoid-mediated inhibition of endotoxin-induced cytokine production (636). Obviously, more studies are needed to establish the precise role of this interesting cytokine in sepsis.

E. Granulocyte Colony-Stimulating Factor

G-CSF was originally identified as a neutrophil-specific hemopoietin. Analysis of the nucleotide sequence of cDNA coding for G-CSF predicts that G-CSF consists of a single peptide chain of 174 amino acid residues (637). G-CSF can be produced by a number of different cells, for example, macrophages or endothelial cells, in response to endotoxin, TNF, or IL1 (638, 639). Levels of G-CSF increase after an intravenous administration of endotoxin to humans, the highest levels occurring 6 hr after the challenge (270, 275, 282, 640). The precise role of G-CSF in sepsis is not known. On the one hand, it may be involved in the leukocytosis and contribute to the activation of neutrophils (641, 642) and the release of cytokines such as TNF (643) and aggravate acute lung injury (644). On the other hand, G-CSF induces the release of anti-inflammatory cytokines and receptors, such as IL1ra and sTNF-R (643), and attenuates sepsis in some animal models possibly by reducing circulating TNF levels (645–648) or by improved recruitment of neutrophils to infected sites (649). A similar protective effect of G-CSF was also observed in a model for peritonitis induced by cecal perforation (650) and in a model for T cell-mediated lethal shock triggered by superantigens (651). Furthermore, G-CSF was able to reduce mortality in neutropenic rabbits challenged transtracheally with *Pasteurella multocida* (652). In other models, however, no significant effect of G-CSF was observed on the systemic and pulmonary responses to endotoxin (653).

A few studies have reported on G-CSF levels in patients with sepsis. Levels are increased in patients with sepsis compared to patients with trauma and remained elevated in nonsurviving patients (492). In patients with meningococcal sepsis, G-CSF levels were elevated, particularly in patients with shock. More precisely, levels exceeding 10 ng/ml are associ-

ated with shock, disseminated intravascular coagulation, fulminant infection, and fatal outcome (654).

F. Granulocyte-Macrophage Colony-Stimulating Factor

A few observations suggest that GM-CSF is also involved in the pathogensis of sepsis. The administration of GM-CSF to healthy volunteers induced activation and degranulation of neutrophils as well as the release of IL8, but not that of TNF or IL6 (655). In addition, pretreatment with GM-CSF enhanced the release of TNF, IL1, and IL6 and increased mortality in mice challenged with endotoxin (647, 656). Administration of GM-CSF 3 hr after the onset of peritonitis induced by cecal ligation and puncture to rats resulted in an accelerated course of sepsis, presumably because of inhibition of peritoneal infiltration of neutrophils and hence impaired local defense against the infecting microorganisms (657). In contrast, pretreatment of rats suffering from burns for 5 days with GM-CSF before induction of sepsis by cecal ligation and puncture resulted in improved survival (658). Finally, increased circulating levels of GM-CSF briefly occurred in patients with life-threatening shock associated with meningococcal sepsis (654).

X. Perspectives

Following the observation by Cerami and co-workers (16) that TNF is involved in the pathogenesis of sepsis, a large number of papers have shown expression and release of various cytokines in human and animal sepsis. However, their precise role in the pathogenesis of human sepsis is not fully understood as is illustrated, for example, by the failure of large multicenter trials to show efficacy of inhibitors of TNF or IL1 in reducing mortality of septic patients. One might therefore postulate that cytokines are not important in the pathogenesis of human sepsis. Although it cannot be definitely ruled out, a strong argument against this possibility is that the administration of cytokines, such as TNF or IL1, to human beings induces a sepsis-like syndrome. For example, studies in (cancer) patients have shown that septic shock-like syndromes can be induced by IL2 (19, 20, 659, 660), which in turn induces the release of most cytokines discussed above (247, 516, 661–665). Another explanation for the failure of anticytokine therapies in sepsis is that the release of most cytokines occurs rather early in the septic process and, hence, that treatment with anticytokines is only efficacious when applied at a very early stage of sepsis. In agreement herewith, anti-IL1 and anti-TNF therapies are particularly efficacious in animal models when administered before or shortly after the challenge. In this respect it is to be noted that studies in cancer patients

have shown that the repeated administration of IL2 can induce severe hypotension and multiple organ failure, both of which resolve within 2 or 3 days upon discontinuation of IL2 therapy. Because most patients with sepsis do not die within 2 or 3 days upon admission to a hospital, it seems justified to expect that appropriate cytokine-modulating therapy should be able to prevent mortality in most patients with full-blown sepsis. A third reason for the failure of anticytokine therapies in human sepsis may be that in most animal models cytokines are synthesized and released in an ordered fashion due to a standardized way of challenging with endotoxin or bacteria. In human sepsis the challenge will be nonstandardized and quite variable. Therefore, the synthesis and release of cytokines in human sepsis will occur in a less standardized fashion, and, in addition, will be modified by underlying disease processes. This will allow synergisms and antagonisms between cytokines and other mediators that are not observed in animal models for sepsis and, hence, make anticytokine therapies in patients unpredictable. A fourth reason for the failure of anticytokine therapies in human sepsis is that cytokines not only have harmful effects in sepsis but also help in the defense against the invading microorganisms. These opposing effects may vary from patient to patient and in the various stages of the septic process. Therefore, successful cytokine-modulating therapy in sepsis likely will require (i) further appreciation of the precise role of each cytokine in the pathogenesis of sepsis, (ii) development of methods that allow rapid bedside assessment of the contribution of various cytokines to the septic process in individual patients, and (iii) determination of patient-specific cytokine profiles, which can be used to fine-tune cytokine-modulating therapies. It is hoped that such an approach will help reduce mortality of sepsis.

REFERENCES

1. Bone, R. C. (1992). Sepsis and multiple organ failure: Consensus and controversy. *In* "Mediators of Sepsis. *Update Int. Care Emergency Medicine*" (M. Lamy and L. G. Thijs, eds.) Vol. 16, pp. 3. Springer Verlag, Berlin.
2. Morrison, D. C., and R. J. Ulevitch (1978). The effects of bacterial endotoxins on host mediation systems. *Am. J. Pathol.* **93,** 527–617.
3. McCabe, W. R., T. L. Treadwell, and A. De Maria,Jr. (1983). Pathophysiology of bacteremia. *Am. J. Med.* **75,** 7–18.
4. Hack, C. E., and L. G. Thijs (1991). The orchestra of mediators in the pathogenesis of sepsis. *In* "Update on Intensive Care and Emergency Medicine" (J. L. Vincent, ed.), Vol. 14, pp. 232–246. Springer Verlag, Berlin.
5. ACCP/SCCM Censensus Conference Committee (1992). Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Chest* **101,** 1644–1655.
6. Suffredini, A. F. (1992). Endotoxin administration to humans: A model of inflammatory responses relevant to sepsis. *In* "Mediators of Sepsis. Update in Intensive Care and

Emergency Medicine" (M. Lamy and L. G. Thijs, eds.), Vol. 15, pp. 13–30. Springer-Verlag, Berlin.

7. Suffredini, A. F., R. E. Fromm, M. M. Parker, M. Brenner, J. A. Kovacs, R. A. Wesley, and J. E. Parrillo (1989). The cardiovascular response of normal humans to the administration of endotoxin. *N. Engl. J. Med.* **321,** 280–287.
8. Mohlen, M. A. M. von der, A. N. Kimmings, N. I. Wedel, M. L. C. M. Mevissen, J. Jansen, N. Friedmann, T. J. Lorenz, B. J. Nelson, M. L. White, R. Bauer, C. E. Hack, A. J. M. Eerenberg, and S. J. H. van Deventer (1995). Inhibition of endotoxin-induced cytokine release and neutrophil activation in humans by use of recombinant bactericidal/permeability-increasing protein. *J. Infect. Dis.* **172,** 144–151.
9. Levi, M., H. ten Cate, K. A. Bauer, T. van der Poll, T. S. Edgington, H. R. Buller, S. J. H. Van Deventer, C. E. Hack, J. W. Ten Cate, and R. D. Rosenberg (1994). Inhibition of endotoxin-induced activation of coagulation and fibrinolysis by pentoxifylline or by a monoclonal anti-tissue factor antibody in chimpanzees. *J. Clin. Invest.* **93,** 114–120.
10. Cross, A. S., S. M. Opal, J. C. Sadoff, and P. Gemski (1993). Choice of bacteria in animal models of sepsis. *Infect. Immun.* **61,** 2741–2747.
11. De Boer, J. P., A. A. Creasey, A. Chang, D. Roem, A. J. M. Eerenberg, C. E. Hack, and F. B. Taylor, Jr. (1993). Activation of the complement system in baboons challenged with live E. coli: Correlation with mortality and evidence for a biphasic activation pattern. *Infect. Immun.* **61,** 4293–4301.
12. Larrick, J. W., and S. C. Wright (1990). Cytotoxic mechanism of tumor necrosis factor-α. *FASEB J.* **4,** 3215–3223.
13. Taga, T. (1992). IL6 signalling through IL6 receptor and receptor-associated signal transducer, gp130. *Res. Immunol.* **143,** 737–739.
14. Kishimoto, T., S. Akira, and T. Taga (1992). Interleukin-6 and its receptor: A paradigm for cytokines. *Science* **258,** 593–597.
15. Dinarello, C. A. (1991). Interleukin-1 and interleukin-1 antagonism. *Blood* **77,** 1627–1652.
16. Beutler, B., I. W. Milsark, and A. Cerami (1985). Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science* **229,** 869–871.
17. Beutler, B., and A. Cerami (1987). Cachectin: More than a tumor necrosis factor. *N. Engl. J. Med.* **316,** 379–385.
18. Cerami, A., and B. Beutler (1988). The role of cachectin/TNF in endotoxic shock and cachexia. *Immunol. Today* **9,** 28–31.
19. Ognibene, F. P., S. A. Rosenberg, M. Lotze, J. Skibber, M. M. Parker, J. H. Shelhamer, and J. E. Parillo (1988). Interleukin-2 administration causes reversible hemodynamic changes and left ventricular dysfunction similar to those seen in septic shock. *Chest* **94,** 750–754.
20. Gaynor, E. R., L. Vitek, L. Sticklin, S. P. Creekmore, M. E. Ferraro, J. X. Thomas, S. G. Fisher, and R. I. Fisher (1988). The hemodynamic effects of treatment with interleukin-2 and lymphokine activated killer cells. *Ann. Intern. Med.* **109,** 953–958.
21. Bone, R. C. (1991). The pathogenesis of sepsis. *Ann. Intern. Med.* **115,** 457–469.
22. Parrillo, J. E. (1991). Management of septic shock: Present and future. *Ann. Intern. Med.* **115,** 491–493.
23. Erickson, L., F. J. de Sauvage, K. Kikly, K. Carver-Moore, S. Pitts-Meek, N. Gillett, K. C. F. Sheehan, R. D. Schreiber, D. V. Goeddel, and M. W. Moore (1994). Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature* **372,** 560–563.

24. Fischer, C. J., Jr., J. M. Agosti, S. M. Opal, S. F. Lowry, R. A. Balk, J. C. Sadoff, E. Abraham, R. M. H. Schein, and E. Benjamin (1996). Treatment of septic shock with the tumor necrosis factor:Fc fusion protein. *N. Eng. J. Med* **334,** 1697–1702.
25. Engelberts, I., S. Stephens, G. J. M. Francot, C. J. Van der Linden, and W. A. Buurman (1991). Evidence for different effects of soluble TNF-receptors on various TNF measurements in human biological fluids. *Lancet* **338,** 515–516.
26. Parsons, P. E., F. A. Moore, E. E. Moore, D. N. Ikle, P. M. Henson, and G. S. Worthen (1992). Studies on the role of tumor necrosis factor in adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **146,** 694–700.
27. Le, J., and J. Vilçek (1987). Biology of disease; tumor necrosis factor and interleukin 1: Cytokines with multiple overlapping biological activities. *Lab. Invest.* **56,** 234–248.
28. Movat, H. Z., C. E. Burrowes, M. I. Cybulsky, and C. A. Dinarello (1987). Acute inflammation and a Shwartzman-like reaction induced by interleukin-1 and tumor necrosis factor. *Am. J. Pathol.* **129,** 463–476.
29. Aggarwal, B. B., W. J. Kohr, P. E. Hass, B. Moffet, C. A. Spencer, W. J. Heuzel, T. S. Bringman, C. E. Nedwin, D. V. Soeddel, and R. N. Harkins (1985). Human tumor necrosis factor. Production, purification and characterization. *J. Biol. Chem.* **260,** 2345–2354.
30. Beutler, B., J. Mahony, N. Letrang, P. Pekala, and A. Cerami (1985). Purification of cachectin, a lipoprotein lipase suppressing hormone secreted by endotoxin-induced RAW 264.7 cells. *J. Exp. Med.* **161,** 984–995.
31. Beutler, B., D. Greenwald, J. D. Hulmes, M. Chang, C. E. Pan, J. Mathison, R. Ulevitch, and A. Cerami (1985). Identity of tumor necrosis factor and the macrophage-secreted factor cachectin. *Nature* **316,** 552–554.
32. Pennica, D., G. E. Nedwin, J. S. Hayflick, P. H. Seeburg, R. Derynck, M. A. Palladino, W. J. Kohr, B. B. Aggarwal, and D. V. Goeddel (1984). Human tumor necrosis factor: Precursor structure, expression and homology to lymphotoxin. *Nature* **312,** 724–729.
33. Ruddle, N. H. (1992). Tumor necrosis factor (TNF-alpha) and lymphotoxin (TNF-beta). *Curr. Opin. Immunol.* **4,** 327–332.
34. Jones, E. Y., D. I. Stuart, and N. P. C. Walker (1989). Structure of tumour necrosis factor. *Nature* **338,** 225–228.
35. Smith, R. A., and C. Baglioni (1987). The active form of tumor necrosis factor is a trimer. *J. Biol. Chem.* **262,** 6951–6954.
36. Kriegler, M., C. Perez, K. DeFay, I. Albert, and S. D. Lu (1988). A novel form of TNF/Cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. *Cell* **53,** 45–53.
37. Decker, T., M. L. Lohmann-Matthes, and G. E. Gifford (1987). Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. *J. Immunol.* **138,** 957–962.
38. Mohler, K. M., P. R. Sleath, J. N. Fitzner, D. P. Cerretti, M. Alderson, S. S. Kerwar, D. S. Torrance, C. Otten-Evans, T. Greenstreet, K. Weerawarna, S. R. Kronheim, M. Petersen, M. Gerhart, C. J. Kozlosky, C. J. March, and R. A. Black (1994). Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature* **370,** 218–220.
39. Gearing, A. J. H., P. Beckett, M. Christodoulou, M. Churchill, J. Clements, A. H. Davidson, A. H. Drummond, W. A. Galloway, R. Gilbert, J. L. Gordon, T. M. Leber, M. Mangan, K. Miller, P. Nayee, K. Owen, S. Patel, W. Thomas, G. Wells, L. M. Wood, and K. Woolley (1994). Processing of tumour necrosis factor-alpha precursor by metalloproteinases. *Nature* **370,** 555–557.

40. McGeehan, G. M., J. D. Becherer, R. C. Bast, Jr., C. M. Boyer, B. Champion, K. M. Connolly, J. G. Conway, P. Furdon, S. Karp, S. Kidao, A. B. McElroy, J. Nichols, K. M. Pryzwansky, F. Schoenen, L. Sekut, A. Truesdale, M. Verghese, J. Warner, and J. P. Ways (1994). Regulation of tumour necrosis factor-alpha processing by metalloproteinase inhibitor. *Nature* **370,** 558–561.
41. Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K. A. Schooley, M. Gerhart, R. Davis, J. N. Fitzner, R. S. Johnson, R. J. Paxton, C. J. March, and D. P. Cerretti (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* **385,** 729–732.
42. Moss, M. L., S. L. Jin, M. E. Milla, W. Burkhart, H. L. Carter, W. J. Chen, W. C. Clay, J. R. Didsbury, D. Hassler, C. R. Hoffman, T. A. Kost, M. H. Lambert, M. A. Leesnitzer, P. McCauley, G. McGeehan, J. Mitchell, M. Moyer, G. Pahel, W. Rocque, L. K. Overton, F. Schoenen, T. Seaton, J. L. Su, J. Warner, D. Willard, and J. D. Becherer (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* **385,** 733–736.
43. Black, R. A., F. H. Durie, C. Otten-Evans, R. Miller, J. L. Slack, D. H. Lynch, B. Castner, K. M. Mohler, M. Gerhart, R. S. Johnson, Y. Itoh, Y. Okada, and H. Nagase (1996). Relaxed specificity of matrix metalloproteinases (MMPS) and TIMP insensitivity of tumor necrosis factor-alpha (TNF-alpha) production suggest the major TNF-alpha converting enzyme is not an MMP. *Biochem. Biophys. Res. Commun.* **225,** 400–405.
44. Robache-Gallea, S., V. Morand, J. M. Bruneau, B. Schoot, E. Tagat, E. Realo, S. Chouaib, and S. Roman-Roman (1995). In vitro processing of human tumor necrosis factor α. *J. Biol. Chem.* **270,** 23688–23692.
45. Spies, T., C. C. Morton, S. A. Nedospasov, W. Fiers, D. Pious, and J. L. Strominger (1986). Genes for the tumor necrosis factors alpha and beta are linked to the human major histocompability complex. *Proc. Natl. Acad. Sci. USA* **83,** 8699–8702.
46. Dinarello, C. A. (1988). Biology of interleukin 1. *FASEB J.* **2,** 108–115.
47. Kronheim, S. R., C. J. March, S. K. Erb, P. J. Conlon, D. Y. Mochizuki, and T. P. Hopp (1985). Human interleukin 1: Purification to homogeneity. *J. Exp. Med.* **161,** 490–500.
48. Schmid, J. A. (1984). Purification and partial characterization of normal human interleukin-1. *J. Exp. Med.* **160,** 772–781.
49. Wood, D. D., E. K. Bayne, M. B. Goldring, M. Gaven, D. Hamerman, J. L. Humes, E. J. Ihrie, P. E. Lipsky, and M. J. Staruch (1985). The four biochemical distinct species of human interleukin 1 all exhibit similar biological activities. *J. Immunol.* **134,** 895–902.
50. Auron, P. E., A. C. Webb, L. J. Rosenwasser, S. F. Mucci, A. Rich, K. R. Wolff, and C. A. Dinarello (1984). Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *Proc. Natl. Acad. Sci. USA* **81,** 7907–7911.
51. March, C. J., B. Mosley, A. Larsen, D. P. Cerretti, G. Braedt, V. Price, S. Gillis, C. S. Henney, S. R. Kronheim, K. Grabstein, P. J. Conlon, T. P. Hopp, and D. Cosman (1985). Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* **315,** 641–647.
52. Dinarello, C. A. (1996). Biologic basis for interleukin-1 in disease. *Blood* **87,** 2095–2147.
53. Rubartelli, A., F. Cozzolino, M. Talio, and R. Sitia (1990). A novel secretory pathway for interleukin-1β, a protein lacking a signal sequence. *EMBO J.* **9,** 1503–1510.
54. Dinarello, C. A. (1993). Modalities for reducing interleukin 1 activity in disease. *Immunol. Today* **14,** 260–264.

55. Dinarello, C. A. (1992). Role of interleukin-1 in infectious diseases. *Immunol. Rev.* **127,** 119–146.
56. Kostura, M. J., M. J. Tocci, G. Limjuco, J. Chin, P. Cameron, A. G. Hillman, N. A. Chartrain, and J. A. Schmidt (1989). Identification of a monocyte specific pre-interleukin 1β convertase activity. *Proc. Natl. Acad. Sci. USA* **86,** 5227–5231.
57. Black, R. A., S. R. Kronheim, M. Cantrell, M. C. Deeley, C. J. March, K. S. Prickett, J. Wignall, P. J. Conlon, D. Cosman, T. P. Hopp, and D. Y. Mochizuki (1988). Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. *J. Biol. Chem.* **263,** 9437–9442.
58. Sleath, P. R., R. C. Hendrickson, S. R. Kronheim, C. J. March, and R. A. Black (1990). Substrate specificity of the protease that processes human interleukin-1β. *J. Biol. Chem.* **265,** 14526–14528.
59. Cerretti, D. P., C. J. Kozlosky, B. Mosley, N. Nelson, K. Van Ness, T. A. Greenstreet, C. J. March, S. R. Kronheim, T. Druck, L. A. Cannizzaro, K. Huebner, and R. A. Black (1992). Molecular cloning of the interleukin-1-beta converting enzyme. *Science* **256,** 97–100.
60. Walker, N. P. C., R. V. Talanian, K. D. Brady, L. C. Dang, N. J. Bump, C. R. Ferenz, S. Franklin, T. Ghayur, M. C. Hackett, L. D. Hammill, L. Herzog, M. Hugunin, W. Houy, J. A. Mankovich, L. McGuiness, E. Orlewicz, M. Paskind, C. A. Pratt, P. Reis, A. Summani, M. Terranova, J. P. Welch, L. Xiong, A. Moller, D. E. Tracey, R. Kamen, and W. W. Wong (1994). Crystal structure of the cysteine protease interleukin-1-beta-converting enzyme: A (p20/p10)2 homodimer. *Cell* **78,** 343–352.
61. Wilson, K. P., J.-A. F. Black, J. A. Thomson, E. E. Kim, J. P. Griffith, M. A. Navia, M. A. Murcko, S. P. Chambers, R. A. Aldape, S. A. Raybuck, and D. J. Livingston (1994). Structure and mechanism of interleukin-1-beta converting enzyme. *Nature* **370,** 270–275.
62. Barinaga, M. (1994). Cell suicide: By ice, not fire. *Science* **263,** 754–756.
63. Thornberry, N. A. (1994). Key mediator takes shape. *Nature* **370,** 251–252.
64. Miller, B. E., P. A. Krasney, D. M. Gauvin, K. B. Holbrook, D. J. Koonz, R. V. Abruzzese, R. E. Miller, K. A. Pagani, R. E. Dolle, M. A. Ator, and S. C. Gilman (1995). Inhibition of mature IL-1β production in murine macrophages and a murine model of inflammation by WIN 67694, an inhibitor of IL-1β converting enzyme. *J. Immunol.* **154,** 1331–1338.
65. Fletcher, D. S., L. Agarwal, K. T. Chapman, J. Chin, L. A. Egger, G. Limjuco, S. Luell, D. E. MacIntyre, E. P. Peterson, N. A. Thornberry, and M. J. Kostura (1995). A synthetic inhibitor of interleukin-1beta converting enzyme prevents endotoxin-induced interleukin-1beta production in vitro and in vivo. *J. Interferon Res.* **15,** 243–248.
66. Eisenberg, S. P., R. J. Evans, W. P. Arend, E. Verderber, M. T. Brewer, C. H. Hannum, and R. C. Thompson (1990). Primary structure and functional expression from complementary DNA of a human interleukin-1 receptor antagonist. *Nature* **343,** 341–346.
67. Carter, D. B., M. R. Deibel, Jr., C. J. Dunn, C.-S. C. Tomich, A. L. Laborde, J. L. Slightom, A. E. Berger, M. J. Bienkowski, F. F. Sun, R. N. McEwan, P. K. W. Harris, A. W. Yem, G. A. Waszak, J. G. Chosay, L. C. Sieu, M. M. Hardee, H. A. Zurcher-Neely, I. M. Reardon, R. L. Heinrikson, S. E. Truesdell, J. A. Shelly, T. E. Eessalu, B. M. Taylor, and D. E. Tracey (1990). Purification, cloning, expression and biological characterization of an interleukin-1 receptor antagonist protein. *Nature* **344,** 633–638.
68. Arend, W. P. (1993). Interleukin-1 receptor antagonist. *Adv. Immunol.* **54,** 167–227.
69. Muzio, M., N. Polentarutti, M. Sironi, G. Poli, L. de Gioia, M. Introna, A. Mantovani, and F. Colotta (1995). Cloning and characterization of a new isoform of the interleukin 1 receptor antagonist. *J. Exp. Med.* **182,** 623–628.

70. Tartaglia, L. A., and D. V. Goeddel (1992). Two TNF receptors. *Immunol. Today* **13,** 151–153.
71. Loetscher, H., Y.-C. E. Pan, H.-W. Lahm, R. Gentz, M. Brockhaus, H. Tabuchi, and W. Lesslauer (1990). Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell* **61,** 351–359.
72. Olsson, I., T. Gatanaga, U. Gullberg, M. Lantz, and G. A. Granger (1993). Tumour necrosis factor (TNF) binding proteins (soluble TNF receptor forms) with possible roles in inflammation and malignancy. *Eur. Cytokine Network.* **4,** 169–180.
73. Brockhaus, M., H.-J. Schoenfeld, E.-J. Schlaeger, W. Hunziker, W. Lesslauer, and H. Loetscher (1990). Identification of two types of TNF receptors on human cell lines by monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* **874,** 3127–3131.
74. Schall, T. J., M. Lewis, K. J. Koller, A. Lee, G. C. Rice, G. H. W. Wong, T. Gatanaga, G. A. Granger, R. Lentz, H. Raab, W. J. Kohr, and D. V. Goeddel (1990). Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell* **61,** 361–370.
75. Bazzoni, F., and B. Beutler (1996). The tumor necrosis factor ligand and receptor families. *N. Eng. J. Med.* **334,** 1717–1725.
76. Engelmann, H., D. Aderka, Y. Nophar, O. Kemper, C. Brakebusch, H. Holtmann, and D. Wallach (1993). Soluble and cell surface receptors for tumor necrosis factor. *In* "Host Defense Dysfunction in Trauma, Shock and Sepsis" (E. Faist, G. Meakins, and F. M. Schildberg, eds.), pp. 599–607. Springer-Verlag, Berlin.
77. Fiers, W. (1991). Tumor necrosis factor: Characterization at the molecular, cellular and in vivo level. *FEBS Lett.* **285,** 199–212.
78. Kirchhofer, D., T. B. Tschopp, P. Hadvary, and H. R. Baumgartner (1994). Endothelial cells stimulated with tumor necrosis factor-alpha express varying amounts of tissue factor resulting in inhomogenous fibrin deposition in a native blood flow system. Effects of thrombin inhibitors. *J. Clin. Invest.* **93,** 2073–2083.
79. Mackay, F., H. Loetscher, D. Stueber, G. Gehr, and W. Lesslauer (1993). Tumor necrosis factor alpha (TNF-alpha)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. *J. Exp. Med.* **177,** 1277–1286.
80. Menegazzi, R., R. Cramer, P. Patriarca, P. Scheurich, and P. Dri (1994). Evidence that tumor necrosis factor alpha (TNF)-induced activation of neutrophil respiratory burst on biologic surfaces is mediated by the p55 TNF receptor. *Blood* **84,** 287–293.
81. Grell, M., E. Douni, H. Wajant, M. Lohden, M. Clauss, B. Maxeiner, S. Georgopoulos, W. Lesslauer, G. Kollias, K. Pfizenmaier, and P. Scheurich (1995). The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* **83,** 793–802.
82. Chih-Hsueh Chen, P., G. C. DuBois, and M. Y. Chen (1995). Mapping the domain(s) critical for the binding of human tumor necrosis factor-α to its two receptors. *J. Biol. Chem.* **270,** 2874–2878.
83. Van Zee, K. J., S. A. Stackpole, W. J. Montegut, M. A. Rogy, S. E. Calvano, K. C. Hsu, M. Chao, C. L. Meschter, H. Loetscher, D. Stuber, U. Ettlin, B. Wipf, W. Lesslauer, S. F. Lowry, and L. L. Moldawer (1994). A human tumor necrosis factor (TNF) alpha mutant that binds exclusively to the p55 TNF receptor produces toxicity in the baboon. *J. Exp. Med.* **179,** 1185–1191.
84. Burress Welborn, M., III, K. van Zee, P. D. Edwards, J. H. Pruitt, A. Kaibara, J. N. Vauthey, M. Rogy, W. L. Castleman, S. F. Lowry, J. S. Kenney, D. Stüber, U. Ettlin, B. Wipf, H. Loetscher, E. M. Copeland, III, W. Lesslauer, and L. L. Moldawer (1996). A human tumor necrosis factor p75 receptor agonist stimulates in vitro T cell

proliferation but does not produce inflammation or shock in the baboon. *J. Exp. Med.* **184,** 165–171.

85. Sims, J. E., C. J. March, D. Cosman, M. B. Widmer, H. R. MacDonald, C. J. McMahan, C. E. Grubin, J. M. Wignall, J. L. Jackson, S. M. Call, D. Friend, A. R. Alpert, S. Gillis, D. L. Urdal, and S. K. Dower (1988). cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science* **241,** 585–589.
86. Chizzonite, R., T. Truitt, P. L. Kilian, A. S. Stern, P. Nunes, K. P. Parker, K. L. Kaffka, A. O. Chua, D. K. Lugg, and U. Gubler (1989). Two high-affinity interleukin 1 receptors represent separate gene products. *Proc. Natl. Acad. Sci. USA* **86,** 8029–8033.
87. Dinarello, C. A., and S. M. Wolff (1993). The role of interleukin-1 in disease. *N. Engl. J. Med.* **328,** 106–113.
88. Dinarello, C. A. (1992). Role of interleukin-1 and tumor necrosis factor in systemic responses to infection and inflammation. *In* "Inflammation: Basic Principles and Clinical Correlates" (J. I. Gallin, I. M. Goldstein, and R. Snyderman, eds.), 2nd ed., pp. 211–232. Raven Press, New York.
89. Sims, J. E., J. G. Giri, and S. K. Dower (1994). Short analytical review. The two interleukin-1 receptors play different roles in IL-1 actions. *Clin. Immunol. Immunopathol.* **72,** 9–14.
90. Colotta, F., S. K. Dower, J. E. Sims, and A. Mantovani (1994). The type II 'decoy' receptor: A novel regulatory pathway for interleukin 1. *Immunol. Today* **15,** 562–566.
91. Collart, M. A., D. Belin, J. D. Vassali, S. deKoddoso, and P. Vassali (1986). Gamma-interferon enhances macrophage transcription of tumor necrosis factor/cachectin, interleukin 1 and urokinase genes, which are controlled by short-lived repressors. *J. Exp. Med.* **164,** 2113–2118.
92. Caput, D., B. Beutler, K. Hartog, R. Thayer, S. S. Brown, and A. Cerami (1985). Identification of a common nucleotide sequence in the 3′-untranslated region of mRNA molecules specifying inflammatory mediators. *Proc. Natl. Acad. Sci. USA* **83,** 1670–1674.
93. Stuber, F., M. Petersen, F. Bokelmann, and U. Schade (1996). A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-alpha concentrations and outcome of patients with severe sepsis. *Crit. Care Med.* **24,** 381–384.
94. Van Kessel, K. P. M., J. A. G. Van Strijp, and J. Verhoef (1991). Inactivation of recombinant human tumor necrosis factor-alpha by proteolytic enzymes released from stimulated human neutrophils. *J. Immunol.* **147,** 3862–3868.
95. Ito, A., A. Mukaiyama, Y. Itoh, H. Nagase, I. B. Thogersen, J. J. Enghild, Y. Sasaguri, and Y. Mori (1996). Degradation of interleukin 1β by matrix metalloproteinases. *J. Biol. Chem.* **271,** 14657–14660.
96. Lantz, M., U. Gullberg, E. Nilsson, and I. Olsson (1990). Characterization in vitro of a human tumor necrosis factor-binding protein. A soluble form of a tumor necrosis factor receptor. *J. Clin. Invest.* **86,** 1396–1402.
97. Himmler, A., I. Maurer-Fogy, M. Kronke, P. Scheurich, K. Phizenmaier, M. Lantz, I. Olsson, R. Hauptman, C. Stratowa, and G. Adolf (1990). Molecular cloning and expression of human and rat tumour necrosis factor receptor chain (p60) and its soluble derivative, tumour necrosis factor-binding protein. *DNA Cell Biol.* **9,** 705–715.
98. Lien, E., N. B. Liabakk, A. C. Johnsen, U. Nonstad, A. Sundan, and T. Espevik (1995). Polymorphonuclear granulocytes enhance lipopolysaccharide-induced soluble p75 tumor necrosis factor receptor release from mononuclear cells. *Eur. J. Immunol.* **25,** 2714–2717.

99. Porteu, F., M. Brockhaus, D. Wallach, H. Engelmann, and C. F. Nathan (1991). Human neutrophil elastase releases a ligand-binding fragment from the 75-kDa tumor necrosis factor (TNF) receptor. Comparison with the proteolytic activity responsible for shedding of TNF receptors from stimulated neutrophils. *J. Biol. Chem.* **266,** 18846–18850.
100. Crowe, P.D., B. N. Walter, K. M. Mohler, C. Otten-Evans, R. A. Black, and C. F. Ware (1995). A metalloprotease inhibitor blocks shedding of the 80-KD TNF receptor and TNF processing in T lymphocytes. *J. Exp. Med.* **181,** 1205–1210.
101. Mullberg, J., F. H. Durie, C. Otten-Evans, M. R. Alderson, S. Rose-John, D. Cosman, R. A. Black, and K. M. Mohler (1995). A metalloprotease inhibitor blocks shedding of the IL-6 receptor and the p60 TNF receptor. *J. Immunol.* **155,** 5198–5205.
102. Spinas, G.A., U. Keller, and M. Brockhaus (1992). Release of soluble receptors for tumor necrosis factor (TNF) in relation to circulating TNF during experimental endotoxinemia. *J. Clin. Invest.* **90,** 533–536.
103. Peppel, K., D. Crawford, and B. Beutler (1991). A tumor necrosis factor (TNF) receptor-IgG heavy chain chimeric protein as a bivalent antagonist of TNF activity. *J. Exp. Med.* **174,** 1483–1489.
104. Evans, T. J., D. Moyes, A. Carpenter, R. Martin, H. Loetscher, W. Lesslauer, and J. Cohen (1994). Protective effect of 55- but not 75-kD soluble tumor necrosis factor receptor-immunoglobulin G fusion proteins in an animal model of gram-negative sepsis. *J. Exp. Med.* **180,** 2173–2179.
105. Girardin, E., P. Roux-Lombard, G. E. Grau, P. Suter, H. Gallati, the J5 Study Group, and J. M. Dayer (1992). Imbalance between tumour necrosis factor-alpha and soluble TNF receptor concentrations in severe meningococcaemia. *Immunology* **76,** 20–23.
106. Van Zee, K. J., T. Kohno, E. Fischer, C. S. Rock, L. L. Moldawer, and S. F. Lowry (1992). Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor alpha in vitro and in vivo. *Proc. Natl. Acad. Sci. USA* **89,** 4845–4849.
107. Aderka, D., H. Engelmann, Y. Maor, C. Brakebusch, and D. Wallach (1992). Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. *J. Exp. Med.* **175,** 323–329.
108. Symons, J. A., J. A. Eastgate, and G. W. Duff (1991). Purification and characterization of a novel soluble receptor for interleukin 1. *J. Exp. Med.* **174,** 1251–1260.
109. Symons, J. A., P. R. Young, and G. W. Duff (1995). Soluble type II interleukin 1 (IL-1) receptor binds and blocks processing of IL-1beta precursor and loses affinity for IL-1 receptor antagonist. *Immunology* **92,** 1714–1718.
110. Giri, J. G., J. Wells, S. K. Dower, C. E. McCall, R. N. Guzman, J. Slack, T. A. Bird, K. Shanebeck, K. H. Grabstein, J. E. Sims, and M. R. Alderson (1994). Elevated levels of shed type II IL-1 receptor in sepsis. *J. Immunol.* **153,** 5802–5809.
111. Poutsiaka, D. D., B. D. Clark, E. Vannier, and C. A. Dinarello (1991). Production of interleukin-1 receptor antagonist and interleukin-1-beta by peripheral blood mononuclear cells is differentially regulated. *Blood* **78,** 1275–1281.
112. Arend, W. P., M. F. Smith, Jr., R. W. Janson, and F. G. Joslin (1991). IL-1 receptor antagonist and IL-1β production in human monocytes are regulated differently. *J. Immunol.* **147,** 1530–1536.
113. Kline, J. N., M. M. Monick, and G. W. Hunninghake (1992). IL-1 receptor antagonist release is regulated differently in human alveolar macrophages than in monocytes. *J. Appl. Physiol.* **73,** 1686–1692.
114. Vogels, M. T. E., E. J. B. M. Mensink, K. Ye, O. C. Boerman, C. M. M. Verschueren, C. A. Dinarello, and J. W. M. van der Meer (1994). Differential gene expression for

IL-1 receptor antagonist, IL-1, and TNF receptors and IL-1 and TNF synthesis may explain IL-1-induced resistance to infection. *J. Immunol.* **153,** 5772–5779.

115. Arend, W. P., H. G. Welgus, R. C. Thompson, and S. P. Eisenberg (1990). Biological properties of recombinant human monocyte-derived interleukin 1 receptor antagonist. *J. Clin. Invest.* **85,** 1694–1697.
116. Knudsen, P. J., C. A. Dinarello, and T. B. Strom (1986). Prostaglandins posttranscriptionally inhibit monocyte expression of interleukin 1 activity by increasing intracellular cyclic adenosine monophosphate. *J. Immunol.* **137,** 3189–3194.
117. Knudsen, P. J., C. A. Dinarello, and T. B. Strom (1987). Glucocorticoids inhibit transcriptional and post-transcriptional expression of interleukin 1 in U 937 cells. *J. Immunol.* **139,** 4129–4134.
118. Dubois, C. M., F. W. Ruscetti, E. W. Palaszynski, L. A. Falk, J. J. Oppenheim, and J. R. Keller (1990). Transforming growth factor b is a potent inhibitor of interleukin 1 (IL-1) receptor expression: Proposed mechanism of inhibition of IL-1 action. *J. Exp. Med.* **172,** 737–744.
119. Essner, R., K. Rhoades, W. H. McBride, D. L. Morton, and J. S. Economou (1989). IL-4 down regulates IL-1 and TNF gene expression in human monocytes. *J. Immunol.* **142,** 3857–3861.
120. van der Poll, T., and H. P. Sauerwein (1993). Tumour necrosis factor-alpha: Its role in the metabolic response to sepsis. *Clin. Sci.* **84,** 247–256.
121. Barber, A. E., S. M. Coyle, M. A. Marano, E. Fischer, S. E. Calvano, Y. Fong, L. L. Moldawer, and S. F. Lowry (1993). Glucocorticoid therapy alters hormonal and cytokine responses to endotoxin in man. *J. Immunol.* **150,** 1999–2006.
122. Brouckaert, P., B. Everaerdt, and W. Fiers (1992). The glucocorticoid antagonist RU38486 mimics interleukin-1 in its sensitization to the lethal and interleukin-6-inducing properties of tumor necrosis factor. *Eur. J. Immunol.* **22,** 981–986.
123. Hosford, D., M. Koltai, and P. Braquet (1992). PAF, cytokines and cell to cell interactions in shock and sepsis. *In* "Update in Intensive Care Medicine, Vol. 16: Mediators of Sepsis" (M. Lamy and L. G. Thijs, eds.), pp. 152–173. Springer-Verlag, Berlin.
124. Heidenreich, S., J.-H. Gong, A. Schmidt, M. Nain, and D. Gemsa (1989). Macrophage activation by granulocyte/macrophage colony-stimulating factor: Priming for enhanced release of tumor necrosis factor-α and prostaglandin E_2. *J. Immunol.* **143,** 1198–1205.
125. Okusawa, S., C. A. Dinarello, K. B. Yancey, S. Endres, T. J. Lawley, M. M. Frank, J. F. Burke, and J. A. Gelfand (1987). C5a induction of human interleukin 1. Synergistic effect with endotoxin or interferon-gamma. *J. Immunol.* **139,** 2635–2640.
126. Cavaillon, J.-M., C. Fitting, and N. Haeffner-Cavaillon (1990). Recombinant C5a enhances interleukin 1 and tumor necrosis factor release by lipopolysaccharide-stimulated monocytes and macrophages. *Eur. J. Immunol.* **20,** 253–257.
127. Chensue, S. W., P. D. Terebuh, D. G. Remick, W. E. Scales, and S. L. Kunkel (1991). In vivo biologic and immunohistochemical analysis of interleukin-1 alpha, beta and tumor necrosis factor during experimental endotoxemia. Kinetics, kupffer cell expression, and glucocorticoid effects. *Am. J. Pathol.* **138,** 395–402.
128. Chantry, D., M. Turner, E. Abney, and M. Feldmann (1989). Modulation of cytokine production by transforming growth factor-β. *J. Immunol.* **142,** 4295–4300.
129. Espevik, T., I. S. Figari, M. R. Shalaby, G. A. Lackides, G. D. Lewis, H. M. Shepard, and M. A. Palladino. 1987. Inhibition of cytokine production by cyclosporin A and transforming growth factor β. *J. Exp. Med.* **166,** 571–576.
130. Sisson, S. D., and C. A. Dinarello (1988). Production of interleukin-1α, interleukin-1β and tumor necrosis factor by human mononuclear cells stimulated with granulocyte-macrophage colony-stimulating factor. *Blood* **72,** 1368–1374.

131. van der Poll, T., J. Jansen, E. Endert, H. P. Sauerwein, and S. J. H. Van Deventer (1994). Noradrenaline inhibits lipopolysaccharide-induced tumor necrosis factor and interleukin 6 production in human whole blood. *Infect. Immun.* **62,** 2046–2050.

132. Denizot, Y., and N. Nathan (1994). Platelet-activating factor and cytokine production in the perfused rat liver. *Eur. Cytokine Network* **5,** 261–262.

133. Zhang, F., and K. Decker (1994). Platelet-activating factor antagonists suppress the generation of tumor necrosis factor-alpha and superoxide induced by lipopolysaccharide or phorbol ester in rat liver macrophages. *Eur. Cytokine Network* **5,** 311–317.

134. Kunkel, S. L., M. L. Spengler, M. A. May, A. Larsen, and D. G. Remick (1988). Prostaglandin E2 regulates a macrophage-derived tumor necrosis factor gene expression. *J. Biol. Chem.* **263,** 5380–5384.

135. Kunkel, S. L., R. C. Wiggins, S. W. Chensue, and J. W. Larrick (1986). Regulation of macrophage tumor necrosis factor production by prostaglandin E2. *Biochem. Biophys. Res. Commun.* **137,** 404–410.

136. Besedovsky, H., A. Del Rey, E. Sorkin, and C. A. Dinarello (1986). Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. *Science* **233,** 652–654.

137. Minty, A., P. Chalon, J.M. Derocq, X. Dumont, J.C. Guillemot, M. Kaghad, C. Labit, P. Leplatois, P. Liauzun, B. Miloux, C. Minty, P. Casellas, G. Loisson, J. Lupker, D. Shire, P. Ferrara, and D. Caput (1993). Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature* **362,** 248–250.

138. Poll, T. van der, S. M. Coyle, K. Barbosa, C. C. Braxton, and S. F. Lowry (1996). Epinephrine inhibits tumor necrosis factor-a and potentiates interleukin 10 production during human endotoxemia. *J. Clin. Invest.* **97,** 713–719.

139. Pue, C. A., R. F. Mortensen, C. B. Marsh, H. A. Pope, and M. D. Wewers (1996). Acute phase levels of C-reactive protein enhance IL-1β and IL-1ra production by human blood monocytes but inhibit IL-1β and IL-1ra production by alveolar macrophages. *J. Immunol.* **156,** 1594–1600.

140. Ianaro, A., C. A. O'Donnell, M. Di Rosa, and F. Y. Liew (1994). A nitric oxide synthase inhibitor reduces inflammation, down-regulates inflammatory cytokines and enhances interleukin-10 production in carrageenin-induced oedema in mice. *Immunology* **82,** 370–375.

141. Ikeda, N., N. Mukaida, S. Kaneko, N. Fujioka, S. B. Su, H. Nariuchi, M. Unoura, K. I. Harada, Y. Nakanuma, K. I. Kobayashi, and K. Matsushima (1995). Prevention of endotoxin-induced acute lethality in Propionibacterium acnes-primed rabbits by an antibody to leukocyte integrin beta-2 with concomitant reduction of cytokine production. *Infect. Immun.* **63,** 4812–4817.

142. Fan, S. T., and T. S. Edgington (1993). Integrin regulation of leukocyte inflammatory functions CD11b/CD18 enhancement of tumor necrosis factor-a responses of monocytes. *J. Immunol.* **150,** 2972–2980.

143. Bertini, R., M. Bianchi, and P. Ghezzi (1988). Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor. *J. Exp. Med.* **167,** 1708–1712.

144. MacMicking, J. D., C. Nathan, G. Hom, N. Chartrain, D. S. Fletcher, M. Trumbauer, K. Stevens, Q. Xie, K. Sokol, N. Hutchinson, H. Chen, and J. S. Mudgett (1995). Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81,** 641–650.

145. McCall, C. E., L. M. Grosso-Wilmoth, K. LaRue, R. N. Guzman, and S. L. Cousart (1993). Tolerance to endotoxin-induced expression of the interleukin-1-beta gene in blood neutrophils of humans with the sepsis syndrome. *J. Clin. Invest.* **91,** 853–861.

146. Munoz, C., J. Carlet, C. Fitting, B. Misset, J. P. Bleriot, and J. M. Cavaillon (1991). Dysregulation of in vitro cytokine production by monocytes during sepsis. *J. Clin. Invest.* **88,** 1747–1754.

147. Waage, A., and T. Espevik (1988). Interleukin 1 potentiates the lethal effect of tumor necrosis factor a/cachectin in mice. *J. Exp. Med.* **167,** 1987–1992.
148. Polunovsky, V. A., C. H. Wendt, D. H. Ingbar, M. S. Peterson, and P. B. Bitterman (1994). Induction of endothelial cell apoptosis by TNF-α: Modulation by inhibitors of protein sythesis. *Exp. Cell Res.* **214,** 584–594.
149. DeForge, L. E., D. T. Nguyen, S. L. Kunkel, and D. G. Remick (1990). Regulation of the pathophysiology of tumor necrosis factor. *J. Lab. Clin. Med.* **116,** 429–438.
150. Rock, C. S., and S. F. Lowry (1991). Tumor necrosis factor-alpha. *J. Surg. Res.* **51,** 434–445.
151. Koga, S., S. Morris, S. Ogawa, H. Liao, J. P. Bilezikian, G. Chen, W. J. Thompson, T. Ashikaga, J. Brett, and D. M. Stern (1995). TNF modulates endothelial properties by decreasing cAMP. *Am. J. Physiol.* **268,** 1104–1113.
152. Bevilacqua, M. P., J. S. Pober, G. R. Majeau, W. Fiers, R. S. Cotran, and M. A. Gimbrone, Jr. (1986). Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: Characterization and comparison with the actions of interleukin 1. *Proc. Natl. Acad. Sci. USA* **83,** 4533–4537.
153. Nawroth, P. P., and D. M. Stern (1986). Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J. Exp. Med.* **163,** 740–745.
154. Lentz, S. R., M. Tsiang, and J. E. Sadler (1991). Regulation of thrombomodulin by tumor necrosis factor-α: Comparison of transcriptional and posttranscriptional mechanisms. *Blood* **77,** 542–550.
155. Pober, J. S., M. A. Gimbrone Jr., L. A. Lapierre, D. L. Mendrick, W. Fiers, R. Rothlein, and T. A. Springer (1986). Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. *J. Immunol.* **137,** 1893–1896.
156. Parry, G. C., and N. Mackmann (1995). Transcriptional regulation of tissue factor expression in human endothelial cells. *Arteriosclerosis Thromb. Vasc. Biol.* **15,** 612–621.
157. Gamble, J. R., J. M. Harlan, and S. J. Klebanoff (1985). Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc. Natl. Acad. Sci. USA* **82,** 8667–8671.
158. Pohlman, T. H., K. A. Stanness, P. G. Beatty, H. D. Ochs, and J. M. Harlan (1986). An endothelial cell surface factor(s) induced in vitro by lipopolysaccharide, interleukin-1 and tumor necrosis factor-α increases neutrophil adherence by a CDw 18-dependent mechanism. *J. Immunol.* **136,** 4548–4553.
159. Briscoe, D. M., R. S. Cotran, and J. S. Pober (1992). Effects of tumor necrosis factor, lipopolysaccharide, and IL-4 on the expression of vascular cell adhesion molecule-1 in vivo. Correlation with CD3+ T cell infiltration. *J. Immunol.* **149,** 2954–2960.
160. Schleimer, R. P., and B. K. Rutledge (1986). Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin, and tumor-promoting phorbol esters. *J. Immunol.* **136,** 649–654.
161. Dustin, M. L., and T. A. Springer (1988). Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J. Cell Biol.* **107,** 321–331.
162. Mantovani, A., and E. Dejana (1989). Cytokines as communication signals between leukocytes and endothelial cells. *Immunol. Today* **10,** 370–375.
163. Kaushansky, K., V. C. Broudy, J. M. Harlan, and J. W. Adamson (1988). Tumor necrosis factor-α and tumor necrosis factor-β (lymphotoxin) stimulate the production

of granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and IL-1 in vivo. *J. Immunol.* **141,** 3410–3415.

164. Libby, P., J. M. Ordovas, K. R. Auger, A. H. Robbins, L. K. Birinyi, and C. A. Dinarello (1986). Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult human vascular endothelial cells. *Am. J. Pathol.* **124,** 179–185.

165. Nawroth, P. P., I. Bank, D. Handley, J. Cassimeris, L. Chess, and D. Stern (1986). Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. *J. Exp. Med.* **163,** 1363–1375.

166. Strieter, R. M., S. L. Kunkel, H. J. Showell, D. G. Remick, S. H. Phan, P. A. Ward, and R. M. Marks (1989). Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-α, LPS, and IL-1β. *Science* **243,** 1467–1469.

167. Strieter, R. M., R. Wiggins, S. H. Phan, B. L. Wharram, H. J. Showell, D. G. Remick, S. W. Chensue, and S. L. Kunkel (1989). Monocyte chemotactic protein gene expression by cytokine-treated human fibroblasts and endothelial cells. *Biochem. Biophys. Res. Commun.* **162,** 694–700.

168. Sica, A., J. M. Wang, F. Colotta, E. Dejana, A. Mantovani, J. J. Oppenheim, C. G. Larsen, C. O. C. Zachariae, and K. Matsushima (1990). Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. *J. Immunol.* **144,** 3034–3038.

169. Sica, A., K. Matsushima, J. Van Damme, J. M. Wang, N. Polentarutti, E. Dejana, F. Colotta, and A. Mantovani (1990). IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells. *Immunology* **69,** 548–553.

170. Rossi, V., F. Beviario, P. Ghezzi, E. Dejana, and A. Mantovani (1985). Prostacyclin synthesis induced in vascular cells by interleukin-1. *Science* **229,** 174–176.

171. Kawakami, M., S. Ishibashi, H. Ogawa, T. Murase, F. Takaku, and S. Shibata (1986). Cachectin/TNF as well as interleukin-1 induces prostacylin synthesis in cultured vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **141,** 482–487.

172. Jansen, P. M., I. W. de Jong, M. Hart, K. J. Kim, L. A. Aarden, L. B. Hinshaw, F. B. Taylor, Jr., and C. E. Hack (1996). Release of leukemia inhibitory factor in primate sepsis: Analysis of the role of TNF-alpha. *J. Immunol.* **156,** 4401–4407.

173. Marfaing-Koka, A., O. Devergne, G. Gorgone, A. Portier, T. J. Schall, P. Galanaud, and D. Emilie (1995). Regulation of the production of the RANTES chemokine by endothelial cells. *J. Immunol.* **154,** 1870–1878.

174. Hollenberg, S. M., R. E. Cunnion, and J. E. Parillo (1991). The effect of tumor necrosis factor on vascular smooth muscle. In vitro studies using rat aortic rings. *Chest* **100,** 1133–1137.

175. Beasley, D., J. H. Schwartz, and B. M. Brenner (1991). Interleukin 1 induces prolonged l-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J. Clin. Invest.* **87,** 602–608.

176. Gill, D. J., B. C. Low, and M. R. Grigor (1996). Interleukin-1 beta and tumor necrosis factor-alpha stimulate the cat-2 gene of the L-arginine transporter in cultured vascular smooth muscle cells. *J. Biol. Chem.* **271,** 11280–11283.

177. MacNaul, K. L., and N. I. Hutchinson (1993). Differential expression of iNOS and cNOS mRNA in human vascular smooth muscle cells and endothelial cells under normal and inflammatory conditions. *Biochem. Biophys. Res. Commun.* **196,** 1330–1334.

178. Marsden, P. A., and B. M. Brenner (1992). Transcriptional regulation of the endothelin-1 gene by TNF-alpha. *Am. J. Physiol.* **262,** 854–861.

179. Sawdey, M., T. J. Podor, and D. J. Loskutoff (1989). Regulation of type 1 plasminogen activator inhibitor gene expression in cultured bovine aortic endothelial cells. Induction

by transforming growth factor-β, lipopolysaccharide, and tumor necrosis factor-α. *J. Biol. Chem.* **264,** 10396–10401.

180. Nachman, R. L., K. A. Hajjar, R. L. Silverstein, and C. A. Dinarello (1986). Interleukin 1 induces endothelial cell synthesis of plasminogen activator inhibitor. *J. Exp. Med.* **163,** 1595–1600.
181. Schleef, R. R., M. P. Bevilacqua, M. Sawdey, M. A. Gimbrone, Jr., and D. J. Loskutoff (1988). Cytokine activation of vascular endothelium. *J. Biol. Chem.* **263,** 5797–5803.
182. Van Hinsbergh, V. W. M., E. A. van den Berg, W. Fiers, and G. Dooijewaard (1990). Tumor necrosis factor induces the production of urokinase-type plasminogen activator by human endothelial cells. *Blood* **75,** 1991–1998.
183. Van Hinsbergh, V. W. M., T. Kooistra, E. A. van den Berg, H. M. G. Princen, W. Fiers, and J. J. Emeis (1988). Tumor necrosis factor increases the production of plasminogen activator inhibitor in human endothelial cells in vitro and rats in vivo. *Blood* **72,** 1467–1473.
184. Niedbala, M. J., and M. S. Picarella (1992). Tumor necrosis factor induction of endothelial cell urokinase-type plasminogen activator mediated proteolysis of extracellular matrix and its antagonism by gamma-interferon. *Blood* **79,** 678–687.
185. Beasley, D., and M. McGuiggin (1994). Interleukin 1 activates soluble guanylate cyclase in human vascular smooth muscle cells through a novel nitric oxide-independent pathway. *J. Exp. Med.* **179,** 71–80.
186. Caterina, R. de, P. Libby, H. B. Peng, V. J. Thannickal, T. B. Rajavashisth, M. A. Gimbrone, Jr., W. Soo Shin, and J. K. Liao (1995). Nitric oxide decreases cytokine-induced endothelial activation. *J. Clin. Invest.* **96,** 60–68.
187. Renesto, P., and M. Chignard (1991). Tumor necrosis factor-α enhances platelet activation via cathepsin G released from neutrophils. *J. Immunol.* **146,** 2305–2309.
188. Klebanoff, S. J., M. A. Vadas, J. M. Harlan, L. H. Sparks, J. R. Gamble, J. M. Agosti, and A. M. Waltersdorph (1986). Stimulation of neutrophils by tumor necrosis factor. *J. Immunol.* **136,** 4220–4225.
189. Larrick, J. W., D. Graham, K. Toy, L. S. Lin, G. Senyk, and B. M. Fendly (1987). Recombinant tumor necrosis factor activation of human granulocytes. *Blood* **69,** 640–644.
190. Atkinson, Y. H., W. A. Marasco, A. F. Lopez, and M. A. Vadas (1988). Recombinant human tumor necrosis factor-alpha. Regulation of N-formylmethionylleucylphenylalanine receptor affinity and function on human neutrophils. *J. Clin. Invest.* **81,** 759–765.
191. Ahmed, N. A., J. Yee, B. Giannias, B. Kapadia, and N. V. Christou (1996). Expression of human neutrophil L-selection during the systemic inflammatory response syndrome is partly mediated by tumor necrosis factor alpha. *Arch. Surg.* **131,** 31–35.
192. Elbim, C., S. Bailly, S. Chollet-Martin, J. Hakim, and M. A. Gougerot-Pocidalo (1994). Differential priming effects of proinflammatory cytokines on human neutrophil oxidative burst in response to bacterial N-formyl peptides. *Infect. Immun.* **62,** 2195–2201.
193. Tetsuka, T., D. Daphna-Iken, B. W. Miller, Z. Guan, L. D. Baier, and A. R. Morrison (1996). Nitric oxide amplifies interleukin 1-induced cyclooxygenase-2 expression in rat mesangial cells. *J. Clin. Invest.* **97,** 2051–2056.
194. Arias-Negrete, S., K. Keller, and K. Chadee (1995). Proinflammatory cytokines regulate cyclooxygenase-2 mRNA expression in human macrophages. *Biochem. Biophys. Res. Commun.* **208,** 582–589.
195. Wisniewski, H. G., J. C. Hua, D. M. Poppers, D. Naime, J. Vilcek, and B. N. Cronstein (1996). TNF/IL-1-inducible protein TSG-6 potentiates plasmin inhibition by inter-a-inhibitor and exerts a strong anti-inflammatory effect in vivo. *J. Immunol.* **156,** 1609–1615.

196. Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. Hariri, T. J. Fahey,III, A. Zentella, J. D. Albert, G. T. Shires, and A. Cerami (1986). Shock and tissue injury induced by human recombinant cachectin. *Science* **234,** 470–474.

197. Tracey, K. J., S. F. Lowry, T. J. Fahey,III, J. D. Albert, Y. Fong, D. Hesse, B. Beutler, K. B. Manogue, S. E. Calvano, H. Wei, A. Cerami, and G. T. Shires (1987). Cachectin tumor necrosis factor induces lethal shock and stress hormone responses in the dog. *Surg. Gynecol. Obstet.* **164,** 415–422.

198. Natanson, C., P. W. Eichenholz, R. L. Danner, P. Q. Eichacker, W. D. Hoffman, G. C. Kuo, S. M. Banks, T. J. MacVittie, and J. E. Parrillo (1989). Endotoxin and tumor necrosis factor challenges in dogs simulate the cardiovascular profile of human septic shock. *J. Exp. Med.* **169,** 823–832.

199. Bauss, F., W. Droge, and D. N. Mannel (1987). Tumor necrosis factor mediates endotoxic effects in mice. *Infect. Immun.* **55,** 1622–1625.

200. Remick, D. G., R. G. Kunkel, J. W. Larrick, and S. L. Kunkel (1987). Acute in vivo effects of human recombinant tumor necrosis factor. *Lab. Invest.* **56,** 583–590.

201. Okusawa, S., J. A. Gelfland, T. Ikejima, R. J. Connolly, and C. A. Dinarello (1988). Interleukin 1 induces a shock-like state in rabbits. *J. Clin. Invest.* **81,** 1162–1172.

202. Fischer, E., M. A. Marano, A. E. Barber, A. Hudson, K. Lee, C. S. Rock, A. S. Hawes, R. C. Thompson, T. J. Hayes, T. D. Anderson, W. R. Benjamin, S. F. Lowry, and L. L. Moldawer (1991). Comparison between effects of interleukin-1α administration and sublethal endotoxemia in primates. *Am. J. Physiol.* **261,** R442–R452.

203. Schuger, L., J. Varani, R. M. Marks, S. L. Kunkel, K. J. Johnson, and P. A. Ward (1989). Cytotoxicity of tumor necrosis factor-alpha for human umbilical vein endothelial cells. *Lab. Invest.* **61,** 62–68.

204. Martin, S., K. Maruta, V. Burkart, S. Gillis, and H. Kolb (1988). IL-1 and IFN-gamma increase vascular permeability. *Immunology* **64,** 301–305.

205. Robaye, B., R. Mosselmans, W. Fiers, J. E. Dumont, and P. Galand (1991). Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells in vitro. *Am. J. Pathol.* **138,** 447–453.

206. Kilbourn, R. G., S. S. Gross, A. Jubran, J. Adams, O. W. Griffith, R. Levi, and R. F. Lodato (1990). N^G-Methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: Implications for the involvement of nitric oxide. *Proc. Natl. Acad. Sci. USA* **87,** 3629–3632.

207. Kettelhut, I. C., W. Fiers, and A. L. Goldberg (1987). The toxic effects of tumor necrosis factor in vivo and their prevention by cyclooxygenase inhibitors. *Proc. Natl. Acad. Sci. USA* **84,** 4273–4277.

208. Jaeschke, H., N. A. Essani, M. A. Fisher, S. L. Vonderfecht, A. Farhood, and C. W. Smith (1996). Release of soluble intercellular adhesion molecule 1 into bile and serum in murine endotoxin shock. *Hepatology* **23,** 530–536.

209. Remick, D. G., R. M. Strieter, M. K. Eskandari, D. T. Nguyen, M. A. Genord, C. L. Raiford, and S. L. Kunkel (1990). Role of tumor necrosis factor-alpha in lipopolysaccharide- induced pathologic alterations. *Am. J. Pathol.* **136,** 49–60.

210. Piguet, P. F., G. E. Grau, and P. Vassalli (1990). Subcutaneous perfusion of tumor necrosis factor induces local proliferation of fibroblasts, capillaries, and epidermal cells, or massive tissue necrosis. *Am. J. Pathol.* **136,** 103–110.

211. Butler, L. D., N. K. Layman, R. L. Cain, P. E.. Riedl, K. M. Mohler, J. L Bobbitt, R. Belagajie, J. Sharp, and A. M. Bendele (1989). Interleukin 1-induced pathophysiology: induction of cytokines, development of histopathologic changes, and immunopharmacologic intervention. *Clin. Immunol. Immunopathol.* **53,** 400–421.

212. Johnson, J., K. L. Brigham, G. Jesmok, and B. Meyrick (1991). Morphologic changes in lungs of anesthetized sheep following intravenous infusion of recombinant tumor necrosis factor alpha. *Am. Rev. Respir. Dis.* **144,** 179–186.
213. Stephens, K. E., A. Ishizaka, J. W. Larrick, and T. A. Raffin (1988). Tumor necrosis factor causes increased pulmonary permeability and edema. Comparison to septic acute lung injury. *Am. Rev. Respir. Dis.* **137,** 1364–1370.
214. Movat, H. Z., M. I. Cybulsky, I. G. Colditz, M. K. W. Chan, and C. A. Dinarello (1987). Acute inflammation in gram-negative infection: Endotoxin, interleukin 1, tumor necrosis factor, and neutrophils. *FASEB J.* **46,** 97–104.
215. Drake, W. T., N. N. Lopes, J. W. Fenton, and A. C. Issekutz (1992). Thrombin enhancement of interleukin-1 and tumor necrosis factor-alpha induced polymorphonuclear leukocyte migration. *Lab. Invest.* **67,** 617–627.
216. Baggiolini, M., A. Walz, and S. L. Kunkel (1989). Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J. Clin. Invest.* **84,** 1045–1049.
217. Eichacker, P. Q., A. Farese, W. D. Hoffman, S. M. Banks, T. Mouginis, S. Richmond, G. C. Kuo, T. J. MacVittie, and C. Natanson (1992). Leukocyte CD11b/18 antigen-directed monoclonal antibody improves early survival and decreases hypoxemia in dogs challenged with tumor necrosis factor. *Am. Rev. Respir. Dis.* **145,** 1023–1029.
218. Abe, Y., S. Sekiya, T. Yamasita, and F. Sendo (1990). Vascular hyperpermeability induced by tumor necrosis factor and its augmentation by IL-1 and IFN-gamma is inhibited by selective depletion of neutrophils with a monoclonal antibody. *J. Immunol.* **145,** 2902–2907.
219. Stephens, K. E., A. Ishizaka, Z. Wu, J. W. Larrick, and T. A. Raffin (1988). Granulocyte depletion prevents tumor necrosis factor-mediated acute lung injury in guinea pigs. *Am. Rev. Respir. Dis.* **138,** 1300–1307.
220. Hauser, G. J., J. K. McIntosh, W. D. Travis, and S. A. Rosenberg (1990). Manipulation of oxygen radical-scavenging capacity in mice alters host sensitivity to tumor necrosis factor toxicity but does not interfere with its antitumor efficacy. *Cancer Res.* **50,** 3503–3508.
221. De Graaf, T. W., M. E. Van der Stelt, M. G. Anbergen, and W. Van Dijk (1993). Inflammation-induced expression of sialyl lewis X-containing glycan structures on alpha-1-acid glycoprotein (orosomucoid) in human sera. *J. Exp. Med.* **177,** 657–666.
222. Libert, C., P. Brouckaert, and W. Fiers (1994). Protection by alpha-1-acid glycoprotein against tumor necrosis factor-induced lethality. *J. Exp. Med.* **180,** 1571–1575.
223. van der Poll, T., S. J. H. Van Deventer, C. E. Hack, G. J. Wolbink, L. A. Aarden, H. R. Büller, and J. W. Ten Cate (1992). Effects on leukocytes after injection of tumor necrosis factor into healthy humans. *Blood* **79,** 693–698.
224. Otsuka, Y., K. Nagano, K. Hori, K. Nagano *et al.* (1990). Inhibition of neutrophil migration by tumor necrosis factor. Ex vivo and in vivo studies in comparison with in vitro effect. *J. Immunol.* **145,** 2639–2643.
225. Ogilvie, A. C., C. E. Hack, J. Wagstaff, G. J. van Mierlo, A. J. M. Eerenberg, L. L. Thomsen, K. Hoekman, and E. M. Rankin (1996). IL-1beta does not cause neutrophil degranulation but does lead to IL-6, IL-8, and nitrite/nitrate release when used in patients with cancer. *J. Immunol.* **156,** 389–394.
226. Jansen, P. M., M. A. Boermeester, E. Fischer, I. W. de Jong, T. van der Poll, L. L. Moldawer, C. E. Hack, and S. F. Lowry (1995). Contribution of interleuking-1 to activation of coagulation and fibrinolysis, neutrophil degranulation, and the release of secretory-type phospholipase a2 in sepsis: Studies in nonhuman primates after interleuking-1α administration and during lethal bacteremia. *Blood* **86,** 1027–1034.

227. van der Poll, T., H. R. Büller, H. ten Cate, C. H. Wortel, K. A. Bauer, S. J. H. Van Deventer, C. E. Hack, H. P. Sauerwein, R. D. Rosenberg, and J. W. Ten Cate (1990). Activation of coagulation after administration of tumor necrosis factor to normal subjects. *N. Engl. J. Med.* **322,** 1622–1626.

228. van der Poll, T., M. Levi, H. R. Buller, S. J. H. Van Deventer, J. P. De Boer, C. E. Hack, and J. W. Ten Cate (1991). Fibrinolytic response to tumor necrosis factor in healthy subjects. *J. Exp. Med.* **174,** 729–732.

229. Bauer, K. A., H. ten Cate, S. Barzegar, D. R. Spriggs, M. L. Sherman, and R. D. Rosenberg (1989). Tumor necrosis factor infusions have a procoagulant effect on hemostatic mechanism of humans. *Blood* **74,** 165–172.

230. Van Hinsbergh, V. W. M., K. A. Bauer, T. Kooistra, C. Kluft, G. Dooijewaard, M. L. Sherman, and W. Nieuwenhuizen (1990). Progress of fibrinolysis during tumor necrosis factor infusions in humans. Concomitant increase in tissue-type plasminogen activator, plasminogen activator inhibitor type-1, and fibrin(ogen) degradation products. *Blood* **76,** 2284–2289.

231. Taylor, F. B., S. E. He, A. C. K. Chang, J. Box, G. Ferrell, D. Lee, M. Lockhart, G. Peer, and C. T. Esmon (1996). Infusion of phospholipid vesicles amplifies the local thrombotic response to TNF and anti-protein C into a consumptive response. *Thromb Haemost* **75,** 578–584.

232. Naito, Y., J. Fukata, K. Shindo, O. Ebisui, N. Murakami, T. Tominaga, Y. Nakai, K. Mori, N. W. Kasting, and H. Imura (1991). Effects of interleukins on plasma arginine vasopressin and oxytocin levels in conscious freely moving rats. *Biochem. Biophys. Res. Commun.* **174,** 1189–1195.

233. Levi, M., J. P. De Boer, D. Roem, J. W. Ten Cate, and C. E. Hack (1992). Plasminogen activation in vivo upon intravenous infusion of DDAVP: Quantitative assessment of plasmin-α2-antiplasmin complexes with a novel monoclonal antibody based radioimmunoassay. *Thromb. Haemost.* **67,** 111–116.

234. Van Deventer, S. J. H., M. Hart, T. van der Poll, C. E. Hack, and L. A. Aarden (1993). Endotoxin and tumor necrosis factor-alpha-induced interleukin-8 release in humans. *J. Infect. Dis.* **167,** 461–464.

235. van der Poll, T., J. Jansen, M. Levi, H. ten Cate, J. W. Ten Cate, and S. J. H. Van Deventer (1994). Regulation of interleukin 10 release by tumor necrosis factor in humans and chimpanzees. *J. Exp. Med.* **180,** 1985–1988.

236. van der Poll, T., S. J. H. Van Deventer, H. ten Cate, M. Levi, and J. W. Ten Cate (1994). Tumor necrosis factor is involved in the appearance of interleukin-1 receptor antagonist in endotoxemia. *J. Infect. Dis.* **169,** 665–667.

237. Fischer, E., K. J. Van Zee, M. A. Marano, C. S. Rock, J. S. Kenney, D. D. Poutsiaka, C. A. Dinarello, S. F. Lowry, and L. L. Moldawer (1992). Interleukin-1 receptor antagonist circulates in experimental inflammation and in human disease. *Blood* **79,** 2196–2200.

238. Nurnberger, W., S. Holthausen, K. Schirlau, I. Michelmann, S. Burdach, and U. Gobel (1993). Activation of the complement and contact system during rTNF-alpha/rIFN-gamma therapy. *Mol. Immunol.* **30** (Suppl):39. [Abstract].

239. Van Deventer, S. J. H., H. R. Büller, J. W. Ten Cate, L. A. Aarden, C. E. Hack, and A. Sturk (1990). Experimental endotoxemia in humans: Analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. *Blood* **76,** 2520–2526.

240. Van Deventer, S. J. H., C. E. Hack, G. J. Wolbink, H. J. Voerman, R. J. M. Strack van Schijndel, J. W. Ten Cate, and L. G. Thijs (1991). Endotoxin-induced neutrophil activation. The role of complement revisited. *Progr.Clin.Biol.Res.* **367,** 101–108.

241. Moore, F. D.,Jr., N. A. Moss, A. Revhaug, D. W. Wilmore, J. A. Mannick, and M. L. Rodrick (1987). A single dose of endotoxin activates neutrophils without activating complement. *Surgery* **102,** 200–205.
242. Van Leenen, D., T. van der Poll, M. Levi, H. ten Cate, S. J. H. Van Deventer, C. E. Hack, L. A. Aarden, and J. W. Ten Cate (1993). Pentoxifylline attenuates neutrophil activation in experimental endotoxemia in chimpanzees. *J. Immunol.* **151,** 2318–2325.
243. Thijs, L. G., C. E. Hack, R. J. M. Strack van Schijndel, J. H. Nuijens, G. J. Wolbink, A. J. M. Eerenberg-Belmer, van der H. Vall, and J. Wagstaff (1989). Complement activation and high-dose of interleukin-2. *Lancet* **2,** 395.
244. Thijs, L. G., C. E. Hack, R. J. M. Strack van Schijndel, J. H. Nuijens, G. J. Wolbink, A. J. M. Eerenberg-Belmer, H. van der Vall, and J. Wagstaff (1990). Activation of the complement system during immunotherapy with recombinant Interleukin-2: Relation to the development of side effects. *J. Immunol.* **144,** 2419–2424.
245. Baars, J. W., C. E. Hack, J. Wagstaff, A. J. M. Eerenberg-Belmer, G. J. Wolbink, L. G. Thijs, R. J. M. Strack van Schijndel, H. L. J. A. Van der Vall, and H. M. Pinedo (1992). The activation of polymorphonuclear neutrophils and the complement system during immunotherapy with recombinant interleukin-2. *Br. J. Cancer* **65,** 96–101.
246. Mier, J. W., G. Vachino, M. S. Klempner, F. R. Aronson, R. Noring, S. Smith, E. P. Brandon, W. Laird, and M. B. Atkins (1990). Inhibition of interleukin-2-induced tumor necrosis factor release by dexamethasone: Prevention of an acquired neutrophil chemotaxis defect and differential suppression of interleukin-2-associated side effects. *Blood* **10,** 1933–1940.
247. Mier, J. W., G. Vachino, J. W. Van der Meer, R. P. Numerof, S. Adams, J. G. Cannon, H. A. Bernheim, M. B. Atkins, D. R. Parkinson, and C. A. Dinarello (1988). Induction of circulating tumor necrosis factor (TNF alpha) as the mechanism for the febrile response to interleukin-2 (IL-2) in cancer patients. *J. Clin. Immunol.* **8,** 426–436.
248. Barton, P. A., and J. S. Warren (1993). Complement component C5 modulates the systemic tumor necrosis factor response in murine endotoxic shock. *Infect. Immun.* **61,** 1474–1481.
249. Hsueh, W., X. Sun, L. N. Rioja, and F. Gonzalez-Crussi (1990). The role of the complement system in shock and tissue injury induced by tumour necrosis factor and endotoxin. *Immunology* **70,** 309–314.
250. Rothstein, J. L., T. F. Lint, and H. Schreiber (1988). Tumor necrosis factor/cachectin. Induction of hemorrhagic necrosis in normal tissue requires the fifth component of complement (C5). *J. Exp. Med.* **168,** 2007–2021.
251. Yokoyama, T., L. Vaca, R. D. Rossen, W. Durante, P. Hazarika, and D. L. Mann (1993). Cellular basis for the negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian heart. *J. Clin. Invest.* **92,** 2303–2312.
252. Pinsky, D. J., B. Cai, X. Yang, C. Rodriguez, R. R. Sciacca, and P. J. Cannon (1995). The lethal effects of cytokine-induced nitric oxide on cardiac myocytes are blocked by nitric oxide synthase antagonism or transforming growth factor beta. *J. Clin. Invest.* **95,** 677–685.
253. Oddis, C. V., and M. S. Finkel (1995). Cytokine-stimulated nitric oxide production inhibits mitochondrial activity in cardiac myocytes. *Biochem. Biophys. Res. Commun.* **213,** 1002–1009.
254. Murray, D. R., and G. L. Freeman (1996). Tumor necrosis factor-alpha induces a biphasic effect on myocardial contractility in conscious digs. *Circ. Res.* **78,** 154–160.
255. Fahey, T. J., III, T. Yoshioka, G. T. Shires, and G. A. Fantini (1996). The role of tumor necrosis factor and nitric oxide in the acute cardiovascular response to endotoxin. *Ann. Surg.* **223,** 63–69.

256. Herbertson, M. J., H. A. Werner, C. M. Goddard, J. A. Russel, A. Wheeler, R. Coxon, and K. R. Walley (1995). Anti-tumor necrosis factor-α prevents decreased ventricular contractility in endotoxemic pigs. *Am. J. Respir. Crit. Care. Med.* **152,** 480–488.
257. Klabunde, R. E., and A. F. Coston (1995). Nitric oxide synthase inhibition does not prevent cardiac depression in endotoxic shock. *Shock* **3,** 73–78.
258. Finkel, M. S., C. V. Oddis, T. D. Jacob, S. C. Watkins, B. G. Hattler, and R. L. Simmons (1992). Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* **257,** 387–389.
259. Schulz, R., D. L. Panas, R. Catena, S. Moncada, P. M. Olley, and G. D. Lopaschuk (1995). The role of nitric oxide in cardiac depression induced by interleukin-1 beta and tumour necrosis factor-alpha. *Br. J. Pharmacol.* **114,** 27–34.
260. Leist, M., F. Gantner, I. Bohlinger, G. Tiegs, P. G. Germann, and A. Wendel (1995). Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. *Am. J. Pathol.* **146,** 1220–1234.
261. Berkenbosch, F., D. E. C. De Goeij, A. Del Rey, and H. O. Besedovsky (1989). Neuroendocrine, sympathetic and metabolic responses induced by interleukin-1. *Neuroendocrinology* **50,** 570–576.
262. Michie, H. R., K. R. Manogue, D. R. Spriggs, A. Revhaug, S. O'Dwyer, C. A. Dinarello, A. Cerami, S. M. Wolff, and D. W. Wilmore (1988). Detection of circulating tumor necrosis factor after endotoxin administration. *N. Engl. J. Med.* **318,** 1481–1486.
263. Martich, G. D., R. L. Danner, M. Ceska, and A. F. Suffredini (1991). Detection of IL-8 and TNF in normal humans after intravenous endotoxin: The effect of antiinflammatory agents. *J. Exp. Med.* **173,** 1021–1024.
264. Hesse, D. G., K. J. Tracey, Y. Fong, K. R. Manogue, M. A. Palladino,Jr., A. Cerami, G. T. Shires, and S. F. Lowry (1988). Cytokine appearance in human endotoxemia and primate bacteremia. *Surg. Gynecol. Obstet.* **166,** 147–153.
265. Fong, Y., M. A. Marano, L. L. Moldawer, H. Wei, S. E. Calvano, J. S. Kenney, A. C. Allison, A. Cerami, G. T. Shires, and S. F. Lowry (1990). The acute splanchnic and peripheral tissue metabolic response to endotoxin in humans. *J. Clin. Invest.* **85,** 1896–1904.
266. Cannon, J. G., R. G. Tompkins, J. A. Gelfand, H. R. Michie, G. G. Stanford, J. W. M. Van der Meer, S. Endres, G. Lonnemann, J. Corsetti, B. Chernow, D. W. Wilmore, S. M. Wolff, J. F. Burke, and C. A. Dinarello (1990). Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever. *J. Infect. Dis.* **161,** 79–84.
267. Spinas, G. A., D. Bloesch, U. Keller, W. Zimmerli, and S. Cammisuli (1991). Pretreatment with ibuprofen augments circulating tumor necrosis factor-α, interleukin-6, and elastase during acute endotoxinemia. *J. Infect. Dis.* **163,** 89–95.
268. Spinas, G. A., D. Bloesch, M. T. Kaufmann, U. Keller, and J. M. Dayer (1990). Induction of plasma inhibitors of interleukin 1 and TNF-alpha by endotoxin administration to normal humans. *Am. J. Physiol.* **259,** R993–R997.
269. Zabel, P., D. T. Wolter, M. M. Schonhartig, and U. F. Schade (1989). Oxpentifylline in endotoxaemia. *Lancet* **334,** 1474–1477.
270. Taveira da Silva, A. M., H. C. Kaulbach, F. S. Chuidian, D. R. Lambert, A. F. Suffredini, and R. L. Danner (1993). Shock and multiple-organ dysfunction after self-administration of salmonella endotoxin. *N. Engl. J. Med.* **328,** 1457–1460.
271. Leeper-Woodford, S. K., P. D. Carey, K. Byrne, B. J. Fisher, C. Blocher, H. J. Sugerman, and A. A. Fowler, III (1991). Ibuprofen attenuates plasma tumor necrosis factor activity during sepsis-induced acute lung injury. *J. Appl. Physiol.* **71,** 915–923.

272. Neuner, P., G. Klosner, E. Schauer, M. Pourmojib, W. Macheiner, C. Grunwald, R. Knobler, A. Schwartz, T. A. Luger, and T. Schwartz (1994). Pentoxifylline in vivo down-regulates the release of IL-1beta, IL-6, IL-8 and tumour necrosis factor-alpha by human peripheral blood mononuclear cells. *Immunology* **83,** 262–267.
273. Biemond, B. J., M. Levi, H. ten Cate, T. van der Poll, H. R. Buller, C. E. Hack, and J. W. ten Cate (1995). Plasminogen activator and plasminogen activator inhibitor I release during experimental endotoxaemia in chimpanzees: Effect of interventions in the cytokine and coagulation cascades. *Clin. Sci.* **88,** 587–594.
274. van der Poll, T., M. Levi, S. J. H. Van Deventer, H. ten Cate, B. L. Haagmans, B. J. Biemond, H. R. Buller, C. E. Hack, and J. W. Ten Cate (1994). Differential effects of anti-tumor necrosis factor monoclonal antibodies on systemic inflammatory responses in experimental endotoxemia in chimpanzees. *Blood* **83,** 446–451.
275. Suffredini, A. F., D. Reda, S. M. Banks, M. Tropea, J. M. Agosti, and R. Miller (1995). Effects of recombinant dimeric TNF receptor on human inflammatory responses following intravenous endotoxin administration. *J. Immunol.* **155,** 5038–5045.
276. Kuipers, B., T. van der Poll, M. Levi, S. J. H. Van Deventer, H. ten Cate, Y. Imai, C. E. Hack, and J. W. Ten Cate (1994). Platelet-activating factor antagonist TCV-309 attenuates the induction of the cytokine network in experimental endotoxemia in chimpanzees. *J. Immunol.* **152,** 2438–2446.
277. van der Poll, T., J. Jansen, D. Van Leenen, M. Von der Mohlen, M. Levi, H. ten Cate, H. Gallati, J. W. Ten Cate, and S. J. H. Van Deventer (1993). Release of soluble receptors for tumor necrosis factor in clinical sepsis and experimental endotoxemia. *J. Infect. Dis.* **168,** 955–960.
278. Shapiro, L., B. D. Clark, S. F. Orencole, D. D. Poutsiaka, E. V. Granowitz, and C. A. Dinarello (1993). Detection of soluble tumor necrosis factor (TNF) receptor (p55) in blood samples from healthy and endotoxemic humans. *J. Infect. Dis.* **167,** 1344–1350.
279. Zee, K. J. van, S. M. Coyle, S. E. Calvano, H. S. A. Oldenburg, D. M. Stiles, J. Pribble, M. Catalano, L. L. Moldawer, and S. F. Lowry (1995). Influence of IL-1 receptor blockade on the human response to endotoxemia. *J. Immunol.* **154,** 1499–1507.
280. Bemelmans, M. H. A., D. J. Gouma, and W. A. Buurman (1993). LPS-induced sTNF-receptor release in vivo in a murine model. Investigation of the role of tumor necrosis factor, IL-1, leukemia inhibiting factor, and IFN-gamma. *J. Immunol.* **151,** 5554–5562.
281. Granowitz, E. V., A. A. Santos, D. D. Poutsiaka, J. G. Cannon, D. W. Wilmore, S. M. Wolff, and C. A. Dinarello (1991). Production of interleukin-1-receptor antagonist during experimental endotoxaemia. *Lancet* **338,** 1423–1424.
282. Granowitz, E. V., R. Porat, J. W. Mier, S. F. Orencole, M. V. Callahan, J. G. Cannon, E. A. Lynch, K. Ye, D. D. Poutsiaka, E. Vannier, L. Shapiro, J. P. Pribble, D. M. Stiles, M. A. Catalano, S. M. Wollf, and C. A. Dinarello (1993). Hematologic and immunomodulatory effects of an interleukin-1 receptor antagonist coinfusion during low-dose endotoxemia in healthy humans. *Blood* **82,** 2985–2990.
283. Kawasaki, H., M. Moriyama, Y. Ohtani, M. Naitoh, A. Tanaka, and H. Nariuchi (1989). Analysis of endotoxin fever in rabbits by using a monoclonal antibody to tumor necrosis factor (cachectin). *Infect. Immun.* **57,** 3131–3135.
284. Mathison, J. C., E. Wolfson, and R. J. Ulevitch (1988). Participation of tumor necrosis factor in the mediation of Gram negative bacterial lipopolysaccharide-induced injury in rabbits. *J. Clin. Invest.* **81,** 1925–1937.
285. Creasey, A. A., P. Stevens, J. Kenney, A. C. Allison, K. Warren, R. Catlett, L. Hinshaw, and F. B. Taylor, Jr. (1991). Endotoxin and cytokine profile in plasma of baboons challenged with lethal and sublethal escherichia coli. *Circ. Shock* **33,** 84–91.

286. Wakabayashi, G., J. A. Gelfand, R. J. Jung, R. J. Connolly, J. F. Burke, and C. A. Dinarello (1991). Staphylococcus epidermidis induces complement activation, tumor necrosis factor and interleukin 1, a shock like state and tissue injury in rabbits without endotoxemia. *J. Clin. Invest.* **87,** 1925–1935.
287. Fiedler, V. B., I. Loof, E. Sander, V. Voehringer, C. Galanos, and M. A. Fournel (1992). Monoclonal antibody to tumor necrosis factor-alpha prevents lethal endotoxin sepsis in adult rhesus monkeys. *J. Lab. Clin. Med.* **120,** 574–588.
288. Miethke, T., C. Wahl, K. Heeg, B. Echtenacher, P. H. Krammer, and H. Wagner (1992). T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: Critical role of tumor necrosis factor. *J. Exp. Med.* **175,** 91–98.
289. Mohler, K. M., D. S. Torrance, C. A. Smith, R. G. Goodwin, K. E. Stremler, V. P. Fung, H. Madani, and M. B. Widmer (1993). Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists. *J. Immunol.* **151,** 1548–1561.
290. Redl, H., G. Schlag, A. Schiesser, and J. Davies (1993). Tumor necrosis factor is a mediator of phospholipase release during bacteremia in baboons. *Am. J. Physiol.* **264,** H2119–H2123.
291. Wakabayashi, G., J. A. Gelfand, J. F. Burke, R. C. Thompson, and C. A. Dinarello (1991). A specific receptor antagonist for interleukin 1 prevents escherichia coli-induced shock in rabbits. *FASEB J.* **5,** 338–343.
292. Fischer, E., M. A. Marano, K. J. Van Zee, C. S. Rock, A. S. Hawes, W. A. Thompson, L. DeForge, J. S. Kenney, D. G. Remick, D. C. Bloedow, R. C. Thompson, S. F. Lowry, and L. L. Moldawer (1992). Interleukin-1 receptor blockade improves survival and hemodynamic performance in Escherichia coli Septic shock, but fails to alter host responses to sublethal endotoxemia. *J. Clin. Invest.* **89,** 1551–1557.
293. Aiura, K., J. A. Gelfand, J. F. Burke, R. C. Thompson, and C. A. Dinarello (1993). Interleukin-1 (IL-1) receptor antagonist prevents staphylococcus epidermidis-induced hypotension and reduces circulating levels of tumor necrosis factor and IL-1-beta in rabbits. *Infect. Immun.* **61,** 3342–3350.
294. Cirelli, R. A., L. A. Carey, J. K. Fisher, D. L. Rosolia, T. H. Elsasser, T. J. Caperna, M. H. Gee, and K. H. Albertine (1995). Endotoxin infusion in anesthetized sheep is associated with intrapulmonary sequestration of leukocytes that immunohistochemically express tumor necrosis factor-a. *J. Leukocyte Biol.* **57,** 820–826.
295. Redl, H., G. Schlag, A. Schieser, and J. Davies (1995). Thrombomodulin release in baboon sepsis is dependent on the dose of Escherichia coli and the presence of tumor necrosis factor. *J. Infect. Dis.* **171,** 1522–1527.
296. Silva, A. T., K. F. Bayston, and J. Cohen (1990). Prophylactic and therapeutic effects of a monoclonal antibody to tumor necrosis factor-a in experimental gram-negative shock. *J. Infect. Dis.* **162,** 421–427.
297. Suitters, A. J., R. Foulkes, S. M. Opal, J. E. Palardy, J. S. Emtage, M. Rolfe, S. Stephens, A. A. Morgan, A. R. Holt, and L. C. Chaplin (1994). Differential effect of isotype on efficacy of anti-tumor necrosis factor alpha chimeric antibodies in experimental septic shock. *J. Exp. Med.* **179,** 849–856.
298. Hinshaw, L. B., P. Tekamp-Olson, A. C. K. Chang, P. A. Lee, F. B. Taylor, Jr., C. K. Murray, G. T. Peer, T. E. Emerson, Jr., R. B. Passey, and G. C. Kuo (1990). Survival of primates in LD100 septic shock following therapy with antibody to tumor necrosis factor (TNF-alpha). *Circ. Shock* **30,** 279–292.
299. Ferguson-Chanowitz, K. M., A. S. Katocs, Jr., W. C. Pickett, J. B. Kaplan, P. M. Sass, A. L. Oronsky, and S. S. Kerwar (1990). Platelet-activating factor or a platelet-activating

factor antagonist decreases tumor necrosis factor-a in the plasma of mice treated with endotoxin. *J. Infect. Dis.* **162,** 1081–1086.

300. Engelberts, I., E. J. U. Von Asmuth, C. J. Van der Linden, and W. A. Buurman (1991). The interrelation between TNF, IL-6 and PAF secretion induced by LPS in an in vivo and in vitro murine model. *Lymphocyte Cytokine Res.* **10,** 127–131.

301. Fantuzzi, G., H. Zheng, R. Faggioni, F. Benigni, P. Ghezzi, J. D. Sipe, A. R. Shaw, and C. A. Dinarello (1996). Effect of endotoxin in Il-1β-deficient mice. *J. Immunol.* **157,** 291–296.

302. Russel, D. A., K. K. Tucker, N. Chinookoswong, R. C. Thompson, and T. Kohno (1995). Combined inhibition of interleukin-1 and tumor necrosis factor in rodent endotoxemia: Improved survival and organ function. *J. Infect. Dis.* **171,** 1528–1538.

303. Zuckerman, S. H., N. Bryan-Poole, G. F. Evans, L. Short, and A. L. Glasebrook (1995). In vivo modulation of murine serum tumour necrosis factor and interleukin-6 levels during endotoxemia by oestrogen agonists and antagonists. *Immunology* **86,** 18–24.

304. Ertel, W., M. Keel, U. Steckholzer, U. Ungethum, and O. Trentz (1996). Interleukin-10 attenuates the release of proinflammatory cytokines but depresses splenocyte functions in murine endotoxemia. *Arch. Surg.* **131,** 51–56.

305. Sekut, L., J. A., Menius, Jr., M. F. Brackeen, and K. M. Connolly (1994). Evaluation of the significance of elevated levels of systemic and localized tumor necrosis factor in different animal models of inflammation. *J. Lab. Clin. Med.* **124,** 813–820.

306. Redl, H., G. Schlag, G. R. Adolf, B. Natmessnig, and J. Davies (1995). Tumor necrosis factor (TNF)-dependent shedding of the p55 TNF receptor in a baboon model of bacteremia. *Infect. Immun.* **63,** 297–300.

307. Walsh, C. J., H. J. Sugerman, P. G. Mullen, P. D. Carey, S. K. Leeper-Woodford, G. J. Jesmok, E. F. Ellis, and A. A. Fowler (1992). Monoclonal antibody to tumor necrosis factor α attenuates cardiopulmonary dysfunction in porcine gram-negative sepsis. *Arch. Surg.* **127,** 138–145.

308. Miethke, T., K. Duschek, C. Wahl, K. Heeg, and H. Wagner (1993). Pathogenesis of the toxic shock syndrome: T cell mediated lethal shock caused by the superantigen TSST-1. *Eur. J. Immunol.* **23,** 1494–1500.

309. Stevens, D. L., A. E. Bryant, S. P. Hackett, A. Chang, G. Peer, S. Kosanke, T. Emerson, and L. Hinshaw (1996). Group A streptococcal bacteremia: the role of tumor necrosis factor in shock and organ failure. *J. Infect. Dis.* **173,** 619–626.

310. Zheng, H., D. Fletcher, W. Kozak, M. Jiang, K. J. Hofmann, C. A. Conn, D. Soszynski, C. Grabiec, M. E. Trumbauer, A. Shaw, M. J. Kostura, K. Stevens, H. Rosen, R. J. North, H. Y. Chen, M. J. Tocci, M. J. Kluger, and L. H. T. van der Ploeg (1995). Resistance to fever induction and impaired acute-phase response in interleukin-1beta-deficient mice. *Immunity* **3,** 9–19.

311. Lundblad, R., P. Ekstrom, and K. E. Giercksky (1995). Pentoxifylline improves survival and reduces tumor necrosis factor, interleukin-6, and endothelin-1 in fulminant intra-abdominal sepsis in rats. *Shock* **3,** 210–215.

312. Byerley, L. O., N. W. Alcock, and H.F. Starnes, Jr. (1992). Sepsis-induced cascade of cytokine mRNA expression: Correlation with metabolic changes. *Am. J. Physiol.* **262,** E728-E735.

313. Giroir, B. P., J. H. Johnson, T. Brown, G. L. Allen, and B. Beutler (1992). The tissue distribution of tumor necrosis factor biosynthesis during endotoxemia. *J. Clin. Invest.* **90,** 693–698.

314. Cassatella, M. A (1995). The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today* **16,** 21–26.

315. Watanabe, S. I., N. Mukaida, N. Ikeda, M. Akiyama, A. Harada, I. Nakanishi, H. Nariuchi, Y. Watanabe, and K. Matsushima (1995). Prevention of endotoxin shock by an antibody against leukocyte integrin beta2 through inhibiting production and action of TNF. *Int. Immunol.* **7,** 1037–1046.

316. Geoffrey, R., P. Pugh-Humphreys, and A.W. Thomson (1994). Cytokines and their receptors as potential targets. *In* "The Cytokine Handbook" (A. Thomson, ed.), 2nd Ed., pp. 525–566. Academic Press, London.

317. Carpenter, A., T. J. Evans, W. A. Buurman, M. H. Bemelmans, D. Moyes, and J. Cohen (1995). Differences in the shedding of soluble TNF receptors between endotoxin-sensitive and endotoxin-resistant mice in response to lipopolysaccharide or live bacterial challenge. *J. Immunol.* **155,** 2005–2012.

318. Bemelmans, M. H. A., D. J. Gouma, and W. A. Buurman (1993). LPS-induced sTNF-receptor release in vivo in a murine model. *J. Immunol.* **151,** 5554–5562.

319. Poll, T. van der, E. Fischer, S. M. Coyle, K. J. van Zee, J. P. Pribble, D. M. Stiles, P. S. Barie, W. A. Buurman, L. L. Moldawer, and S. F. Lowry (1995). Interleukin-1 contributes to increased concentrations of soluble tumor necrosis factor receptor type I in sepsis. *J. Infect. Dis.* **172,** 577–580.

320. Steinshamn, S., M. H. Bemelmans, W. A. Buurman, and A. Waage (1995). Granulocytopenia reduces release of soluble TNF receptor p75 in endotoxin-stimulated mice: A possible mechanism of enhanced TNF activity. *Cytokine* **7,** 50–56.

321. Tracey, K. J., Y. Fong, D. G. Hesse, K. R. Monogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. Cerami (1987). Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature* **330,** 662–664.

322. Opal, S. M., A. S. Cross, J. C. Sadoff, H. H. Collins, N. M. Kelly, G. H. Victor, J. E. Palardy, and M. W. Bodmer (1991). Efficacy of antilipopolysaccharide and anti-tumor necrosis factor monoclonal antibodies in a neutropenic rat model of *Pseudomonas sepsis. J. Clin. Invest.* **88,** 885–890.

323. Hinshaw, L. B., T. E. Emerson, Jr., F. B. Taylor,Jr., A. C. Chang, M. Duerr, G. T. Peer, D. J. Flournay, G. L. White, S. D. Kosanke, and C. K. Murray (1992). Lethal staphylococcus aureus-induced shock in primates: Prevention of death with anti-TNF antibody. *J. Trauma.* **33,** 568–573.

324. Opal, S. M., A. S. Cross, N. M. Kelly, J. C. Sadoff, M. W. Bodmer, J. E. Palardy, and G. H. Victor (1990). Efficacy of a monoclonal antibody directed against tumor necrosis factor in protecting neutropenic rats from lethal infection with *Pseudomonas aeruginosa. J. Infect. Dis.* **161,** 1148–1152.

325. Eskandari, M. K., G. Bolgos, C. Miller, D. T. Nguyen, L. E. DeForge, and D. G. Remick (1992). Anti-tumor necrosis factor antibody therapy fails to prevent lethality after cecal ligation and puncture or endotoxemia. *J. Immunol.* **148,** 2724–2730.

326. Chorinchath, B. B., L. Y. Kong, L. Mao, and R. E. McCallum (1996). Age-associated differences in TNF-alpha and nitric oxide production in endotoxic mice. *J. Immunol.* **156,** 1525–1530.

327. Roy, D. le, P. Morand, S. Lengacher, M. Celio, G. E. Grau, M. P. Glauser, and D. Heumann (1996). Streptococcus mitis cell walls and lipopolysaccharide induce lethality in D-galactosamine-sensitzed mice by a tumor necrosis factor-dependent pathway. *Infect. Immun.* **64,** 1846–1849.

328. Remick, D., P. Manohar, G. Bolgos, J. Rodriguez, L. Moldawer, and G. Wollenberg (1995). Blockade of tumor necrosis factor reduces lipopolysaccharide lethality, but not the lethality of cecal ligation and puncture. *Shock* **4,** 89–95.

329. Ashkenazi, A., S. A. Marsters, D. J. Capon, S. M. Chamow, I. S. Figari, D. Pennica, D. V. Goeddel, M. A. Palladino, and D. H. Smith (1991). Protection against endotoxic

shock by tumor necrosis factor receptor immunoadhesin. *Proc. Natl. Acad. Sci. USA* **88,** 10535–10539.

330. Jin, H., R. Yang, S. A. Marsters, S. A. Bunting, F. M. Wurm, S. M. Chamow, and A. Ashkenazi (1994). Protection against rat endotoxic shock by p55 tumor necrosis factor (TNF) receptor immunoadhesin. *J. Infect. Dis.* **170,** 1323–1326.
331. Porat, R., H. N. Paddock, S. D. Schwaitzberg, R. J. Connolly, T. Wilkens, J. R. Dasch, M. P. Gascon, J. S. Hutchison, A. Ythier, and D. Wallach (1995). Glycosylated recombinant human tumor necrosis factor binding protein-1 reduces mortality, shock, and production of tumor necrosis factor in rabbit escherichia coli sepsis. *Crit. Care Med.* **23,** 1080–1089.
332. Garcia, I., Y. Miyazaki, K. Araki, M. Araki, R. Lucas, G. E. Grau, G. Milon, Y. Belkaid, C. Montixi, and W. Lesslauer (1995). Transgenic mice expressing high levels of soluble TNF-R1 fusion protein are protected from lethal septic shock and cerebral malaria, and are highly sensitive to listeria monocytogenes and leishmania major infections. *Eur. J. Immunol.* **25,** 2401–2407.
333. Sheehan, K. C. F., J. K. Pinckard, C. D. Arthur, L. P. Dehner, D. V. Goeddel, and R. D. Schreiber (1995). Monoclonal antibodies specific for murine p55 and p75 tumor necrosis factor receptors: Identification of a novel in vivo role for p75. *J. Exp. Med.* **181,** 607–617.
334. Rothe, J., W. Lesslauer, H. Lotscher, Y. Lang, P. Koebel, F. Kontgen, A. Athage, R. Zinkernagel, M. Steinmetz, and H. Bluethmann (1993). Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. *Nature* **364,** 798–802.
335. Pfeffer, K., T. Matsuyama, T. M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P. S. Ohashi, M. Kronke, and T. W. Mak (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell* **73,** 457–467.
336. Tartaglia, L. A., D. Pennica, and D. V. Goeddel (1993). Ligand passing: The 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. *J. Biol. Chem.* **268,** 18542–18548.
337. Slowik, M. R., L. G. De Luca, W. Fiers, and J. S. Pober (1993). Tumor necrosis factor activates human endothelial cells through the p55 tumor necrosis factor receptor but the p75 receptor contributes to activation at low tumor necrosis factor concentration. *Am. J. Pathol.* **143,** 1724–1730.
338. Echtenacher, B., W. Falk, D. N. Mannel, and P. H. Krammer (1990). Requirement of endogenous tumor necrosis factor/cachectin for recovery from experimental peritonitis. *J. Immunol.* **145,** 3762–3766.
339. Bagby, G. J., K. J. Plessala, L. A. Wilson, J. J. Thompson, and S. Nelson (1991). Divergent efficacy of antibody to tumor necrosis factor-α in intravascular and peritonitis models of sepsis. *J. Infect. Dis.* **163,** 83–88.
340. Kato, T., A. Murata, H. Ishida, H. Toda, N. Tanaka, H. Hayashida, M. Monden, and N. Matsuura (1995). Interleukin 10 reduces mortality from severe peritonitis in mice. *Antimicrobiol. Agents Chemother.* **39,** 1336–1340.
341. Echtenacher, B., D. N. Mannel, and L. Hultner (1996). Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* **381,** 75–77.
342. Poll, T. van der, A. Marchant, W. A. Buurman, L. Berman, C. V. Keogh, D. D. Lazarus, L. Nguyen, M. Goldman, L. L. Moldawer, and S. F. Lowry (1995). Endogenous IL-10 protects mice from death during septic peritonitis. *J. Immunol.* **155,** 5397–5401.
343. Stack, A. M., R. A. Saladino, C. Thompson, F. Sattler, D. L. Weiner, J. Parsonnet, H. Nariuchi, G. R. Siber, and G. R. Fleischer (1995). Failure of prophylactic and

therapeutic use of a murine anti-tumor necrosis factor monoclonal antibody in escherichia coli sepsis in the rabbit. *Crit. Care Med.* **23,** 1512–1518.

344. Nakane, A., M. Okamoto, M. Asano, M. Kohanawa, and T. Minagawa (1995). Endogenous gamma interferon, tumor necrosis factor, and interleukin-6 in staphylococcus aureus infection in mice. *Infect. Immun.* **63,** 1165–1172.
345. Alexander, H. R., G. M. Doherty, M. I. Block, P. J. Kragel, J. C. Jensen, H. N. Langstein, E. Walker, and J. A. Norton (1991). Single-dose tumor necrosis factor protection against endotoxin-induced shock and tissue injury in rats. *Infect. Immun.* **59,** 3889–3894.
346. Cross, A. S., J. C. Sadoff, N. Kelly, E. Bernton, and P. Gemski (1989). Pretreatment with recombinant murine tumor necrosis factor α/cachectin and murine interleukin 1α protects mice from lethal bacterial infection. *J. Exp. Med.* **169,** 2021–2027.
347. Fong, Y., K. J. Tracey, L. L. Moldawer, D. G. Hesse, K. B. Manogue, J. S. Kenney, A. T. Lee, G. C. Kuo, A. C. Allison, S. F. Lowry, and A. Cerami (1989). Antibodies to cachectin/tumor necrosis factor reduce interleukin 1b and interleukin 6 appearance during lethal bacteremia. *J. Exp. Med.* **170,** 1627–1633.
348. Espat, N. J., J. C. Cendan, E. A. Beierle, T. A. Auffenberg, J. Rosenberg, D. Russel, J. S. Kenney, E. Fischer, W. Montegut, and S. F. Lowry (1995). PEG-BP-30 monotherapy attenuates the cytokine-mediated inflammatory cascade in baboon Escherichia coli septic shock. *J. Surg. Res.* **59,** 153–158.
349. Otteren, G. M. van, R. M. Strieter, S. L. Kunkel, R. Paine, III, M. J. Greenberger, J. M. Danforth, M. D. Burdick, and T. J. Standiford (1995). Compartmentalized expression of rantes in a murine model of endotoxemia 1. *J. Immunol.* **154,** 1900–1908.
350. Redl, H., G. Schlag, S. Bahrami, R. Kargl, W. Hartter, W. Woloszczuk, and J. Davies (1994). Big-endothelin release in baboon bacteremia is partially TNF dependent. *J. Lab. Clin. Med.* **124,** 796–801.
351. Junger, W. G., D. B. Hoyt, H. Redl, F. C. Liu, W. H. Loomis, J. Davies, and G. Schlag (1995). Tumor necrosis factor antibody treatment of septic baboons reduces the production of sustained T-cell suppressive factors. *Shock* **3,** 173–178.
352. Standiford, T. J., S. L. Kunkel, N. W. Lukacs, M. J. Greenberger, J. M. Danforth, R. G. Kunkel, and R. M. Strieter (1995). Macrophage inflammatory protein-1 alpha mediates lung leukocyte recruitment, lung capillary leak, and early mortality in murine endotoxemia. *J. Immunol.* **155,** 1515–1524.
353. Wang, P., T. J. Wood, M. Zhou, Z. F. Ba, and I. H. Chaudry (1996). Inhibition of the biologic activity of tumor necrosis factor maintains vascular endothelial cell function during hyperdynamic sepsis. *J. Trauma.* **40,** 694–700.
354. Windsor, A. C. J., C. J. Walsh, P. G. Mullen, D. J. Cook, B. J. Fisher, C. R. Blocher, S. K. Leeper-Woodford, H. J. Sugerman, and A. A. Fowler,III (1993). Tumor necrosis factor-alpha blockade prevents neutrophil CD18 receptor upregulation and attenuates acute lung injury in porcine sepsis without inhibition of neutrophil oxygen radical generation. *J. Clin. Invest.* **91,** 1459–1468.
355. Gershenwald, J. E., Y. Fong, T. J. Fahey,III, S. E. Calvano, R. Chizzonite, P. L. Kilian, S. F. Lowry, and L. L. Moldawer (1990). Interleukin 1 receptor blockade attenuates the host inflammatory response. *Proc. Natl. Acad. Sci. USA* **87,** 4966–4970.
356. McIntyre, K. W., G. J. Stepan, K. D. Kolinsky, W. R. Benjamin, J. M. Plocinski, K. L. Kaffka, C. A. Campen, R. A. Chizzonite, and P. L. Kilian (1991). Inhibition of interleukin 1 (IL-1) binding and bioactivity in vitro and modulation of acute inflammation in vivo by IL-1 receptor antagonist and anti-IL-1 receptor monoclonal antibody. *J. Exp. Med.* **173,** 931–939.

357. Ulich, T. R., S. M. Yin, K. Z. Guo, J. del Castillo, S. P. Eisenberg, and R. C. Thompson (1991). The intratracheal administration of endotoxin and cytokines. III. The interleukin-1 (IL-1) receptor antagonist inhibits endotoxin- and IL-1-induced acute inflammation. *Am. J. Pathol.* **138,** 521–524.

358. Ohlsson, K., P. Björk, M. Bergenfeldt, R. Hageman, and R. C. Thompson (1990). Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature* **348,** 550–552.

359. Alexander, H. R., G. M. Doherty, C. M. Buresh, D. J. Venzon, and J. A. Norton (1991). A recombinant human receptor antagonist to IL-1 improves survival after lethal endotoxemia in mice. *J. Exp. Med.* **173,** 1029–1032.

360. Havell, E. A., L. L. Moldawer, D. Helfgott, P. L. Kilian, and P. B. Sehgal (1992). Type I IL-1 receptor blockade exacerbates murine listeriosis. *J. Immunol.* **148,** 1486–1492.

361. Granowitz, E. V., E. Vannier, D. D. Poutsiaka, and C. A. Dinarello (1992). Effect of interleukin-1 (IL-1) blockade on cytokine synthesis: II. IL-1 receptor antagonist inhibits lipopolysaccharide-induced cytokine synthesis by human monocytes. *Blood* **79,** 2364–2369.

362. Granowitz, E. V., B. D. Clark, E. Vannier, M. V. Callahan, and C. A. Dinarello (1992). Effect of interleukin-1 (IL-1) blockade on cytokine synthesis: I. IL-1 receptor antagonist inhibits IL-1-induced cytokine synthesis and blocks the binding of IL-1 to its type II receptor on human monocytes. *Blood* **79,** 2356–2363.

363. Waage, A., T. Haltensen, and T. Espevik (1987). Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease. *Lancet* **1,** 355–357.

364. Girardin, E., G. E. Grau, J.M. Dayer, P. Roux-Lambard, the J5 Study Group, and P.-H. Lambert (1988). Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. *N. Engl. J. Med.* **319,** 397–400.

365. Pinsky, M. R., J. L. Vincent, J. Deviere, M. Alegre, R. J. Kahn, and E. Dupont (1993). Serum cytokine levels in human septic shock. Relation to multiple-system organ failure and mortality. *Chest* **103,** 565–575.

366. Marks, J. D., C. B. Marks, J. M. Luce, A. B. Montgomery, J. Turner, C. A. Metz, and J. F. Murray (1990). Plasma tumor necrosis factor in patients with septic shock: Mortality rate, incidence of adult respiratory distress syndrome, and effects of methylprednisolone administration. *Am. Rev. Respir. Dis.* **141,** 94–97.

367. Waage, A., P. Brandtzaeg, A. Halstensen, P. Kierulf, and T. Espevik (1989). The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1, and fatal outcome. *J. Exp. Med.* **169,** 333–338.

368. Marano, M. A., Y. Fong, L. L. Moldawer, H. Wei, S. E. Calvano, K. J. Tracey, P. S. Barie, K. Manogue, A. Cerami, G. T. Shires, and S. Lowry (1990). Serum cachectin/tumor necrosis factor in critically ill patients with burns correlates with infection and mortality. *Surg. Gynecol. Obstet.* **170,** 32–38.

369. Offner, F., J. Philippe, D. Vogelaers, F. Colardyn, G. Baele, M. Baudrihaye, A. Vermeulen, and G. Leroux-Roels (1990). Serum tumor necrosis factor levels in patients with infectious disease and septic shock. *J. Lab. Clin. Med.* **116,** 100–105.

370. Damas, P., A. Reuter, P. Gysen, J. Demonty, M. Lamy, and P. Franchimont (1989). Tumor necrosis factor and interleukin-1 serum levels during severe sepsis in humans. *Crit. Care Med.* **17,** 975–978.

371. Philippe, J., G. Dooijewaard, F. Offner, P. Turion, G. Baele, and G. Leroux-Roels (1992). Granulocyte elastase, tumor necrosis factor-alpha and urokinase levels as prognostic markers in severe infection. *Thromb. Haemost.* **68,** 19–23.

372. Calandra, T., J.-D. Baumgartner, G. E. Grau, M.-M. Wu, P.-H. Lambert, J. Schellekens, J. Verhoef, M. P. Blauser, and the Swiss–Dutch J5 Immunoglobulin Study Group (1990). Prognostic values of tumor necrosis factor/cachectin, interleukin- 1, interferon-alpha, and interferon-gamma in the serum of patients with septic shock. *J. Infect. Dis.* **161,** 982–987.
373. Gardlund, B., J. Sjolin, A. Nilsson, M. Roll, C. J. Wickerts, and B. Wretlind (1995). Plasma levels of cytokines in primary septic shock in humans: Correlation with disease severity. *J. Infect. Dis.* **172,** 296–301.
374. Kornelisse, R. F., J. A. Hazelzet, H. F. Savelkoul, W. C. Hop, M. H. Suur, A. N. Borsboom, I. M. Risseeuw-Appel, E. van der Voort, and R. de Groot (1996). The relationship between plasminogen activator inhibitor-1 and proinflammatory and counterinflammatory mediators in children with meningococcal septic shock. *J. Infect. Dis.* **173,** 1148–1156.
375. Nurnberger, W., A. Platonov, H. Stannigel, V. B. Beloborodov, I. Michelmann, R. von Kries, S. Burdach, and U. Gobel (1995). Definition of a new score for severity of generalized neisseria meningitidis infection. *Eur. J. Pediatr.* **154,** 896–900.
376. de Groote, M. A., M. A. Martin, P. Densen, M. A. Pfaller, and R. P. Wenzel (1989). Plasma tumor necrosis factor levels in patients with presumed sepsis. *J. Am. Med. Assoc.* **262,** 249–251.
377. Casey, L. C., R. A. Balk, and R. C. Bone (1993). Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. *Ann. Intern. Med.* **119,** 771–778.
378. Baud, L., J. Cadranel, G. Offenstadt, L. Luquel, B. Guidet, and P. Amstutz (1990). Tumor necrosis factor and septic-shock. *Crit. Care Med.* **18,** 349–350.
379. Fisher, C. J., Jr., S. M. Opal, J. F. Dhainaut, S. Stephens, J. L. Zimmerman, P. Nightingale, S. J. Harris, R. M. H. Schein, E. A. Panacek, J. L. Vincent, G. E. Foulke, E. L. Warren, C. Garrard, G. Park, M. W. Bodmer, J. Cohen, C. Van der Linden, A. S. Cross, J. C. Sadoff, and the CB0006 Sepsis Syndrome Study Group (1993). Influence of an anti-tumor necrosis factor monoclonal antibody on cytokine levels in patients with sepsis. *Crit. Care Med.* **21,** 318–327.
380. Munoz, C., B. Misset, C. Fitting, J. P. Bleriot, J. Carlet, and J. M. Cavaillon (1991). Dissociation between plasma and monocyte-associated cytokines during sepsis. *Eur. J. Immunol.* **21,** 2177–2184.
381. Rosenbloom, J., M. R. Pinsky, J. L. Bryant, A. Shin, T. Tran, and T. Whiteside (1995). Leukocyte activation in the peripheral blood of patients with cirrhosis of the liver and SIRS. *J. Am. Med. Assoc.* **274,** 58–65.
382. Goldie, A. S., K. C. H. Fearon, J. A. Ross, G. R. Barclay, R. E. Jackson, I. S. Grant, G. Ramsay, A. S. Blyth, and J. C. Howie (1995). Natural cytokine antagonists and endogenous antiendotoxin core antibodies in sepsis syndrome. *J. Am. Med. Assoc.* **274,** 172–177.
383. Schroder, J., F. Stuber, H. Gallati, F. U. Schade, and B. Kremer (1995). Pattern of soluble TNF receptors I and II in sepsis. *Infection* **23,** 143–148.
384. James, K. (1990). Interactions between cytokines and α_2-macroglobulin. *Immunol. Today* **11,** 163–166.
385. Dinarello, C. A., and J. G. Cannon (1993). Cytokine measurements in septic shock. *Ann. Intern. Med.* **119,** 853–854.
386. Goldie, A. S., K. C. Fearon, J. A. Ross, G. R. Barclay, R. E. Jackson, I. S. Grant, G. Ramsay, A. S. Blyth, and J. C. Howie (1995). Natural cytokine antagonists and endogenous anti-endotixin core antibodies in sepsis syndrome. The sepsis intervention group. *J. Am. Med. Assoc.* **274,** 172–177.

387. Leroux-Roels, G., and F. Oftner (1990). Tumor necrosis factor in sepsis. *J. Am. Med. Assoc.* **263,** 1494–1495.
388. Dofferhoff, A. S., H. J. De Jong, V. J. Bom, J. Van der Meer, P. C. Limburg, H. G. De Vries, J. Marrink, P. O. Mulder, and J. Weits (1992). Complement activation and the production of inflammatory mediators during the treatment of severe sepsis. *Scand. J. Infect. Dis.* **24,** 197–204.
389. Dofferhoff, A. S., V. J. Bom, H. G. De Vries, J. Van Ingen, J. Van der Meer, B. P. Hazenberg, P. O. Mulder, and J. Weits (1992). Patterns of cytokines, plasma endotoxin, plasminogen activator inhibitor, and acute phase proteins during the treatment of severe sepsis in humans. *Crit. Care Med.* **20,** 185–192.
390. Roten, R., M. Markert, F. Feihl, M.-D. Schaller, M.-C. Tagan, and C. Perret (1991). Plasma levels of tumor necrosis factor in the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **143,** 590–592.
391. Millar, A. B., N. M. Foley, M. Singer, N. McI. Johnson, A. Meager, and G. A. W. Rook (1989). Tumour necrosis factor in bronchopulmonary secretions of patients with adult respiratory distress syndrome. *Lancet* **3,** 712–714.
392. Suter, P. M., S. Suter, E. Girardin, P. Roux-Lombard, G. E. Grau, and J.-M. Dayer (1992). High bronchoalveolar levels of tumor necrosis factor and its inhibitors, interleukin-1, interferon, and elastase, in patients with adult respiratory distress syndrome after trauma, shock, or sepsis. *Am. Rev. Respir. Dis.* **145,** 1016–1022.
393. Meduri, G. U., G. Kohler, S. Headley, E. Tolley, F. Stentz, and A. Postlethwaite (1995). Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome. *Chest* **108,** 1303–1314.
394. Tran van Nhieu, J., B. Misset, F. Lebargy, J. Carlet, and J. F. Bernaudin (1993). Expression of tumor necrosis factor-alpha gene in alveolar macrophages from patients with the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **147,** 1585–1589.
395. Jacobs, R. F., D. R. Tabor, A. W. Burks, and G. D. Campbell (1989). Elevated interleukin-1 release by human alveolar macrophages during the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **140,** 1686–1692.
396. Frieling, J. T. M., M. van Deuren, J. Wijdenes, J. W. M. van der Meer, C. Clement, C. J. van der Linden, and R. W. Sauerwein (1995). Circulating interleukin-6 receptor in patients with sepsis syndrome. *J. Infect. Dis.* **171,** 469–472.
397. Calvano, S. E., T. van der Poll, S. M. Coyle, P. S. Barie, L. L. Moldawer, and S. F. Lowry (1996). Monocyte tumor necrosis factor receptor levels as a predictor of risk in human sepsis. *Arch. Surg.* **131,** 434–437.
398. Pilz, G., P. Fraunberger, R. Appel, E. Kreuzer, K. Werdan, A. Walli, and D. Seidel (1996). Early prediction of outcome in score-identified, postcardiac surgical patients at high risk for sepsis, using soluble tumor necrosis factor receptor-p55 concentrations. *Crit. Care Med.* **24,** 596–600.
399. Pruitt, J. H., M. B. Welborn, P. D. Edwards, T. R. Harward, J. W. Seeger, T. D. Martin, C. Smith, J. A. Kenney, R. I. Wesdorp, S. Meijer, M. A. Cuesta, A. Abouhanze, E. M. Copeland, III, J. Giri, J. E. Sims, L. L. Moldawer, and H. S. Oldenburg (1996). Increased soluble interleukin-1 type II receptor concentrations in postoperative patients and in patients with sepsis syndrome. *Blood* **87,** 3282–3288.
400. Zeni, F., P. Pain, M. Vindimian, J. P. Gay, P. Gery, M. Bertrand, Y. Page, D. Page, R. Vermesch, and J. C. Bertrand (1996). Effects of pentoxifylline on circulating cytokine concentrations and hemodynamics in patients with septic shock: Results from a double-blind, randomized, placebo-controlled study. *Crit. Care Med.* **24,** 207–214.
401. Exley, A. R., J. Cohen, W. Buurman, R. Owen, G. Hanson, J. Lumley, J. M. Aulakh, M. Bodmer, A. Riddell, S. Stephens, and M. Perry (1990). Monoclonal antibody to TNF in severe septic shock. *Lancet* **335,** 1275–1277.

402. Dhainaut, J. F., J. L. Vincent, C. Richard, P. Lejeune, C. Martin, L. Fierobe, S. Stephens, U. M. Ney, and M. Sopwith (1995). CDP571, a humanized antibody to human tumor necrosis factor-alpha: Safety, pharmacokinetics, immune response, and influence of the antibody on cytokine concentrations in patients with septic shock. CPD571 sepsis study group. *Crit. Care Med.* **23,** 1461–1469.

403. Vincent, J. L., J. Bakker, G. Mareceaux, L. Schandene, R. J. Kahn, and E. Dupont (1992). Anti-TNF antibodies administration increases myocardial contractility in septic shock patients. *Chest* **101,** 810–815.

404. Abraham, E., R. Wunderink, H. Silverman, T. M. Perl, S. Nasraway, H. Levy, R. Bone, R. P. Wenzel, R. Balk, and R. Allred (1995). Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome. A randomized controlled, double-blind, multicenter clinical trial. TNF-alpha MAb sepsis study group. *J. Am. Med. Assoc.* **273,** 934–941.

405. Fisher, C. J., Jr., G. J. Slotman, S. M. Opal, J. P. Pribble, R. C. Bone, G. Emmanuel, D. C. Bloedow, and M. A. Catalano (1994). Initial evaluation of human recombinant interleukin-1 receptor antagonist in the treatment of sepsis syndrome: A randomized, open-label, placebo-controlled multicenter trial. *Crit. Care Med.* **22,** 12–21.

406. Fisher, C. J., Jr., J.-F. A. Dhainaut, S. M. Opal, J. P. Pribble, R. A. Balk, G. J. Slotman, T. J. Iberti, E. C. Rackow, M. J. Shapiro, R. L. Greenman, H. D. Reines, M. P. Shelly, B. W. Thompson, J. F. LaBrecque, M. A. Catalano, W. A. Knaus, and J. C. Sadoff (1994). Recombinant human interleukin 1 receptor antagonist in the treatment of patients with sepsis syndrome. Results from a randomized, double-blind, placebo-controlled trial. *J. Am. Med. Assoc.* **271,** 1836–1843.

407. Knaus, W. A., F. E. Harrell, Jr., J. F. la Brecque, D. P. Wagner, J. P. Pribble, E. A. Draper, C. J. Fisher, Jr., and L. Soll (1996). Use of predicted risk of mortality to evaluate the efficacy of anticytokine therapy in sepsis. The rhIL-1ra phase III sepsis syndrome study group. *Crit. Care Med.* **24,** 46–56.

408. Boermeester, M. A., P. A. M. van Leeuwen, S. M. Coyle, G. J. Wolbink, C. E. Hack, and S. F. Lowry (1995). Interleukin-1 blockade attenuates mediator release and dysregulation of the hemostatic mechanism during human sepsis. *Arch. Surg.* **130,** 739–748.

409. Slotman, G. J., B. Friedman, C. Brathwaite, A. J. Mure, J. V. Quinn, and E. Shapiro (1995). Interleukin-1 mediates increased plasma levels of eicosanoids and cytokines in patients with septis syndrome. *Shock* **4,** 318–323.

410. Van der Meer, J. W. M., M. Barza, S. M. Wolff, and C. A. Dinarello (1988). A low dose of recombinant interleukin 1 protects granulocytopenic mice from lethal Gram-negative infection. *Proc. Natl. Acad. Sci. USA* **85,** 1620–1623.

411. Ozaki, Y., T. Ohashi, A. Minami, and S. Nakamura (1987). Enhanced resistance of mice to bacterial infection by recombinant human interleukin-1. *Infect. Immun.* **55,** 1436–1442.

412. Akira, S., T. Taga, and T. Kishimoto (1993). Interleukin-6 in biology and medicine. *Adv. Immunol.* **54,** 1–78.

413. Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwamura, K. Nakajima, K. Koyama, A. Iwamatsu, S. Tsunasawa, F. Sakiyama, H. Matsui, Y. Takahara, T. Taniguchi, and T. Kishimoto (1986). Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* **324,** 73–76.

414. Leutz, A., K. Damm, E. Sterneck, S. Ness, R. Frank, H. Gausepohl, Y.-C. E. Pan, J. Smart, M. Hayman, and T. Graf (1989). Molecular cloning of the chicken myelomono-

cytic growth factor (cMGF) reveals relationship to interleukin 6 and granulocyte colony stimulating factor. *EMBO J.* **8,** 175–181.

415. Rose, T. M., and A. G. Bruce (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. USA* **88,** 8641–8645.

416. Sehgal, P. B., A. Zilberstein, R. Ruggieri, L. T. May, A. Ferguson-Smith, D. L. Slate, M. Revel, and F. H. Ruddle (1986). Human chromosome 7 carries the $\beta 2$ interferon gene. *Proc. Natl. Acad. Sci. USA* **83,** 5219–5222.

417. Yamasaki, K., T. Taga, Y. Hirata, H. Yawata, Y. Kawanishi, B. Seed, T. Taniguchi, T. Hirano, and T. Kishimoto (1988). Cloning and expression of the human interleukin-6 (BSF-2/IFNβ 2) receptor. *Science* **241,** 825–828.

418. Bazan, J. F. (1990). Haemopoietic receptors and helical cytokines. *Immunol. Today* **11,** 350–354.

419. Taga, T., M. Hibi, Y. Hirata, K. Yamasaki, K. Yasukawa, T. Matsuda, T. Hirano, and T. Kishimoto (1989). Interleukin 6 (IL-6) triggers the association of its receptor (IL-6-R) with a possible signal transducer, gp 130. *Cell* **58,** 573–581.

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

421. Paonessa, G., R. Graziani, A. De Serio, R. Savino, L. Ciapponi, A. Lahm, A. L. Salvati, C. Toniatti, and G. Ciliberto (1995). Two distinct and independent sites on IL-6 trigger gp130 dimer formation and signalling. *EMBO J.* **14,** 1942–1951.

422. Ward, L. D., G. J. Howlett, G. Discolo, K. Yasukawa, A. Hammacher, R. L. Moritz, and R. J. Simpson (1994). The high affinity interleukin-6 (IL-6) receptor is a hexameric complex consisting of two molecules of each IL-6, IL-6 receptor and gp-130. *J. Biol. Chem.* **269,** 23286–23289.

423. Brakenhoff, J. P. J., M. Hart, E. R. De Groot, F. Di Padova, and L. A. Aarden (1990). Structure–funtion analysis of human IL-6: Epitope mapping of neutralizing monoclonal antibodies with amino- and carboxyl-terminal deletion mutants. *J. Immunol.* **145,** 561–568.

424. Brakenhoff, J. P. J., F. D. de Hon, V. Fontaine, M. Hart, E. R. De Groot, J. Content, and L. A. Aarden (1992). Two different sites on the IL6 molecule are involved in biological activity. *In* "IL6: Physiopathology and Clinical Potentials" (M. Revèl, ed.), Vol. 88, pp. 33-41. Serono symposia, Raven Press, New York.

425. Zhang, X.-G., J.-J. Gu, Z.-Y. Lu, K. Yasukawa, G. D. Yancopoulos, K. Turner, M. Shoyab, T. Taga, T. Kishimoto, R. Bataille, and B. Klein (1994). Ciliary neurotrophic factor, interleukin 11, leukemia inhibitory factor, and oncostatin M are growth factors for human myeloma cell lines using the interleukin 6 signal transducer gp130. *J. Exp. Med.* **177,** 1337–1342.

426. Nishimoto, N., A. Ogata, Y. Shima, Y. Tani, H. Ogawa, M. Nakagawa, H. Sugiyama, K. Yoshizaki, and T. Kishimoto (1994). Oncostatin M, leukemia inhibitory factor and interleukin 6 induce the proliferation of human plasmacytoma cells via the common signal transducer gp130. *J. Exp. Med.* **179,** 1343–1347.

427. Suzuki, H., K. Yasukawa, T. Saito, M. Narazaki, A. Hasegawa, T. Taga, and T. Kishimoto (1993). Serum soluble interleukin-6 receptor in MRL/lpr mice is elevated with age and mediates the interleukin-6 signal. *Eur. J. Immunol.* **23,** 1078–1082.

428. Brakenhoff, J. P. J., F. D. de Hon, V. Fontaine, E. ten Boekel, H. Schooltink, S. Rose-John, P. C. Heinrich, J. Content, and L. A. Aarden (1994). Development of a human interleukin-6 receptor antagonist. *J. Biol. Chem.* **269,** 86–93.

429. Kohase, M., D. Henriksen-Destefano, L. T. May, J. Vilçek, and P. B. Sehgal (1986). Induction of β2-interferon by tumor necrosis factor: A homeostatic mechanism in the control of cell proliferation. *Cell* **45,** 659–666.

430. Walther, Z., L. T. May, and P. B. Sehgal (1988). Transcriptional regulation of the interferon-β2/B-cell differentiation factor BSF-2/hepatocyte stimulating factor gene in human fibroblasts by other cytokines. *J. Immunol.* **140,** 974–977.

431. Van Damme, J., G. Opdenakker, R. J. Simpson, M. R. Rubira, S. Cayphas, A. Vink, A. Billiau, and J. Van Snick (1987). Identification of the human 26kDa protein, interferon b2 (IFNb2) as a B cell hybridoma/plasmacytoma growth factor induced by interleukin 1 and tumor necrosis factor. *J. Exp. Med.* **165,** 914–919.

432. Hirano, T., and T. Kishimoto (1990). Interleukin-6 (IL-6). *In* "Handbook of Experimental Pharmacology Vol. 95:I Peptide Growth Factors and Their Receptors" (M.B. Sporn and A.B. Roberts, eds.) pp. 633–665. Springer-Verlag, Berlin.

433. Helle, M., L. Boeije, and L. A. Aarden (1989). Interleukin-6 is an intermediate in interleukin-1 induced thymocyte proliferation. *J. Immunol.* **142,** 4335–4338.

434. Helle, M., J. P. J. Brakenhoff, E. R. De Groot, and L. A. Aarden (1988). Interleukin 6 is involved in interleukin-1-induced activities. *Eur. J. Immunol.* **18,** 957–959.

435. Gauldie, J., C. Richards, D. Harnish, P. M. Lansdorp, and H. Baumann (1987). Interferon β2/B-cell stimulatory 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. USA* **84,** 7251–7255.

436. Geiger, T., T. Andus, J. Klapproth, T. Hirano, T. Kishimoto, and P. C. Heinrich (1988). Induction of rat acute-phase proteins by interleukin 6 in vivo. *Eur. J. Immunol.* **18,** 717–721.

437. Heinrich, P. C., J. V. Castell, and T. Andus (1990). Interleukin-6 and the acute phase response. *Biochem. J.* **269**(Suppl.), 51–66.

438. Wong, G. G., and S. C. Clark (1988). Multiple actions of interleukin 6 within a cytokine network. *Immunol. Today* **9,** 137–139.

439. Fey, G. H., and J. Gauldie (1990). The acute phase response of the liver in inflammation. *In* "Progress in Liver Disease" (H. Popper and F. Schaffner, eds.), Vol. 9. Philadelphia. Saunders, pp. 89–116.

440. Baumann, H., K.-A. Won, and G. P. Jahreis (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.

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

442. Kopf, M., H. Baumann, G. Freer, M. Freudenberg, M. Lamers, T. Kishimoto, R. Zinkernagel, H. Bluethmann, and G. Köhler (1994). Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* **368,** 339–342.

443. Fattori, E., M. Cappelletti, P. Costa, C. Sellitto, L. Cantoni, M. Carelli, R. Faggioni, G. Fantuzzi, P. Ghezzi, and V. Poli (1994). Defective inflammatory response in interleukin 6-deficient mice. *J. Exp. Med.* **180,** 1243–1250.

444. Nijsten, M. W. N., E. R. De Groot, H. J. ten Duis, H. J. Klasen, C. E. Hack, and L. A. Aarden (1987). Serum levels of interleukin-6 and acute phase responses. *Lancet* **2,** 921.

445. Nijsten, M. W. N., C. E. Hack, M. Helle, H. J. ten Duis, H. J. Klasen, and L. A. Aarden (1991). Interleukin-6 and its relation to the humoral immune response and clinical parameters in burned patients. *Surgery* **109,** 761–767.

446. Borish, L., R. Rosenbaum, L. Albury, and S. Clark (1989). Activation of neutrophils by recombinant interleukin 6. *Cell. Immunol.* **121,** 280–289.
447. Tilg, H., E. Trehu, M. B. Atkins, C. A. Dinarello, and J. W. Mier (1994). Interleukin-6 (IL-6) as an anti-inflammatory cytokine: Induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood* **83,** 113–118.
448. Tilg, H., E. Vannier, G. Vachino, C. A. Dinarello, and J. W. Mier (1993). Antiinflammatory properties of hepatic acute phase proteins: Preferential induction of interleukin 1 (IL-1) receptor antagonist over IL-1-beta synthesis by human peripheral blood mononuclear cells. *J. Exp. Med.* **178,** 1629–1636.
449. Aderka, D., J. Le, and J. Vilcek (1989). IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. *J. Immunol.* **143,** 3517–3523.
450. Schindler, R., J. Mancilla, S. Endres, R. Ghorbani, S. C. Clark, and C. A. Dinarello (1990). Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. *Blood* **75,** 40–47.
451. Ulich, T. R., S. Yin, K. Guo, E. S. Yi, D. Remick, and J. del Castillo (1991). Intratracheal injection of endotoxin and cytokines. II. Interleukin-6 and transforming growth factor beta inhibit acute inflammation. *Am. J. Pathol.* **138,** 1097–1101.
452. Dinarello, C. A., J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, M. A. Palladino, and J. V. O'Connor (1986). Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *J. Exp. Med.* **163,** 1433–1450.
453. Nijsten, M. W. N., E. R. De Groot, H. J. ten Duis, H. J. Klasen, C. E. Hack, and L. A. Aarden (1987). Serum levels of interleukin-6 and acute phase responses. *Lancet* **2,** 921.
454. Kinugawa, K., T. Takahashi, O. Kohmoto, A. Yao, T. Aoyagi, S. Momomura, Y. Hirata, and T. Serizawa (1994). Nitric oxide-mediated effects of interleukin-6 on [Ca2+]i and cell contraction in cultured chick ventricular myocytes. *Circ. Res.* **75,** 285–295.
455. Asano, S., A. Okano, K. Ozawa, T. Nakahata, T. Ishibashi, K. Koike, H. Kimura, Y. Tanioka, A. Shibuya, T. Hirano, T. Kishimoto, F. Takaku, and Y. Akiyama (1990). In vivo effects of recombinant human interleukin-6 in primates: Stimulated production of platelets. *Blood* **75,** 1602–1605.
456. Preiser, J. C., D. Schmartz, P. Van der Linden, J. Content, P. Van den Bussche, W. Buurman, W. Sebald, E. Dupont, M. R. Pinsky, and J. L. Vincent (1991). Interleukin-6 administration has no acute hemodynamic or hematologic effect in the dog. *Cytokine* **3,** 1–4.
457. Ryffel, B., B. D. Car, G. Woerly, M. Weber, F. DiPadova, M. Kammuller, S. Klug, R. Neubert, and D. Neubert (1994). Long-term interleukin-6 administration stimulates sustained thrombopoiesis and acute-phase protein synthesis in a small primate—The marmoset. *Blood* **83,** 2093–2102.
458. Mestries, J.-C., E. K. O. Kruithof, M.-P. Gascon, F. Herodin, D. Agay, and A. Ythier (1994). In vivo modulation of coagulation and fibrinolysis by recombinant glycosylated human interleukin-6 in baboons. *Eur. Cytokine Network* **5,** 275–281.
459. Van der Meer, J. W. M., M. Helle, and L. A. Aarden (1989). Comparison of the effects of recombinant interleukin-6 and recombinant interleukin-1 on nonspecific resistance to infection. *Eur. J. Immunol.* **19,** 413–416.
460. Barton, B. E., and J. V. Jackson (1993). Protective role of interleukin-6 in the lipopolysaccharide-galactosamine septic shock model. *Infect. Immun.* **61,** 1496–1499.

461. Bucklin, S. E., R. Silverstein, and D. C. Morrison (1993). An interleukin-6-induced acute-phase response does not confer protection against lipopolysaccharide lethality. *Infect. Immun.* **61,** 3184–3189.

462. Crowl, R. M., J. T. Stoller, R. R. Conroy, and C. R. Stoner (1991). Induction of phospholipase A2 gene expression in human hepatoma cells by mediators of the acute phase response. *J. Biol. Chem.* **226,** 2647–2651.

463. Mold, C., T. W. Du Clos, S. Nakayama, K. M. Edwards, and H. Gewurz (1982). C-reactive protein reactivity with complement and effects on phagocytosis. *Ann. N.Y. Acad. Sci.* **389,** 251–259.

464. Vachino, G., J. A. Gelfand, M. B. Atkins, J. D. Tamerius, P. Demchak, and J. W. Mier (1991). Complement activation in cancer patients undergoing immunotherapy with interleukin-2 (IL-2): Binding of complement and C-reactive protein by IL-2-activated lymphocytes. *Blood* **78,** 2505–2513.

465. van der Poll, T., M. Levi, C. E. Hack, H. ten Cate, S. J. H. Van Deventer, A. J. M. Eerenberg, E. R. De Groot, J. Jansen, H. Gallati, H. R. Büller, J. W. Ten Cate, and L. A. Aarden (1994). Elimination of interleukin 6 attenuates coagulation activation in experimental endotoxemia in chimpanzees. *J. Exp. Med.* **179,** 1253–1259.

466. Fong, Y., L. L. Moldawer, M. Marano, H. Wei, S. B. Tatter, R. H. Clarick, U. Santhanam, D. Sherris, L. T. May, P. B. Sehgal, and S. F. Lowry (1989). Endotoxemia elicits increased circulating β_2-IFN/IL-6 in man. *J. Immunol.* **142,** 2321–2324.

467. Sheron, N., J. N. Lau, J. Hofmann, R. Williams, and G. J. M. Alexander (1990). Dose-dependent increase in plasma IL-6 after recombinant TNF infusion in humans. *Clin. Exp. Immunol.* **82,** 427–428.

468. Shalaby, M. R., A. Waage, L. Aarden, and T. Espevik (1989). Endotoxin, tumor necrosis factor-α/cachectin and interleukin 1 induce interleukin 6 production in vivo. *Clin. Immunol. Immunopathol.* **53,** 488–498.

469. Libert, C., P. Brouckaert, A. Shaw, and W. Fiers (1990). Induction of interleukin 6 by human and murine recombinant interleukin 1 in mice. *Eur. J. Immunol.* **20,** 691–694.

470. Poll, T. van der, J. Jansen, M. Levi, H. ten Cate, C. E. Hack, L. A. Aarden, J. W. ten Cate, and S. J. H. van Deventer (1996). Interleukin 10 release during endotoxaemia in chimpanzees: Role of platelet-activating factor and interleukin 6. *Scand. J. Immunol.* **43,** 122–125.

471. Matthys, P., T. Mitera, H. Heremans, J. van Damme, and A. Billiau (1995). Anti-gamma interferon and anti-interleukin-6 antibodies affect staphylococcal enterotoxin b-induced weight loss, hypoglycemia, and cytokine release in d-galactosamine-sensitized and unsensitized mice. *Infect. Immun.* **63,** 1158–1164.

472. Redl, H., G. Schlag, S. Bahrami, U. Schade, M. Ceska, and P. Stütz (1991). Plasma neutrophil-activating peptide-1/interleukin-8 and neutrophil elastase in a primate bacteremia model. *J. Infect. Dis.* **164,** 383–388.

473. Ray, A., S. B. Tatter, L. T. May, and P. B. Sehgal (1988). Activation of the human "β_2-interferon/hepatocyte-stimulating factor/interleukin 6" promoter by cytokines, viruses, and second messenger agonists. *Proc. Natl. Acad. Sci. USA* **85,** 6701–6705.

474. Creasey, A. A., A. C. Chang, L. Feigen, T. C. Wun, F. B. Taylor,Jr., and L. B. Hinshaw (1993). Tissue factor pathway inhibitor reduces mortality from Escherichia Coli septic shock. *J. Clin. Invest.* **91,** 2850–2856.

475. Carr, C., G. S. Bild, A. C. K. Chang, G. T. Peer, M. O. Palmier, R. B. Frazier, M. E. Gustafson, T. C. Wun, A. A. Creasey, L. B. Hinshaw, F. B. Taylor, Jr., and G. R. Galluppi (1995). Recombinant E.coli derived tissue factor pathway inhibitor reduces coagulopathic and lethal effects in the baboon Gram-negative model of septic shock. *Circ. Shock* **44,** 126–135.

476. Johnson, K., L. Aarden, Y. Choi, E. de Groot, and A. Creasey (1996). The proinflammatory cytokine response to coagulation and endotoxin in whole blood. *Blood* **87,** 5051–5060.

477. Höpken, U., M. Mohn, A. Strüber, H. Montz, H. Burchardi, O. Götze, and M. Oppermann (1996). Inhibition of interleukin-6 synthesis in an animal model of septic shock by anti-C5a monoclonal antibodies. *Eur. J. Immunol.* **26,** 1103–1109.

478. Starnes, H. F., Jr., M. K. Pearce, A. Tewari, J. H. Yim, J.-C. Zou, and J. S. Abrams (1990). Anti-IL-6 monoclonal antibodies protect against lethal Escherichia coli infection and lethal tumor necrosis factor-α challenge in mice. *J. Immunol.* **145,** 4185–4191.

479. Heremans, H., C. Dillen, W. Put, J. Van Damme, and A. Billiau (1992). Protective effect of anti-interleukin (IL)-6 antibody against endotoxin, associated with paradoxically increased IL-6 levels. *Eur. J. Immunol.* **22,** 2395–2401.

480. Matthys, P., C. Dillen, P. Proost, H. Heremans, J. Van Damme, and A. Billiau (1993). Modification of the anti-CD3-induced cytokine release syndrome by anti-interferon-gamma or anti-interleukin-6 antibody treatment: Protective effects and biphasic changes in blood cytokine levels. *Eur. J. Immunol.* **23,** 2209–2216.

481. Gennari, R., and J. W. Alexander (1995). Anti-interleukin-6 antibody treatment improves survival during gut-derived sepsis in a time-dependent manner by enhancing host defense. *Crit. Care Med.* **23,** 1945–1953.

482. Libert, C., A. Vink, P. Coulie, P. Brouckaert, B. Everaerdt, J. Van Snick, and W. Fiers (1992). Limited involvement of interleukin-6 in the pathogenesis of lethal septic shock as revealed by the effect of monoclonal antibodies against interleukin-6 or its receptor in various murine models. *Eur. J. Immunol.* **22,** 2625–2630.

483. Barton, B. E., J. Shortall, and J. V. Jackson (1996). Interleukins 6 and 11 protect mice from mortality in a staphylococcal enterotixin-induced toxic shock model. *Infect. Immun.* **64,** 714–718.

484. Dalrymple, S. A., R. Slattery, D. M. Aud, M. Krishna, L. A. Lucian, and R. Murray (1996). Interleukin-6 is required for a protective immune response to systemic escherichia coli infection. *Infect. Immun.* **64,** 3231–3235.

485. Aarden, L. A., P. M. Lansdorp, and E. R. De Groot (1985). A growth factor for B cell hybridomas produced by human monocytes. *Lymphokines* **10,** 175–185.

486. Helfgott, D. C., S. B. Tatter, U. Santhanam, R. H. Clarick, N. Bhardwaj, L. T. May, and P. B. Sehgal (1989). Multiple forms of IFN-β_2/IL-6 in serum and body fluids during acute bacterial infection. *J. Immunol.* **142,** 948–953.

487. Hack, C. E., E. R. De Groot, R. J. F. Felt-Bersma, J. H. Nuijens, R. J. M. S. Van Schijndel, A. J. M. Eerenberg-Belmer, L. G. Thijs, and L. A. Aarden (1989). Increased plasma levels of interleukin-6 in sepsis. *Blood* **74,** 1704–1710.

488. Calandra, T., J. Gerain, D. Heumann, J.-D. Baumgartner, M. P. Glauser, and the Swiss–Dutch J5 Immunoglobulin Study Group (1991). High circulating levels of interleukin-6 in patients with septic shock: Evolution during sepsis, prognostic value, and interplay with other cytokines. *Am. J. Med.* **91,** 23–29.

489. Friedland, J. S., Y. Suputtamongkol, D. G. Remick, W. Chaowagul, R. M. Strieter, S. L. Kunkel, N. J. White, and G. E. Griffin (1992). Prolonged elevation of interleukin-8 and interleukin-6 concentrations in plasma and of leucocyte interleukin-8 mRNA levels during septicemic and localized psendomonas pseudomallei infection. *Infect. Immun.* **60,** 2402–2408.

490. Wortel, C. H., M. A. M. Von der Mohlen, S. J. H. Van Deventer, C. L. Spring, M. Jastremski, M. J. Lubbers, C. R. Smith, I. E. Allan, and J. W. Ten Cate (1992). Effectiveness of a human monoclonal anti-endotoxin antibody (HA-1A) in gram-

negative sepsis: Relationship to endotoxin and cytokine levels. *J. Infect. Dis.* **166,** 1367–1374.

491. Damas, P., D. Le doux, M. Nijs, Y. Vrindts, D. De Groote, P. Franchimont, and M. Lamy (1992). Cytokine serum levels during severe sepsis in humans. IL-6 as a marker of severity. *Ann. Surg.* **215,** 356–362.

492. Tanaka, H., K. Ishikawa, M. Nishino, T. Shimazu, and T. Yoshioka (1996). Changes in granulocyte colony-stimulating factor concentration in patients with trauma and sepsis. *J. Trauma.* **40,** 718–725.

493. Martin, C., P. Saux, J.-L. Mege, G. Perrin, L. Papazian, and F. Gouin (1994). Prognostic values of serum cytokines in septic shock. *Intensive Care Med.* **20,** 272–277.

494. Moscovitz, H., F. Shofer, H. Mignott, A. Behrman, and L. Kilpatrick (1994). Plasma cytokine determinations in emergency department patients as a predictor of bacteremia and infectious disease severity. *Crit. Care Med.* **22,** 1102–1107.

495. Fekade, D., K. Knox, K. Hussein, A. Melka, D. G. Lalloo, R. E. Coxon, and D. A. Warrell (1996). Prevention of Jarisch–Herxheimer reactions by treatment with antibodies against tumor necrosis factor alpha. *N. Engl. J. Med.* **335,** 311–315.

496. Strieter, R. M., A. E. Koch, V. B. Antony, R. B. Fick,Jr., T. J. Standiford, and S. L. Kunkel (1994). The immunopathology of chemotactic cytokines: The role of interleukin-8 and monocyte chemoattractant protein-1. *J. Lab. Clin. Med.* **123,** 183–197.

497. Westwick, J., S. W. Li, and R. D. Camp (1989). Novel neutrophil-stimulating peptides. *Immunol. Today* **10,** 146–147.

498. Van Damme, J (1991). Interleukin-8 and related molecules. *In* "The Cytokine Handbook" (A.W. Thomson, ed.), pp. 201-214. Academic Press, London.

499. Matsushima, K., K. Morishita, T. Yoshimura, S. Lavu, Y. Kobayashi, W. Lew, E. Appella, H. F. Kung, E. J. Leonard, and J. J. Oppenheim (1988). Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumor necrosis factor. *J. Exp. Med.* **167,** 1883–1893.

500. Van Damme, J., M. Rampart, R. Conings, B. Decock, N. Van Osselaer, J. Willems, and A. Billiau (1990). The neutrophil-activating proteins (NAP) interleukin-8 and β-thromboglobuline: In vitro and in vivo comparison of NH2-terminally processed forms. *Eur. J. Immunol.* **20,** 2113-2118.

501. Baggiolini, M., B. Dewald, and A. Walz (1992). Interleukin-8 and related chemotactic cytokines. *In* "Inflammation: Basic Principles and Clinical Correlates" (J. I. Gallin, I. M. Goldstein, and R. Snyderman, eds.), pp. 247-263. Raven Press, New York.

502. Hebert, C. A., F. W. Luscinskas, J.-M. Kiely, E. A. Luis, W. C. Darbonne, G. L. Bennett, C. C. Liu, M. S. Obin, M. A. Gimbrone,Jr., and J. B. Baker (1990). Endothelial and leukocyte forms of IL-8. Conversion by thrombin and interactions with neutrophils. *J. Immunol.* **145,** 3033–3040.

503. Murphy, P. M., and H. L. Tiffany (1991). Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science* **253,** 1280–1283.

504. Holmes, W. E., J. Lee, W.-J. Kuang, G. C. Rice, and W. I. Wood (1991). Structure and functional expression of a human interleukin-8 receptor. *Science* **253,** 1278–1280.

505. Moser, B., C. Schumacher, V. von Tscharner, I. Clark-Lewis, and M. Baggiolini (1991). Neutrophil-activating peptide 2 and gro/melanoma growth-stimulatory activity interact with neutrophil-activating peptide-1/interleukin-8 receptors on human neutrophils. *J. Biol. Chem.* **266,** 10666–10671.

506. Lee, J., R. Horuk, G. C. Rice, G. L. Bennett, T. Camerato, and W. I. Wood (1992). Characterization of two high affinity human interleukin-8 receptors. *J. Biol. Chem.* **267,** 16283–16289.

507. Geiser, T., B. Dewald, M. U. Ehrengruber, I. Clark-Lewis, and M. Baggiolini (1993). The interleukin-8-related chemotactic cytokines GRO-alpha, GRO-beta, and GRO-gamma activate human neutrophil and basophil leukocytes. *J. Biol. Chem.* **268,** 15419–15424.

508. Schumacher, C., I. Clark-Lewis, M. Baggiolini, and B. Moser (1992). High- and low-affinity binding of GRO-alpha and neutrophil-activating peptide 2 to interleukin 8 receptors on human neutrophils. *Proc. Natl. Acad. Sci. USA* **89,** 10542–10546.

509. Villarete, L. H., and D. G. Remick (1995). Nitric oxide regulation of IL-8 expression in human endothelial cells. *Biochem. Biophys. Res. Commun.* **211,** 671–676.

510. Murakami, K., A. Ueno, K. Yamanouchi, and T. Kondo (1995). Thrombin induces GROalpha/MGSA production in human umbilical vein endothelial cells. *Thromb. Res.* **79,** 387–394.

511. Colotta, F., F. L. Sciacca, M. Sironi, W. Luini, M. J. Rabiet, and A. Mantovani (1994). Expression of monocyte chemotactic protein-1 by monocytes and endothelial cells exposed to thrombin. *Am. J. Pathol.* **144,** 975–985.

512. Weyrich, A. S., M. R. Elstad, R. P. McEver, T. M. McIntyre, K. L. Moore, J. H. Morrissey, S. M. Prescott, and G. A. Zimmerman (1996). Activated platelets signal chemokine synthesis by human monocytes. *J. Clin. Invest.* **97,** 1525–1534.

513. Metinko, A. P., S. L. Kunkel, T. J. Standiford, and R. M. Strieter (1992). Anoxia-hyperoxia induces monocyte-derived interleukin-8. *J. Clin. Invest.* **90,** 791–798.

514. Kilgore, K. S., C. M. Flory, B. F. Miller, V. M. Evans, and J. S. Warren (1996). The membrane attack complex of complement induces interleukin-8 and monocyte chemoattractant protein-1 secretion from human umbilical vein endothelial cells. *Am. J. Pathol.* **149,** 953–961.

515. Darbonne, W. C., G. C. Rice, M. A. Mohler, T. Apple, C. A. Hebert, A. J. Valente, and J. B. Baker (1991). Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin. *J. Clin. Invest.* **88,** 1362–1369.

516. Tilg, H., L. Shapiro, M. B. Atkins, C. A. Dinarello, and J. W. Mier (1993). Induction of circulating and erythrocyte-bound IL-8 by IL-2 immunotherapy and suppression of its in vitro production by IL-1 receptor antagonist and soluble tumor necrosis factor receptor (p75) chimera. *J. Immunol.* **151,** 3299–3307.

517. Sylvester, I., T. Yoshimura, M. Sticherling, J.-M. Schroder, M. Ceska, P. Peichi, and E. J. Leonard (1992). Neutrophil attractant protein-1-immunoglobulin G immune complexes and free anti-NAP-1 antibody in normal human serum. *J. Clin. Invest.* **90,** 471–481.

518. Schröder, J.-M., U. Mrowietz, E. Morita, and E. Christophers (1987). Purification and parital biochemical characterization of a human monocyte-derived, neutrophil-activating peptide that lacks interleukin-1 activity. *J. Immunol.* **139,** 3473–3483.

519. Yoshimura, T., K. Matsushima, S. Tanaka, E. A. Robinson, E. Appella, J. J. Oppenheim, and E. J. Leonard (1987). Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc. Natl. Acad. Sci. USA* **84,** 9233–9237.

520. Colditz, I., R. Zwahlen, B. Dewald, and M. Baggiolini (1989). In vivo inflammatory activity of neutrophil-activating factor, a novel chemotactic peptide derived from human monocytes. *Am. J. Pathol.* **134,** 755–760.

521. Van Damme, J., J. Van Beumen, G. Opdenakker, and A. Billiau (1988). A novel, NH2-terminal sequence-characterized human monokine possessing neutrophil chemotactic, skin-reactive, and granulocytosis-promoting activity. *J. Exp. Med.* **167,** 1364–1376.

522. Collins, P. D., P. J. Jose, and T. J. Williams (1991). The sequential generation of neutrophil chemoattractant proteins in acute inflammation in the rabbit in vivo: Rela-

tionship between C5a and proteins with the characteristics of IL- 8/neutrophil-activating protein 1. *J. Immunol.* **146,** 677–684.

523. Schröder, J.-M (1989). The monocyte-derived neutrophil activating peptide (NAP/interleukin 8) stimulates human neutrophil arachidonate-5-lipoxygenase, but not the release of cellular arachidonate. *J. Exp. Med.* **170,** 847–863.
524. Carveth, H. J., J. F. Bohnsack, T. M. McIntyre, M. Baggiolini, S. M. Prescott, and G. A. Zimmerman (1989). Neutrophil activating factor (NAF) induces polymorphonuclear leukocyte adherence to endothelial cells and to subendothelial matrix proteins. *Biochem. Biophys. Res. Commun.* **162,** 387–393.
525. Huber, A. R., S. L. Kunkel, R. F. Todd, III, and S. J. Weiss (1991). Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science* **254,** 99–102.
526. Gimbrone, M. A., Jr., M. S. Obin, A. F. Brock, E. A. Luis, P. E. Hass, C. A. H'bert, Y. K. Yip, D. W. Leung, D. G. Lowe, W. J. Kohr, W. C. Darbonne, K. B. Bechtol, and J. B. Baker (1989). Endothelial interleukin-8: A novel inhibitor of leukocyte–endothelial interactions. *Science* **246,** 1601–1603.
527. Leonard, E. J., T. Yoshimura, S. Tanaka, and M. Raffeld (1991). Neutrophil recruitment by intradermally injected neutrophil attractant/activation protein-1. *J. Invest. Dermatol.* **96,** 690–694.
528. Hechtman, D. H., M. I. Cybulsky, H. J. Fuchs, J. B. Baker, and M. A. Gimbrone, Jr. (1991). Intravascular IL-8. Inhibitor of polymorphonuclear leukocyte accumulation at sites of acute inflammation. *J. Immunol.* **147,** 883–892.
529. Van Zee, K. J., E. Fischer, A. S. Hawes, C. A. Hebert, T. G. Terrell, J. B. Baker, S. F. Lowry, and L. L. Moldawer (1992). Effects of intravenous IL-8 administration in nonhuman primates. *J. Immunol.* **148,** 1746–1752.
530. Van Zee, K. J., L. E. DeForge, E. Fischer, M. A. Marano, J. S. Kenney, D. G. Remick, S. F. Lowry, and L. L. Moldawer (1991). IL-8 in septic shock, endotoxemia, and after IL-1 administration. *J. Immunol.* **146,** 3478–3482.
531. Sheron, N., and R. Williams (1992). IL-8 as a circulating cytokine: Induction by recombinant tumour necrosis factor-alpha. *Clin. Exp. Immunol.* **89,** 100–103.
532. Harada, A., N. Sekido, T. Akahoshi, T. Wada, N. Mukaida, and K. Matsushima (1994). Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J. Leukocyte Biol.* **56,** 559–564.
533. Solomkin, J. S., R. C. Bass, H. S. Bjornson, C. J. Tindal, and G. F. Babcock (1994). Alterations of neutrophil responses to tumor necrosis factor alpha and interleukin-8 following human endotoxemia. *Infect. Immun.* **62,** 943–947.
534. Sylvester, I., A. F. Suffredini, A. J. Boujoukos, G. D. Martich, R. L. Danner, T. Yoshimura, and E. J. Leonard (1993). Neutrophil attractant protein-1 and monocyte chemoattractant protein-1 in human serum. *J. Immunol.* **151,** 3292–3298.
535. Schmal, H., T. P. Shanley, M. L. Jones, H. P. Friedl, and P. A. Ward (1996). Role for macrophage inflammatory protein-2 in lipopolysaccharide-induced lung injury in rats. *J. Immunol.* **156,** 1963–1972.
536. DeForge, L. E., J. S. Kenney, M. L. Jones, J. S. Warren, and D. G. Remick (1992). Biphasic production of IL-8 in lipopolysaccharide (LPS)-stimulated human whole blood: Separation of LPS- and cytokine-stimulated components using anti-tumor necrosis factor and anti-IL-1 antibodies. *J. Immunol.* **148,** 2133–2141.
537. Cassatella, M. A., L. Meda, S. Bonora, M. Ceska, and G. Constantin (1993). Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-

1-beta in mediating the production of IL-8 triggered by lipopolysaccharide. *J. Exp. Med.* **178,** 2207–2211.

538. Jansen, P. M., J. Van Damme, W. Put, I. W. De Jong, F. B. Taylor,Jr., and C. E. Hack (1995). Monocyte chemotactic protein 1 (MCP-1) is released during lethal and sublethal bacteremia in baboons. *J. Infect. Dis.* **171,** 1640–1642.
539. Sekido, N., N. Mukaida, A. Harada, I. Nakanishi, Y. Watanabe, and K. Matsushima (1993). Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8. *Nature* **365,** 654–657.
540. Hack, C. E., M. Hart, R. J. M. Strack van Schijndel, A. J. M. Eerenberg, J. H. Nuijens, L. G. Thijs, and L. A. Aarden (1992). Interleukin-8 in sepsis: Relation to shock and inflammatory mediators. *Infect. Immun.* **60,** 2853–2842.
541. Danner, R. L., A. F. Suffredini, A. L. Van Dervort, M. Ceska, P. L. Stuetz, J. A. Zablotny, and E. A. Patterson (1990). Detection of interleukin 6 (IL6) and interleukin 8 (IL8) during septic shock in humans. *Clin. Res.* **38,** 352a.
542. Endo, S., K. Inada, M. Ceska, T. Takakuwa, Y. Yamada, H. Nakae, T. Kasai, H. Yamashita, K. Taki, and M. Yoshida (1995). Plasma interleukin 8 and polymorphonuclear leukocyte elastase concentrations in patients with septic shock. *J. Inflamm.* **45,** 136–142.
543. Halstensen, A., M. Ceska, P. Brandtzaeg, H. Redl, A. Naess, and A. Waage (1993). Interleukin-8 in serum and cerebrospinal fluid from patients with meningococcal disease. *J. Infect. Dis.* **167,** 471–475.
544. Vindenes, H., E. Ulvestad, and R. Bjerknes (1995). Increased levels of circulating interleukin-8 in patients with large burns: Relation to burn size and sepsis. *J. Trauma.* **39,** 635–640.
545. Raijmakers, P. G. H. M., A. B. J. Groeneveld, J. A. Rauwerda, A. J. Schneider, G. J. J. Teule, C. E. Hack, and L. G. Thijs (1995). Transient increase in interleukin-8 and pulmonary micro-vascular permeability following aortic surgery. *Am. J. Respir. Crit. Care. Med.* **151,** 698–705.
546. Groeneveld, A. B. J., P. G. H. M. Raijmakers, C. E. Hack, and L. G. Thijs (1995). Interleukin 8-related neutrophil elastase and the severity of the adult respiratory distress syndrome. *Cytokine* **7,** 746–752.
547. Cohen, A. B., C. MacArthur, S. Idell, R. Maunder, T. Martin, C. A. Dinarello, D. Griffith, and J. Mclarty (1988). A peptide from alveolar macrophages that releases neutrophil enzymes into the lungs in patients with the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **137,** 1151–1158.
548. Donnelly, S. C., R. M. Strieter, S. L. Kunkel, A. Walz, C. R. Robertson, D. C. Carter, I. S. Grant, A. J. Pollok, and C. Haslett (1993). Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet* **341,** 643–647.
549. Miller, E. J., A. B. Cohen, S. Nagao, D. Griffith, R. J. Maunder, T. R. Martin, J. P. Weiner-Kronish, M. Sticherling, E. Christophers, and M. A. Matthay (1992). Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. *Am. Rev. Respir. Dis.* **146,** 427–432.
550. Chollet-Martin, S., P. Montravers, C. Gibert, C. Elbim, J. M. Desmonts, J. Y. Fagon, and M. A. Gougerot-Pocidalo (1993). High levels of interleukin-8 in the blood and alveolar spaces of patients with pneumonia and adult respiratory distress syndrome. *Infect. Immun.* **61,** 4553–4559.
551. Torre, D., C. Zeroli, M. Giola, G. P. Fiori, G. Minoja, D. Maraggia, and M. Chiaranda (1993). Levels of interleukin-8 in patients with adult respiratory distress syndrome. *J. Infect. Dis.* **167,** 505–506.

552. Donnelly, S. C., C. Haslett, I. Dransfield, C. E. Robertson, D. C. Carter, J. A. Ross, I. S. Grant, and T. F. Tedder (1994). Role of selectins in development of adult respiratory distress syndrome. *Lancet* **344,** 215–219.
553. Nakano, Y., T. Kasahara, N. Mukaida, Y. C. Ko, M. Nakano, and K. Matsushima (1994). Protection against lethal bacterial infection in mice by monocyte-chemotactic and -activating factor. *Infect. Immun.* **62,** 377–383.
554. Bossink, A. W. J., L. Paemen, P. M. Jansen, C. E. Hack, L. G. Thijs, and J. van Damme (1995). Plasma levels of the chemokines monocyte chemotactic proteins-1 and -2 are elevated in human sepsis. *Blood* **86,** 3841–3847.
555. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* **170,** 2081–2095.
556. Vieira, P., R. De Waal Malefijt, M. N. Dang, K. E. Johnson, R. Kastelein, D. F. Fiorentino, J. E. De Vries, M. G. Roncarolo, T. R. Mosman, and K. W. Moore (1991). Isolation and expression of human cytokine synthesis inhibitor factor (CSIF/IL-10) cDNA clones: Homology to Epstein–Barr virus open reading frame BCRFI. *Proc. Natl. Acad. Sci. USA* **88,** 1172–1178.
557. Liu, Y., S. H.-Y. Wei, A. S.-Y. Ho, R. De Waal Malefijt, and K. W. Moore (1994). Expression cloning and characterization of a human IL-10 receptor. *J. Immunol.* **152,** 1821–1829.
558. De Waal Malefijt, R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. De Vries (1991). Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: An autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* **174,** 1209–1220.
559. Fiorentino, D. F., A. Zlotnik, T. R. Mosman, M. Howard, and A. O'Garra (1991). IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* **147,** 3815–3822.
560. Bogdan, C., Y. Vodovitz, and C. Nathan (1991). Macrophage deactivation by interleukin-10. *J. Exp. Med.* **174,** 1549–1555.
561. Marchant, A., C. Bruyns, P. Vandenabeele, M. Ducarme, C. Gerard, A. Delvaux, D. De Groote, D. Abramowicz, T. Velu, and M. Goldman (1994). Interleukin-10 controls interferon-gamma and tumor necrosis factor production during experimental endotoxemia. *Eur. J. Immunol.* **24,** 1167–1171.
562. Wang, P., P. Wu, J. C. Anthes, M. I. Siegel, R. W. Egan, and M. M. Billah (1994). Interleukin-10 inhibits interleukin-8 production in human neutrophils. *Blood* **83,** 2678–2683.
563. Wang, P., P. Wu, M. I. Siegel, R. W. Egan, and M. M. Billah (1994). IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells. *J. Immunol.* **153,** 811–816.
564. Sironi, M., C. Munoz, T. Pollicino, A. Siboni, F. L. Sciacca, S. Bernasconi, A. Vecchi, F. Colotta, and A. Mantovani (1993). Divergent effects of interleukin-10 on cytokine production by mononuclear phagocytes and endothelial cells. *Eur. J. Immunol.* **23,** 2692–2695.
565. Ramani, M., V. Ollivier, F. Khechai, T. Vu, C. Ternisien, F. Bridey, and D. De Prost (1993). Interleukin-10 inhibits endotoxin-induced tissue factor mRNA production by human monocytes. *FEBS Lett.* **334,** 114–116.
566. Pradier, O., C. Gerard, A. Delvaux, M. Lybin, D. Abramowicz, P. Capel, T. Velu, and M. Goldman (1993). Interleukin-10 inhibits the induction of monocyte procoagulant activity by bacterial lipopolysaccharide. *Eur. J. Immunol.* **23,** 2700–2703.
567. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75,** 263–274.

568. Berg, D. J., M. W. Leach, R. Kuhn, K. Rajewsky, W. Muller, N. J. Davidson, and D. Rennick (1995). Interleukin 10 but not interleukin 4 is a natural suppressant of cutaneous inflammatory responses. *J. Exp. Med.* **182,** 99–108.

569. Berg, D. J., R. Kuhn, K. Rajewsky, W. Muller, S. Menon, N. Davidson, G. Grunig, and D. Rennick (1995). Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. *J. Clin. Invest.* **96,** 2339–2347.

570. Howard, M., T. Muchamuel, S. Andrade, and S. Menon (1993). Interleukin 10 protects mice from lethal endotoxemia. *J. Exp. Med.* **177,** 1205–1208.

571. Gerard, C., C. Bruyns, A. Marchant, D. Abramowicz, P. Vandenabeele, A. Delvaux, W. Fiers, M. Goldman, and T. Velu (1993). Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. *J. Exp. Med.* **177,** 547–550.

572. Bean, A. G. D., R. A. Freiberg, S. Andrade, S. Menon, and A. Zlotnik (1993). Interleukin 10 protects mice against staphylococcal enterotoxin B-induced lethal shock. *Infect. Immun.* **61,** 4937–4939.

573. Zheng, X. X., A. W. Steele, P. W. Nickerson, W. Steurer, J. Steiger, and T. B. Strom (1995). Administration of noncytolyctic IL-10/Fc in murine models of lipopolysaccharide-induced septic shock and allogeneic islet transplantation. *J. Immunol.* **154,** 5590–5600.

574. Rogy, M. A., T. Auffenberg, N. J. Espat, R. Philip, D. Remick, G. K. Wollenberg, E. M. Copeland, III, and L. L. Moldawer (1995). Human tumor necrosis factor receptor (p55) and interleukin 10 gene transfer in the mouse reduces mortality to lethal endotoxemia and also attenuates local inflammatory responses. *J. Exp. Med.* **181,** 2289–2293.

575. Marchant, A., J. Deviere, B. Byl, D. De Groote, J.-L. Vincent, and M. Goldman (1994). Interleukin-10 production during septicaemia. *Lancet* **343,** 707–708.

576. Gomez-Jimenez, J., M. C. Martin, R. Sauri, R. M. Segura, F. Esteban, J. C. Ruiz, X. Nuvials, J. L. Boveda, R. Peracaula, and A. Salgado (1995). Interleukin-10 and the monocyte/macrophage-induced inflammatory response in septic shock. *J. Infect. Dis.* **171,** 472–475.

577. Derkx, B., A. Marchant, M. Goldman, R. Bijlmer, and S. van Deventer (1995). High levels of interleukin-10 during the initial phase of fulminant meningococcal septic shock. *J. Infect. Dis.* **171,** 229–232.

578. Brandtzaeg, P., L. Osnes, R. Ovstebo, G. Britt Joo, A. B. Westvik, and P. Kierulf (1996). Net inflammatory capacity of human septic shock plasma evaluated by a monocyte-based target cell assay: Identification of interleukin-10 as a major functional deactivator of human monocytes. *J. Exp. Med.* **184,** 51–60.

579. Lehmann, A. K., A. Halstensen, S. Sornes, O. Rokke, and A. Waage (1995). High levels of interleukin 10 in serum are associated with fatality in meningococcal disease. *Infect. Immun.* **63,** 2109–2112.

580. Deuren, M. van, J. van der Ven-Jongekrijg, A. K. M. Bartelink, R. van Dalen, R. W. Sauerwein, and J. W. M. van der Meer (1995). Correlation between proinflammatory cytokines and antiinflammatory mediators and the severity of disease in meningococcal infections. *J. Infect. Dis.* **172,** 433–439.

581. Marchant, A., M. L. Alegre, A. Hakim, G. Pierard, G. Marecaux, G. Friedman, D. de Groote, R. J. Kahn, J. L. Vincent, and M. Goldman (1995). Clinical and biological significance of interleukin-10 plasma levels in patients with septic shock. *J. Clin. Immunol.* **15**:266-273.

582. Sherry, R. M., J. I. Cue, J. K. Goddard, J. B. Parramore, and J. T. DiPiro (1996). Interleukin-10 is associated with the development of sepsis in trauma patients. *J. Trauma.* **40,** 613–616.
583. Huhn, R. D., E. Radwanski, S. M. O'Connell, M. G. Sturgill, L. Clarke, R. P. Cody, M. B. Affrime, and D. L. Cutler (1996). Pharmacokinetics and immunomodulatory properties of intravenously administered recombinant human interleukin-10 in healthy volunteers. *Blood* **87,** 699–705.
584. Chernoff, A. E., E. V. Granowitz, L. Shapiro, E. Vannier, G. Lonnemann, J. B. Angel, J. S. Kennedy, A. R. Rabson, S. M. Wolff, and C. A. Dinarello (1995). A randomized, controlled trial of IL-10 in humans. Inhibition of inflammatory cytokine production and immune responses. *J. Immunol.* **154,** 5492–5499.
585. Ertel, W., J. P. Kremer, J. Kenney, U. Steckholzer, D. Jarrar, O. Trentz, and F. W. Schildberg (1995). Downregulation of proinflammatory cytokine release in whole blood from septic patients. *Blood* **85,** 1341–1347.
586. Mulligan, M. S., M. L. Jones, A. A. Vaporciyan, M. C. Howard, and P. A. Ward (1993). Protective effects of IL-4 and IL-10 against immune complex-induced lung injury. *J. Immunol.* **151,** 5666–5674.
587. Ramani, M., V. Ollivier, C. Ternisien, T. Vu, C. Elbim, J. Hakim, and D. De Prost (1993). Interleukin 4 prevents the induction of tissue factor mRNA in human monocytes in response to LPS or PMA stimulation. *Br. J. Haematol.* **85,** 462–468.
588. Te Velde, A. A., R. J. F. Huijbens, K. Heije, J. E. De Vries, and C. G. Figdor (1990). Interleukin-4 (IL-4) inhibits secretion of IL-1beta, tumor necrosis factor alpha, and IL-6 by human monocytes. *Blood* **76,** 1392–1397.
589. Redmond, H. P., K. D. Chavin, J. S. Bromberg, and J. M. Daly (1991). Inhibition of macrophage-activating cytokines is beneficial in acute septic response. *Ann. Surg.* **214,** 508–509.
590. Jungi, T. W., M. Brcic, S. Eperon, and S. Albrecht (1994). Transforming growth factor-beta and interleukin-10, but not interleukin-4, down regulate procoagulant activity and tissue factor expression in human monocyte-derived macrophages. *Thromb. Res.* **76,** 463–474.
591. DiPiro, J. T., T. R. Howdieshell, J. K. Goddard, D. B. Callaway, R. G. Hamilton, and A. R. Mansberger, Jr. (1995). Association of interleukin-4 plasma levels with traumatic injury and clinical course. *Arch. Surg.* **130,** 1159–1162.
592. Nathan, C. (1992). Interferon and inflammation. *In* "Inflammation: Basic Principles and Clinical Correlates (J. I. Gallin, I. M. Goldstein, and R. Snyderman, eds.), 2nd Ed. pp. 265–290. Raven Press, New York.
593. Gray, P., and D. V. Goeddel (1982). Structure of the human immune interferon gene. *Nature* **298,** 859–863.
594. Kitbourn, R. G., and P. Belloni (1990). Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1, or endotoxin. *J. Natl. Cancer Inst.* **82,** 772–776.
595. Aulitzky, W. E., W. K. Aulitzky, J. Frick, M. Herold, G. Gastl, H. Tilg, M. Berger, and C. Huber (1990). Treatment of cancer patients with recombinant interferon-gamma induces release of endogenous tumor necrosis factor-alpha. *Immunobiology* **180,** 385–394.
596. Jansen, P. M., T. C. T. M. van der Pouw Kraan, I. W. de Jong, G. van Mierlo, J. Wijdenes, A. A. Chang, L. A. Aarden, F. B. Taylor, Jr., and C. E. Hack (1996). Release of interleukin-12 in experimental Escherichia coli septic shock in baboons: Relation to plasma levels of interleukin-10 and interferon-gamma. *Blood* **87,** 5144–5151.

597. Heinzel, F. P. (1990). The role of IFN-gamma in the pathology of experimental endotoxemia. *J. Immunol.* **145,** 2920–2924.
598. Billiau, A., H. Heremans, F. Vandekerckhove, and C. Dillen (1987). Anti-interferon-gamma antibody protects mice against the generalized Shwartzman reaction. *Eur. J. Immunol.* **17,** 1851–1858.
599. Heremans, H., J. Van Damme, C. Dillen, R. Dijkmans, and A. Billiau (1990). Interferon gamma, a mediator of lethal lipopolysaccharide-induced Shwartzman-like shock reactions in mice. *J. Exp. Med.* **171,** 1853–1869.
600. Bucklin, S. E., S. W. Russell, and D. C. Morrison (1994). Participation of IFN-gamma in the pathogenesis of LPS lethality. *Prog. Clin. Biol. Res.* **388,** 399–406.
601. Kohler, J., D. Heumann, G. Garotta, D. LeRoy, S. Bailat, C. Barras, J.-D. Baumgartner, and M. P. Glauser (1993). IFN-gamma involvement in the severity of gram-negative infections in mice. *J. Immunol.* **151,** 916–921.
602. Faggioni, R., S. Gatti, M. T. Demitri, R. Delgado, B. Echtenacher, P. Gnocchi, H. Heremans, and P. Ghezzi (1994). Role of xanthine oxidase and reactive oxygen intermediates in LPS- and TNF-induced pulmonary edema. *J. Lab. Clin. Med.* **123,** 394–399.
603. Zhao, Y. X., and A. Tarkowski (1995). Impact of interferon-gamma receptor deficiency on experimental Staphylococcus aureus septicemia and arthritis. *J. Immunol.* **155,** 5736–5742.
604. Cusumano, V., G. Mancuso, F. Genovese, D. Delfino, C. Beninati, E. Losi, and G. Teti (1996). Role of gamma interferon in an neonatal mouse model of group b streptococcal disease. *Infect. Immun.* **64,** 2941–2944.
605. Heremans, H., C. Dillen, J. Van Damme, and A. Billiau (1994). Essential role for natural killer cells in the lethal lipopolysaccharide-induced Shwartzman-like reaction in mice. *Eur. J. Immunol.* **24,** 1155–1160.
606. Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf, and A. Sher (1993). Interleukin 12 is required for the T-lymphocyte-independent induction of interferon-gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. *Proc. Natl. Acad. Sci. USA* **90,** 6115–6119.
607. Banks, R. E., P. M. Patel, and P. J. Selby (1995). Interleukin 12: A new clinical player in cytokine therapy. *Br. J. Cancer* **71,** 655–659.
608. Chua, A. O., R. Chizzonite, B. B. Desai, T. P. Truitt, P. Nunes, L. J. Minetti, R. R. Warrier, D. H. Presky, J. F. Levine, M. K. Gately, and U. Gubler (1994). Expression cloning of a human IL-12 receptor component. *J. Immunol.* **153,** 128–136.
609. Presky, D. H., H. Yang, L. J. Minetti, A. O. Chua, N. Nabavi, C.-Y. Wu, M. K. Gately, and E. Gubler (1996). A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. *Proc. Natl. Acad. Sci. USA* **93,** 14002–14007.
610. Nakamura, K., H. Okamura, K. Nagata, T. Komatsu, and T. Tamura (1993). Purification of a factor which provides a costimulatory signal for gamma interferon production. *Infect. Immun.* **61,** 64–70.
611. Heinzel, F. P., R. M. Rerko, P. Ling, J. Hakimi, and D. S. Schoenhaut (1994). Interleukin 12 is produced in vivo during endotoxemia and stimulates synthesis of gamma interferon. *Infect. Immun.* **62,** 4244–4249.
612. Wysocka, M., M. Kubin, L. Q. Vieira, L. Ozmen, G. Garotta, P. Scott, and G. Trinchieri (1995). Interleukin-12 is required for interferon gamma production and lethality in lipopolysaccharide-induced shock in mice. *Eur. J. Immunol.* **25,** 672–676.
613. Carson, W. E., M. E. Ross, R. A. Baiocchi, M. J. Marien, N. Boiani, K. Grabstein, and M. A. Caligiuri (1995). Endogenous production of interleukin 15 by activated

human monocytes is critical for optimal production of interferon-gamma by natural killer cells in vitro. *J. Clin. Invest.* **96,** 2578–2582.

614. Micallef, M. J., T. Ohtsuki, K. Kohno, F. Tanabe, S. Ushio, M. Namba, T. Tanimoto, K. Torigoe, M. Fujii, M. Ikeda, S. Fukuda, and M. Kurimoto (1996). Interferon-gamma-inducing factor enhances t helper 1 cytokine production by stimulated human T cells. *Eur. J. Immunol.* **26,** 1647–1651.

615. Okamura, H., H. Tsutsui, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, K. Akita, M. Namba, F. Tanabe, K. Konishi, S. Fukuda, and M. Kurimoto (1995). Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* **378,** 88–91.

616. Ushio, S., M. Namba, T. Okura, K. Hattori, Y. Nukada, K. Akita, F. Tanabe, K. Konishi, M. Micallef, M. Fujii, K. Torigoe, T. Tanimoto, S. Fukuda, M. Ikeda, H. Okamura, and M. Kurimoto (1996). Cloning of the cDNA for human IFN-gamma-inducing factor, expression in escherichia coli, and studies on the biologic activities of the protein. *J. Immunol.* **156,** 4274–4279.

617. Trinchieri, G., and P. Scott (1994). The role of interleukin 12 in the immune response, disease and therapy. *Immunol. Today* **15,** 460–463.

618. Sarmiento, U. M., J. H. Riley, P. A. Knaack, J. M. Lipman, J. M. Becker, M. K. Gately, R. Chizzonite, and T. D. Anderson (1994). Biologic effects of recombinant human interleukin-12 in squirrel monkeys (sciureus saimiri). *Lab. Invest.* **71,** 862–873.

619. Takahashi, I., I. Nakagawa, L. Xu, and S. Hamada (1995). IL-12 rescues galactosamine-loaded mice from lethal shock triggered by Staphylococcal enterotoxin. *Biochem. Biophys. Res. Commun.* **217,** 74–80.

620. Tzung, T.-P., T. C. Mahl, P. Lance, V. Andersen, and S. A. Cohen (1992). Interferon-alpha prevents endotoxin-induced mortality in mice. *Eur. J. Immunol.* **22,** 3097–3101.

621. Yaegashi, Y., P. Nielsen, A. Sing, C. Galanos, and M. A. Freudenberg (1995). Interferon β, a cofactor in the interferon gamma production induced by gram-negative bacteria in mice. *J. Exp. Med.* **181,** 953–960.

622. Hilton, D. J. (1992). LIF: Lots of interesting functions. *Trends Biochem. Sci.* **17,** 72–76.

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

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

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

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

627. Alexander, H. R., G. G. H. Wong, G. M. Doherty, D. J. Venzon, D. L. Fraker, and J. A. Norton (1992). Differentiation factor/leukemia inhibitory factor protection against lethal endotoxemia in mice: Synergistic effect with interleukin 1 and tumor necrosis factor. *J. Exp. Med.* **175,** 1139–1142.

628. Waring, P. M., L. J. Waring, T. Billington, and D. Metcalf (1995). Leukemia inhibitory factor protects against experimental lethal Escherichia coli septic shock in mice. *Proc. Natl. Acad. Sci. USA* **92,** 1337–1341.

629. Mayer, P., K. Geissler, M. Ward, and D. Metcalf (1993). Recombinant human leukemia inhibitory factor induces acute phase proteins and raises the blood platelet counts in nonhuman primates. *Blood* **81,** 3226–3233.
630. Block, M. I., M. Berg, M. J. McNamara, J. A. Norton, D. L. Fraker, and H. R. Alexander (1993). Passive immunization of mice against D factor blocks lethality and cytokine release during endotoxemia. *J. Exp. Med.* **178,** 1085–1090.
631. Villiger, P. M., Y. Geng, and M. Lotz (1993). Induction of cytokine expression by leukemia inhibitory factor. *J. Clin. Invest.* **91,**1575–1581.
632. Waring, P., K. Wycherley, D. Cary, N. Nicola, and D. Metcalf (1992). Leukemia inhibitory factor levels are elevated in septic shock and various inflammatory body fluids. *J. Clin. Invest.* **90,** 2031–2037.
633. Waring, P. M., L. J. Waring, and D. Metcalf (1994). Circulating leukemia inhibitory factor levels correlate with disease severity in meningococcemia. *J. Infect. Dis.* **170,** 1224–1228.
634. Villers, D., T. Dao, J. M. Nguyen, E. Bironneau, A. Godard, M. Moreau, D. de Groote, F. Nicolas, J. P. Soulillou, and I. Anegon (1995). Increased plasma levels of human interleukin for DA1.a cells/leukemia inhibitory factor in sepsis correlate with shock and poor prognosis. *J. Infect. Dis.* **171,** 232–236.
635. Bernhagen, J., T. Calandra, R. A. Mitchell, S. B. Martin, K. J. Tracey, W. Voelter, K. R. Manogue, A. Cerami, and R. Bucala (1993). MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature* **365,** 756–759.
636. Calandra, T., J. Bernhagen, C. N. Metz, L. A. Spiegel, M. Bacher, T. Donnelly, A. Cerami, and R. Bucala (1995). MIF as a glucocorticoid-induced modulator of cytokine production. *Nature* **376,** 68–71.
637. Nagata, S., M. Tsuchiya, S. Asano, Y. Kaziro, T. Yamazaki, O. Yamamoto, Y. Hirata, N. Kubota, M. Oheda, H. Nomura *et. al.* (1986). Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. *Nature* **319,** 415–418.
638. Rennick, D., G. Yang, L. Gemmell, and F. Lee (1987). Control of hemopoiesis by a bone marrow stronal cell clone: Lipopolysaccharide and interleukin-1-inducible production of colony-stimulating factors GM-CSF and G-CSF. *Blood* **69,** 682–691.
639. Golde, D. W., and G. C. Baldwin (1992). Myeloid growth factors. *In* "Inflammation: Basic Principles and Clinical Correlates" (J. I. Gallin, I. M. Goldstein, and R. Snyderman, eds.), 2nd Ed., pp. 291–301. Raven Press, New York.
640. Mackensen, A., C. Galanos, and R. Engelhardt (1991). Treatment of cancer patients with endotoxin induces release of endogenous cytokines. *Pathobiology* **59,** 264–267.
641. Yuo, A., G. Fey, S. Kitagawa, A. Ohsaka, M. Ohta, K. Miyazao, T. Okabe, A. Urabe, M. Saito, and F. Takaku (1989). Recombinant human granulocyte colony-stimulating factor as an activator of human granulocytes: Potentiation of responses triggered by receptor-mediated agonists and stimulation of C3bi receptor expression and adherence. *Blood* **74,** 2144–2149.
642. de Haas, M., J. M. Kerst, C. E. Van der Schoot, J. Calafat, C. E. Hack, J. H. Nuijens, D. Roos, R. H. J. Van Oers, and A. E. G. Kr. Von dem Borne (1994). Granulocyte colony stimulating factor administration to healthy volunteers: Analysis of the immediate activating effects on circulating neutrophils. *Blood* **84,** 3885–3894.
643. Pollmacher, T., C. Korth, J. Mullington, W. Schreiber, J. Sauer, H. Vedder, C. Galanos, and F. Holsboer (1996). Effects of granulocyte colony-stimulating factor on plasma cytokine and cytokine receptor levels and on the in vivo host response to endotoxin in healthy men. *Blood* **87,** 900–905.
644. King, J., B. P. Deboisblanc, C. M. Mason, J. M. Onofrio, G. Lipscomb, D. E. Mercante, W. R. Summer, and S. Nelson (1995). Effect of granulocyte colony-stimulating factor on acute lung injury in the rat. *Am. J. Respir. Crit. Care Med.* **151,** 302–309.

645. Matsumoto, M., S. Matsubara, T. Matsuno, M. Tamura, K. Hattori, H. Nomura, M. Ono, and T. Yokota (1987). Protective effect of human granulocyte colony-stimulating factor on microbial infection in neutropenic mice. *Infect. Immun.* **55,** 2715–2720.

646. Kanazawa, M., A. Ishizaka, N. Hasegawa, Y. Suzuki, and T. Yokoyama (1992). Granulocyte colony-stimulating factor does not enhance endotoxin-induced acute lung injury in guinea pigs. *Am. Rev. Respir. Dis.* **145,** 1030–1035.

647. Gorgen, I., T. Hartung, M. Leist, M. Niehorster, G. Tiegs, S. Uhlig, F. Weitzel, and A. Wendel (1992). Granulocyte colony-stimulating factor treatment protects rodents against lipopolysaccharide-induced toxicity via suppression of systemic tumor necrosis factor-alpha. *J. Immunol.* **149,** 918–924.

648. Haberstroh, J., H. Breuer, I. Lucke, K. Massarrat, R. Fruh, U. Mand, P. Hagedorn, L. Brunnberg, and B. U. von Specht (1995). Effect of recombinant human granulocyte colony-stimulating factor on hemodynamic and cytokine response in a porcine model of Pseudomonas sepsis. *Shock* **4,** 216–224.

649. Lang, C. H., G. J. Bagby, C. Dobrescu, S. Nelson, and J. J. Spitzer (1992). Effect of granulocyte colony-stimulating factor on sepsis-induced changes in neutrophil accumulation and organ glucose uptake. *J. Infect. Dis.* **166,** 336–343.

650. Lundblad, R., M. Y. Wang, G. Kvalheim, E. Lingaas, and K. E. Giercksky (1995). Granulocyte colony-stimulating factor improves myelopoiesis and host defense in fulminant intra-abdominal sepsis in rats. *Shock* **4,** 68–73.

651. Aoki, Y., K. Hiromatsu, N. Kobayashi, T. Hotta, H. Saito, H. Igarashi, Y. Niho, and Y. Yoshikai (1995). Protective effect of granulocyte colony-stimulating factor against T-cell-mediated lethal shock triggered by superantigens. *Blood* **86,** 1420–1427.

652. Smith, W. S., G. E. Sumnicht, R. W. Sharpe, D. Samuelson, and F. E. Millard (1995). Granulocyte colony-stimulating factor versus placebo in addition to penicillin G in a randomized blinded study of gram-negative pneumonia sepsis: Analysis of survival and multisystem organ failure. *Blood* **86,** 1301–1309.

653. Fink, M. P., B. P. O'Sullivan, M. J. Menconi, S. P. Wollert, H. Wang, M. E. Youssef, and J. M. Bellelsle (1993). Effect of granulocyte colony-stimulating factor on systemic and pulmonary responses to endotoxin in pigs. *J. Trauma.* **34,** 571–577.

654. Waring, P. M., J. Presneill, D. W. Maher, J. E. Layton, J. Cebon, L. J. Waring, and D. Metcalf (1995). Differential alterations in plasma colony-stimulating factor concentrations in meningococcaemia. *Clin. Exp. Immunol.* **102,** 501–506.

655. Pelt, L. J. van, M. V. Huisman, R. S. Weening, A. E. G. Kr. von dem Borne, D. Roos, and R. H. J. van Oers (1996). Single dose of granulocyte-macrophage colony-stimulating factor induces systemic interleukin-8 release and neutrophil activation in healthy volunteers. *Blood* **87,** 5305–5313.

656. Brissette, W. H., D. A. Baker, E. J. Stam, J. P. Umlaud, and R. J. Griffiths (1995). GM-CSF rapidly primes mice for enhanced cytokine production in response to LPS and TNF. *Cytokine* **7,** 291–295.

657. Toda, H., A. Murata, Y. Oka, K. Uda, N. Tanaka, I. Ohashi, T. Mori, and N. Matsuura (1994). Effect of granulocyte-macrophage colony-stimulating factor on sepsis-induced organ injury in rats. *Blood* **83,** 2893–2898.

658. Molloy, R. G., R. Holzheimer, M. Nestor, K. Collins, J. A. Mannick, and M. L. Rodrick (1995). Granulocyte-macrophage colony-stimulating factor modulates immune function and improves survival after experimental thermal injury. *Br. J. Surg.* **82,** 770–776.

659. Rosenberg, S. A., M. T. Lotze, and J. J. Mule (1988). New approaches to the immunotherapy of cancer using interleukin-2. *Ann. Intern. Med.* **108,** 853–864.

660. Rosenberg, S. A., M. T. Lotze, L. M. Muul, A. E. Chang, F. P. Avis, S. Leitman, W. Marston Linehan, C. N. Robertson, R. A. Lee, J. T. Rubin, C. Seipp, C. G. Simpson, and D. E. White (1987). A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N. Engl. J. Med.* **316,** 889–897.

661. Boccoli, G., R. Masciulli, E. M. Ruggeri, P. Carlini, G. Giannella, E. Montesoro, G. Mastroberardino, G. Isacchi, U. Testa, F. Calabresi, and C. Peschle (1990). Adoptive immunotherapy of human cancer: The cytokine cascade and monocyte activation following high-dose interleukin 2 bolus treatment. *Cancer Res.* **50,** 5795–5800.

662. Fraker, D. L., H. N. Langstein, and J. A. Norton (1989). Passive immunization against tumor necrosis factor partially abrogates interleukin 2 toxicity. *J. Exp. Med.* **170,** 1015–1020.

663. Kovacs, E. J., B. Brock, L. Varesio, and H. A. Young (1989). IL-2 induction of IL-1β mRNA expression in monocytes. *J. Immunol.* **143,** 3532–3537.

664. Jablons, D. M., J. J. Mul, J. K. McIntosh, P. B. Sehgal, L. T. May, C. M. Huang, S. A. Rosenberg, and M. T. Lotze (1989). IL-6/IFN-β-2 as a circulating hormone. Induction by cytokine administration in humans. *J. Immunol.* **142,** 1542–1547.

665. Baars, J. W., G. J. Wolbink, M. H. L. Hart, C. E. Hack, A. J. M. Eerenberg, H. M. Pinedo, and J. Wagstaff (1994). The release of interleukin-8 during intravenous bolus treatment with interleukin-2. *Ann. Oncol.* **5,** 929–934.

This article was accepted for publication on March 19, 1997.

ADVANCES IN IMMUNOLOGY, VOL. 66

Role of Macrophage Migration Inhibitory Factor in the Regulation of the Immune Response

CHRISTINE N. METZ and RICHARD BUCALA

The Picower Institute for Medical Research, Manhasset, New York 11030

I. Introduction

The development of an inflammatory response is an essential component of the host response to infection or tissue invasion. Cytokines, released primarily by leukocytes, play a pivotal role in this process by recruiting and activating the various cell types that participate in an inflammatory reaction. The regulation of inflammation is by necessity under tight control because an overwhelming response by the host can be detrimental, as exemplified by the frequently fatal manifestations of endotoxemia and the toxic shock syndromes (1, 2).

Glucocorticoid hormones are among the most potent regulators of host inflammation and act to suppress the production of cytokines and other proinflammatory mediators (3–6). Recent studies have led to the discovery that the mediator historically termed "macrophage migration inhibitory factor" (MIF) acts as a physiological counterregulator of glucocorticoid action within the immune system. MIF has the unique property of being released from macrophages and T cells in response to glucocorticoids. Once released, MIF overrides or counterregulates the inhibitory effect of glucocorticoids on immune and inflammatory responses (7, 8). In this review, we summarize data that have led to the conceptual development of the MIF/glucocorticoid regulatory system and discuss the implications of MIF action in the overall control of host inflammatory and immune responses.

II. Background

Infection or tissue invasion initiates a localized inflammatory response that serves to contain and ultimately eliminate the offending stimulus. Although this occurs primarily by the action of infiltrating immune effector cells, there is also a systemic response to tissue invasion. Initially, this is elicited by pain (or fear) and consists of an activation of the hypothalamic–pituitary–adrenal axis (HPA), leading to the adrenal release of glucocorticoid hormones. Glucocorticoids are extremely potent, endogenous modulators of inflammation and immunity and will affect the course and magnitude

0065-2776/97 $25.00

of the ensuing host response (3, 4, 6). Baseline glucocorticoid levels also appear to control the "set-point" of the inflammatory reaction (9, 10). One dramatic example of the central role of glucocorticoids in host immunity is the extreme compromise exhibited by animals that have had their pituitary or adrenal glands removed (11, 12). For instance, rodents that have been hypophysectomized so as to remove their source of adrenocorticotrophic hormone (ACTH) are extremely sensitive to bacterial infection and endotoxic shock. These mice readily succumb to nanogram quantities of endotoxin (lipopolysaccharide), whereas up to 1000-fold higher doses are required to achieve comparable lethality in normal, intact animals (12). Pharmacologically, glucocorticoids remain the most powerful anti-inflammatory and immunosuppressive substances identified and their widespread clinical utility is limited only by dose-dependent side effects leading to diabetes, hypertension, osteoporosis, cataracts, and growth arrest (4).

Several years ago, we made note of the long-standing observation that in contrast to other hormonal networks that regulate carbohydrate, electrolyte, or blood pressure homeostasis, no systemic mediator(s) had been identified that could counterregulate the powerful effects of glucocorticoids on the immune system. This situation prompted us to engage in exploratory studies to find potentially new mediators that might be released systemically and that could counter the suppressive effects of glucocorticoids on inflammation and immunity. As part of this experimental program, we cloned an apparently novel 12.5-kDa protein that was identified to be secreted by the corticotrophic pituitary cell line AtT-20 (7). Upon sequencing, we were astonished to find that this pituitary peptide shared very high homology with the previously identified human protein, MIF.

By way of background, the existence of a mediator called MIF was first proposed in the early 1960s based on the observation that a product associated with activated lymphocytes could inhibit the random movement or migration of cultured macrophages (13–15). This engendered significant interest as MIF became one of the first soluble, nonimmunoglobulin factors that could be studied *in vitro*. Over the next decade, MIF activity was reported to be associated with various macrophage functions including adherence, phagocytosis, spreading, and enhanced tumoricidal activity (16–18). Nearly 25 years passed before human MIF was cloned (19), however, and this was due largely to difficulties in identifying an abundant cellular or tissue source of the protein for amino acid sequence determination. During this period, additional mediators with the "negative" chemotactic activity of MIF were also found, such as interferon-γ (IFN-γ) and interleukin-4 (IL-4), and this served to diminish somewhat the general interest in MIF (20, 21). It also must be noted that several published reports that utilized the original preparations of human MIF have been

withdrawn. The "MIF" used in these studies was present in an unpurified form that was subsequently found to contain significant quantities of the mitogen phytohemagglutinin (an immune cell activator) (22, 23).

III. Properties of the MIF Protein

We cloned mouse MIF from the cDNA of the AtT-20 pituitary cell line and human MIF from the Jurkat T cell line. The recombinant proteins were expressed in *Escherichia coli* and purified in an lipopolysaccharide (LPS)-free form by a two-step procedure consisting of Mono-Q anion exchange followed by reverse-phase chromatography. Both mouse and human recombinant MIF exhibit macrophage migration inhibitory activity when assayed in a modified Boyden chamber system (7, 24).

Mouse and human MIF show 90% identity in their primary amino acid sequence (115 amino acids), the highest known homology described to date for a mouse/human "cytokine" pair. All the MIF homologs described to date, which include human, mouse, rat, chicken, and bovine proteins, lack a classical N-terminal leader sequence (19, 25–28, 88) and appear to be released from cells either by granular secretion or by a nonconventional pathway. Although two potential N-glycosylation sites exist in the primary sequence, mass spectroscopic analysis of a preparation of purified native MIF has ruled out any covalent, posttranslational modification (24).

IV. Structure of the MIF Gene

The mouse and human MIF genes are both small (<1 kb), display a similar intron/exon organization, and show a high degree of homology in their coding regions (70.4, 86.4, and 67.5% for exons 1, 2, and 3, respectively) (25–27). With the exception of a 27% sequence homology with the enzyme L-dopachrome tautomerase (*described below*), neither the mouse nor the human MIF DNA sequences display significant homologies with any other known genes. The mouse MIF gene is located on chromosome 10 and maps to a position coincident with several recessive mutations, including the gray lethal (*gl*), mocha (*mh*), and grizzled (*gr*) (26). Currently, however, there is no indication that mutations of the MIF gene result in a known mouse defect or disease. Ongoing efforts to create an MIF knockout mouse *(in collaboration with Dr. Glen Dranoff, Dana Farber Cancer Institute)* have so far been unsuccessful. Although the MIF(+/−) phenotype has been obtained, no homozygous MIF(-/-) progeny have yet been bred.

As shown in Fig. 1, several consensus sequences that may be involved in the transcriptional regulation of MIF gene have been identified. These

FIG. 1. Sequence of mouse MIF gene and potential promoter/enhancer motifs (26).

+241 cgacgtgtgaggagggatggggctggaagccaaggtgtgccggcgggtggcggctggagctctccggaagacctgtggccctgtaggcagtctttcaggcggtctaacagtgtgtctgta +360

Exon 2 **TyrIleAlaValHisValValProAspGlnLeuMetThrPheSerGlyThrAsnAspProCysAlaLeuCysSerLeuHisSerIl**

+361 tccctcccgcctcgccgcccctcccccacccagTACATCGCAGTGCACGTGGTCCCGGACCAGCTCATGACTTTTAGCGGCACGAACGATCCCTGCGCCCTCTGCAGCCTGCACAGCAT +480

eGlyLysIleGlyGlyAlaGlnAsnArgAsnTyrSerLysLeuLeuCysGlyLeuLeuSerAspArgLeuHisIleSerProAspAr

+481 CGGCAAGATCGGTGGTGCCCAGAACCGCAACTACAGTAAGCTGCTGTGTGGCCTGCTGTCCGATCGCCTGCACATCAGCCCGGACCGgtacgtgggggacgaggggaggagggagaggag +600

Exon 3 **gValTyrIle**

+601 *gggcactgggaggtcaccagccaaagaggggggggcgttcagaggacactggcacgcagcgcgctctcctagaccacgtgcttagctgagccaggctttcattttctcagGGTCTACATC* +720

AsnTyrTyrAspMetAsnAlaAlaAsnValGlyTrpAsnGlySerThrPheAla---

+721 AACTATTACGACATGAACGCTGCCAACGTGGGCTGGAACGGTTCCACCTTCGCTTGAGTCCTGGCCCCACTTACCTGCACCGCTGTTCTTTGAGCCTCGCTCCACGTAGTGTTCTGTGTT +840

+841 TATCCACCGGTAGCGATGCCCACCTTCCAGCCGGGAGAAATAAATGGTTTATAAGAGACCacggttgcctcagcttctgcttccttggcttgcggaggaattgggtgcagggtggggacc +960

+961 ttgaatggaagcccaggctctgaacgtgggattggggtgtgatggcagggtcgaagctggccagtttggtcctctctaaactagtccagtggttcagatgtcctttcttgtggcctctcc +1080

+1081 tatgcagccttatgtataaaagaggccgacttattcacctggtcaggagtgttaattaggtccttaccacagtggcaaatggagccggtaatatgtgtcatggaaggggagtgcttggat +1200

+1201 acagggga +1208

FIG. 1.—*Continued*

enhancer/regulatory binding domains, many of which exist in the promoters of both the mouse and the human MIF genes (26, 27), include a sequence motif implicated in the basal expression of the protooncogene *c-fos*, an Sp-1 site, a cAMP responsive element (CRE), an AP-2 site, and a possible "negative" glucocorticoid responsive element (nGRE), all located in close proximity to the RNA transcription start site. A cytokine-1 (CK-1) site and a nuclear factor-κB (NF-κB) site also were identified further upstream on the minus DNA strand. Functional promoter studies are needed to determine the role of these and other promoter elements in the expression of the MIF gene. Nevertheless, it appears that the MIF promoter contains regulatory sequences that are characteristic of both cytokine (NF-κB) and endocrine hormone (CRE and nGRE) genes (26, 27).

In contrast to the human MIF gene, which is expressed as a single functional gene, analyses of mouse genomic DNA have revealed the existence of multiple pseudogenes (25, 26). Northern blotting of RNA from various mouse tissues has shown that the MIF gene is transcribed as a single, 0.6-kb mRNA species and that in many tissues and cell types MIF is consitutively expressed (26). This has been confirmed by recent immunohistochemistry and *in situ* hybridization methods that show that MIF is present in many nonimmune and endocrine cells, where it may play an autocrine or paracrine role in cell regulation (29).

V. Cell and Tissue Localization of MIF

MIF circulates normally at a basal level in serum that has been determined to be 3–5 ng/ml in rodents and human subjects (7; C. N. Metz, unpublished observations). MIF protein exists preformed in macrophages, T cells, and the corticotrophic cells of the anterior pituitary gland (7, 29–31). Following stimulation, cellular MIF content declines rapidly (<1 hr) and this is followed by an induction in the expression of MIF mRNA (>3 hr) (29). As detailed in Table I, significant quantities of preformed MIF are also detected in various tissues and cell types, including the adrenal glands, kidney, lung, liver, and the skin.

A. Anterior Pituitary Gland

As mentioned earlier, MIF was discovered to be an abundant, preformed constituent of the anterior pituitary gland (7). Immunocytochemical and ELISA analyses have shown that MIF accounts for approximately 0.5% of the total pituitary protein content (7). By comparison, the values for the "classical" pituitary hormones ACTH and prolactin are 0.2 and 0.08%, respectively (32, 33). Both the administration of endotoxin (LPS) *in vivo* and the stimulation of pituitary cells *in vitro* with LPS results in

TABLE I
TISSUE AND CELLULAR LOCALIZATION OF MIF

Organ	Primary cell type/region	Reference
Anterior pituitary	ACTH and some TSH secreting cells	7, 34
Immune system	Monocytes/macrophages, T cells (TH2 > TH1)	30, 31
	Eosinophils	87
Adrenal gland	Cortex—zona glomerulosa, zona fasiculata	29
Lung	Bronchial epithelium, alveolar macrophages (type II pneumocytes)	29, 36, 37
Kidney	Tubular and glomerular epithelial cells and some endothelial cells	29
Liver	Hepatocytes surrounding central veins and Kupffer cells following LPS administration	29
Skin	Keratinocytes, sebaceous glands, and outer root sheath of hair follicle	29, 38
Testes	Leydig cells	40
Pancreas	Islets, β cells	43
Eye	Corneal epithelial and endothelial cells, differentiating cells of the lens	41, 42
Brain	Cortex, hypothalmus, and cerebellum—neurons	44, 88

the release of MIF protein and an increase in MIF message levels. Corticotrophic pituitary cells also release MIF after stimulation with corticotrophin-releasing factor (CRF), albeit MIF secretion appears to occur in response to lower levels of CRF than are necessary to stimulate ACTH release (34). Further studies utilizing immunogold electron microscopy have localized MIF to the granules present exclusively in ACTH and TSH secreting cells (34). These initial observations on the pituitary localization of MIF have led us to explore the role of MIF in infection and stress using various experimental models (discussed below).

B. MONOCYTES/MACROPHAGES

For almost 30 years MIF activity had been considered to be a product of the activated T lymphocyte and its target of action the monocyte/macrophage. We discovered, however, that the macrophage is also an important source of MIF production (8, 30). Moreover, in the classic tuberculin-induced delayed-type hypersensitivity (DTH) model in mice, the macrophage is the predominant source of MIF (35). Macrophage MIF is released upon stimulation with various proinflammatory agents, including gram-negative endotoxin (LPS), the gram-positive exotoxins, toxic shock syndrome toxin 1, and the cytokines TNF-α, and IFN-γ, as well as hemozoin or malaria pigment (8, 30, 64; T. Calandra, unpublished observations). Of note, the LPS concentration required to upregulate MIF expression is at

least two orders of magnitude lower than that needed to induce the expression of TNF-α, suggesting that the MIF release response is extremely sensitive to proinflammatory stimuli. Furthermore, the production of MIF in response to increasing concentrations of bacterial LPS follows a bell-shaped curve, decreasing at higher levels. This may be an important protective mechanism of the host to prevent detrimental effects of excessive amounts of MIF release, as discussed in the following section. At high levels (>100 ng/ml) MIF was also found to induce TNF-α secretion by macrophages and to synergize with IFN-γ to promote nitric oxide production (30). These data indicate that at the local level, MIF may act in concert with TNF-α and IFN-γ to amplify the proinflammatory responses of macrophages.

C. T Lymphocytes

Using specific molecular probes for MIF, we recently examined the expression of MIF by T cells and explored its role in T cell physiology (31). T cells purified from mouse spleens or human peripheral blood constitutively express MIF mRNA and MIF in preformed stores. T cells release significant quantities of MIF protein upon stimulation with αCD3 antibody, mitogens (PMA, ionomycin, and PHA), or glucocorticoids and this is followed over time by an increase in the levels of MIF mRNA expression (discussed below). Both TH1 cells, which favor the development of cell-mediated immunity, and TH2 cells, which promote humoral immunity, constitutively express MIF. However, a prominent induction of MIF mRNA and protein was observed only when TH2 clones were stimulated with concanavalin A (Con A) (31). The observation that there is an induction of MIF expression and release of MIF protein by T cells following activation in turn led to studies examining the potential role of MIF in T cell immunity (discussed below).

D. Adrenal Gland

Immunoreactive MIF protein is present under normal conditions in the zona glomerulosa and in selected areas of the zona fasiculata of the rat adrenal cortex and is absent in the adrenal medulla (29). MIF mRNA was present constitutively and the strongest hybridization signals were observed in the zona glomerulosa. Following the systemic administration of LPS, the adrenal content of MIF decreased, and this was followed by a significant increase in the expression of MIF message.

E. Lung

Analysis of rat lung sections revealed a basal level of expression of MIF mRNA and protein within the bronchial epithelium and in some alveolar

macrophages (29). There was an induction in MIF mRNA levels and a slight decrease in the MIF protein content following LPS injection. Immunohistochemical staining of human lung tissue obtained from patients suffering from acute respiratory distress syndrome (ARDS) revealed significant quantities of MIF present not only in macrophages but also in type II pneumocytes (36, 37).

F. Liver

Immunoreactive MIF was localized to the hepatocytes surrounding the central veins and to the endothelial cells of both the sinus and central venules (29). Whereas MIF mRNA expression was barely detectable in hepatocytes and Kupffer cells under normal conditions, the systemic administration of LPS produced a marked increase in MIF mRNA expression in Kupffer cells (29).

G. Skin

MIF is expressed predominantly in the keratinocytes of the epidermis (29, 38), in the cells of sebaceous glands, and in the outer root sheath of the hair follicles (29). Similar to the observations made in other tissues, MIF protein expression declined while mRNA expression increased following the administration of LPS *in vivo*. The rapid loss of MIF protein immunoreactivity presumably reflects active secretion. This is then accompanied by a coordinate induction of MIF mRNA expression to replace lost protein stores. Several other cytokines, including TNF-α, IL-1, IL-6, lL-8, and TGF-β, have been shown to be present in the epidermal keratinocytes (39). Currently, very little is known about the role of cytokines in the pathogenesis of inflammatory skin disease. However, this finding suggests the potential role of MIF in immune reactions and inflammation of the skin.

H. Testes

A systematic analysis of tissue/organ expression of MIF has also led to the identification of MIF expression in the rat testis (40). Immunoreactive MIF was localized to the intertubular regions of the testis, specifically to the Leydig cells. Northern blotting analysis then confirmed that the testis is a site of MIF production and not simply a site of protein uptake. The addition of recombinant MIF to cultured Leydig cells results in a dose-dependent suppression of inhibin production by seminiferous epithelium, suggesting a further role of MIF in the regulation of testicular function.

I. Eye

MIF is also abundantly expressed in the early stages of the embryonic chicken lens and expression strongly correlated with cell differentiation

(41). Two potential functions of MIF in the lens have been postulated—It may play a role during eye infection by interacting with the few macrophage-like cells (halocytes) in the vitreous body of the eye or it may mediate the communication between the lens and the retina during normal eye development. In addition, MIF (mRNA and protein) expression has been localized to the human cornea (42). MIF was present in endothelial cells and the basal cells of the corneal epithelium, suggesting that MIF may be involved in corneal cell immunity and cellular differentiation.

J. Pancreatic Islets

An immunohistochemical survey of the tissue distribution of MIF also revealed significant quantities in the endocrine pancreas. Further studies using the differentiated insulin-secreting β cell line, INS-1, and isolated rat pancreatic islets demonstrated that the expression of MIF is regulated by glucose. Moreover, secreted MIF acts to regulate insulin secretion in an autocrine manner, indicating that MIF may play a central role in the regulation of carbohydrate metabolism (43).

K. Brain

We have recently mapped the distribution of MIF transcripts and protein in the rat brain (44). MIF expression was found primarily in the neurons of the cortex, hypothalmus, cerebellum, and pons. *In situ* hybridization revealed the highest levels of MIF mRNA in the cell bodies of neurons, whereas by immunohistochemistry, most of the MIF protein was detected within the terminal fields associated with neurons. Following the intracisternal injection of LPS, MIF is rapidly released into the cerebral spinal fluid (CSF). The presence of MIF within the hippocampus and hypothalamus and the release of MIF into the CSF after LPS administration complement our previous findings of MIF as an important neuroendocrine mediator.

VI. Biological Properties of MIF

As previously stated, MIF was originally described as a lymphokine that inhibited the random migration of guinea pig macrophages (13–15). However, over the past several years MIF has been discovered to exhibit a number of pleiotropic effects, as shown in Table II.

A. Mediator Secreted in Response to Inflammatory Stimuli

The first *in vivo* studies of MIF in rodents showed that the pituitary release of MIF is an integral part of the host's systemic stress response. When mice received an intraperitoneal injection of endotoxin, there was a dramatic decrease in the pituitary content of MIF protein, a concomitant

TABLE II
BIOLOGICAL ACTIVITIES OF MIF

Activity	Reference
In vitro (≥100 ng/ml)	
Inhibitor of macrophage migration	13, 24
Stimulator of TNFα and costimulator of NO production	30
In vitro (<100 ng/ml)	
Counterregulator of the anti-inflammatory and immunosuppressive effects of glucocorticoid hormones on immune cells	8, 31
Mediator of T cell activation	31
In vivo/regulatory effects	
Pituitary, macrophage, and T cell mediator secreted in response to inflammatory stimuli	7, 8, 31
Required for antigen-dependent T cell priming, antibody production by B cells, and DTH	31, 35
Mediator of septic shock, as well as other inflammatory infectious diseases including glomerulonephritis, arthritis, malaria, and ARDS	7, 37, 55, 56, 58, 64

rise in plasma MIF levels, and a slower, time-dependent increase in the expression of pituitary MIF mRNA (7, 8). MIF was found to circulate normally in serum at concentrations of ~3–5 ng/ml in rodents or in normal human volunteers (7, 8; C. N. Metz, unpublished observations). Activation of the HPA axis in rodents by handling or other physiological stress also produced a time-dependent elevation in plasma MIF that reached levels of ~50 ng/ml (8) and occurred over a similar time course (~3 hr) as the more classically described, stress-related increases in circulating ACTH and glucocorticoid levels. These early data provided the first evidence for the concept that MIF circulates normally and is released by the pituitary gland in response to stress or systemic inflammatory stimuli (*discussed further below*).

The first biological studies of purified, recombinant MIF (rMIF) showed that this protein exhibited a number of proinflammatory functions of critical importance to the host. MIF was found to potentiate endotoxemia when co-injected with LPS into mice (7). Conversely, neutralizing anti-MIF antibodies fully protected mice from a lethal LPS injection, demonstrating that MIF, like TNF-α, IL-1, and IFN-γ, plays a critical role in the inflammatory network leading to endotoxic shock and death (7).

B. MIF AS A GLUCOCORTICOID COUNTERREGULATOR

The proinflammatory effects of MIF were somewhat unexpected when considered in the context of the observation that MIF is released from the same pituitary cell type that releases ACTH, a mediator that stimulates

the adrenal secretion of glucocorticoids—potent anti-inflammatory hormones. This apparent paradox was resolved by experiments in which it was shown that glucocorticoids, in low concentrations, directly induce MIF release from macrophages and T cells (8). Initially, this finding was also surprising because it had been generally considered that glucocorticoids inhibit the release of proinflammatory mediators (45–47). MIF thus became the first mediator to be described that is actively released from cells upon glucocorticoid stimulation (8). The observation that glucocorticoids induce the secretion of immune cell MIF, a "proinflammatory" factor, led us to propose that the primary role of MIF might be to regulate the anti-inflammatory effects of glucocorticoids (8). This possibility was tested first by examining the effect of rMIF on glucocorticoid inhibition of cytokine production *in vitro* and on endotoxin-induced lethality *in vivo*. As shown in Fig. 2, when added to cultured monocytes, rMIF was found to override, in a dose-dependent fashion, glucocorticoid inhibition of TNF-α, IL-1β, IL-6, and IL-8 secretion (8). The ability of MIF to overcome the inhibition of cytokine secretion varied directly with the concentration of glucocorticoid and decreased with increasing dexamethasone concentrations.

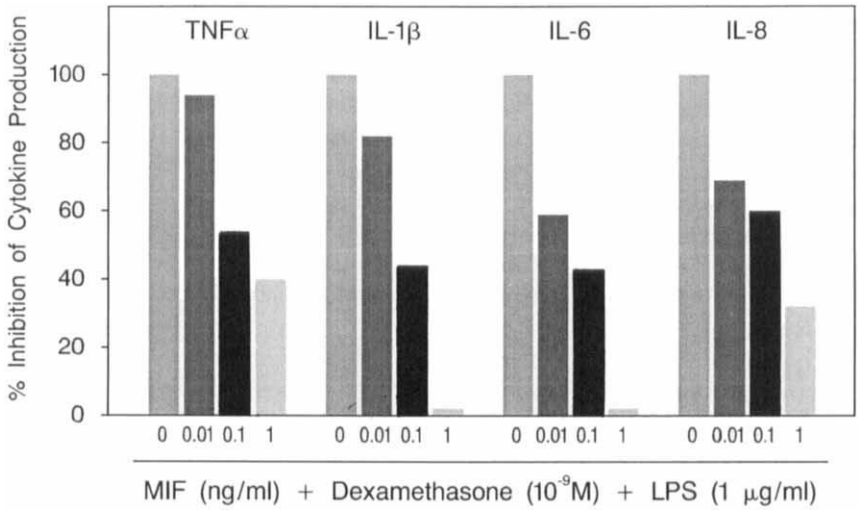


FIG. 2. MIF overrides glucocorticoid-mediated suppression of cytokine production by human monocytes *in vitro*. Human mononuclear cells isolated from peripheral blood were preincubated with dexamethasone (10^{-9} *M*) or dexamethasone with rMIF (0.01, and 0.1, 1.0 ng/ml) before the addition of LPS (1 μg/ml) to stimulate cytokine production. Culture supernatants were collected after 12 hr and the secreted cytokines were measured by ELISA (8).

The counterregulatory activity of MIF in glucocorticoid-mediated immunosuppression *in vivo* was confirmed by experiments in which we studied the effect of exogenously administered MIF on protection conferred by dexamethasone in a mouse model of endotoxic shock. As illustrated in Fig. 3, the administration of rMIF together with dexamethasone to LPS-treated mice completely blocked the protective effect of dexamethasone on LPS lethality (8). By counteracting glucocorticoids, MIF allows for the development of acute inflammatory response and, subsequently, of an antigen-specific immune response. The release of MIF "centrally," from the pituitary, indicates that the host also has the capacity to antagonize the anti-inflammatory properties of glucocorticoids at the systemic level (7, 8).

On first examination, the fact that inflammation or stress result in the production of both anti-inflammatory (glucocorticoid) and proinflammatory (MIF) mediators may appear paradoxical. However, in common with other physiological systems, it is apparent that a counterregulatory mechanism is essential to provide the balance and regulation that are necessary to control the inflammatory cascade. Both MIF and glucocorticoids circulate at concentrations that have regulatory activity, indicating that the baseline state of the MIF/glucocorticoid diad is one of an "active" balance between pro- and anti-inflammatory effects. As for glucocorticoids, serum concentrations of MIF increase many-fold during stress, inflammation, or infection (7, 8, 29). The observation that MIF release follows a bell-shaped, dose–response curve and that its overriding capacity is diminished at high gluco-

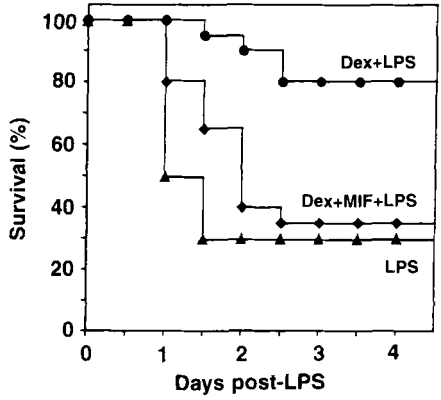


FIG. 3. MIF overrides glucocorticoid-mediated inhibition of LPS lethality *in vivo*. BALB/c mice were injected intraperitoneally with dexamethasone (1.25 mg/kg) with or without rMIF (0.6 mg/kg) or saline as control. After 2 hr, all mice were injected intraperitoneally with LPS (22.5 mg/kg). MIF-treated mice received an additional MIF injection at the time of LPS challenge and 17 hr thereafter (8).

corticoid concentrations further suggests the existence of important physiological "control points" within the MIF/glucocorticoid counterregulatory system.

C. T Cell Activation and Antigen-Specific Immunity

Further studies have established that the MIF/glucocorticoid counterregulatory relationship plays a critical role in T cell activation and in antigen-specific T cell and B cell responses. As in the macrophage system, MIF is released by T cells activated by mitogens, receptor cross-linking agents, or specific antigen (31). Importantly, glucocorticoids in low concentrations will also initiate the release of MIF from T cells (8). This MIF then acts in a regulatory fashion to override glucocorticoid inhibition of T cell activation (31). When primed human T cells were incubated with either mitogen or antigen together with glucocorticoid, MIF was found to override, in a dose-dependent manner, the glucocorticoid-mediated suppression of T cell proliferation and cytokine (IL-2 and IFN-γ) production, as shown in Fig. 4. The important role of the MIF/glucocorticoid regulatory system on T cell responses *in vivo* was substantiated by administering neutralizing anti-MIF antibodies to mice at the time of immunization with a soluble antigen. Anti-MIF significantly inhibited the development of both antigen-specific T cells and the primary antibody response, an effect that was attributed to the increased immunosuppressive effects of glucocorticoids after neutralization of endogenous MIF (31).

D. Disease Pathogenesis

The biological properties of MIF as a physiological counterregulator of glucocorticoid action in inflammation and immunity have prompted much interest into the potential role of MIF in various inflammatory disease states.

1. Septic Shock

The pituitary plays an important role in the host response to endotoxemia (48). Hypophysectomized animals (lacking their pituitary) succumb to LPS lethality at doses at least 1000-fold less than their normal counterparts (12). It has been hypothesized that this is due to the fact that pituitary-deprived animals fail to secrete ACTH and thus exhibit an impaired glucocorticoid stress response. However, other observations suggest that stress glucocorticoid responses cannot completely explain the extreme sensitivity of the hypophysectomized animals to endotoxin (12).

Because the pituitary is ideally situated to integrate central and peripheral stimuli and initiate the increase in systemic glucocorticoids that accompanies host stress responses, we examined the role of pituitary MIF in shock

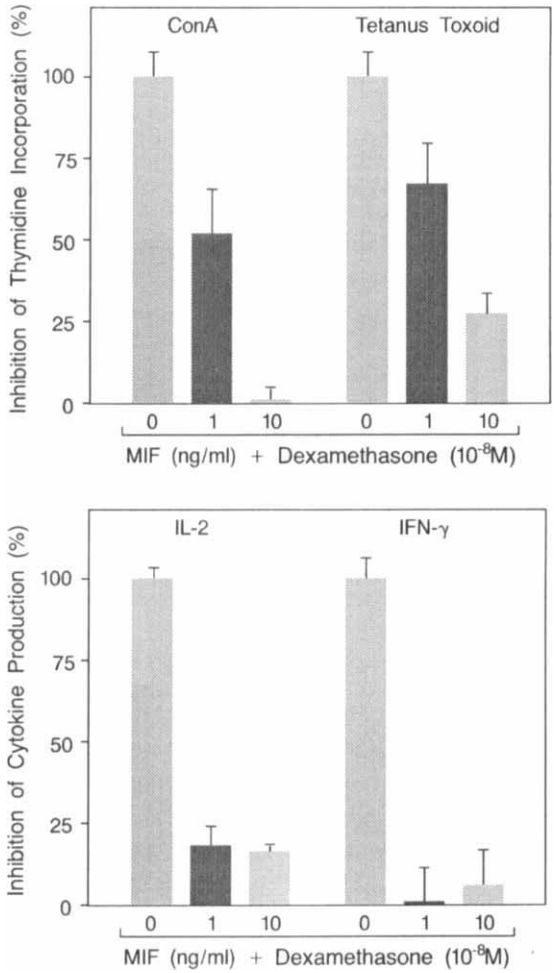


FIG. 4. MIF overrides glucocorticoid-mediated inhibition of T cell activation. Human T cells were purified from the peripheral blood and cultured with dexamethasone (10^{-8} *M*) alone or with dexamethasone (10^{-8} *M*) plus rMIF prior to stimulation with either Con A or tetanus toxoid together with antigen presenting cells. The cultures were then pulsed with [^{3}H]thymidine (top) or supernatants were collected for cytokine determination by ELISA (bottom) (31).

using a rodent model of endotoxemia (7). Following the intraperitoneal injection of LPS into mice, pituitary MIF mRNA levels increase, pituitary MIF protein decreases, and circulating concentrations of MIF increase. Like TNF-α and IL-1β, MIF is a critical mediator of the acute inflammatory

response. When coinjected into mice with LPS, recombinant MIF enhances lethal endotoxemia, whereas neutralizing anti-MIF antibodies provides full protection from endotoxic shock (7).

2. *Delayed-Type Hypersensitivity*

MIF was originally described to mediate macrophage accumulation and activation in DTH reactions (14, 15). The tuberculin skin reaction is a classical model of DTH. It is specific to the sensitizing stimulus, is "delayed" in onset, and is mediated by an infiltrating cellular immune response. As such, DTH is often considered to be a model immunological response for the study of normal and pathological processes *in vivo*. We examined the expression of MIF (mRNA and protein) and investigated its role in the DTH response by studying the tuberculin reaction in mice (35). Both MIF mRNA and protein were expressed in DTH lesions, although primarily by monocytes/macrophages and not by T cells as originally hypothesized. These studies also were the first to reveal the expression of MIF within endothelial cells in areas of active inflammation. The administration of neutralizing MIF antibodies to mice significantly inhibited the development of DTH, confirming the central role of MIF in this classical immunological response.

3. *Glomerulonephritis*

Macrophage activation and accumulation contribute to glomerular crescent formation in various types of human and experimental glomerulonephritis (49–54). The expression of MIF in the kidney during the development of rat anti-glomerular basement membrane glomerulonephritis, a model of macrophage-mediated renal injury, has been explored (55). In this model, renal injury is induced by the administration of rabbit anti-glomerular basement membrane antibody (anti-GBM) into rabbit IgG-sensitized rat hosts. This results in a severe inflammatory reaction, leading to crescentic glomerulonephritis, proteinurea, and renal failure.

MIF mRNA and protein were found to be constitutively expressed in the normal kidney, specifically in the tubular epithelial cells and in some glomerular visceral and parietal epithelial cells (55). MIF expression by intrinsic kidney cells, as well as the endothelium, increased during the development of rat crescentic glomerulonephritis. Macrophage accumulation was observed in the areas of highest MIF expression and was associated with focal glomerular and tubulointerstitial lesion formation, as well as with macrophage accumulation in Bowman's space and crescent formation. Glomerular MIF expression during the progression of anti-GBM glomerulonephritis also correlated with increased urinary protein excretion and decreased creatinine clearance.

Further experiments have examined the role of neutralizing anti-MIF antibodies in rat glomerulonephritis (56). Animals treated with a neutralizing anti-MIF monoclonal antibody had significantly reduced proteinurea, enhanced renal function, reduced histological renal tissue damage, and substantially decreased renal leukocytic infiltration and activation compared to animals treated with an isotype-matched control antibody. Blocking MIF activity with monoclonal antibody treatment also resulted in a substantial decrease of MIF mRNA and protein expression by both intrinsic kidney cells and infiltrating macrophages. Anti-MIF antibody treatment was associated with significant decreases in the expression of IL-1β, leukocyte adhesion molecules, and the inducible form of nitric oxide synthase. In this disease model, anti-MIF did not affect the humoral immune response, nor did it alter the immune deposition within the kidney. Thus, the role of MIF in suppressing rat crescentic glomerulonephritis appeared to be related primarily to its ability to inhibit cell-based mechanisms of tissue injury.

4. Arthritis

Rheumatoid arthritis is a chronic, remitting and relapsing autoimmune disease characterized by synovial proliferation leading to pannus formation, cartilage destruction, and bone erosion. Macrophages contribute to the pathogenesis of arthritis by secreting various chemoattractants and by upregulating vascular adhesion molecules and integrins via IL-1 and TNF-α secretion. Together with other immune cell types, macrophages release factors that promote local tissue inflammation, enhance the growth and proliferation of synovial lining cells, induce neovascularization, and cause joint destruction by matrix-degrading enzymes (57).

We have measured MIF levels in synovial fluids obtained from the knee joints of humans suffering from rheumatoid arthritis (RA) or osteoarthritis (OA) generally a less inflammatory and nonerosive form of arthritis. As shown in Fig. 5, there was threefold more MIF in the joint fluid from RA patients than in that obtained from OA patients.

Immunohistochemical studies in the rat have established that MIF protein is present constitutively within the epithelial lining cells of the synovium of the joint (C. Metz and R. Bucala, unpublished observations). In joints obtained from animals with collagen-induced arthritis, MIF expression was increased in the synovial lining cells. MIF was also readily detected in the infiltrating macrophages (and to a lesser extent in T cells) as well as in endothelial cells bordering areas of inflammation.

Depletion of MIF by the administration of neutralizing antibodies during the immunization phase of collagen-induced arthritis in mice led to delayed arthritis onset and markedly reduced severity of arthritic pathology (58).

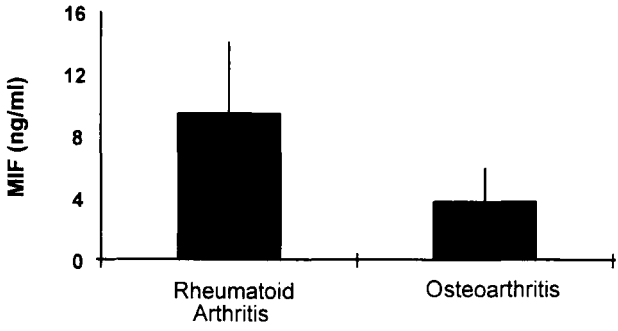


FIG. 5. MIF levels in synovial fluids obtained from patients with either rheumatoid arthrititis or osteoarthritis.

The suppressive effects were associated with a decrease in circulating anti-collagen type II IgG_{2a} autoantibodies and a decrease in the T cell proliferative response to collagen II.

5. *Acute Respiratory Distress Syndrome*

ARDS is a life-threatening condition of lung injury caused by the infiltration and activation of inflammatory cells, particularly neutrophils, within the pulmonary airspaces (59). ARDS is a prime example of an inflammatory disease state in which the regulatory balance within the immune response shifts toward acute inflammation and tissue injury. The clinical presentation of ARDS is associated with the production of numerous proinflammatory cytokines and chemokines (IL-1, TNF-α, and IL-8) (60, 61) that initiate a cascade of events leading to damage of the alveolar/capillary interface and subsequent leakage of fluid into the alveolar space (59). The onset of the condition is rapid and the disease course is rapid, unpredictable, and often fatal. Various pharmacologic therapies, including glucocorticoids, have been tested in ARDS in an attempt to inhibit the overproduction of inflammatory mediators (62, 63). Given the potential role of MIF as both a proinflammatory mediator and a downregulator of the glucocorticoid action, it was natural to consider that this mediator may play a critical role in the regulatory imbalance associated with ARDS (37).

As shown in Fig. 6, the MIF levels were higher in the bronchoalveolar lavage fluids (BALs) obtained from ARDS patients than control subjects. By immunohistochemistry, alveolar macrophages and type II alveolar epithelial cells appeared to be the major sites of MIF production within the pulmonary airspaces (36, 37).

To examine the potential effects of MIF in the regulation of cytokine production during ARDS, cells obtained from the BALs of ARDS patients

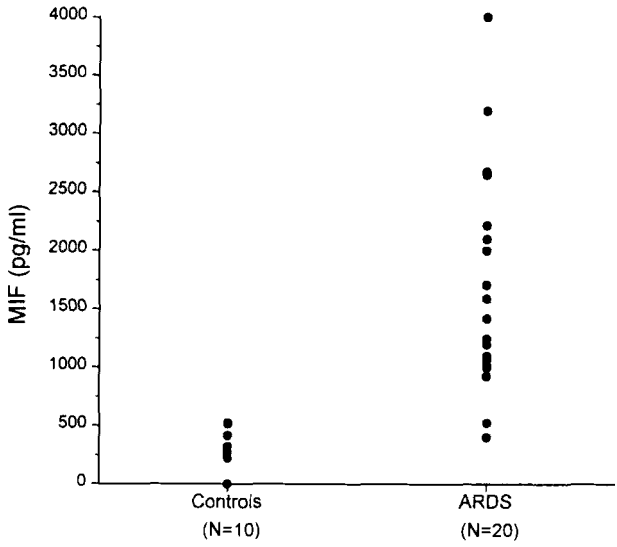


FIG. 6. MIF levels in bronchoalveolar lavage obtained from normal controls vs ARDS patients (37).

were incubated with neutralizing anti-MIF antibodies (37). A significant reduction in both TNF-α (30%) and IL-8 (60%) secretion was found compared to controls. Conversely, the addition of recombinant MIF to *in vivo*-activated BAL monocytes cultures increased even further the spontaneous release of cytokines by these cells.

We next investigated whether MIF could override the inhibitory effects of steroids on BAL cells obtained from patients with ARDS (37). In a series of overriding experiments, it was shown that recombinant MIF, when added to corticosteroid-treated ARDS cells, overcame the inhibitory effects of the steroids on cytokine production. These data were the first to establish that MIF could function as an antagonist of the anti-inflammatory actions of glucocorticoids in cells obtained from inflammatory lesions that had been activated *in situ*.

6. *Malaria*

Approximately 1–2 million people die each year of malaria infection. We investigated the potential role of MIF in the pathophysiology of malaria by studying a mouse model, *P. chabaudi* infection. We observed that macrophages secrete large quantities of MIF after phagocytosis of parasitized erythrocytes or malaria pigment (hemazoin) *in vitro* and that there is a disease-dependent increase in circulating MIF levels during *P. chabaudi* infection in mice *in vivo* (64).

An important manifestation of malaria infection is a severe anemia that contributes significantly to the malaria-related deaths of children before the age of 2. The pathogenesis of anemia in malaria is not well understood but appears to result from enhanced erythrocyte destruction (65, 66) and defective erythropoiesis (67, 68). Recent studies have shown that decreased erythropoietin production does not contribute to malaria-associated anemia (69) and that the circulating immune mediators, IL-1β, IL-1α, TNF-α, and IFN-γ, do not contribute significantly to the suppression of erythropoiesis during malaria (70). Therefore, there remains an interest in identifying inhibitors of erythropoiesis that may be expressed during malaria infection.

Preliminary studies indicate that recombinant MIF has a direct inhibitory effect on the formation of erythrocyte progenitors *in vitro* (H. Broxmeyer, unpublished observations). This effect was blocked specifically by the presence of neutralizing anti-MIF antibodies. Given the observation that circulating levels of MIF increase dramatically during malaria infection, it is likely that MIF plays a role in the defective red blood cell production that frequently accompanies the disease. Nevertheless, further studies will be required to elucidate the precise function of MIF during the host response to malaria infection.

VII. Recent Structure–Function Studies

The human and rat MIF protein structures have been solved by X-ray crystallography (71–73). The structures of the two proteins are almost identical, differing in the positioning of 11 carboxy-terminal residues, which could not be visualized in the rat MIF crystal. As shown in Fig. 7, MIF forms a homotrimer of approximately 35 × 50 × 50 Å. Each monomer subunit consists of two antiparallel α helices and six β strands. Several hydrogen bonding sites between the monomers and a hydrophobic core stabilize the MIF trimer.

At first glance, the overall structure of the MIF molecule—two α helices running parallel to a β sheet plane—appears to be similar to that of the human class I histocompatibility antigen HLA-A2 (74) or IL-8 (75); however, the folding topology of MIF is entirely unique. It forms an α/β structure consisting of three β sheets surrounded by six α helices to form a unique solvent-accessible channel that runs the length of the molecule. This channel, which spans from 3 or 4 to 15 Å, contains a region of positive potential, suggesting that it might interact with negatively charged moieties. Although MIF is a secreted protein, all three cysteines exist as free thiols.

MIF has been postulated to have glutathione-*S*-transferase activity (76, 77). Although MIF shares 25–30% sequence homology with the first 30 amino acids of glutathione *S*-transferase and MIF may bind to reduced

FIG. 7. Crystal structure of MIF. Three-dimensional structure of human MIF viewed down its threefold axis of symmetry [adapted from Ref. (71)].

glutathione with low affinity (28, 78), the three-dimensional structures of the two proteins are quite different. Moreover, attempts to demonstrate glutathione S-transferase activity using recombinant MIF have been unsuccessful (24).

In addition to the weak primary sequence homology with the enzyme D-dopachrome tautomerase, MIF displays significant three-dimensional structural homology with two bacterial enzymes with isomerase activity—4-oxalocrotonate tautomerase (4-OT) and 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) (79). CHMI exists as a trimer similar to MIF. In contrast, 4-OT is organized as a hexamer (i.e., a dimer of trimers), but the overall three-dimensional shape of each trimer is highly homologous to MIF. Interestingly, we have also observed a tautomerase activity associated with MIF in the conversion of D-dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (80). Although the substrate tested (D-dopachrome) does not exist naturally, the observation that MIF has tautomerase activity suggests that an enzymatic activity may underlie at least part of its biological function.

Interestingly, the architecture of the MIF molecule reveals several atypical features for a cytokine, including a unique α/β barrel fold and a solvent-accessible channel. Elucidation of the three-dimensional structure of MIF now makes it possible to design and analyze mutants to study the role of the central channel, the isomerase activity, and the domain(s) responsible for glucocorticoid counterregulatory activity.

VIII. Therapeutic Implications and Future Directions

Despite the widespread use of glucocorticoids as immunosuppressive or anti-inflammatory therapies, there is only partial knowledge of the molecular mechanisms by which glucocorticoids exert their effects. Glucocorticoids are potent inhibitors of cytokine production and are known to inhibit gene transcription (45, 47, 81, 82). The interaction between the cytokine MIF and the glucocorticoid hormones presumably occurs via post receptor signaling pathways that converge upon a common subset of transcriptional regulators. Of note, it has been proposed recently that glucocorticoid inhibition of the transcription activator, NF-κB, may account for some of the anti-inflammatory effects of glucocorticoids (83, 84). Dexamethasone induces the transcription of the IkBα gene, thereby increasing IkBα protein concentrations in the cytoplasm, decreasing NF-κB translocation to the nucleus, and reducing NF-κB-mediated activation of cytokine genes. Elucidation of the molecular basis of the MIF/glucocorticoid interaction is an important future goal and may ultimately add to our understanding of the complex sequence of events responsible for acute and life-threatening inflammatory responses. Within the clinical setting, the ability of MIF to override glucocorticoid effects may explain in part why the administration of high doses of glucocorticoids to patients already in septic shock, and in whom both the stress response and the cytokine cascade have been activated, has not been shown to be efficacious (85, 86).

An emerging body of data indicates that MIF is a critical component of the immune system that acts in concert with glucocorticoids to regulate inflammation and immunity. An elevation in the blood or tissue level of MIF would act to counterregulate the action of glucocorticoids, and it is likely that this action would extend to glucocorticoids administered pharmacologically as part of a therapeutic regimen. The clincial development of steroid resistance in patients treated for autoimmune or inflammatory disease may result in part from an induction of MIF expression, thereby producing a systemic antiglucocorticoid effect. It is interesting to consider that an antibody or small molecule-based anti-MIF strategy might prove useful in increasing the immunosuppressive and anti-inflammatory properties of glucocorticoids, thereby decreasing and possibly eliminating

the requirement for steroid therapy in a variety of autoimmune and inflammatory diseases.

ACKNOWLEDGMENTS

We are grateful to our numerous colleagues for their contributions to this work: R. C. Atkins, M. Bacher, J. Bernhagen, T. Calandra, S. C. Donnelly, R. Holmdahl, Y. H. Lan, E. Lolis, A. Meinhardt, J. Martiney, and R. Mitchell.

REFERENCES

1. Kellum, J., and Decker, J. M. (1996). The immune system: Relation to sepsis and multiple organ failure. *AACN Clin. Issues* **7,** 339–350.
2. Parillo, J. E. (1990). Septic shock in humans: Advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy. *Ann. Intern. Med.* **113,** 227–242.
3. Orth, D. N., Kovacs, W. J., DeBold, C. R., Wilson, J. D., and Foster, D. W. (1992). "Williams Textbook of Endocrinology," 4th ed. Saunders, Philadelphia.
4. Goldstein, I. M., Bowen, D. L., and Fauci, A. S. (1992). Adrenal corticosteroids. *In* "Inflammation: Basic Principles and Clinical Correlates" (J. I. Gallin, I. M. Goldstein, and R. Snyderman, Eds.), pp. 1061–1081. Raven Press, New York.
5. Chrousos, G. P. (1995). The hypothalamic–pituitary–adrenal axis and immune-mediated inflammation. *N. Engl. J. Med.* **332,** 1351–1362.
6. Munck, A., Guyre, P. M., and Holbrook, N. J. (1984). Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrinol. Rev.* **5,** 25–44.
7. Bernhagen, J., Calandra, T., Mitchell, R. A., Martin, S. B., Tracey, K. J., Voelter, W., Manogue, K. R., Cerami, A., and Bucala, R. (1993). Macrophage migration inhibitory factor (MIF) is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature* **365,** 756–759.
8. Calandra, T., Bernhagen, J., Metz, C. N., Spiegel, L. A., Bacher, M., Donnelly, T., Cerami, A., and Bucala, R. (1995). MIF as a glucocorticoid-induced counter-regulator of cytokine production. *Nature* **377,** 68–71.
9. Hawes, A. S., Rock, C. S., Keogh, C. V., Lowry, S. F., and Calvano, S. E. (1992). *In vivo* effects of the antiglucocorticoid RU 486 on glucocorticoid and cytokine responses to *Escherichia coli* endotoxin. *Infect. Immun.* **60,** 2641–2647.
10. Fantuzzi, G., Di Santo, E., Sacco, S., Benigni, F., and Ghezzi, P. (1995). Role of the hypothalamus–pituitary–adrenal axis in the regulation of TNF production in mice. *J. Immunol.* **155,** 3552–3555.
11. Bertini, R., Bianchi, M., and Ghezzi, P. (1988). Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor. *J. Exp. Med.* **167,** 1708–1712.
12. Silverstein, R., Turley, B. R., Cristofferson, C. A., Johnson, D. C., and Morrison, D. C. (1991). Hydrazine sulfate protects D-galactosamine-sensitized mice against endotoxin and tumor necrosis factor/cachectin lethality: Evidence of a role for the pituitary. *J. Exp. Med.* **173,** 357–365.
13. George, M., and Vaughan, J. H. (1962). *In vitro* cell migration as a model for delayed hypersensitivity. *Proc. Soc. Exp. Biol. Med.* **111,** 514–521.
14. Bloom, B. R., and Bennett, B. (1966). Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity. *Science* **153,** 80–82.
15. David, J. (1966). Delayed hypersensitivity *in vitro:* Its mediation by cell-free substances formed by lymphoid cell–antigen interaction. *Proc. Natl. Acad. Sci. USA* **56,** 72–77.
16. Nathan, C. F., Remold, H. G., and David, J. R. (1973). Characterization of a lymphocyte factor which alters macrophage function. *J. Exp. Med.* **137,** 275–288.

17. Nathan, C. F., Karnovsky, M. L., and David, J. R. (1971). Alterations of macrophage functions by mediators from lymphocytes. *J. Exp. Med.* **133,** 1356–1376.
18. Churchill, W. H., Piessens, W. F., Sulis, C. A., and David, J. R. (1975). Macrophages activated as suspension cultures with lymphocyte mediators devoid of antigen become cytotoxic for tumor cells. *J. Immunol.* **115,** 781–786.
19. Weiser, W. Y., Temple, P. A., Witek-Gianotti, J. S., Remold, H. G., Clark, S. C., and David, J. R. (1989). Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor. *Proc. Natl. Acad. Sci. USA* **86,** 7522–7526.
20. Thurman, G. B., Braude, I. A., Gray, P. W., Oldham, R. K., and Stevenson, H. C. (1985). MIF-like activity of natural and recombinant human interferon-gamma and their neutralization by monoclonal antibody. *J. Immunol.* **134,** 305–309.
21. McInnes, A., and Rennick, D. M. (1988). Interleukin 4 induces cultured monocytes/macrophages to form giant multinucleated cells. *J. Exp. Med.* **167,** 598–611.
22. David, J. R. (1993). Erratum: Important note on recombinant macrophage migration inhibition factor. *J. Immunol.* **151.**
23. David, J. R. (1993). Corrections, Immunology. *Proc. Natl. Acad. Sci. USA* **90,** 12056.
24. Bernhagen, J., Mitchell, R. A., Calandra, T., Voelter, W., Cerami, A., and Bucala, R. (1994). Purification, bioactivity, and secondary structure analysis of mouse and human macrophage migration inhibitory factor (MIF). *Biochemistry* **33,** 14144–14155.
25. Kosak, C. A., Adamson, M. C., Buckler, C. E., Segovia, L., Paralkar, V., and Wistow, G. (1995). Genomic cloning of mouse MIF (macrophage inhibitory factor) and genetic mapping of the human and mouse expressed gene and nine mouse pseudogenes. *Genomics* **27,** 405–411.
26. Mitchell, R., Bacher, M., Bernhagen, J., Pushkarskaya, T., Seldin, M., and Bucala, R. (1995). Cloning and characterization of the gene for mouse macrophage migration inhibitory factor (MIF). *J. Immunol.* **154,** 3863–3870.
27. Parkalkar, V., and Wistow, G. (1994). Cloning the human gene for macrophage migration inhibitory factor (MIF). *Genomics* **19,** 48–51.
28. Sakai, M., Nishihara, J., Hibiya, Y., Koyama, Y., and Nishi, S. (1994). Glutathione binding rat liver 13K protein is the homologue of the macrophage migration inhibitory factor. *Biochem. Mol. Biol. Int.* **33,** 439–446.
29. Bacher, M., Meinhardt, A., Lan, H. Y., Mu, W., Metz, C. N., Chesney, J. A., Calandra, T., Gemsa, D., Donnelly, T., Atkins, R. C., and Bucala, R. (1997). Migration inhibitory factor expression in experimentally induced endotoxemia. *Am. J. Pathol.* **150,** 235–246.
30. Calandra, T., Bernhagen, J., Mitchell, R. A., and Bucala, R. (1994). The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. *J. Exp. Med.* **179,** 1895–1902.
31. Bacher, M., Metz, C. N., Calandra, T., Mayer, K., Chesney, J., Lohoff, M., Gemsa, D., Donnelly, T., and Bucala, R. (1996). An essential regulatory role for MIF in T-cell activation. *Proc. Natl. Acad. Sci. USA* **93,** 7849–7854.
32. Thorner, M. O., Vance, M. L., Horvath, E. T.,and Kovacs, K. (1992). *In* "Williams Textbook of Endocrinology," 8th ed., Chap. 6, pp. 221–310. Saunders, Philadelphia.
33. Frantz, A. G., and Wilson, J. D. (1992). *In* "Williams Textbook of Endocrinology," 8th ed., Chap. 15, pp. 953–975. Saunders, Philadelphia.
34. Nishino, T., Bernhagen, J., Shiiki, H., Calandra, T., Dohi, K., and Bucala, R. (1995). Localization of macrophage migration inhibitory factor (MIF) to secretory granules within the corticotrophic and thyrotrophic cells of the pituitary gland. *Mol. Med.* **1,** 781–788.
35. Bernhagen, J., Bacher, M., Calandra, T., Metz, C. N., Doty, S., Donnelly, T., and Bucala, R. (1996). An essential role for macrophage migration inhibitory factor (MIF) in the tuberculin delayed-type hypersensitivity reaction. *J. Exp. Med.* **183,** 277–282.

36. Rahman, I., Haslett, C., Rossi, A., Wallace, W., Bucala, R., Metz, C., and Donnelly, S. C. (1996). Epithelial cells are a source of macrophage migration inhibitory factor (MIF)—Potential role in acute respiratory distress syndrome (ARDS). *Thorax* **51,** A54.
37. Donnelly, S. C., Haslett, C., Reid, P. T., Grant, I. S., Wallace, W. A. H., Metz, C. N., Bruce, L. J., and Bucala, R. (1997). Regulatory role for macrophage migration inhibitory factor (MIF) in the acute respiratory distress syndrome (ARDS). *Nature Med.*, **3,** 320–323.
38. Shimizu, T., Ohkawara, A., Nishihira, J., and Sakamoto, W. (1996). Identification of macrophage migration inhibitory factor (MIF) in human skin and its immunohistochemical localization. *FEBS Lett.* **381,** 199–202.
39. Barker, J. N., Mitra, R. S., Griffiths, C. E., Dixit, V. M., and Nickoloff, B. J. (1991). Keratinocytes as initiators of inflammation. *Lancet* **337,** 211–214.
40. Meinhardt, A., Bacher, M., McFarlane, J. R., Metz, C. N., Seitz, J., Hedger, M. P., de Kretser, D. M., and Bucala, R. (1996). Macrophage migration inhibitory factor (MIF) production by leydig cells: Evidence for a role in the regulation of testicular function. *Endocrinology* **137,** 5090–5095.
41. Wistow, G., Shaughnessy, M. P., Lee, D. C., Hodin, J., and Zelenka, P. S. (1993). A macrophage migration inhibitory factor is expressed in the differentiating cells of the eye lens. *Proc. Natl. Acad. Sci. USA* **90,** 1272–1275.
42. Matsuda, A., Tagawa, Y., Matsuda, H., and Nishihara, J. (1996). Identification and immunolocalization of macrophage migration inhibitory factor in human cornea. *FEBS Lett.* **385,** 225–228.
43. Waeber, G., Calandra, T., Roduit, R., Haefliger, J. A., Thompson, N., Thorens, B., Temler, E., Meinhardt, A., Bacher, M., Metz, C. N., Nicod, P., and Bucala, R. (1997). Insulin secretion is regulated by the glucose-dependent production of islet β-cell MIF. *Proc. Natl. Acad. Sci. USA,* **94,** 4782–4787.
44. Bacher, M., *et al.* (1997). MIF expression in the rat brain. Manuscript in preparation.
45. Gillis, S., Crabtree, G. R., and Smith, K. A. (1979). Glucocorticoid-induced inhibition of T cell growth factor production. *J. Immunol.* **123,** 1624–1631.
46. Beutler, B., Krochin, N., Milsark, I. W., Luedke, C., and Cerami, A. (1986). Control of cachectin (tumor necrosis factor) synthesis: Mechanisms of endotoxin resistance. *Science* **232,** 977–980.
47. Knudsen, P. J., Dinarello, C. A., and Strom, T. B. (1987). Glucocorticoids inhibit transcriptional and post-transcriptional expression of interleukin 1 in U937 cells. *J. Immunol.* **139,** 4129–4134.
48. Besedovsky, H., Del Rey, A., Sorkin, E., and Dinarello, C. A. (1986). Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. *Science* **233,** 652–654.
49. Hooke, D. H., Gee, D. C., and Atkins, R. C. (1987). Leukocyte analysis using monoclonal antibodies in human glomerulonephritis. *Kidney Int.* **31,** 964–972.
50. Main, I. W., Nikolic-Paterson, D. J., and Atkins, R. C. (1992). T cells and macrophages and their role in renal injury. *Sem. in Nephrol.* **12,** 428–440.
51. Cattell, V. (1994). Macrophages in acute glomerular inflammation. *Kidney Int.* **45,** 945–952.
52. Van Goor, H., Ding, G., Kees-Folts, D., Grond, J., Schreiner, G. F., and Diamond, J. R. (1994). Macrophages and renal disease. *Lab. Invest.* **71,** 456–464.
53. Lan, H. Y., Nikolic-Paterson, D. J., Mu, W., and Atkins, R. (1995). Local macrophage proliferation in the progression of glomerular tubulointerstitial injury in rat anti-GBM glomerulonephritis. *Kidney Int.* **48,** 753–760.
54. Lan, H. Y., Nikolic-Paterson, D. J., and Atkins, R. C. (1995). Local macrophage porliferation in experimental Goodpasture's syndrome. *Nephrol.* **1,** 151–156.

55. Lan, H. Y., Mu, W., Yang, N., Meinhardt, A., Nikolic-Paterson, D. J., Ng, Y. Y., Bacher, M., Atkins, R. C., and Bucala, R. (1996). De novo renal expression of macrophage migration inhibitory factor during the development of rat crescentic glomerulonephritis. *Am. J. Pathol.* **149,** 1119–1127.
56. Lan, H. Y., Bacher, M., Yang, N., Mu, W., Nikolic-Paterson, D. J., Metz, C., Meinhardt, A., Bucala, R., and Atkins, R. C. (1997). The pathogenic role of MIF in immunologically-induced kidney disease in the rat. *J. Exp. Med.,* **185,** 1455–1465.
57. Zvaifler, N. J. (1995). Macrophages and the synovial lining. *Scand. J. Rheumatol. Suppl.* **101,** 67–75.
58. Mikulowska, A., Metz, C., Bucala, R., and Holmdahl, R. (1997). Macrophage migration inhibitory factor is involved in the pathogenesis of collagen type II arthritis in mice. *J. Immunol.,* **158,** 5514–5517.
59. Kollef, M. H., and Schuster, D. P. (1995). The acute respiratory distress syndrome. *N. Engl. J. Med.* **332,** 27–37.
60. Suter, P. M., Suter, S., Girardin, E., Roux-Lombard, P., Grau, G. E., and Dayer, J. M. (1992). High bronchoalveolar levels of tumor necrosis factor and its inhibitors, interleukin 1, interferon, and elastase in patients with adult respiratory distress syndrome after trauma, shock, or sepsis. *Am. Rev. Respir. Dis.* **145,** 1016–1022.
61. Donnelly, S. C., Streiter, R. M., Kunkel, S. L., Walz, A., Robertson, C. R., Carter, D. C., Grant, I. S., Pollak, A. J., and Haslett, C. (1993). Interleukin-8 (IL-8) and the development of adult respiratory distress syndrome (ARDS) in at-risk patient groups. *Lancet* **341,** 643–647.
62. Cross, C., Frei, B., and Louie, S. (1990). The adult respiratory distress syndrome (ARDS) and oxidative stress: Therapeutic implications. *Adv. Exp. Med. Biol.* **264,** 435–438.
63. Sibbald, W. J., Anderson, R. R., Reid, B., Holliday, R. L., and Driedger,A. A. (1981). Alveolar–capillary permeability in human septic ARDS: Effect of high dose corticosteroid therapy. *Chest* **79,** 133–142.
64. Martiney, J. A., Metz, C., Espinoza, M., Calandra, T., and Bucala, R. (1996). Role of macrophage migration inhibitory factor (MIF) in the pathophysiology of lethal mouse malaria infection. ASBMB/ASIP/AAI Joint Meeting. [Abstract.]
65. Balcerzak, S. P., Arnold, J. D., and Martin, D. C. (1972). Anatomy of red cell damage by *Plasmodium falciparum in man. Blood* **40,** 98–104.
66. Looaresuwan, S., Merry, A. H., Phillips, R. E., Pleehachinda, R., Wattanagoon, M., Ho, M., Charoenlarp, P., Warrell, D. A., and Weatherall, D. (1987). Reduced erythrocyte survival following clearance of malarial parasitemia in Thai patients. *Br. J. Hematol.* **67,** 473–478.
67. Abdalla, S. (1984). Hematopoiesis in human malaria. *Blood Cells* **16,** 401–416.
68. Dormer, P., Dietrich, M., Kern, P., and Horstmann, R. D. (1983). Ineffective erythropoiesis in acute human *Plasmodium falciparum malaria. Blut.* **46,** 279–288.
69. Buchard, G.-D., Radloff, P., Philipps, J., Nkeyi, M., Knobloch, J., and Kremsner, P. G. (1995). Increased erythropoietin production in children with severe malarial anemia. *Am. J. Trop. Med. Hyg.* **53,** 547–551.
70. Yap, G. S., and Stevenson, M. M. (1994). Inhibition of in vitro erythropoiesis by soluble mediators in *Plasmodium chabaudi AS* malaria: Lack of a major role for interleukin-1, tumor necrosis factor alpha, and gamma interferon. *Infect. Immun.* **62,** 357–362.
71. Sun, H., Bernhagen, J., Bucala, R., and Lolis, E. (1996). Crystal structure at 2.6Å resolution of human macrophage migration inhibitory factor. *Proc. Natl. Acad. Sci. USA* **93,** 5191–5196.
72. Kato, Y., Muto, T., Tomura, T., Tsumura, H., Watarai, H., Mikayama, T., Ishizaka, K., and Kuroki, R. (1996). The crystal structure of human glycosylation-inhibiting factor

is a trimeric barrel with three 6-stranded B-sheets. *Proc. Natl. Acad. Sci. USA* **93,** 3007–3010.

73. Suzuki, M., Sugimoto, H., Nakagawa, A., Tanaka, I., Nishihira, J., and Sakai, M. (1996). Crystal structure of the macrophage migration inhibitory factor from rat liver. *Nature Struct. Biol.* **3,** 259–266.
74. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329,** 506–512.
75. Baldwin, E. T., Weber, I. T., St. Charles, R., Xuan, J. C., Appella, E., Yamada, M., Matsushima, K., Edwards, B. F., Clore, G. M., Gronenborn, A. M., and Wlodawer, A. (1991). Crystal structure of interleukin 8: Symbiosis of NMR and crystallography. *Proc. Natl. Acad. Sci. USA* **88,** 502–506.
76. Blocki, F. A., Schlievert, P. M., and Wackett, L. P. (1992). Rat liver protein linking chemical and immunological detoxification system. *Nature* **360,** 269–270.
77. Pearson, W. R. (1994). MIF proteins are not glutathione transferase homologs. *Prot. Sci.* **3,** 525–527.
78. Nishihira, J., Kuriyama, T., Sakai, M., Nishi, S., Ohki, S.-K., and Hikichi, K. (1995). The structure and physiochemical properties of rat liver macrophage migration inhibitory factor. *Biochem. Biophys. Acta* **1247,** 159–162.
79. Subramanya, H. S., Roper, D. I., Dauter, Z., Dodson, E. J., Davies, G. J., Wilson, K. S., and Wigley, D. B. (1996). Enzymatic ketonization of 2-hydroxymuconate: Specificity and mechanism investigated by the crystal structures of two isomerases. *Biochemistry* **35,** 792–802.
80. Rosengren, E., Bucala, R., Aman, P., Jacobsson, L., Odh, G., Metz, C. N., and Rorsman, H. (1996). The immunoregulatory mediator macrophage migration inhibitory factor (MIF) catalyzes a tautomerization reaction. *Mol. Med.* **2,** 143–149.
81. Arya, S. K., Wong-Staal, F., and Gallo, R. C. (1984). Dexamethasone-mediated inhibition of human T cell growth factor and gamma interferon messenger RNA. *J. Immunol.* **133,** 273–377.
82. Lew, W., Oppenheim, J. J., and Matsushima, K. A. (1988). Analysis of the suppression of IL-1 alpha and IL-1 beta production in human peripheral blood mononuclear adherent cells by a glucocorticoid hormone. *J. Immunol.* **140,** 1895–1902.
83. Scheinman, R. I., Cogswell, P. C., Lofquist, A. K., and Baldwin, A. S. (1995). Role of transcriptional activation of IκBα in mediation of immunosuppression of glucocorticoids. *Science* **270,** 283–286.
84. Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A., and Karin, M. (1995). Immunosuppression by glucocorticoids: Inhibition of NF-κB activity through induction of IκB synthesis. *Science* **270,** 286–290.
85. Bone, R. C., Fisher, C. J., Clemmer, T. P., Slotman, G. J., Metz, C. A., and Balk, R. A. (1987). A controlled clinical trial of high-dose methylprednisone in the treatment of severe sepsis and septic shock. *N. Engl. J. Med.* **317,** 653–658.
86. The Veterans Administration Systemic Sepsis Cooperative Study Group (1987). Effect of high-dose glucocorticoid therapy on mortality in patients with clinical signs of systemic sepsis. *N. Engl. J. Med.* **317,** 659–665.
87. Rossi, A. G., Haslett, C., Metz, C. N., Rahman, I., Bucala, R., and Donnelly, S. C. (1997). Eosinophils secrete macrophage migration inhibitory factor (MIF)—Potential role in asthma. ALA/ATS International Conference. [Abstract]
88. Galat, A., Riviere, S., Bouet, F., and Menez, A. (1994). A diversified family of 12 kDa proteins with a high amino acid sequence similarity to macrophage migration inhibitory factor (MIF). *Eur. J. Biochem.* **224,** 417–421.

This article was accepted for publication on March 19, 1997.

ADVANCES IN IMMUNOLOGY, VOL. 66

The Intrinsic Coagulation/Kinin-Forming Cascade: Assembly in Plasma and Cell Surfaces in Inflammation

ALLEN P. KAPLAN, KUSUMAM JOSEPH, YOJI SHIBAYAMA,[1] SESHA REDDIGARI, BERHANE GHEBREHIWET, and MICHAEL SILVERBERG

Division of Allergy and Clinical Immunology, Department of Medicine, State University of New York, Stony Brook, New York 11794-8161

I. Introduction

The plasma kinin-forming system consists of three essential plasma proteins that interact upon binding to certain negatively charged surfaces or macromolecular complexes. These are coagulation factor XII (Hageman factor or HF), prekallikrein, and high-molecular-weight kininogen (HK). Once factor XII is activated to factor XIIa, it converts plasma prekallikrein to kallikrein and kallikrein digests HK to liberate the vasoactive peptide, bradykinin. Factor XIIa also converts coagulation factor XI to factor XIa to continue the intrinsic coagulation cascade. The interactions of all four of these proteins to initiate blood clotting is known as "contact activation"; thus, the formation of bradykinin is a cleavage product of the initiating step of this cascade (Fig. 1).

For optimal activation of factor XII, however, both prekallikrein and HK must be present. Thus, the rate of factor XII conversion to factor XIIa is dependent on each protein of the kinin-forming pathway and, in this sense, all the proteins are coagulation factors. This intrinsic coagulation/kinin-forming cascade appears to be in equilibrium in plasma even in the absence of any exogenous surface, i.e., there is a finite rate at which activation occurs continuously, but plasma inhibitors hold the system in check. The addition of a macromolecular surface augments the activation rate such that significant generation of bradykinin occurs; then a combination of kininases and protease inhibitors reestablish the equilibrium. In recent years, it has become evident that certain blood cells and vascular wall endothelial cells are capable of binding the proteins of the kinin-forming cascade. These reactions require zinc ion. Thus, activation may preferentially occur along the cell surface and some of the binding proteins may actually function as endogenous activators. These new concepts will be reviewed, the structure and functions of the individual components will be summarized, and their role in inflammation discussed.

[1] Visiting Research Associate, Nippon Zoki Pharmaceuticals, Osaka, Japan.

0065-2776/97 $25.00

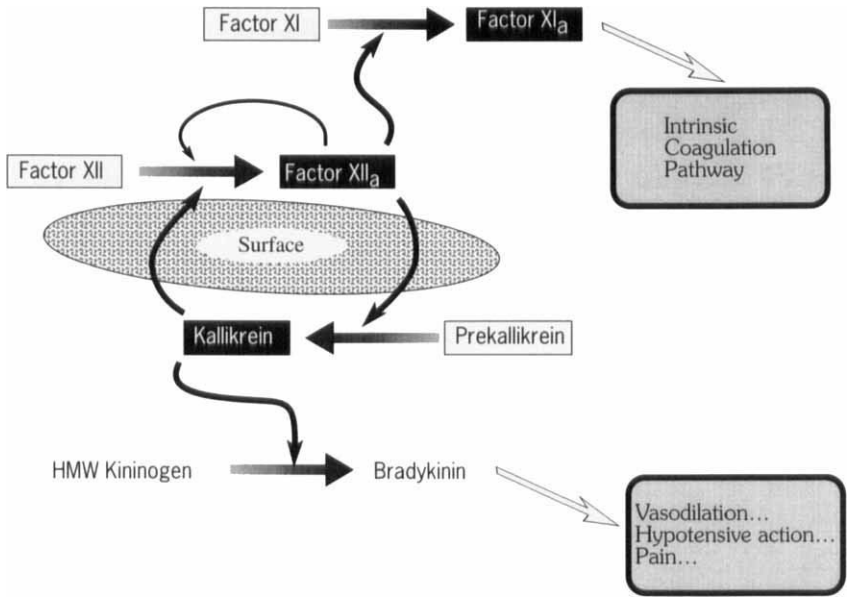


FIG. 1. Relationship of bradykinin formation to the initiation of the intrinsic coagulation cascade.

II. Protein Constituents

A. FACTOR XII

Factor XII circulates as a single-chain zymogen with no detectable enzymatic activity (Silverberg and Kaplan, 1982). It has a molecular weight of 80,000 on SDS gel electrophoresis (Revak *et al.*, 1974) (Table I), is synthe-

TABLE I
PHYSICO-CHEMICAL PROPERTIES OF PROTEINS OF THE CONTACT ACTIVATION CASCADE

Protein	Factor XII	Prekallikrein	Factor XI	High-molecular-weight kininogen
Molecular weight (Da; calculated)	80,427	79,545	140,000	116,643
Carbohydrate (w/w) (%)	16.8	15	5	40
Isoelectric point	6.3	8.7	8.6	4.7
Extinction coefficient ($E^{1\%}$ 280/nm)	14.2	11.7	13.4	7.0
Plasma concentration				
μg/ml	30–45	35–50	4–6	70–90
nmol/liter (average)	400	534	36	686

sized in the liver, and circulates in plasma at a concentration of 30–35 μg/ml. Its primary sequence has been deduced from cDNA analysis (Cool *et al.*, 1985; Que and Davie, 1986) and from direct protein sequence data (Fujikawa and McMullen, 1983; McMullen and Fujikawa, 1985). The 596 amino acids present account for a molecular weight of 66,915; the remainder (16.8%) is carbohydrate. The protein has distinct domains homologous to fibronectin, plasminogen, and plasminogen activators (Castellino and Beals, 1987; Cool *et al.*, 1985) at its N-terminal end, whereas the C terminus has the catalytic domain. This latter portion is homologous to serine proteases such as pancreatic trypsin and even more so to the catalytic domain of plasminogen activators.

Factor XII is unusual because it is capable of autoactivating once bound to initiating "surfaces" (Silverberg *et al.*, 1980a; Tankersley and Finlayson, 1984). Thus, factor XII that is bound undergoes a conformational change that renders it a substrate for factor XIIa (Griffin, 1978). Gradually, all the bound factor XII can be converted to factor XIIa. Whether plasma normally has a trace of factor XIIa present is unknown, but if so its concentration is less than 0.01% of that of factor XII. The alternative is that the first molecule of factor XIIa is formed by interaction of two factor XII zymogen molecules on the surface, but this presumes some minimal activity present in the zymogen. If so, it is below our limits of detection and we favor the former scenario (Silverberg and Kaplan, 1982).

Activation of factor XII is due to cleavage of the molecule at a critical Arg-Val bond (Fujikawa and McMullen, 1983) contained within a disulfide bridge such that the resultant factor XIIa is a two-chain, disulfide-linked 80-kDa enzyme consisting of a heavy chain of 50 kDa and a light chain of 28 kDa (Revak *et al.*, 1977). The light chain contains the enzymatic active site (Meier *et al.*, 1977) and is at the carboxy-terminal end, whereas the heavy chain contains the binding site for the surface and is at the amino-terminal end (Pixley *et al.*, 1987). Further cleavage can occur at the C-terminal end of the heavy chain to produce a series of fragments of activated factor XII that retain enzymatic activity (Kaplan and Austen, 1970; 1971). The most prominent of these is a 30-kDa species termed factor XII fragment (factor XII_f). Careful examination of Factor XII_f on SDS gels under nonreducing conditions reveals a doublet in which the higher band at 30 kDa is gradually converted to the lower band, which has a molecular weight of 28.5 kDa (Dunn and Kaplan, 1982). Reduced gels demonstrate that these two forms of factor XII_f are composed of the light chain of factor XIIa and a very small piece of the original heavy chain. Factor XII_f lacks the binding site to the surface as well as the ability of factor XIIa to convert factor XI to factor XIa, and does not participate in factor XII autoactivation. However, these fragments remain potent activa-

tors of prekallikrein (Kaplan and Austen, 1970). Thus, formation of factor XII_f allows bradykinin production to continue until the enzyme is inactivated and the reactions can proceed at sites distant from the initiating surface. A diagrammatic representation of the cleavages in factor XII to generate factor XIIa and the two forms of factor XII_f is shown in Fig. 2.

Once factor XIIa interacts with prekallikrein, rapid conversion to kallikrein ensues followed by an important positive feedback in which kallikrein digests surface-bound factor XII to form factor XIIa and then factor XII_f (Cochrane *et al.*, 1973; Dunn *et al.*, 1982; Meier *et al.*, 1977). This reaction is 50–2000 times more rapid than the autoactivation reaction (Dunn *et al.*, 1982; Tankersley and Finlayson, 1984) depending on the particular surface utilized. Thus, quantitatively, most of the factor XIIa or factor XII_f activity generated when plasma is activated is a result of kallikrein activation of factor XII. However, the autoactivation phenomenon can be demonstrated in plasma that is congenitally deficient in prekallikrein (Fletcher trait) and cannot therefore generate any bradykinin (Saito *et al.*, 1974; Weiss *et al.*, 1974; Wuepper, 1973). Blood coagulation (i.e., conversion of factor XI to factor XIa by factor XIIa) does proceed, albeit at a much

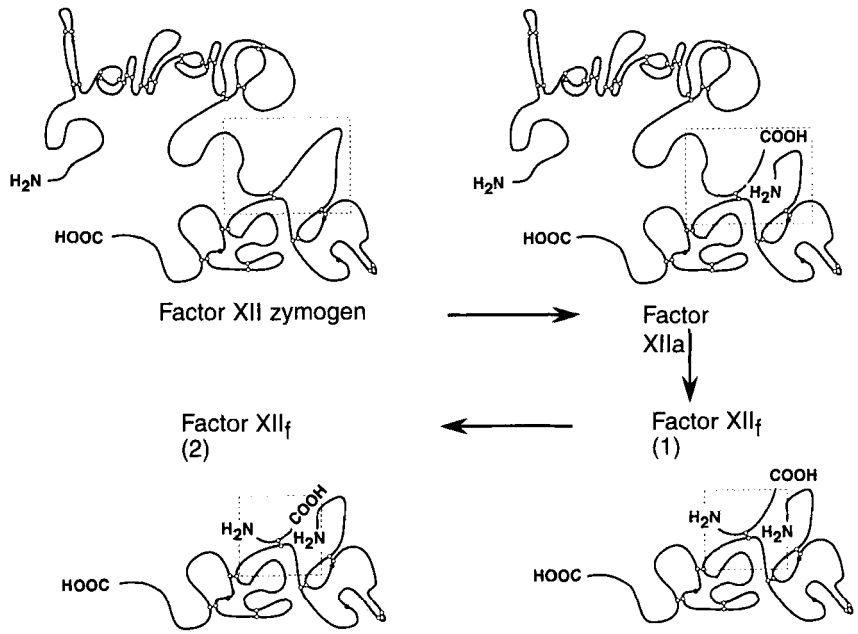


FIG. 2. Cleavage sites upon activation of human Hageman factor (factor XII) to form factor XIIa and then factor XIIf.

slower rate, and the partial thromboplastin time (PTT) can be shown to progressively shorten as the time of incubation of the plasma with the surface is increased prior to recalcification. As more factor XIIa forms by autoactivation, the rate of factor XI activation increases and the PTT approaches normal.

B. Prekallikrein

Prekallikrein is also a zymogen without detectable proteolytic activity that is converted to kallikrein by cleavage during contact activation (Mandle and Kaplan, 1977). On SDS gels, it has two bands at 88 and 85 kDa. The entire amino acid sequence of the protein has been determined by a combination of direct protein sequencing and amino acid sequence prediction from cDNAs isolated from a λgt-11 expression library (Chung *et al.*, 1986). A signal peptide of 19 residues (which is cleaved off prior to secretion) is followed by the sequence of the mature plasma prekallikrein, which has 619 amino acids with a calculated molecular weight of 69,710. There is 15% carbohydrate as well. The heterogeneity observed by SDS gel electrophoresis is not reflected in the amino acid sequence; thus, it may be due to variation in glycosylation. Activation of prekallikrein by factor XIIa or factor XII_f is due to cleavage of a single Arg-Ile bond within a disulfide bridge such that a heavy chain of 56 kDa is disulfide linked to a light chain of either 33 or 36 kDa, each of which has a DFP-inhibitable active site (Bouma *et al.*, 1980; Mandle and Kaplan, 1977). This light chain heterogeneity reflects the two forms of the zymogen.

The amino acid sequence of the kallikrein heavy chain is unusual and is homologous only to the corresponding portion of factor XI. It has four tandem repeats, each of which contains approximately 90 or 91 amino acids. The presence of six cysteines per repeat suggests a repeating structure with three disulfide loops. It is postulated that a gene coding for the ancestor of this repeat sequence duplicated and then the entire segment duplicated again to give the current structure. The light chain, containing the active site, is homologous to many of the catalytic domains of other enzymes of the coagulation cascade.

In contrast to factor XII, prekallikrein does not circulate as a separate protein. It is bound to HK in a 1 : 1 bimolecular complex through a site on its heavy chain. The binding is firm, with a dissociation constant of 12–15 n*M* (Bock *et al.*, 1985; Mandle *et al.*, 1976), and this is unchanged upon conversion of prekallikrein to kallikrein. Thus, about 80–90% of prekallikrein is normally complexed to HK in plasma (Reddigari and Kaplan, 1989b). It is the prekallikrein–HK complex that binds to surfaces during contact activation and the binding is primarily through HK (Wiggins *et al.*, 1977), although some interaction of prekallikrein with the surface

can be inferred (McMillin *et al.*, 1974). The dissociation of 10–20% of the kallikrein that forms along the surface may serve to propagate the formation of bradykinin in the fluid phase and at sites distant from the initiating reaction (Cochrane and Revak, 1980; Silverberg *et al.*, 1980b).

C. Factor XI

Coagulation factor XI is the second substrate of factor XIIa (Fig. 1), but it has no role in bradykinin formation. Factor XI is unique among the clotting factors because the circulating zymogen consists of two identical chains linked by disulfide bonds (Bouma and Griffin, 1977; Kurachi and Davie, 1977). The dimer has an apparent molecular weight of 160 kDa on SDS gel electrophoresis but reveals a single 80-kDa protein upon reduction. Factor XI activation follows the familiar pattern of cleavage of a single peptide band (Arg-Ile) within a disulfide bridge to yield an amino terminal heavy chain of 50 kDa and a disulfide-linked light chain of 33 kDa. Because both subunits can be cleaved by factor XIIa and each resultant light chain bears a functional active site, factor XIa is a four-chain protein with two active sites. The concentration in plasma is only 4–8 μg/ml, the lowest among the contact proteins, and its heavy chain(s), like that of kallikrein, binds to the light chain of HK. Thus, factor XI and HK also circulate as a complex (Thompson *et al.*, 1977). The dissociation constant is 70 n*M* (Tait and Fujikawa, 1987), which is high enough to ensure that virtually all the Factor XI is complexed. The molar ratio of the complex can consist of one or two molecules of HK per factor XI because of the dimeric nature of factor XI (Warn-Cramer and Bajaj, 1985). The binding site for HK on factor XI has been localized to the first (N-terminal) tandem repeat (Baglia *et al.*, 1989). The factor XI–HK complex binds to the surface and conversion to factor XIa must occur on the surface; fluid phase conversion by factor XII_f is only 2–4% of that of surface-bound factor XIIa (Kaplan and Austen, 1971). The primary function of factor XIa is to activate factor IX to IXa, which is the first calcium-dependent reaction in the intrinsic coagulation cascade.

The amino acid sequence of human factor XI has been determined by cDNA analysis. It has an 18-amino-acid leader peptide followed by a 607-amino acid sequence for each of the two chains of the mature protein. The amino acid sequence of the heavy chain of factor XIa, like that of kallikrein, has four tandem repeats of about 90 amino acids with six cysteines/repeats implying three disulfide bands. Unpaired cysteines in the first and fourth repeats are postulated to form the interchain disulfide bridges between monomers to produce the homodimer.

D. High-Molecular-Weight Kininogen

HK circulates in plasma as a 115-kDa nonenzymatic glycoprotein at a concentration of 70–90 μg/ml (Adam *et al.*, 1985; Berrettini *et al.*, 1986;

Colman and Muller-Esterl, 1988; Proud *et al.*, 1980; Reddigari and Kaplan, 1989b). Its apparent molecular weight by gel filtration is aberrant at about 200,000, indicative of a large partial specific volume due to its conformation in solution (Mandle *et al.*, 1976). It forms noncovalent complexes with both prekallikrein and factor XI with dissociation constants of 15 n*M* (Bock and Shore, 1983; Bock *et al.*, 1985) and 70 n*M* (Tait and Fujikawa, 1987; Thompson *et al.*, 1979), respectively. There is sufficient HK in plasma to theoretically bind both factor XII substrates, and the excess HK (about 10–20%) circulates uncomplexed. The complexes of HK with prekallikrein or factor XI are formed with the light chain region of HK; the isolated light chain (after reduction and alkylation) possesses the same binding characteristics as the whole molecule (Thompson *et al.*, 1978, 1979). HK functions as a coagulation cofactor and this activity resides in the light chain (Thompson *et al.*, 1978), which consists of a basic (histidine-rich) amino-terminal domain that binds to initiating surfaces (Ikari *et al.*, 1981) and a carboxy-terminal domain that binds prekallikrein or factor XI (Tait and Fujikawa, 1986). The one cysteine in the light chain links it to the heavy chain. The prekallikrein binding site maps to residues 194–224 (Tait and Fujikawa, 1986, 1987) and the factor XI site to residues 185–242 (Tait and Fujikawa, 1987). Because these sites overlap, one molecule of HK can bind only one molecule of either prekallikrein or factor XI at a time.

During contact activation, kallikrein cleaves HK at two positions within a disulfide bridge; first at the C-terminal Arg-Ser (Mori and Nagasawa, 1981; Mori *et al.*, 1981) and then at the N-terminal Lys-Arg to release the nonapeptide bradykinin (ArgProProGlyPheSerProPheArg). A two-chain disulfide-linked kinin-free HK results that consists of a heavy chain of 65,000 and a light chain variously reported at molecular weights of 56–62 kDa. A subsequent further cleavage of the light chain yields a final product of 46–49 kDa (Bock and Shore, 1983; Mori and Nagasawa, 1981; Mori *et al.*, 1981; Reddigari and Kaplan, 1988; Schiffman *et al.*, 1980), which retains all light chain functions. Tissue kallikrein can also digest HK to liberate kallidin or Lys-bradykinin, leaving the heavy chain disulfide linked to the 56- to 62-kDa light chain; the additional cleavage of the light chain is not made by this enzyme (Reddigari and Kaplan, 1988). It is important to note that tissue kallikrein is immunologically and structurally unrelated to plasma kallikrein. It is secreted by various organs or cells, such as salivary glands, kidney, pancreas, prostate, pituitary gland, and neutrophils, and is found in high concentrations in saliva, urine, and prostatic fluid. Its primary substrate is low-molecular-weight kininogen (LK), but it can release kallidin from either HK or LK. Kallidin is functionally very similar to bradykinin, albeit slightly less potent. A plasma aminopeptidase (Guimaraes *et al.*, 1973) removes the N-terminal Lys to convert it to bradykinin.

The very unusual domain structure of HK is shown in Fig. 3. Domain 5, the histidine-rich region at the N-terminal end of the light chain, binds to initiating surfaces, whereas the binding of prekallikrein or factor XI at the C-terminal domain 6 of the light chain accounts for the cofactor function of HK in intrinsic coagulation and kinin generation. The complete amino acid sequence of HK has been determined as translated from the cDNA as well as by direct sequence analysis of the purified protein (Kellermann *et al.*, 1986; Kitamura *et al.*, 1985; Lottspeich *et al.*, 1985; Takagaki *et al.*, 1985). HK has 626 amino acids with a calculated molecular weight of 69,896. An unusually high content of carbohydrate accounts for 40% of the observed molecular weight of 115 kDa. The heavy chain of 362 residues is derived from the N terminus. This is followed by the 9-residue bradykinin (domain 4) sequence and then the light chain of 265 residues. The N-terminal end is blocked with pyroglutamic acid (cyclic glutamate). The carbohydrate is distributed via three N-linked glycosidic linkages on the H chain and nine O-linked glycosidic linkages on the L chain. The H chain has three contiguous and homologous "apple"-type domains consisting of residues 1–116, 117–238, and 239–360 (Fig. 3). There are 17 cysteines, 1 of which is disulfide linked to the L chain and the others are linked from 8 disulfide loops within these domains (Kellermann *et al.*, 1986). The three domains on the heavy chain are homologous to the cystatin family of protease inhibitors (includes sulfhydryl proteases such as cathepsins B, H, and L). Domains 2 and 3 (but not 1) retain this inhibitory function; for example, native HK can bind and inactivate two molecules of papain (Gounaris *et al.*, 1984; Higashiyama *et al.*, 1986;

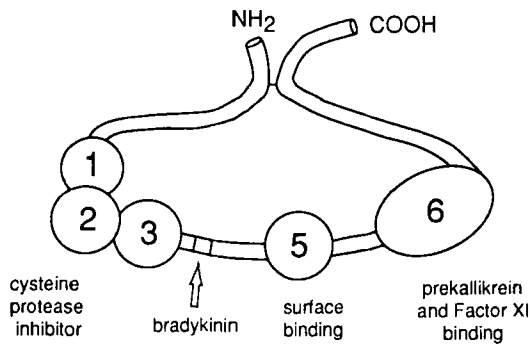


FIG. 3. The structure of high-molecular weight (HMW) kininogen. The heavy chain region consists of three homologous domains (1–3) of which the latter two are sulfhydryl protease inhibition sites. Domain 4 contains the bradykinin moiety. The light chain region contains the surface binding site (domain 5) and overlapping binding sites for prekallikrein and factor XII (domain 6).

Ishiguro *et al.*, 1987; Müller-Esterl *et al.*, 1985a). Limited proteolysis of the heavy chain can occur at susceptible bonds that separate the domains so that individual domains can be isolated. Cleavage at these sites may occur under certain pathologic conditions.

E. Low-Molecular-Weight Kininogen and Kininogen Genes

Plasma contains another precursor of bradykinin (MW 68 kDa) known as LK. Its digestion by tissue kallikrein yields Lys-bradykinin and a kinin-free two-chain molecule consisting of a 65-kDa heavy chain disulfide linked to a light chain of only 4 kDa (Jacobsen and Kritz, 1967; Johnson *et al.*, 1987; Kellermann *et al.*, 1986; Lottspeich *et al.*, 1985; Müller-Esterl *et al.*, 1985b). LK is not cleaved by plasma kallikrein. The amino acid sequences of HK and LK are identical from the amino terminus through the bradykinin sequence plus the next 12 residues (Müller-Esterl *et al.*, 1985b), after which the two sequences diverge. Thus, LK does not bind to surfaces or to prekallikrein or factor XI. The kininogens are produced from a single gene thought to have originated by two successive duplications of a primordial cystatin-like gene (Kitamura *et al.*, 1985). As shown in Fig. 4, there are 11 exons. The first 9 code for the heavy chain and each of the three domains in this portion of the protein is represented by 3 exons. The tenth exon codes for bradykinin and the light chain of HK, whereas the light chain of LK is encoded by exon 11. The mRNAs for HK and LK are produced by alternative splicing at a point 12 amino acids beyond the bradykinin sequence, thus enabling the two proteins to have different light chains (Fig. 4).

III. Mechanisms of Bradykinin Formation

A. Contact Activation

Contact activation was initially observed by the interaction of blood with glass surfaces (Margolis, 1958); subsequently, finely divided kaolin was used extensively as an experimental surface and for coagulation assays such as the partial thromboplastin time (Proctor and Rapaport, 1961). Ellagic acid (Ratnoff and Crum, 1964), a tannin-like substance used as a component of many commercial assay systems, was purported to be a soluble initiator but was later shown to form large sedimentable aggregates catalyzed by trace heavy metal ions, so it too is particulate (Bock *et al.*, 1981). Subsequently, dextran sulfate (Fujikawa *et al.*, 1980; Kluft, 1978) and sulfatide (Fujikawa *et al.*, 1980) have been used to study contact activation. Although sulfatide, a galactose sulfate sphingolipid found in nerve tissue, is an activator, it occurs in quantities too small to be an effective activator. However, when purified, it can form highly charged micelles that are very efficient

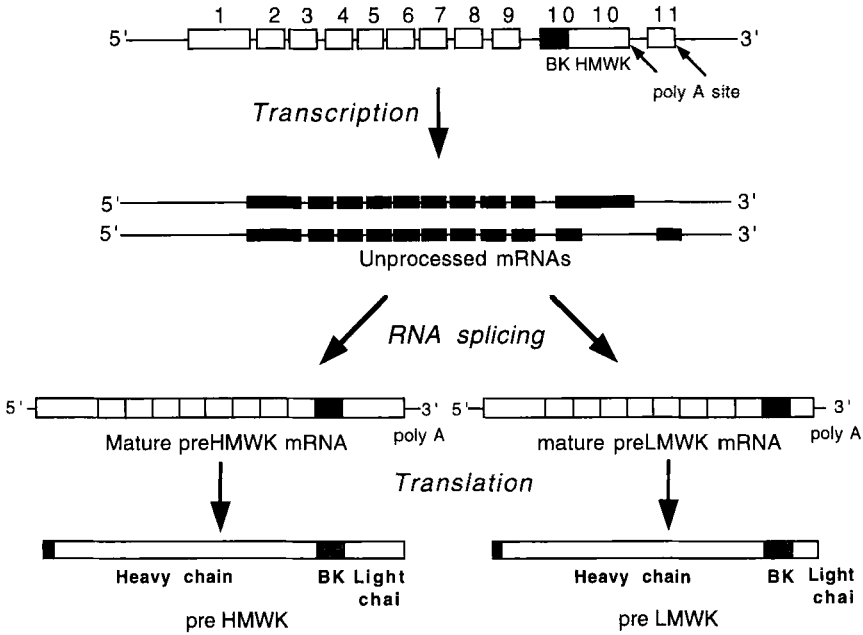


FIG. 4. The gene for high-molecular-weight kininogen (HMWK). The boxes labeled 1–9 represent the exon coding for the heavy chain of both HMWK and low-molecular-weight kininogen (LMWK). Exon 10 codes for the bradykinin (BK) sequence and the light chain of HMWK, whereas exon 11 codes for the light chain of LMWK. The mature mRNAs are assembled by alternative splicing events in which the light-chain sequences are attached to the 3′ end of the 13-amino acid common sequence C terminal to BK.

initiators (Griep *et al.*, 1985; Tans and Griffin, 1982). Dextran sulfate is a truly soluble activator and a close homolog of naturally occurring sulfated mucopolysaccharides. High-molecular-weight preparations of 500 kDa are typically used (Fujikawa *et al.*, 1980; Silverberg and Kaplan, 1982; Tankersley and Finlayson, 1984), but in a study of factor XII autoactivation (Silverberg and Diehl, 1987b), much smaller fractions were effective down to as low as 5 kDa. The rate of factor XII activation increased markedly with dextran sulfate at 10 kD (or more) where the theoretical number of factor XII molecules capable of binding per particle increased from one to two, and similar results were seen with heparin. This presumably provides a critical intermolecular interaction required for optimal autoactivation.

Naturally occurring polysaccharides are effective if they are highly sulfated and these include heparin and chondroitin sulfate E (described in rodent mucosal mast cells) (Hojima *et al.*, 1984). Other mucopolysaccharides known to catalyze factor XII autoactivation are dermatan sulfate,

keratin polysulfate, or chondroitin sulfate C (Brunnée *et al.*, submitted). The basement membrane of endothelial cell matrix may support contact activation, but this has not been demonstrated *in vivo*. Collagen, long thought to be an initiator, was proven to be ineffective and the activity reported was possibly due to contaminating matrix proteins. One pathophysiologic substance very likely to initiate contact activation *in vivo* is endotoxin (Morrison and Cochrane, 1974; Pettinger and Young, 1970; Roeise *et al.*, 1988) and there is good reason to believe that the contact cascade is activated in septic shock and the observed symptoms are due, in part, to the generation of bradykinin (Kaufman *et al.*, 1991; Mason *et al.*, 1970). Crystals of uric acid and pyrophosphate can also initiate kinin formation via this pathway (Ginsberg *et al.*, 1980; Kellermeyer and Breckenridge, 1965).

B. Regulation of Contact Activation

The various interactions of the bradykinin-forming pathway are shown in Fig. 5, which also includes the steps inhibited by C1 Inhibitor (C1 INH). The autoactivation of factor XII as shown is very slow. However, the reciprocal reactions involving kallikrein contribute to a tremendously fast activation of factor XII as illustrated by the finding that if one molecule each of FXIIa and kallikrein per milliliter are present in a mixture of factor XII and prekallikrein at their plasma concentrations, 50% of the factor

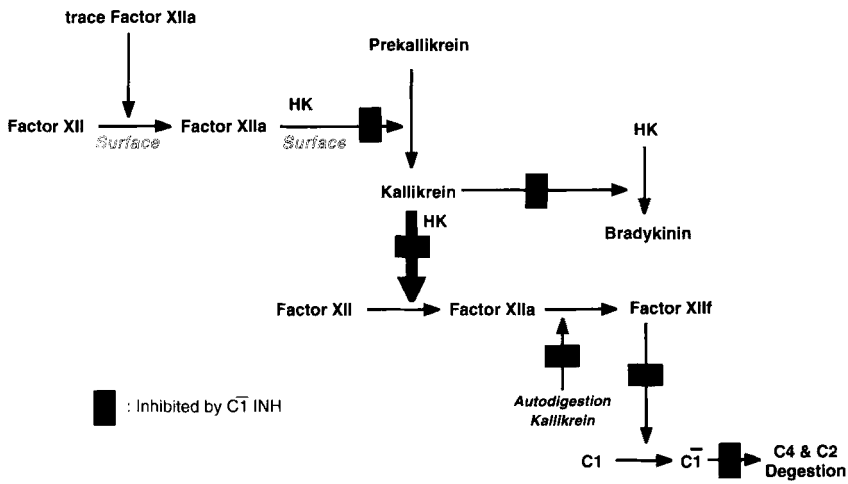


Fig. 5. Pathway for bradykinin formation indicating the autoactivation of Hageman factor (HF or factor XII), the positive feedback by which kallikrein activates HF, cleavage of HMW kininogen to release bradykinin, formation of Hageman factor fragment (HFf), and enzymatic activation of C1. The steps inhibitable by C1 INH are indicated.

XII would be activated in 13 sec (Tankersley and Finlayson, 1984). This corresponds to less than 5×10^{-13} *M* of active enzyme in the preparations. The *in vivo* source of the active enzyme is unknown, but it may be formed by other plasma proteases, e.g., plasmin, proteases secreted by cells, or limited autoactivation along cell surfaces. In fact, very slow turnover of the cascade may always be occurring (Bernardo *et al.*, 1993a,b; Reddigari *et al.*, 1993b; Shibayama *et al.*, 1994; Silverberg and Kaplan, 1982) and controlled by plasma inhibitors (Weiss *et al.*, 1986). Introduction of a surface or other polyanionic substances accelerates many thousandfold the baseline turnover of factor XII and prekallikrein to ignite the cascade. The addition of the cofactor HK (which was not included in the aforementioned kinetic analysis) accelerates these reactions even further but requires the surface to be present. The surface appears to create a local milieu in the contiguous fluid phase (Griep *et al.*, 1985; Griffin and Cochran, 1976; Silverberg and Diehl, 1987a), in which the local concentrations of reactants are greatly increased, which increases the rates of the reciprocal interaction. In addition, surface–bound factor XII undergoes a conformational change that renders it more susceptible to cleavage (Griffin, 1978). The alternative idea (Kurachi *et al.*, 1980; McMillin *et al.*, 1974; Ratnoff and Saito, 1979) that binding of factor XII induces a conformation change that exposes an active site has essentially been disproved. Inhibitors such as C1 INH are not bound to the surface; thus, the balance between activation and inactivation is upset. The effect of dilution on plasma also diminishes the effect of inhibitors far more than any slowing of enzymatic reaction rates. The net effect is, therefore, a marked augmentation of reaction rate.

Using dextran sulfate, the effect of the surface on the rate of factor XIIa conversion of prekallikrein to kallikrein was augmented 70-fold (Tankersley and Finlayson, 1984), whereas the effect on digestion of factor XII by kallikrein was as much as 3000- to 12,000-fold (Rosing *et al.*, 1985; Tankersley and Finlayson, 1984). This latter reaction is about 2000-fold more rapid than the rate of factor XII autoactivation, and this kinetic dominance means that prekallikrein must be considered to be a coagulation factor. As indicated earlier, the PTT of prekallikrein-deficient plasma is much prolonged but does autocorrect as factor XII autoactivates on the surface. On the other hand, factor XII-deficient plasma has a markedly abnormal PTT and does not autocorrect and is essentially devoid of intrinsic clotting or kinin formation. Alternatively, purified factor XII preparations activate when tested with a surface or polyanion under physiologic conditions (Silverberg *et al.*, 1980a; Tankersley and Finlayson, 1984; Tans *et al.*, 1983), whereas, prekallikrein does not. Hence, factor XII is considered absolutely requisite for intrinsic coagulation, whereas prekallikrein acts as an accelerator.

In plasma, the involvement of HK was indicated by the discovery of persons whose plasma had a very prolonged PTT and who generated no bradykinin upon incubation with kaolin, but who were not deficient in factor XII or prekallikrein (Colman *et al.*, 1975; Donaldson *et al.*, 1976; Wuepper *et al.*, 1975). This phenomenon was explained by the identification of HK as a nonenzymatic cofactor in contact activation. It appeared to accelerate activation of both factor XI and prekallikrein as well as factor XI (Griffin and Cochrane, 1976; Meier *et al.*, 1977; Revak *et al.*, 1977; Wiggins *et al.*, 1977). The discovery that prekallikrein and factor XII circulate bound to HK provided the mechanistic key to the explanation (Thompson *et al.*, 1977). One function of HK is to present the substrates of factor XIIa in a conformation that facilitates their activation. Thus, prekallikrein that is bound to a surface in the absence of HK is not subsequently activated by factor XIIa (Silverberg *et al.*, 1980b). A synthetic peptide encompassing the HK binding site for prekallikrein can interfere with contact activation by competitively interfering with the binding of prekallikrein to the HK light chain (Tait and Fujikawa, 1987); similarly, a monoclonal antibody to this binding site inhibits coagulation and kinin formation in plasma (Reddigari and Kaplan, 1989a). Factor XI activation is almost totally dependent on the formation of a surface binding complex with HK. HK also augments the rate of factor XII activation in plasma (Revak *et al.*, 1977; Wiggins *et al.*, 1977), although it does not augment the activity of kallikrein against synthetic substrates. The effect seems to be largely indirect. First, it is required for efficient formation of kallikrein in surface-activated plasma. Second, because kallikrein can dissociate from surface-bound HK, it can interact with surface-bound factor XII on an adjacent particle, thereby disseminating the reaction (Cochrane and Revak, 1980; Silverberg *et al.*, 1980a; Wiggins *et al.*, 1977). As a result, the effective kallikrein/factor XII ratio is increased in the presence of HK. Finally, in plasma, HK can displace other adhesive glycoproteins such as fibrinogen from binding to the surface (Schmaier *et al.*, 1984). These data indicate that HK must also be considered to be a coagulation cofactor because it is required for the generation of kallikrein (a factor XII activator) as well as the activation of factor XI. HK-deficient plasma has a profoundly prolonged activated PTT that is almost as abnormal as that of factor XII deficiency (Colman *et al.*, 1975; Donaldson *et al.*, 1976; Wuepper *et al.*, 1975), although persons with congenital HK deficiency have no bleeding diathesis.

Regulation of contact activation also occurs via plasma protease inhibitors. A summary of the major control proteins of this pathway is given in Table II. The C1 INH is a major inhibitor of factor XIIa or factor XII_f (de Agostini *et al.*, 1984; Forbes *et al.*, 1970; Pixley *et al.*, 1985; Schreiber *et al.*, 1973) and it is not active against other coagulation enzymes except

TABLE II
PLASMA INHIBITORS OF ENZYMES OF CONTACT ACTIVATION: RELATIVE CONTRIBUTIONS TO INHIBITION IN NORMAL PLASMA

Inhibitor	Enzyme			
	Factors XIIa and XII_f		Kallikrein	Factor XIa
C1 inhibitor	91.3	93	52 (84)[a]	47
Antithrombin III[b]	1.5	4	nd	5
α_2-Macroglobulin	4.3	—	35 (16)[a]	—
α_1-Protease inhibitor	—	—	nd	23
α_2-Antiplasmin	3.0	3	nd	25

Note. nd, not determined separately.
[a] Data obtained from generation of kallikrein *in situ*.
[b] Data are for results obtained in the absence of added heparin.

kallikrein and factor XIa. The inhibitor is cleaved by the protease and then binds at the active site of the protease in a 1:1 molar covalent complex that completely inactivates the enzyme (Travis and Salvesen, 1983). Antithrombin III, which is a critical control protein for much of the coagulation cascade, makes a minor contribution to factor XIIa or factor XII_f inactivation (Cameron *et al.*, 1989; de Agostini *et al.*, 1984; Pixley *et al.*, 1985; Stead *et al.*, 1976). Heparin can augment the inhibition by AT III, although there is some variance reported as to the magnitude of augmentation. Heparin can also function as an activating polyanion for contact activation (Hojima *et al.*, 1984; Silverberg and Deihl, 1987b). Curiously, α_2-macroglobulin, which is an inhibitor of broad reactivity with enzymes, does not significantly inhibit any forms of activated factor XII.

Kallikrein is inhibited by both C1 INH and α_2-macroglobulin (Gigli *et al.*, 1970; Harpel, 1974; McConnell, 1972), which together account for more than 90% of the inhibitory activity of plasma (Schapira *et al.*, 1982b; van der Graaf *et al.*, 1983). α_2-Macroglobulin does not bind to the active site but traps the protease within its structure so as to sterically interfere with its ability to cleave large protein substrates (Barrett and Starkey, 1973). About one-third of the enzyme's activity on small synthetic substrates is retained by the complex, whereas the activity on its natural substrates is <1%. Although these two inhibitors contribute roughly equally when kallikrein is added to plasma (Harpel *et al.*, 1985; Schapira *et al.*, 1982b; van der Graaf *et al.*, 1983), when a surface such as kaolin is added, 70–80% of the kallikrein formed is bound to C1 INH (Harpel *et al.*, 1985). The reason for this difference is unknown. Conversely, at low temperatures, C1 INH is less effective, and much of the kallikrein inhibition is mediated by α_2-macroglobulin (Harpel *et al.*, 1985).

Factor XIa is inhibited to a great extent by C1 INH (Wuillemin *et al.*, 1995). When purified FXIa was added to plasma and its distribution among various inhibitors was determined, most of the added XIa was found complexed to C1 INH, even in the presence of heparin, followed by FXIa:α_1-antitrypsin and FXIa:α_2-antiplasmin. However, α_1-antitrypsin was found to be the major inhibitor when chromatographic plasma fractions were tested against FXIa (Heck and Kaplan 1974; Scott *et al.*, 1982). C1 INH and AT III were also previously found to inhibit factor XIa (Meijers *et al.*, 1988; Scott *et al.*, 1982).

Activation on a surface occurs very quickly, whereas inhibition has a slower reaction rate. In plasma of patients with hereditary angioedema (HAE) in which C1 INH is absent or dysfunctional, the amount of surface needed to produce maximal activation is 10- to 20-fold less than that needed to activate normal plasma (Cameron *et al.*, 1989).

IV. Bradykinin: Functions, Control Mechanisms, and Receptors

The functions of bradykinin include venular dilatation, increased vascular permeability (Regoli and Barabe, 1980), constriction of uterine and gastrointestinal smooth muscle, constriction of coronary and pulmonary vasculature, bronchoconstriction, and activation of phospholipase α_2 to augment arachidonic acid metabolism. It acts in most tissues via B_2 receptors, and selective B_2 receptor antagonists have been recently synthesized. In plasma, bradykinin is first digested by carboxypeptidase N (Erdos and Sloane, 1962), which removes the C-terminal Arg leaving des-arg^9 bradykinin (Sheikh and Kaplan, 1986b). This peptide lacks the inflammatory function of bradykinin (vasodilatation and increased permeability) that is evident in skin or smooth muscle, but can interact with B_1 receptors in the vasculature to cause hypotension. It has been reported that B_1 receptors are induced during inflammatory conditions (Regoli and Barabe, 1980), whereas B_2 receptors are synthesized constitutively. However, in cultured bovine pulmonary artery endothelial cells, Smith *et al.* (1995) have shown that both B_1 and B_2 receptors are made constitutively. When serum (rather than plasma) is examined, the rate of removal of the C-terminal Arg is more rapid than can be attributed to carboxypeptidase N (Sheikh and Kaplan, 1989). This may be due to secretion (from cells) or activation of carboxypeptidase U, a newly described exopeptidase (Wang *et al.*, 1994). The next cleavage is by angiotensin converting enzyme, which digests des-Arg9 bradykinin (ArgProProGlyPheSerProPhe) via its tripeptidase activity (Sheikh and Kaplan, 1986a) (dipeptidase activity converts angiotensin I to angiotensin II) to yield ArgProProGlyPhe + SerProPhe. These products are inactive on both B_1 and B_2 receptors. Further slow digestion leads to

the final products of ArgProPro + 1 mol each of the amino acids Gly, Ser, Pro, and Arg, and 2 mol of Phe (Sheikh and Kaplan, 1989).

In vivo, kinin degradation occurs rapidly along endothelial cells of the pulmonary vasculature. However, the predominant enzyme is ACE, which acts as a dipeptidase if the C-terminal Arg is present to remove Phe-Arg. The resultant heptapeptide is cleaved once again at the C-terminal end to liberate SerPro, leaving the pentapeptide ArgProProGlyPhe (Sheikh and Kaplan, 1986a). These peptides are further metabolized to ArgProPro and free amino acids as indicated previously. The cough and angioedema associated with the use of ACE inhibitors for treatment of heart failure, hypertension, diabetes, or scleroderma may be due to inhibition of kinin inactivation and accumulation of bradykinin as conversion to angiotensin II is prevented.

The effects of kinins are dependent on interaction with two types of receptors termed B_1 and B_2 receptors (Hall, 1992; Regoli and Barabe, 1980). B_2 receptors are constitutively expressed on endothelial cells, smooth muscle cells, and neurons, and are, in general, responsible for the vasodilation, increased vascular permeability, and smooth muscle contraction associated with bradykinin or kallidin (Lys-bradykinin). B_1 receptors, on the other hand, are located primarily on smooth muscle cells and are induced *in vivo* and *in vitro* by bacterial endotoxin, cytokines, and growth factors that are released during the inflammatory response or as a result of tissue injury (Deblois *et al.*, 1988, 1989). These receptors are responsive to des-arg^9-bradykinin or des-arg^{10}-kallidin, which are generated by carboxypeptidase N or carboxypeptidase U interaction with bradykinin and kallidin, respectively. Both receptors have been cloned (Hess *et al.*, 1992; Manke *et al.*, 1994; McEachern *et al.*, 1991) and are defined by the use of peptide antagonists that are specific for each type (Stewart *et al.*, 1996). Both receptors are of the seven transmembrane type that transduce signals via G proteins within the cell membrane (Fig. 6).

V. Intrinsic Fibrinolytic Cascade

A factor XII-dependent pathway leading to the conversion of plasminogen to plasmin was described in the 1960s and early 1970s (Iatridis and Ferguson, 1962; McDonagh and Ferguson, 1970; Ogston *et al.*, 1967) and a defect in this pathway has been observed in plasma deficient in factor XII, prekallikrein, or HK (Colman *et al.*, 1975; Donaldson *et al.*, 1976; Saito *et al.*, 1974; Weiss *et al.*, 1974; Wuepper, 1973; Wuepper *et al.*, 1975). The factor XII-dependent fibrinolytic activity is relatively weak and difficult to demonstrate in whole plasma because large quantities of a potent plasminogen activator are not formed. Relatively little plasmin is

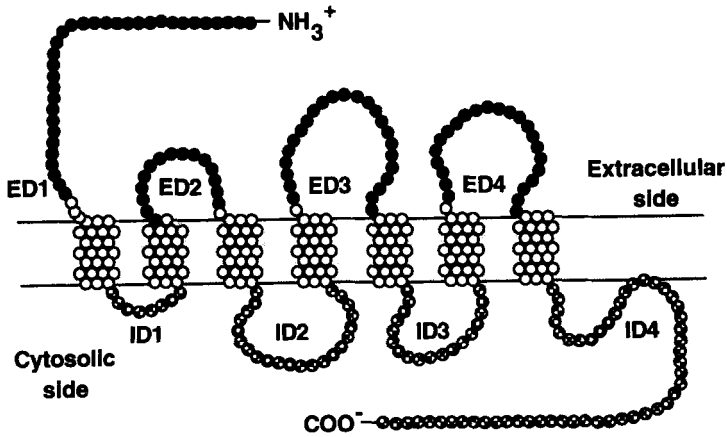


FIG. 6. Diagrammatic representation of a rodent B_2 receptor based on hydrophobicity plots and a transmembrane α-helix hypothesis indicating the seven transmembrane segments, four external domains, and four internal domains. It is a type I membrane protein with the amino terminus extracellular and the C terminus intracellular. Activation via G proteins leads to phosphorylation of critical Ser and Thr residues [adapted with permission from Said, *et al.* (1996). *J. Biol. Chem.* **271,** 1748–1755; copyright 1996 National Academy of Sciences, USA].

generated, which is rapidly inactivated by plasma inhibitors (α_2-antiplasmin and α_2-macroglobulin). Most studies have therefore used diluted, acidified plasma (Ogston *et al.*, 1971) or chloroform-treated plasma (Ogston *et al.*, 1967) in which inhibitors of contact activation and plasmin are rendered inactive. Thus, euglobulin preparations that concentrate the plasma enzymes and cofactors but limit inhibition (Kluft, 1976) or plasma following addition of organic compounds that destroy α_2-antiplasmin and C1 INH (Kluft, 1977; Miles *et al.*, 1981, 1983b) are employed. Such measures are not needed to study blood coagulation or the liberation of bradykinin.

Factor XII-dependent activation of plasminogen was first shown to be accomplished by kallikrein (Colman, 1969) and factor XIa (Mandle and Kaplan, 1977, 1979). When purified preparations are compared, kallikrein and factor XIa directly activate plasminogen with equal potency (Mandle and Kaplan, 1979). However, the plasma concentration of prekallikrein is about 10-fold higher than that of factor XI, and factor XII_f can readily convert prekallikrein to kallikrein in the fluid phase (Kaplan and Austen, 1970; Tankersley *et al.*, 1980), whereas it has minimal activity on factor XI (Kaplan and Austen, 1971). In addition, kallikrein can dissociate from surfaces and act in the fluid phase, whereas factor XIa cannot. Therefore, kallikrein is more important for this pathway.

Activated factor XII (XIIa or XII_f) can also convert plasminogen to plasmin (Goldsmith *et al.*, 1978), but its activity is only 5% of that of kallikrein. These are all weak reactions in that the potencies of kallikrein or factor XIa as plasminogen activators are thousands of times lower than the potency of urokinase (Jorg and Binder, 1985; Mandle and Kaplan, 1979; Miles *et al.*, 1983a). Thus, it can be argued that plasminogen is not a significant substrate for any of them. However, the other blood-clotting enzymes—factor IXa, Xa, and VIIa and thrombin—have no such activity, which argues against this activity being a nonspecific phenomenon and associates it only with those proteins responsible for contact activation.

Other studies of contact-activated fibrinolysis suggest that kallikrein activates the trace quantity of prourokinase in plasma (Hauert *et al.*, 1989) and that urokinase is the main plasminogen activator of plasma (Huisveld *et al.*, 1985). Inhibition by antiurokinase antisera supports this idea, as do zymographic gel studies using plasma euglobulin preparations (Hauert *et al.*, 1989; Miles *et al.*, 1981) (Fig. 7). Other studies have also suggested a role for plasma urokinase in factor XII-independent fibrinolysis (Kluft *et al.*, 1984). Although urokinase is clearly a much more potent plasminogen activator than any of the enzymes associated with contact activation, the

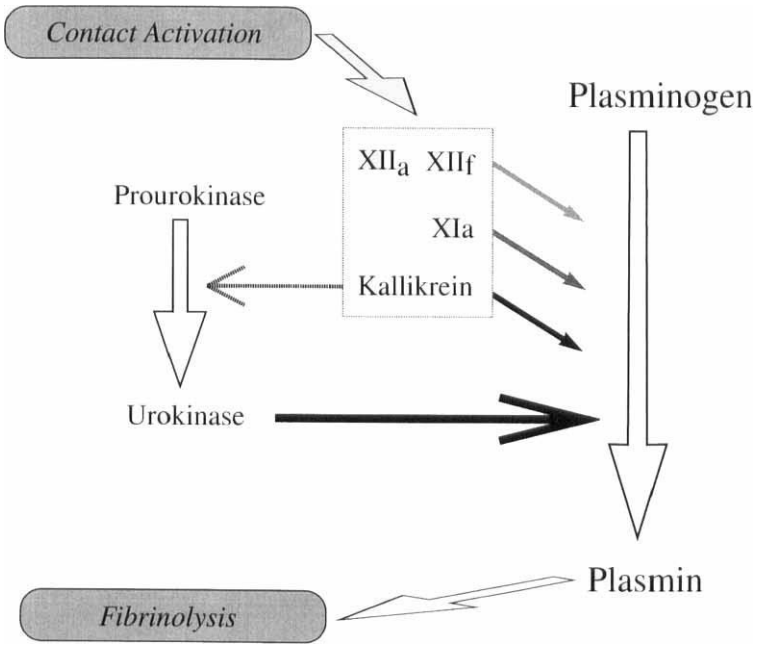


FIG. 7. Pathways of factor XII-dependent fibrinolysis.

quantities of urokinase generated are very small. If the effects of α_2-antiplasmin and C1 INH are abrogated by addition of flufenamic acid derivatives, contact activation results in the formation of about 35 ng/ml of plasmin (Miles *et al.*, 1983a), which represents activation of 0.05% of plasma plasminogen.

However, these studies with whole, diluted, or modified plasma ignore the *in vivo* situation in which platelets and other blood cells are clearly involved in coagulation/fibrinolysis. In this respect, studies suggesting an important role for platelet-bound contact factors in fibrinolysis are noteworthy (Loza *et al.*, 1994). They have shown that the contact-dependent intrinsic pathway of fibrinolysis may be mediated by platelets due to the presence of platelet-bound prekallikrein. When prekallikrein was activated by factor XIIa in a thrombus, local activation of platelet-associated prourokinase occurred, promoting targeted plasminogen activation and fibrinolysis (Gurewich *et al.*, 1993; Loza *et al.*, 1994). During cardiopulmonary bypass surgery, the incidence of thrombus formation was reportedly higher in factor XII-deficient patients than in normals (Burman *et al.*, 1994; Moorman *et al.*, 1993). In a separate study, recurrence of myocardial infarction was found to correlate with lower than normal levels of plasma prekallikrein and factor XII-dependent fibrinolytic activity (Pedersen *et al.*, 1993). Another study has demonstrated that generation of bradykinin was associated with activation of the fibrinolytic system (a positive correlation was found between plasmin : α_2-antiplasmin complexes and cleaved HK) during acute attacks of angioedema in HAE patients (Cugno *et al.*, 1993). These observations suggest that the contact system may really be more critical for fibrinolysis than for thrombosis, and that studies involving the interaction of contact factor with cells may be more valuable than studies of their interactions in whole plasma.

Studies with a baboon sepsis model have also indicated that factor XII and the contact system play an important role in both fibrinolysis and complement activation (Jansen *et al.*, 1994). It was shown that administration of monoclonal anti-factor XII to baboons suffering from lethal sepsis was accompanied by decreased activation of the complement and fibrinolytic system. Another possible interaction between the kallikrein–kinin system and *in vivo* fibrinolysis is suggested by the observation that bradykinin is a potent stimulator of tissue plasminogen activator release from endothelial cells (Smith *et al.*, 1985).

VI. Interactions with Cells

Kallikrein has been reported to interact with human leukocytes in a variety of ways. It is a chemotactic factor for neutrophils (Kaplan *et al.*,

1972) and monocytes (Gallin and Kaplan, 1974) and has been shown to cause neutrophil aggregation (Schapira *et al.*, 1982a) and release of elastase (Wachtfogel *et al.*, 1983). In a rabbit model, kallikrein stimulation of chemotaxis appeared to require cleavage of C5 and release of C5a chemotactic factor (Wiggins *et al.*, 1981). Therefore, C5 bound to the surface of neutrophils may be cleaved in the aforementioned reactions. However, antikallikrein serum was inhibitory, whereas anti-C5 serum had no effect. The investigators therefore concluded that the effect of kallikrein on human neutrophils is independent of complement. Furthermore, a degraded form of kallikrein (β-kallikrein), in which the heavy chain is partially digested, is enzymatically active on kininogen to form kinin, but its reactivity with neutrophils is markedly attenuated (Colman *et al.*, 1985). Factor XIIa has also been shown to stimulate neutrophils; because factor XII_f did not do this, a requirement for a binding site in the heavy chain was also inferred (Wachtfogel *et al.*, 1986). In each instance, the active site of the enzyme is required so that the proenzyme or DFP-treated enzyme is inactive (Kaplan *et al.*, 1972; Schapira *et al.*, 1982a).

The components of the contact activation system also appear to interact with platelets. Apparently, plasma factor XI and factor XIa have separate platelet receptors (Sinha *et al.*, 1984). Platelets also possess an intrinsic protein with factor XI activity, which cross-reacts with plasma factor XI immunologically but differs from it in molecular weight and isoelectric point. This activity is present in patients who are deficient in plasma factor XI (Lipscomb and Walsh, 1969; Tuszynski *et al.*, 1982). Platelet-associated factor XI can be activated by factor XII-dependent and factor XII-independent mechanisms (Walsh *et al.*, 1981). The membrane of activated platelets may also provide a surface on which factor XII can be activated in the presence of prekallikrein and HK (Walsh and Griffin, 1981) so that bound factor XI is then activated. HK has also been shown to be present within the α granules of platelets (Schmaier *et al.*, 1983) that become available during platelet activation (Schmaier *et al.*, 1986a). HK exhibits zinc-dependent (Gustafson *et al.*, 1986) binding to the platelet surface (Greengard *et al.*, 1984; Schmaier *et al.*, 1983) and augments binding of factor XIa (Sinha *et al.*, 1984).

Despite the demonstrated interactions of contact factors with platelets, platelets clearly are not requisite for contact activation as they are for later steps in coagulation. Thus, contact activation proceeds normally in platelet-poor plasma, and the addition of platelets provides little or no augmentation. In fact, evidence exists that platelets may actually inhibit prekallikrein and factor XII activation by secretion of β-thromboglobulin (Kodama *et al.*, 1985; Scully *et al.*, 1980). Nevertheless, platelet activation in periph-

eral tissues due to injury or localized thrombosis may contribute to contact activation and local kinin formation. In some circumstances, the platelets may also provide a factor XII bypass in contact activation (Walsh, 1962).

All the components of the contact activation cascade have been demonstrated to bind to endothelial cells. Schmaier *et al.* (1988) and van Iwaarden *et al.* (1988) first described binding of HK to human umbilical vein endothelial cells in a zinc-dependent reaction. This binding was subsequently demonstrated *in situ* by immunochemical staining of umbilical cord segments following incubation with HK (Nishikawa *et al.*, 1992). The HK–HUVEC interaction was saturable, reversible, dependent on 15–50 μM zinc (normal plasma concentration is 15–25 μM), and fulfilled the characteristics of a receptor-mediated interaction. There are 1 X 10^6 to 1 X 10^7 binding sites (an unusually large number) with a high affinity ($K_d \approx 40$–50 nM). Binding is seen with either chain of HK (i.e., heavy or light chain), thus, a complex interaction with subsites within the receptor seems likely (Reddigari *et al.*, 1993a). A similar complex interaction has been observed with platelets, although the binding site number is far less. Because prekallikrein and factor XI circulate bound to HK, these are brought to the endothelial cell surface via HK (Berrettini *et al.*, 1992). There are no separate receptor sites for either prekallikrein or factor XI. When we examined binding of factor XII to endothelial cells, we found binding characteristics consistent with a receptor that was strikingly similar to that seen with HK, including a requirement for zinc (Reddigari *et al.*, 1993a) (Fig. 8). We then demonstrated that HK and factor XII can compete for binding at a comparable molar basis suggesting that they bind to the same receptor (Reddigari *et al.*, 1993b) (Fig. 9).

We also demonstrated that factor XII can slowly autoactivate when bound to endothelial cells (Reddigari *et al.*, 1993b) (Fig. 10) and that addition of kallikrein can digest bound HK to liberate bradykinin at a rate proportional to the kallikrein concentration and with a final bradykinin level dependent on the amount of bound HK. Thus, activation of the cascade along the endothelial cell surface is likely; bradykinin is liberated and then interacts with the B_2 receptor to increase vascular permeability. Bradykinin can also stimulate cultured endothelial cells to secrete tissue plasminogen activator (Smith *et al.*, 1985), prostaglandin I_2 (prostacyclin), and thromboxane A_2 (Crutchly *et al.*, 1983; Hong, 1980) and can thereby modulate platelet function and stimulate local fibrinolysis.

Neutrophils also bind HK via the $C3b_i$ (CR3) receptor (Wachtfogel *et al.*, 1994), which is absent on endothelial cells, and all components of the kinin-forming cascade can interact at the cell surface (Henderson *et al.*, 1994).

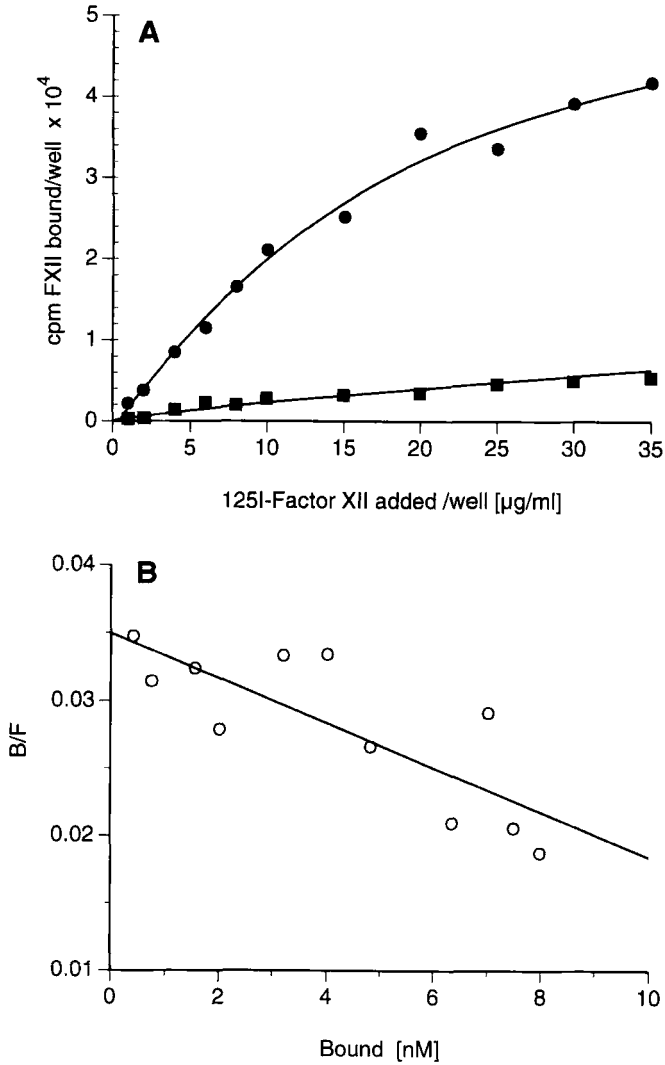


FIG. 8. Concentration-dependent binding of factor XII to HUVEC. HUVECs were incubated in triplicate with 1.0, 2.0, 4.0, 6,0, 8.0, 10, 15, 20, 25, 30, and 35 µg/ml of [^{125}I]FXII in the presence (●) and absence (■) of zinc chloride for 120 min and cell-bound cpm was determined as described previously. (*A*) cpm bound per well plotted against the amount of radioligand added to the wells. (*B*) Scatchard plot of bound cpm.

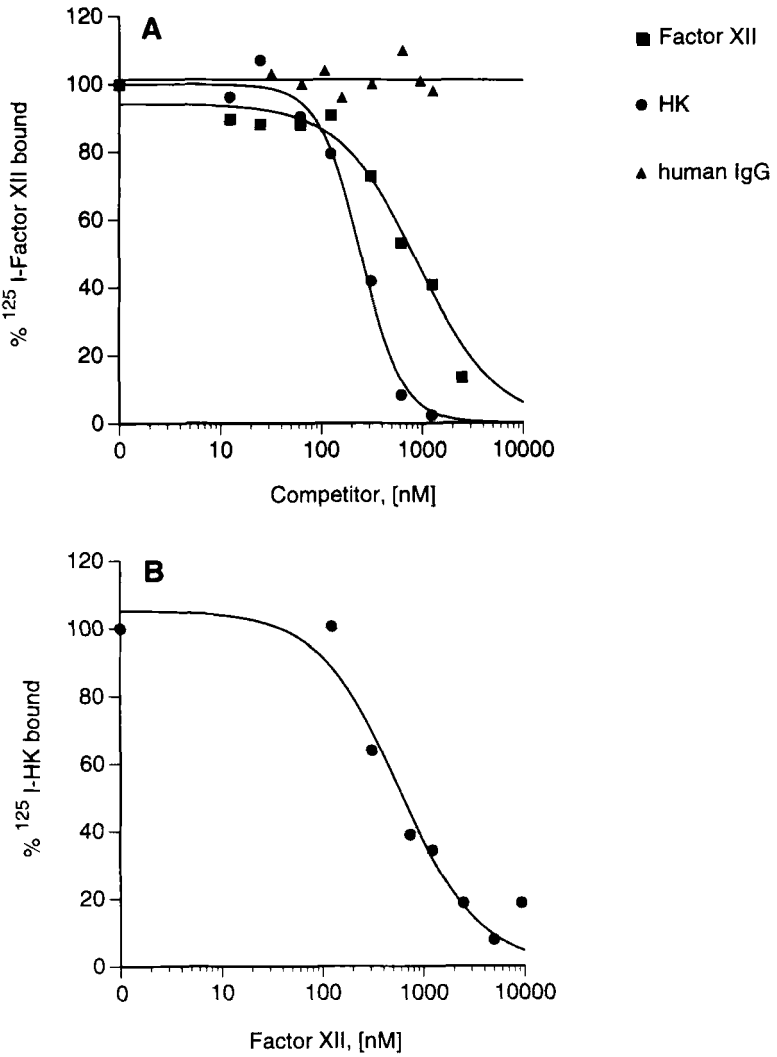
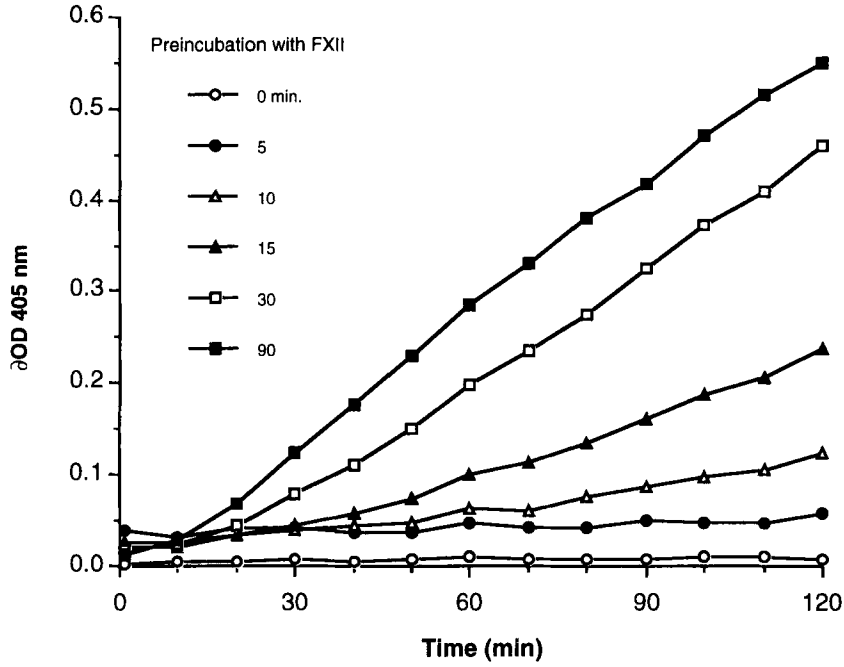
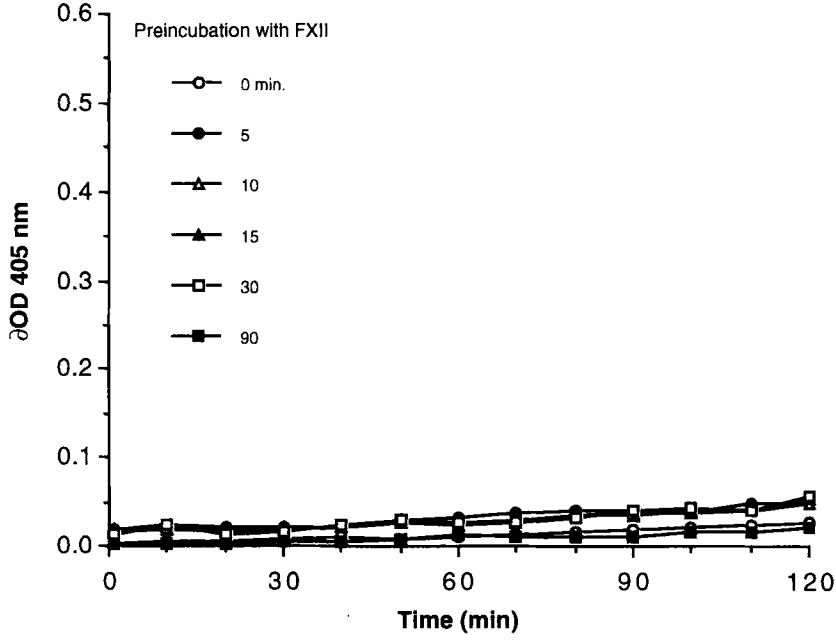


FIG. 9. High-molecular-weight kininogen (HK) competes with factor XII for the same binding sites of HUVEC. (*A*) HUVECs were incubated with 1 μg/ml [^{125}I]FXII in triplicate in the presence of increment concentrations of unlabeled factor XII (■), HK (●), or normal human IgG (▲) for 120 min and bound ligand was determined. The percentage bound in the presence of a competitor is plotted against the concentration of the competitor. (*B*) HUVECs were incubated with 1 μg/liter [^{125}I]HK in triplicate in the presence of increasing concentration of unlabeled factor XII and bound ligand was determined.


Preincubation with FXII
0 min.
5
10
15
30
90
∂OD 405 nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
30
60
90
120
Time (min)


Preincubation with FXII
0 min.
5
10
15
30
90
∂OD 405 nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
30
60
90
120
Time (min)

VII. Identification of gC1qR as the Endothelial Cell Receptor for Factor XII and HK

Our results with endothelial cells suggest interaction of both factor XII and HK with a common cell surface receptor that appear to be present constitutively and with a very high density. We therefore sought to purify and characterize this binding protein; the results were published recently (Joseph *et al.*, 1996) and are summarized below. A solubilized endothelial cell membrane preparation was passed over an HK affinity column in the presence or absence of zinc ion, eluted with glycine–HCl (0.1 *M*, pH 2.5), and the fractions were neutralized. An aliquot of each eluate fraction (with or without zinc) was spotted onto nitrocellulose membrane and blotted with biotinylated HK, developed with alkaline phosphatase-streptavidin, and followed by reaction with nitroblue tetrazolium/5-bromo-4-chloroindolyl phosphate. The results (shown in Fig. 11), aligned to represent comparable fractions, indicate a prominent increase in HK binding after elution in the presence of zinc. Fractions 8–15 were then pooled and concentrated and analyzed by SDS–PAGE using a silver stain or Coomassie brilliant blue as shown in Fig. 12. In the absence of zinc, weak bands are identified at 70and 45 kDa with the silver stain. Although these protein bands become somewhat more prominent when elution is done in the presence of zinc, the main feature was the appearance of a new prominent band at 33 kDa that was visible with Coomassie stain. In addition, ligand blot experiments demonstrated that whereas biotinylated HK bound to the 33-kDa band, no discernible binding was seen to either the 70- or the 45-kDa bands. Based on this information, the 33-kDa protein was subjected for N-terminal amino acid sequence analysis and the first 13 amino acids were found to be identical to the known NH_2 terminus of gC1qR (Ghebrehiwet *et al.*, 1994), a protein that binds to the globular heads of C1q of the complement cascade. We next performed a Western blot using anti-gC1qR monoclonal antibody 60.11 to further assess the identity of these two proteins. Monoclonal antibody 60.11 was able to recognize the 33-kDa HUVEC-derived membrane binding protein (Fig. 12) but not the bands at 45 or 70 kDa.

We next determined whether factor XII could also bind to gC1qR. HUVEC membrane-purified gC1qR or recombinant gC1q-R (rgC1qR) at

FIG. 10. Activation of prekallikrein by HUVEC-bound factor XIIa. HUVECs were incubated with 1 μg/ml FXII for indicated (*inset*) time points in the presence (top) and absence (bottom) of 50 μM zinc chloride. At the end of the incubation, the supernatant from zinc containing wells was removed and the cells were washed. Next, prekallikrein (1 μg/ml) and Chromozym-PK (0.6 mM) were added to both sets of wells, and the change in absorbance at 405 nM was monitored up to 120 min.

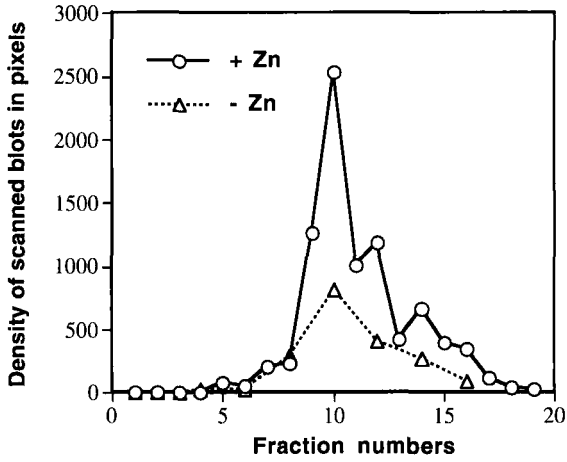


FIG. 11. Elution profile of proteins obtained from an HK-3M Emphaze column. Solubilized endothelial cell membrane preparations were applied to the HK affinity column in the presence and absence of zinc. The bound protein was eluted with glycine–HCl (0.1 *M*, pH 2.5) and 0.5-ml fractions were collected into tubes containing 30 μl each of 1 *M* Tris–HCl, pH 9.0. An aliquot of each fraction was spotted onto the nitrocellulose membrane and probed with biotinylated HK. The intensity of the color was scanned and the values were plotted.

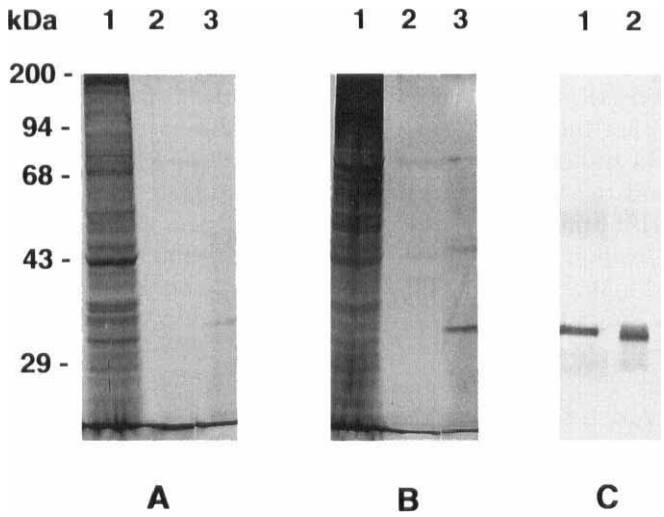


FIG. 12. SDS–PAGE analysis of proteins eluted from HK column. Fractions that reacted with HK were pooled and subjected to a 10% SDS–PAGE (*A* and *B*) as well as Western Blotting (*C*). In *A*, the proteins were stained with Coomassie, whereas in *B* they were stained with silver stain. In both *A* and *B*, lane 1 is the starting endothelial membrane preparation, lane 2 is the eluate without zinc, and lane 3 is the eluate with zinc. (*C*) Western blot of the zinc eluate (lane 3 in *A* or *B*) using anti-gC1q-R 60.11 under reducing (lane 1) and nonreducing (lane 2) conditions.

1.0–2.0 μg were applied to nitrocellulose membranes and blotted with biotinylated HK or factor XII in the presence or absence of 50 μM zinc. Sufficient *o*-phenanthroline was added to bind the zinc in the purified (but not recombinant) gC1qR to allow zinc-independent binding to be assayed. The results, shown in Fig. 13, demonstrate binding of HK or factor XII to either purified or rgC1qR in the presence of zinc and markedly diminished binding in its absence or when 2 mM Ca2+ or $Mg2^{+}$ was used instead of Zn2+ (not shown). Additional controls included biotinylated IgG, which did not bind to gC1qR, and substituting prekallikrein for gC1qR to which biotinylated HK bound, as expected (Mandle *et al.*, 1976). Furthermore, Fig. 14 shows that although excess unlabeled HK almost totally (90%) reverses the ability of factor XII to bind to gC1qR as assessed by scanning, which suggests interaction with a common domain within the protein, factor XII only partially (46%) reverses HK binding. This difference may be due to relative affinity of the two ligands for the gC1qR molecule. Nevertheless, these data completely paralleled those observed on binding of factor XII and HK to endothelial cells.

We next attempted to demonstrate that the interaction with gC1qR is indeed responsible for binding to the cell surface, which was addressed by inhibition experiments. HUVECs were incubated for 30 min with HK (8.7×10^{-8} M), C1q (10.8×10^{-8} M), or anti-gC1qR mAbs 74.5.2 and 60.11. Then, in the presence of 50 μM zinc, [^{125}I]HK was added and

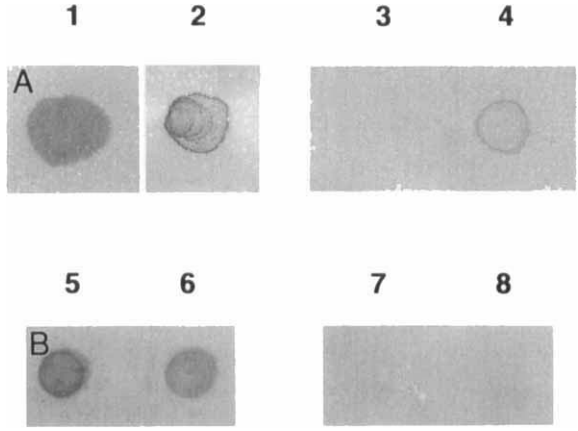


FIG. 13. Dot-blot analysis of HK and factor XII binding to the purified endothelial membrane preparation and recombinant gC1qR. The proteins were spotted onto the nitrocellulose membrane [recombinant protein (2 μg) in lanes 1,3,5, and 7; and purified protein (1 μg) in lanes 2,4,6, and 8] and probed with biotinylated HK (*A*) or biotinylated factor XII (*B*) in the presence (lanes 1,2,5, and 6) or absence (lanes 3, 4, 7, and 8) of 50 μM zinc.

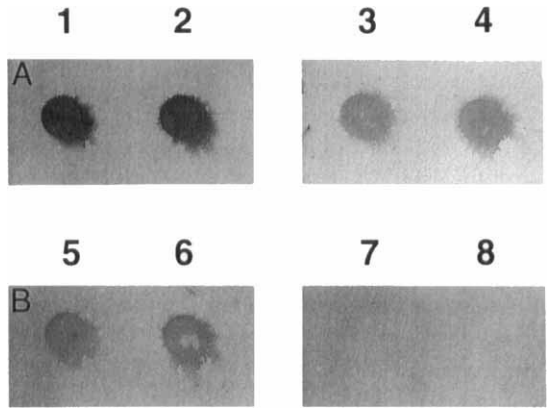


FIG. 14. Dot-blot analysis showing competitive displacement of HK and factor XII binding to gC1qR. gC1qRs (2 μg) were spotted and probed with (*A*) biotinylated HK in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 100 *M* excess of unlabeled factor XII and (*B*) biotinylated factor XII in the absence (lanes 5 and 6) and presence (lanes 7 and 8) of 100 *M* excess of unlabeled HK. Binding was done for 1 hr at room temperature in the presence of 50 m*M* zinc.

incubated for 60 min at 37°C, conditions known to saturate the binding sites (Hasan *et al.*, 1995). For each condition, the percentage inhibition of [^{125}I]HK binding was determined. The results of a representative experiment are shown in Fig. 15. Whereas a 10-mol excess of nonradiolabeled HK inhibited subsequent [^{125}I]HK binding, a comparable concentration of C1q did not, indicating that the binding sites on gC1qR for C1q and for HK do not overlap. Furthermore, although mAb 60.11 did not efficiently inhibit [^{125}I]HK binding to HUVEC, mAb 74.5.2 did. Other mAbs recognizing epitopes in different regions of the molecule were also tested for their inhibitory activity. Of these, only 25.15, which is similar to 74.5.2, was able to inhibit [^{125}I]HK binding to HUVEC. Herwald *et al. et al.* (1996) also demonstrated that gC1qR is a major endothelial cell binding protein for HK but used a domain 5-derived peptide from the light chain rather than whole HK as the ligand. In aggregate these data indicate that gC1qR serves as a zinc-dependent binding protein for factor XII as well as for HK, and that binding to HK occurs via the light chain moiety. The specific location within HK for binding to endothelial cells is within domain 5 (Hasan *et al.*, 1995; Reddigari *et al.*, 1993a) and this also appears to be the site of interaction with gC1qR. It is also clear that the HK heavy chain also binds to endothelial cells. This that interaction has been shown to require domain

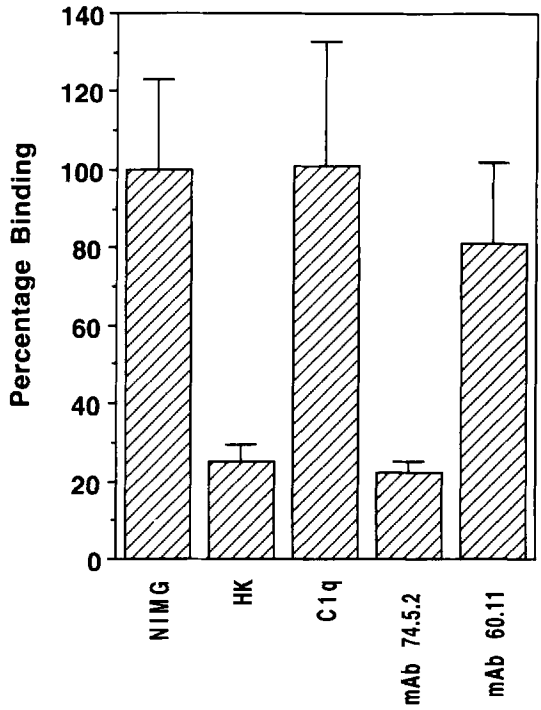


FIG. 15. Inhibition of [^{125}I]HK binding to HUVEC. HUVECs were incubated with HK (8.7×10^{-8} *M*), C1q (10.9×10^{-9} *M*), mAbs 74.5.2 and 60.11(12.5 μg/ml), or nonimmune mouse IgG1 (NIMG, 12.5 μg/ml). After 30 min, [^{125}I]HK (1 μg/ml) was added and further incubated for 1 hr at 37°C. Each bar is a mean (± SD) of two experiments, each performed in triplicate.

3; however, the identity of the cell surface binding protein to this region of HK is unknown. The possibility that the 45- or 72-kDa proteins we have isolated are responsible is currently being pursued. Regardless, our data suggest that the 45- and 72-kDa proteins may be associated with gC1qR within the cell membrane because they copurify. There is no separate receptor for prekallikrein or factor XI on endothelial cells, however, they are brought to the surface by virtue of binding to domain 6 of HK. Thus, all the components necessary for contact activation to proceed can be bound to the surface of vascular endothelial cells (Fig. 16). Activation along the cell surface generates bradykinin, which then interacts with B_2 receptors.

Because factor XII is the initiating protein of the cascade and has been shown to autoactivate upon the cell surface, we questioned whether gC1qR

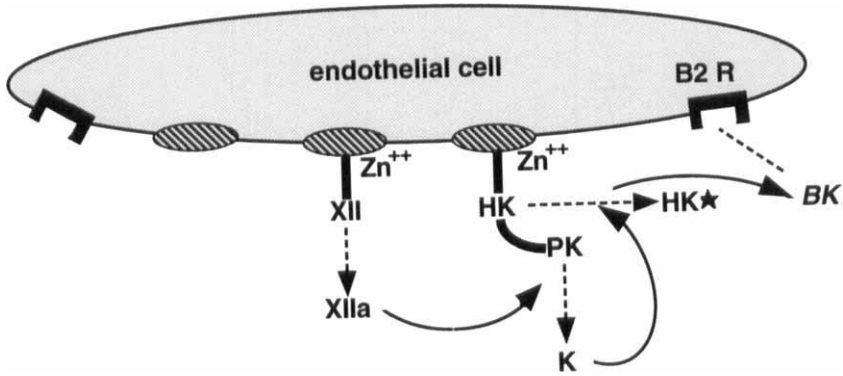


FIG. 16. Dose–response inhibition of low-dose thrombin (560 mU) activation of human platelets by increasing concentrations of HK (*A*) but not factor XII (*B*).

is capable of catalyzing this reaction. We therefore incubated purified factor XII with a wide dose range of gC1qR (0–100 /μl) for a 30-min time period and prepared replicate samples that were incubated in the absence of zinc ion. As shown in Fig. 17, the rate of prekallikrein conversion into kallikrein increased as the concentration of gC1qR increased (Joseph *et al.*, 1996) and there is no activation if zinc is eliminated from the reaction mixture (Hasan *et al.*, 1995). Purified membrane (native) gC1qR yields a response that is indistinguishable from a recombinant protein, indicating that gC1qR glycosylation does not affect its "surface" properties. If gC1qR is incubated directly with prekallikrein or with prekallikrein plus HK, there is no conversion of prekallikrein to kallikrein, again emphasizing the requirement for factor XII. We believe this to be a physiologic phenomenon in that there is continued slow activation of the cascade along cell surfaces which is controlled by C1 INH and α_2-macroglobulin. This may be one source of the minute quantities of factor XIIa that escape inhibition that are requisite for contact activation in plasma or during pathologic processes. The data with cloned gC1qR indicate that secretion of enzymes by endothelial cells that might cleave and activate factor XII to initiate the cascade is not the explanation, although such a contribution by whole cell preparations is possible.

As noted earlier, interaction of the entire intrinsic coagulation/kinin-forming cascade with neutrophils has been observed and binding of all the requisite constituents demonstrated. There is a zinc requirement, both domains 3 and 5 of HK are required, and the binding moiety on the neutrophil surface appears to be the C3bi receptor (CD11b/CD18 or MAC-1) (Henderson *et al.*, 1994; Wachtfogel *et al.*, 1994). The separate interaction of factor XII with the cell surface has not been studied.

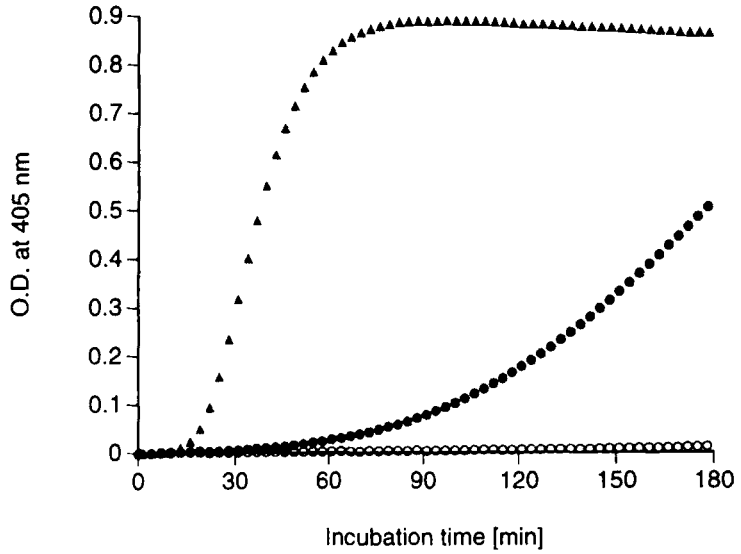


FIG. 17. Autoactivation of FXII by gC1qR; 20 μg/ml FXII, 0.6 m*M* S2222, a synthetic substrate for activated FXII, and 32 μg/ml recombinant gC1qR were incubated in 4-aminophenyl-methysulfonyl fluoride (APMSF)-treated HEPES–saline buffer for 180 min at 25°C. Factor XIIa formation was monitored at 405 n*M*.

There has been considerable work on the interaction of components of the contact activation cascade with platelets and these initial observations actually precede those involving endothelial cells. There are far fewer receptors, i.e., a few thousand rather than a few million, and the nature of the binding proteins has not yet been determined. It is clear that platelets bind HK, and that activated platelets do so better than native platelets, although it is not certain whether this difference is one of induction of binding sites, affinity of binding, or both. The HK interaction requires zinc ion, binding occurs by both domains 3 and 5 (Gustafson *et al.*, 1986; Meloni *et al.*, 1992) but platelets do not have sufficient MAC-1 or gC1qR to account for these observations. Purified thrombospondin has been shown to bind HK (De La Cadena *et al.*, 1994) and is a candidate to account for binding to the platelet surface, but it has not been shown to be responsible for cell surface binding. Preliminary data indicate that glycoprotein Ib is a site of zinc-dependent binding of HK and Factor XII (Joseph *et al.*, 1977). LK, which has a separate light chain from HK, interacts with platelets (as is true of endothelial cells) and, of necessity, does so solely via domain 3 (Herwald *et al.*, 1965; Jiang *et al.*, 1992).

HK has enzyme-inhibiting properties that are located within domains 2 and 3 of the heavy chain and interacts with proteases with a requisite cysteine for activity. For example, HK inhibits papain, Cathepsins B and H, as well as platelet-derived calpains (Ohkubo *et al.*, 1984; Schmaier *et al.*, 1986). It has also been shown that both HK and LK are capable of inhibiting thrombin-induced activation of platelets (Fig. 18a) in a dose dependent fashion (Jiang *et al.*, 1992; Meloni *et al.*, 1991; Puri *et al.*, 1991) if low dose thrombin is utilized. HK does not, however, inhibit thrombin, thus, it appears to interact with the cell surface to inhibit the action of thrombin. This would implicate the thrombin receptor (Schmidt *et al.*, 1996b; Vu *et al.*, 1991), which mediates platelet aggregation, or binding to an associated protein that would interfere with receptor function. The HK site responsible for inhibitory activity is within domain 3, but the specific amino acid sequence that appears to inhibit thrombin action does not appear to be the same as the sequence that, when radiolabeled, accounts for HK binding to the platelet surface (Jiang *et al.*, 1992). Thus, there appears to be two functional areas within domain 3 that interact with platelet with somewhat different, but perhaps complementary, functions. Factor XII does not inhibit thrombin activation of platelets (Fig. 18b); thus, although both bind to platelets in a zinc-dependent reaction, this site of reactivity within HK domain 3 has no corresponding one within factor XII.

VIII. Clinical Considerations

A. Kinin Formation in Hereditary Angioedema

The pathogenesis of HAE suggests liberation of a kinin that has variously been considered to be a product of the second component of complement or produced by contact activation. As shown in Fig. 5, if C1 INH is either absent (type I HAE) or dysfunctional (type II HAE), there is insufficient inhibition of all the activated forms of factor XII, kallikrein, or activated C1 (specifically C1r and C1s, each of which is inhibited by C1 INH). The production of bradykinin is markedly augmented under these conditions and it has been shown that addition of dextran sulfate at concentrations insufficient to activate normal plasma leads to complete digestion of HK in HAE plasma within a few minutes (Cameron *et al.*, 1989). Thus, seemingly insignificant trauma or infections may be sufficient to initiate an attack in such patients.

Soon after C1 INH deficiency was shown to be the cause of HAE, evidence was presented to suggest that cleavage of C2 (Donaldson, 1968) or C2b (Donaldson *et al.*, 1977) would generate a kinin that was responsible for the symptoms. Attempts to produce this kinin by cleavage of C2 or

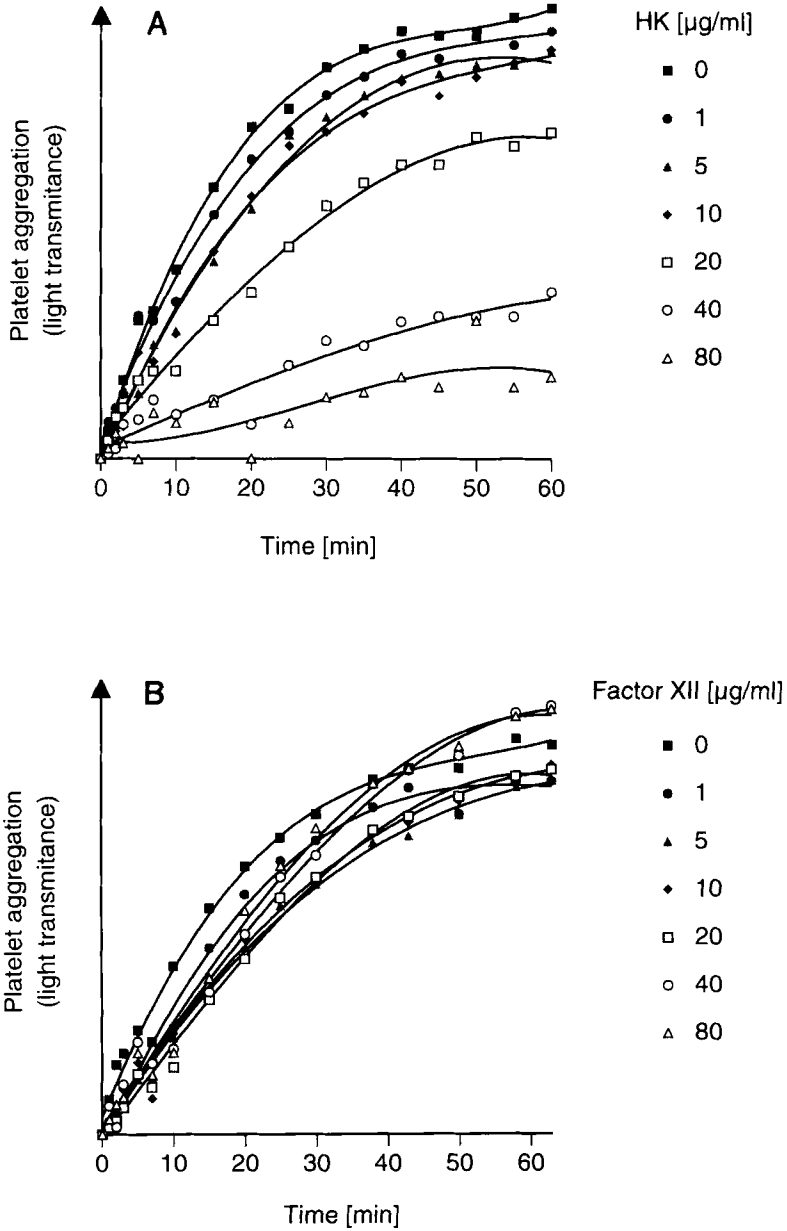


FIG. 18. Diagrammatic representation of the zinc-dependent interaction of both factor XII and HK with gC1qR at the endothelial cell surface leading to activation of factor XII, conversion of prekallikrein (PK) to kallikrein (K), and digestion of HK to generate bradykinin (BK) and leave cleaved kininogen (HK°). BK can then react with the bradykinin B_2 receptor to activate endothelial cells and to cause vasodilation and increased vascular permeability.

C2b have, in general, been negative (Fields *et al.*, 1983), nor has such a kinin been shown to circulate in patients during an attack. On the other hand, synthesis of overlapping peptides within the C2b portion of the molecule revealed a sequence that possessed kinin-like peptides (Strang *et al.*, 1988). However, enzymatic cleavage of the protein to release this peptide has not yet been achieved.

Activated kallikrein has been shown to be present in markedly augmented amounts in blister fluids derived from patients and bradykinin has been reported to be the major kinin found when HAE plasma is activated (Curd *et al.*, 1980). The bulk of evidence favors a major role for bradykinin in causing the symptoms of HAE (Fields *et al.*, 1983). However, the presence of an additional kinin-like fragment derived from C2 by a mechanism that is not yet understood is possible. If so, synergy between the two kinins might occur. Use of B_2 receptor antagonists in such patients, once they are available for human use, should help settle the question.

Complement is nevertheless clearly activated in HAE and this may be due to the autoactivation of C1r when C1 INH is absent. C4 levels are diminished, presumably due to consumption by C1s in HAE patients even when they are asymptomatic; with attacks of swelling, C4 levels approach zero and C2 levels diminish. As seen in Fig. 5, this process may be augmented by factor XII_f (HF_f), which has been shown to enzymatically cleave and activate C1r and, to a lesser degree, C1s (Ghebrehiwet *et al.*, 1983). Use of androgenic therapeutic agents (Danazol and Stanozolol) may increase synthesis of C1 INH sufficiently to prevent swelling. Levels of C4 and C1 INH increase, however, the magnitude may not parallel the clinical effect. Use of agents that inhibit plasminogen activation (*e*-amino caproic acid and tranexamic acid) are also effective therapeutic agents that prevent formation of plasmin; they may also have direct inhibitory effects on C1 activation (Soter *et al.*, 1975). Plasmin is also an enzymatic activator of factor XII (Kaplan and Austen, 1971) and might thereby contribute to HF_f production and bradykinin formation, or plasmin might digest C2b to yield a kinin-like fragment (Strang *et al.*, 1988).

B. Contact Activation in Allergic Diseases

By analogy with observations using dextran sulfate, naturally occurring glycosaminoglycans or proteoglycans may be able to induce contact activation. We have tested heparin proteoglycan from the Furth murine mastocytoma for its ability to activate a mixture of factor XII and prekallikrein. There is progressive conversion of prekallikrein to kallikrein as the concentration of mast cell heparin is increased. The potency of heparin proteoglycan equals that of dextran sulfate and its activity is inhibited by heparinase I or II, but not by heparitinase or chondroitinase ABC. Of the glycosaminoglycans we have tested, heparin, dermatan sulfate, keratin polysulfate, and

chondroitin sulfate C are positive in the assay (in that order), whereas heparan sulfate and chondroitin sulfate A are negative. Collagen types I, III, IV, and V, laminin, fibronectin, and vitronectin are also negative. Activation can then occur by release of heparin and/or other mucopolysaccharides secreted by mast cells and basophils upon exposure to plasma proteins or via interaction of these proteins with exposed connective tissue proteoglycans during tissue injury. The proteins of the kinin-forming system have been shown to be present in interstitial fluid of rabbit skin; thus, the source may not solely be dependent on exudation and activation of plasma.

Any aspect of inflammation that leads to dilution of plasma constituents or exclusion of inhibitors will augment contact activation because inhibitory functions are very dependent on concentration. Thus, the activitability of plasma can be shown to be related directly to dilution. Once levels of C1 INH are less than 25% of normal (i.e., a 1 : 4 dilution), patients with HAE are prone to attacks of swelling.

Activation of the plasma and tissue kinin-forming systems have been observed in allergic reactions in the nose, lungs, and skin, and include the immediate reaction as well as the late phase reaction, although the contributions of the plasma and tissue kallikrein pathways to each aspect of allergic inflammation are likely quite different. Antigen challenge of the nose followed by nasal lavage revealed an increase in TAME esterase activity, which is largely attributable to kallikrein(s) (Proud *et al.*, 1983). The activation was seen during the immediate response as well as the late phase reaction (Creticos *et al.*, 1984). Both LK and HK were shown to be present in nasal lavage fluid (Baumgarten *et al.*, 1985), and fractionation of nasal washings demonstrated evidence of both tissue kallikrein (Baumgarten *et al.*, 1986a) and plasma kallikrein (Baumgarten *et al.*, 1986b). Tissue kallikrein can be secreted by glandular tissue as well as by infiltrating cells, such as neutrophils, and will cleave LK to yield kallidin. Plasma kallikrein will digest HK to yield bradykinin directly. HPLC analysis of kinins in nasal washings revealed both kallidin and bradykinin. The latter can be formed from kallidin by aminopeptidase action, however, a portion of the bradykinin is also likely the direct result of plasma kallikrein activity.

Studies of the allergen-induced late phase reactions in the skin (Atkins *et al.*, 1987) have demonstrated the presence of kallikrein–C1 INH and activated factor XII-C1 INH complexes in induced blisters observed during an 8-hr period. Elevated levels of these complexes were seen between 3 and 6 hr coincident with the late phase response and were specific for the antigen to which the patient was sensitive.

IX. Other Disorders

Endotoxic shock is associated with depletion of contact activation proteins (Hirsh *et al.*, 1974; Mason *et al.*, 1970; O'Donnell *et al.*, 1976; Robin-

son *et al.*, 1975), and serial HK levels have prognostic value because a drop to near zero usually indicates a fatal outcome as do lower prekallikrein levels (O'Donnell *et al.*, 1976). A monoclonal antibody to factor XII markedly diminished the mortality by 50% in a baboon model of endotoxic shock (Pixley *et al.*, 1992, 1993) largely due to effects on hypotension and its sequelae. Parameters of disseminated intravascular coagulation (DIC) were unaffected and likely mediated via tissue thromboplastin, although DIC due to endothelial cell injury and/or endotoxemia is associated with diminished levels of factor XII, prekallikrein, and kallikrein inhibiting activity.

The synovial fluid of patients with rheumatoid arthritis has been shown to contain plasma kallikrein, which can activate stromelysin and convert procollagenase to collagenase (Nagase *et al.*, 1982). Uric acid and pyrophosphate crystals can act as surfaces for contact activation (Ginsberg *et al.*, 1980; Kellermeyer and Breckenridge, 1965) and may contribute to the inflammation seen in gout or pseudogout. However, at least one case of gout (Londino and Luparello, 1984) and one of rheumatoid arthritis (Dolovich and Litle, 1972) have been reported in factor XII-deficient subjects.

Pancreatitis, particularly acute hemorrhagic pancreatitis, is associated with release of large quantities of tissue kallikrein, thus, kallidin and/or bradykinin may contribute to the pooling of fluid within the abdominal cavity and hypotension that can result.

The causes of Alzheimer's disease are not known, although it is associated with deposition of β amyloid protein in the form of plaques as well as τ fibril proteins within neurofibrillary tangles and paired helical filaments. A rare hereditary form of Alzheimer's disease has been associated with a mutation of the amyloid precursor protein. We have demonstrated that, when β-amyloid monomer aggregates as it does within plaques, it is a potent initiator of the plasma kinin-forming cascade. It does so by binding factor XII and HK (Shibayama *et al.*, 1997) and in so doing activates the cascade in a zinc-dependent reaction. Furthermore, the aggregation of β amyloid has been shown to be zinc dependent (Bush *et al.*, 1994). In this fashion, β-amyloid resembles the binding we see to cell membranes, and to gC1qR specifically, becauase the latter protein also activates the cascade. However, activation by aggregated β-amyloid is more rapid than that seen with gC1qR and may have features that are reminiscent of both negatively charged surfaces (which are ion independent) and cell surface initiators. Whether any of the functional disturbances of neurons or glial cells seen in Alzheimer's disease are attributable to the generation of bradykinin remains to be established.

REFERENCES

Adam, A. *et al.* (1985). Human kininogens of low and high molecular mass: Quantification by radioimmunoassay and determination of reference values. *Clin. Chem.* **31,** 423–426.

Atkins, P. C., Miragliotta, G., Talbot, S. F., Zweiman, B., and Kaplan, A. P. (1987). Activation of plasma Hageman factor and kallikrein in ongoing allergic reactions in the skin. *J. Immunol.* **139,** 2744–2748.

Baglia, F. A., Sinha, D., and Walsh, P. N. (1989). Functional domains in the heavy-chain region of factor XI: A high molecular weight kininogen-binding site and a substrate binding site for factor IX. *Blood* **74,** 244–251.

Barrett, A. J., and Starkey, P. M. (1973). The interaction of α_2-macroglobulin with proteinases. *Biochem J* **133,** 709–724.

Baumgarten, C. R., *et al.* (1985). Influx of kininogens into nasal secretions after antigen challenge of allergic individuals. *J. Clin. Invest.* **76,** 191–197.

Baumgarten, C. R., *et al.* (1986a). Plasma kallikrein during experimentally-induced allergic rhinitis: Role in kinin formation and contribution to TAME esterase activity in nasal secretions. *J. Immunol.* **137,** 977–982.

Baumgarten, C. R., Nichols, R. C., Naclerio, R. M., and Proud, D. (1986b). Concentrations of glandular kallikrein in human nasal secretions increase during experimentally-induced allergic rhinitis. *J. Immunol.* **137,** 1323–1328.

Bernardo, M. M., Day, D. E., Halvorson, H. R., Olson, S. T., and Shore, J. D. (1993a). Surface-independent acceleration of factor XII activation by zinc ions. II. Direct binding and fluorescence studies. *J. Biol. Chem.* **268,** 12477–12483.

Bernardo, M. M., Day, D. E., and Olson, S. T. (1993b). Surface-independent acceleration of factor XII activation by zinc ions. I. Kinetic characterization of the metal ion rate enhancement. *J. Biol. Chem.* **268,** 12468.

Berrettini, M., *et al.* (1986). Detection of in vitro and in vivo cleavage of high molecular weight kininogen in human plasma immunoblotting with monoclonal antibodies. *Blood* **68,** 455–462.

Berrettini, M., Schleef, R. R., Heeb, M. J., Hopmeier, P., and Griffin, J. H. (1992). Assembly and expression of an intrinsic factor IX activator complex on the surface of cultured human endothelial cells. *J. Biol. Chem.* **267,** 19833–19839.

Bock, P. E., and Shore, J. D. (1983). Protein–protein interaction of blood coagulation. Characterization of a fluorescein-labeled human high molecular weight kininogen light chain as a probe. *J. Biol. Chem.* **258,** 15079–15086.

Bock, P. E., Srinivasan, K. R., and Shore, J. D. (1981). Activation of intrinsic blood coagulation by ellagic acid: insoluble ellagic acid metal ion complexes are the activating species. *Biochemistry* **20,** 7258–7271.

Bock, P. E., Shore, J. D., Tans, G., and Griffin, J. H. (1985). Protein–protein interactions in contact activation of blood coagulation. Binding of high molecular weight kininogen and the 5-(iodoacetamido) fluorescein-labeled kininogen light chain to prekallikrein, kallikrein, and the separated kallikrein heavy and light chains. *J. Biol. Chem.* **260,** 12434–12443.

Bouma, B. N., and Griffin, J. H. (1977). Human blood coagulation factor XI: Purification, properties, and mechanism of activation by factor XII. *J. Biol. Chem.* **252,** 6432–6437.

Bouma, B. N., Miles, L. A., Barretta, G., and Griffin, J. H. (1980). Human plasma prekallikrein. Studies of its activation by activated factor XII and of its inactivation by diisopropyl phosphofluoridate. *Biochemistry* **19,** 1151–1160.

Brunnée, T., *et al.* Activation of the contact system by glycosaminoglycan derived from mast cell proteoglycan. Submitted for publication.

Burman, J. F., Chung, H. I., and Lane, D. A. (1994). Role of factor XII in thrombin generation and fibrinolysis during cardiopulmonary bypass. *Lancet* **344,** 1192.

Bush, A. I., *et al.* (1994). Rapid inducton of Alzheimer A β amyloid formation by zinc. *Science,* **265,** 1464–1467.

Cameron, C. L., Fisslthaler, B., Sherman, A., Reddigari, S., and Silverberg, M. (1989). Studies on contact activation: Effects of surfaces and inhibitors. *Med. Prog. Tech.* **15,** 53–62.

Castellino, F. J., and Beals, J. M. (1987). The genetic relationships between the kringle domains of human plasminogen, prothrombin, tissue plasminogen activator, urokinase, and coagulation factor XII. *J. Mol. Evol.* **26,** 358–369.

Chung, D. W., Fujikawa, K., McMullen, B. A., and Davie, E. W. (1986). Human plasma prekallikrein, a zymogen to a serine protease that contains four tandem repeats. *Biochemistry* **25,** 2410–2417.

Cochrane, C. G., and Revak, S. D. (1980). Dissemination of contact activation in plasma by plasma kallikrein. *J. Exp. Med.* **152,**: 608–619.

Cochrane, C. G., Revak, S. D., and Wuepper, K. D. (1973). Activation of Hageman factor in solid and fluid phases. *J. Exp. Med.* **138,** 1564–1583.

Colman, R. W. (1969). Activation of plasminogen by plasma kallikrein. *Biochem. Biophys. Res. Commun.* **351,** 273.

Colman, R. W., and Muller-Esterl, W. (1988). Nomenclature of kininogens. *Thromb. Haemost.* **60,** 340–341.

Colman, R. W., *et al.* (1975). Williams trait: Human kininogen deficiency with diminished levels of plasminogen proactivator and prekallikrein associated with abnormalities of the Hageman factor dependent pathways. *J. Clin. Invest.* **56,** 1650–1662.

Colman, R. W., Wachtfogel, Y. T., and Kucich, V. (1985). Effects of cleavage of the heavy chain of human plasma kallikrein on its functional properties. *Blood* **65,** 311.

Cool, D. E., *et al.* (1985). Characterization of human blood coagulation factor XII cDNA. *J. Biol. Chem.* **25,** 13666–13676.

Creticos, P. S., *et al.* (1984). Peptide leucotriene release after antigen challenge in patients sensitive to ragweed. *N. Engl. J. Med.* **310,** 1626–1630.

Crutchly, D. J., Ryan, J. W., Ryan, U. S., and Fisher, G. H. (1983). Bradykinin induced release of prostacyclin and thromboxanes from bovine pulmonary artery endothelial cells. *Biochim. Biophys. Acta* **751,** 99–107.

Cugno, M., Hack, C. E., and de Boer, J. P. (1993). Generation of plasmin during acute attack of hereditary angioedema. *J. Lab. Clin. Med.* **121,** 38.

Curd, J. G., Prograis, L. F., Jr., and Cochrane, C. G. (1980). Detection of active kallikrein in induced blister fluids of hereditary angioedema patients. *J. Exp. Med.* **152,** 742–747.

de Agostini, A., Lijnen, H. R., Pixley, R. A., Colman, R. W., and Schapira, M. (1984). Inactivation of factor XII active fragment in normal plasma. Predominant role of C1-inhibitor. *J Clin Invest* **73,** 1542–1549.

Deblois, D., Bouthillier, J., and Marceau, F. (1988). Effect of glucocorticoids, monokines, and growth factors on the spontaneously developing responses of the rabbit isolated aorta to des-arg^9-bradykinin. *Br. J. Pharmacol.* **93,** 969–977.

Deblois, D., Bouthillier, J., and Marceau, F. (1989). Pharmacological modulation of the upregulated responses to des-arg^9-bradykinin in vivo and in vitro. *Immunopharmacology* **17,** 187–198.

De La Cadena, R. A., *et al.* (1994). Platelet thrombospondin interactions with human high and low molecular weight kininogens. *Thromb. Haemost.* **72,** 125–131.

Dolovich, J., and Little, D. C. (1972). Correlates of skin test reactions to *Bacillus subtilis enzyme preparations. J. Allergy Clin. Immunol.* **49,** 43.

Donaldson, V. H. (1968). Mechanisms of activation of C1 esterase in hereditary angioneurotic edema plasma in vitro: The role of Hageman factor, a clot-promoting agent. *J. Exp. Med.* **127,** 411–429.

Donaldson, V. H., Glueck, H. I., and Miller, M. A. (1976). Kininogen deficiency in Fitzgerald trait: Role of high molecular weight kininogen in clotting and fibrinolysis. *J. Lab. Clin. Med.* **89,** 327–337.

Donaldson, V. H., Rosen, F. S., and Bing, D. H. (1977). Role of the second component of complement (C2) and plasmin in kinin release in hereditary angioneurotic edema (H.A.N.E.). *Plasma Trans. Assoc. Am. Physicians* **40,** 174–183.

Dunn, J. T., and Kaplan, A. P. (1982). Formation and structure of human Hageman factor fragments. *J. Clin. Invest.* **70,** 627–631.

Dunn, J. T., Silverberg, M., and Kaplan, A. P. (1982). The cleavage and formation of activated Hageman factor by autodigestion and by kallikrein. *J. Biol. Chem.* **275,** 1779–1784.

Erdos, E. G., Sloane, G. M. (1962). An enzyme in human plasma that inactivates bradykinin and kallidins. *Biochem Pharmacol* **11,** 585–592.

Fields, T., Ghebrehiwet, B., and Kaplan, A. P. (1983). Kinin formation in hereditary angioedema plasma: Evidence against kinin derivation from C2 and in support of "spontaneous" formation of bradykinin. *J. Allergy Clin. Immunol.* **72,** 54–60.

Forbes, C. O., Pensky, J., and Ratnoff, O. D. (1970). Inhibition of activated Hageman factor and activated plasma thromboplastin antecedent by purified Cl inactivator. *J. Lab. Clin. Med.* **76,** 809–815.

Fujikawa, K., and McMullen, B. A. (1983). Amino acid sequence of Human b-Factor XIIa. *J. Biol. Chem.* **258,** 10924–10933.

Fujikawa, K., Heimark, R. L., Kurachi, K., and Davie, E.W. (1980). Activation of bovine factor XII (Hageman factor) by plasma kallikrein. *Biochemistry* **19,** 1322–1330.

Gallin, J. I., and Kaplan, A. P. (1974). Mononuclear cell chemotactic activity of kallikrein and plasminogen activator and its inhibition by C1 INH and a2 macroglobulin. *J. Immunol.* **113,** 1928.

Ghebrehiwet, B., Randazzo, B. P., Dunn, J. T., Silverberg, M., and Kaplan, A. P. (1983). Mechanism of activation of the classical pathway of complement by Hageman factor fragment. *J. Clin. Invest.* **71,** 1450–1456.

Ghebrehiwet, B., Lim, B.-L., Peerschke, E. I. B., Willis, A. C., and Reid, K. B. M. (1994). Isolation, cDNA cloning and overexpression of a 33-kD cell surface glycoprotein that binds to the globular 'heads' of C1q. *J. Exp. Med.* **179,** 1809–1821.

Gigli, I., Mason, J. W., Colman, R. W., and Austen, K. F. (1970). Interaction of plasma kallikrein with the C1 inhibitor. *J. Immunol.* **104,** 574–581.

Ginsberg, M., Jaques, B., Cochrane, C. G., and Griffin, J. H. (1980). Urate crystal dependent cleavage of Hageman factor in human plasma and synovial fluid. *J. Lab. Clin. Med.* **95,** 497–506.

Goldsmith, G., Saito, H., and Ratnoff, O. D. (1978). The activation of plasminogen by Hageman factor (factor XII) and Hageman factor fragments. *J. Clin. Invest.* **12,** 54.

Gounaris, A. D., Brown, M. A., and Barrett, A. J. (1984). Human plasma α_1-cysteine proteinase inhibitor. Purification by affinity chromatography, characterization, and isolation of an active fragment. *Biochem. J.* **221,** 445–452.

Greengard, J. S., and Griffin, J. H. (1984). Receptor for high molecular kininogen on stimulated washed human platelets. *Biochemistry* **23,** 6863–6869.

Griep, M. A., Fujikawa, K., and Nelsestuen, G. L. (1985). Binding and activation properties of human factor XII, prekallikrein and derived peptides with acidic lipid vesicles. *Biochemistry* **24,** 4124–4130.

Griffin, J. H. (1978). Role of surface in surface-dependent activation of Hageman factor (blood coagulation factor XII). *Proc. Natl. Acad. Sci. USA* **75,** 1998–2002.

Griffin, J. H., and Cochrane, C.G. (1976). Mechanisms for the involvement of high molecular weight kininogen in surface-dependent reactions of Hageman factor. *Proc. Natl. Acad. Sci. USA* **73,** 2554–2558.

Guimaraes, J. A., Borges, D. R., Prado, E. S., and Prado, J. L. (1973). Kinin converting aminopeptidase from human serum. *Biochem. Pharmacol.* **22,** 403–414.

Gurewich, V., Johnstone, M., and Loza, J. P. (1993). Pro-urokinase and prekallikrein are both associated with platelets. Implications for the intrinsic pathway of fibrinolysis and for therapeutic thrombolysis. *FEBS Lett.* **318,** 317.

Gustafson, E. J., Schutsky, D., Knight, L. C., and Schmaier, A. H. (1986). High molecular weight kininogen binds to unstimulated platelets. *J. Clin. Invest.* **78,** 310–318.

Hall, J. M. (1992). Bradykinin receptors: Pharmacological properties and biological roles. *Pharmacol. Ther.* **56,** 131–190.

Harpel, P. C. (1974). In *Chemistry and Biology of the Kallikrein–Kinin System in Health and Disease* (J. J. Pisano and K. F. Austen, Eds.), p. 169. US Government Printing Office, Washington, DC.

Harpel, P. C., Lewin, M. F., and Kaplan, A. P. (1985). Distribution of plasma kallikrein between C1 inactivator and α_2-macroglobulin–kallikrein complexes. *J. Biol. Chem.* **260,** 4257–4263.

Hasan, A. A. K., Cines, D. B., Herwald, H., Schmaier, A. H., and Müller-Esterl, W. (1995). Mapping the cell binding site on high molecular weight kininogen domain 5. *J. Biol. Chem.* **270,** 19256–19261.

Hauert, J., Nicoloso, G., and Schleuning, W. D. (1989). Plasminogen activators in dextran sulfate-activated euglobulin fraction: A molecular analysis of factor XII- and prekallikrein-dependent fibrinolysis. *Blood* **73,** 994.

Heck, L. W., and Kaplan, A. P. (1974). Substrates of Hageman factor: I. Isolation and characterization of human factor XI (PTA) and inhibition of the activated enzyme by α_1-antitrypsin. *J. Exp. Med.* **140,** 1615–1630.

Henderson, L. M., Figueroa, C. D., and Muller-Esterl, W. (1994). Assembly of contact phase factors on the surface of the human neutrophil membrane. *Blood* **84,** 474.

Herwald, H., Hasan, A. A. K., Godovac-Zimmerman, J., Schmaier, A. H., and Müller-Esterl, W. (1965). Identification of an endothelial cell binding site on kininogen domain D3. *J. Biol. Chem.* **270,** 14634–14642.

Herwald, H., Dedio, J., Kellner, R., Loos, M., and Müller-Esterl, W. (1996). Isolation and characterization of the kininogen-binding protein p33 from endothelial cells. *J. Biol. Chem.* **271,** 13040–13047.

Hess, J. R., Borkowski, J. A., Young, G. S., Strader, C. D., and Ransom, R. W. (1992). Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. *Biochem. Biophys. Res. Commun.* **184,** 260–268.

Higashiyama, S., *et al.* (1986). Human high molecular weight kininogen as a thiol protease inhibitor: Presence of the entire inhibition capacity in the native form of heavy chain. *Biochemistry* **25,** 1669–1675.

Hirsh, E. F., Nakajima, T., Oshima, G., Erdos, E. G., and Herman, M. (1974). Kinin-system responses in sepsis after trauma in man. *J. Surg. Res.* **17,** 147–153.

Hojima, Y., Cochrane, C. G., Wiggins, R. C., Austen, K. F., and Stevens, R. L. (1984). In vitro activation of the contact (Hageman factor) system of plasma by heparin and chondroitin sulfate E. *Blood* **63,** 1453–1459.

Hong, S. L. (1980). Effect of bradykinin and thrombin on prostacyclin synthesis in endothelial cells from calf and pig aorta and human umbilical cord vein. *Thromb. Res.* **18,** 787–795.

Huisveld, I. A., Haspers, H., and Van Heeswijk, G. M. (1985). Contribution of contact activation factors to urokinase-related fibrinolytic activator in whole plasma. *Thromb. Haemost.* **54,** 102.

Iatridis, P. G., and Ferguson, J. H. (1962). Active Hageman factor. A plasma lysokinase of the human fibrinolytic system. *J. Clin. Invest.* **41,** 1277.

Ikari, N., Sugo, T., Fuji, S., Kato, H., and Iwanaga, S. (1981). The role of bovine high molecular weight kininogen in contact-mediated activation of bovine factor XII: Interaction of HMW kininogen with kaolin and plasma prekallikrein. *J. Biochem.* **89,** 1699–1709.

Ishiguro, H., Higashiyama, S., Ohkubo, I., and Sasaki, M. (1987). Mapping of functional domains of human high molecular weight and high molecular weight kininogens using murine monoclonal antibodies. *Biochemistry* **26,** 7021–7029.

Jacobsen, S., and Kritz, M. (1967). Some data on two purified kininogens from human plasma. *Br. J. Pharmacol.* **29,** 25–36.

Jansen, P. M., Pixley, R. A., and Chang, A. C. K. (1994). Inhibition of factor XII in septic baboons attenuated activation of complement and fibrinolytic systems and reduced the release of neutrophil elastase. *Intensive Care Med.* **20,** S2.

Jiang, Y., Müller-Esterl, W., and Schmaier, A. H. (1992). Domain 3 of kininogens contains a cell-binding site and a site that modifies thrombin activation of platelets. *J. Biol. Chem.* **267,** 3712–3717.

Johnson, D. A., Salveson, G., Brown, M., and Barrett, A. J. (1987). Rapid isolation of human kininogens. *Thromb. Res.* **48,** 187–193.

Jorg, M., and Binder, B. R. (1985). Kinetic analysis of plasminogen activation by purified plasma kallikrein. *Thromb. Res.* **39,** 323.

Joseph, K., Ghebrehiwet, B., Peerschke, E. I. B., Reid, B. M., and Kaplan, A. P. (1996). Identification of the zinc-dependent endothelial cell binding protein for high molecular weight kininogen and factor XII: Identity with the receptor that binds to the globular "heads" of C1q (gC1qR). *Proc. Natl. Acad. Sci. USA* **93,** 8552–8557.

Joseph, K., Bahou, W., and Kaplan, A. P. (1997). Evidence that the zinc-dependent platelet binding for Factor XII and high molecular weight kininogen is GPIb. *J. Invest. Med.* **45,** 267A.

Kaplan, A. P., and Austen, K. F. (1970). A prealbumin activator of prekallikrein. *J. Immunol.* **105,** 802–811.

Kaplan, A. P., and Austen, K. F. (1971). A prealbumin activator of prekallikrein II: Derivation of activators of prekallikrein from active Hageman factor by digestion with plasmin. *J. Exp. Med.* **133,** 696–712.

Kaplan, A. P., Kay, A. B., and Austen, K. F. (1972). A prealbumin activator of prekallikrein III. Appearance of chemotactic activity for human neutrophils by the conversion of human prekallikrein to kallikrein. *J. Exp. Med.* **135,** 81.

Kaufman, N., *et al.* (1991). α_2 Macroglobulin–kallikrein complexes detect contact system activation in hereditary angioedema and human sepsis. *Blood* **77,** 2660–2667.

Kellermann, J., Lottspeich, F., Henschen, A., and Müller-Esterl, W. (1986). Completion of the primary structure of human high molecular weight kininogen. The amino acid sequence of the entire heavy chain and evidence for its evolution by gene triplication. *Eur. J. Biochem.* **154,** 471–478.

Kellermeyer, R. W., and Breckenridge, R. T. (1965). The inflammatory process in acute gouty arthritis. I. Activation of Hageman factor by sodium urate crystals. *J. Lab. Clin. Med.* **63,** 307–315.

Kitamura, N., *et al.* (1985). Structural organization of the human kininogen gene and a model for its evolution. *J. Biol. Chem.* **260,** 8610–8617.

Kluft, C. (1976). Occurrence of C1-inhibitor and other proteinase inhibitors in euglobulin fractions and their influence of fibrinolytic activity. *Haemostasis* **5,** 136.

Kluft, C. (1977). Elimination of inhibition of euglobulin fibrinolysis by use of flufenamate: Involvement of C1-inactivator. *Haemostasis* **6,** 351.

Kluft, C. (1978). Determination of prekallikrein in human plasma: Optimal conditions for activating prekallikrein. *J. Lab. Clin. Med.* **91,** 83–93.

Kluft, C., Wijngaards, G., and Jie, A. (1984). Intrinsic plasma fibrinolysis: Involvement of urokinase-related activity in the factor XII independent plasminogen proactivator pathway. *J. Lab. Clin. Med.* **103,** 408.

Kodama, K., Kato, H., and Iwanaga, S. (1985). Isolation of bovine platelet cationic proteins which inhibit the surface-mediated activation of factor XII and prekallikrein. *J. Biochem.* **97,** 139.

Kurachi, K., and Davie, E. W. (1977). Activation of human factor XI (plasma thromboplastin antecedent) by factor XIIa (activated Hageman factor). *Biochemistry* **16,** 5831–5839.

Kurachi, K., Fujikawa, K., and Davie, E. W. (1980). Mechanism of activation of bovine factor XI by factor XII and factor XII_a. *Biochemistry* **19,** 1330–1338.

Lipscomb, M. S., and Walsh, P. N. (1969). Human platelets and factor XI. Localization in platelet membranes of factor XI-like activity and its functional distinction from that of factor XI. *J. Clin. Invest.* **63,** 1006.

Londino, A., and Luparello, F. J. (1984). Factor XII deficiency in a man with gout and angioimmunoblastic lymphadenopathy. *Arch. Intern. Med.* **144,** 1497–1498.

Lottspeich, F., Kellermann, J., Henschen, A., Foertsch, B., and Muller-Esterl, W. (1985). The amino acid sequence of the light chain of human high molecular mass kininogen. *Eur. J. Biochem.* **152,** 307–314.

Loza, J. P., Gurewich, V., and Johnstone, M. (1994). Platelet-bound prekallikrein promotes pro-urokinase-induced clot lysis: A mechanism for targeting the factor XII dependent intrinsic pathway of fibrinolysis. *Thromb. Haemost.* **71,** 347.

Mandle, R. J., Jr., and Kaplan, A. P. (1977). Hageman factor substrates. II. Human plasma prekallikrein. Mechanism of activation by Hageman factor and participation in Hageman factor-dependent fibrinolysis. *J. Biol. Chem.* **252,** 6097–6104.

Mandle, R. J., Jr., and Kaplan, A. P. (1979). Hageman factor dependent fibrinolysis. Generation of fibrinolytic activity by the interaction of human activated factor XI and plasminogen. *Blood* **54,** 850.

Mandle, R. J., Jr., Colman, R. W., and Kaplan, A. P. (1976). Identification of prekallikrein and HMW-kininogen as a complex in human plasma. *Proc. Natl. Acad. Sci. USA* **73,** 4179–4183.

Manke, J. G., *et al.* (1994). Expression cloning of a human B_1 bradykinin receptor. *J. Biol. Chem.* **269,** 21583–21586.

Margolis, J. (1958). Activation of plasma by contact with glass: Evidence for a common reaction which releases plasma kinin and initiates coagulation. *J. Physiol.* **144,** 1–22.

Mason, J. W., Kleeberg, U. R., Dolan, P., and Colman, R. W. (1970). Plasma kallikrein and Hageman factor in gram-negative bacteremia. *Ann. Intern. Med.* **73,** 545–551.

McConnell, D. J. (1972). Inhibitors of kallikrein in human plasma. *J. Clin. Invest.* **51,** 1611–1623.

McDonagh, J. S., and Ferguson, J. H. (1970). Studies on the participation of Hageman factor in fibrinolysis. *Thromb. Haemost.* **24,** 1.

McEachern, A. E., *et al.* (1991). Expression cloning of a rat B_2 bradykinin receptor. *Proc. Natl. Acad. Sci. USA* **88,** 7724–7728.

McMillin, C. R., Saito, H., Ratnoff, O. D., and Walton, A. G. (1974). The secondary structure of human Hageman factor (factor XII) and its alteration by activating agents. *J. Clin. Invest.* **54,** 1312–1322.

McMullen, B. A., and Fujikawa, K. (1985). Amino acid sequence of the heavy chain of human a-factor XIIa (activated Hageman factor). *J. Biol. Chem.* **260,** 5328–5341.

Meier, H. L., Pierce, J. V., Colman, R. W., and Kaplan, A. P. (1977). Activation and function of human Hageman factor. The role of high molecular kininogen and prekallikrein. *J. Clin. Invest.* **60,** 18–31.

Meijers, J. C., Vlooswijk, R. A. A., and Bouma, B. N. (1988). Inhibition of human blood coagulation factor XIa by C1 Inhibitor. *Biochemistry* **27,** 959–963.

Meloni, F. J., and Schmaier, A. H. (1991). Low molecular weight kininogen binds to platelets to modulate thrombin-induced platelet activation. *J. Biol. Chem.* **266,** 6786–6794.

Meloni, F. J., Gustafson, E. J., and Schmaier, A. H. (1992). High molecular weight kininogen binds to platelets by its heavy and light chains and when bound has altered susceptibility to kallikrein cleavage. *Blood* **79,** 1233–1244.

Miles, L. A., Rothschild, Z., and Griffin, J. H. (1981). Dextran sulfate stimulated fibrinolytic activity in whole human plasma. Dependence on the contact activation system and a urokinase-related antigen. *Thromb. Haemost.* **46,** 211.

Miles, L. A., Greengard, J. S., and Griffin, J. H. (1983a). A comparison of the abilities of plasma kallikrein, factor XIIa, factor XIa, and urokinase to activate plasminogen. *Thromb. Res.* **29,** 407.

Miles, L. A., Rothschild, Z., and Griffin, J. H. (1983b). Dextran sulfate dependent fibrinolysis in whole human plasma. *J. Lab. Clin. Med.* **101,** 214.

Moorman, R. M., Reynolds, D. S., and Comunale, M. E. (1993). Management of cardiopulmonary bypass in a patient with congenital factor XII deficiency. *J. Cardiothorac. Vasc. Anesth.* **7,** 452.

Mori, K., and Nagasawa, S. (1981). Studies on human high molecular weight (HMW) kininogen. II. Structural change in HMW kininogen by the action of human plasma kallikrein. *J. Biochem.* **89,** 1465–1473.

Mori, K., Sakamoto, W., and Nagasawa, S. (1981). Studies on human high molecular weight (HMW) kininogen III. Cleavage of HMW kininogen by the action human salivary kallikrein. *J. Biochem.* **90,** 503–509.

Morrison, D. C., and Cochrane, C. G. (1974). Direct evidence for Hageman factor (factor XII) activation by bacterial lipopolysaccharides (endotoxins). *J. Exp. Med.* **140,** 797–811.

Müller-Esterl, W., *et al.* (1985a). Human plasma kininogens are identical with α_2-cysteine protease inhibitors. Evidence from immunological, enzymological, and sequence data. *FEBS Lett.* **182,** 310–314.

Müller-Esterl, W., Rauth, G., Lottspeich, F., Kellermann, J., and Henschen, A. (1985b). Limited proteolysis of human low-molecular mass kininogen by tissue kallikrein. Isolation and characterization of the heavy and light chains. *Eur. J. Biochem.* **149,** 15–22.

Nagase, H., Cawston, J. E., DeSilva, M., and Barrett, A. J. (1982). Identification of plasma kallikrein as an activator of latent collagenase in rheumatoid synovial fluid. *Biochim. Biophys. Acta* **707,** 133–142.

Nishikawa, K., Kuna, P., Calcaterra, E., Kaplan, A. P., and Reddigari, S. R. (1992). Generation of the vasoactive peptide bradykinin from human high molecular weight kininogen bound to human umbilical vein endothelial cells. *Blood* **80,** 1980–1988.

O'Donnell, T. F., Clowes, G. H. J., Talamo, R. C., and Colman, R. W. (1976). Kinin activation in the blood of patients with sepsis. *Surg. Gynecol. Obstet.* **143,** 539–545.

Ogston, D., Ogston, L. M., Ratnoff, O. D., and Forbes, C. O. (1967). Studies on a complex mechanism for the activation of plasminogen by kaolin and by chloroform. The participation of Hageman factor and additional cofactors. *J. Clin. Invest.* **48,** 1786.

Ogston, D., Bennett, N. B., and Ogston, C. M. (1971). The assay of a plasma component necessary for the generation of a plasminogen activator in the presence of Hageman factor (Hageman factor cofactor). *Br. J. Haematol.* **20,** 209.

Ohkubo, I., Kurachi, K., Takasawa, T., Shiokawa, H., and Sasaki, M. (1984). Isolation of cDNA for alpha-2-thiol protease inhibitor and its identity with low molecular weight kininogen. *Biochemistry* **23,** 3891.

Pedersen, O. D., Munkvad, S., and Gram, J. (1993). Depression of factor XII-dependent fibrinolytic activity in survivors of acute myocardial infarction at risk of reinfarction. *Eur. Heart J.* **1993.**

Pettinger, M. A., and Young, R. (1970). Endotoxin-induced kinin (bradykinin) formation: Activation of Hageman factor and plasma kallikrein in human plasma. *Life Sci.* **9,** 313–322.

Pixley, R. A., Schapira, M., and Colman, R. W. (1985). The regulation of human factor XII by plasma proteinase inhibitors. *J. Biol. Chem.* **260,** 1723–1729.

Pixley, R. A., Stumpo, L. G., Birkmeyer, K., Silver, L., and Colman, R. W. (1987). A monoclonal antibody recognizing an icosapeptide sequence in the heavy chain of human factor XII inhibits surface-catalyzed activation. *J. Biol. Chem.* **262,** 10140–10145.

Pixley, R. A., *et al.* (1992). Activation of the contact system in lethal hypotensive bacteremia in a baboon model. *Am. J. Pathol.* **140,** 897–906.

Pixley, R.A., *et al.* (1993). The contact system contributes to hypotension but not disseminated intravascular coagulation in lethal baterimia. In vivo use of a monoclonal anti Factor XII antibody to block contact activation in baboons. *J. Clin. Invest.* **92,** 61–68.

Proctor, R. R., and Rapaport, S. J. (1961). The partial thromboplastin time with kaolin: A simple screening test for first stage clotting deficiencies. *Am. J. Clin. Pathol.* **35,** 212–219.

Proud, D., Pierce, J. V., and Pisano, J. J. (1980). Radioimmunoassay of human high molecular weight kininogen in normal and deficient plasma. *J. Lab. Clin. Med.* **95,** 563–574.

Proud, D., *et al.* (1983). Kinins are generated in vivo following nasal airway challenge of allergic individuals with allergen. *J. Clin. Invest.* **72,** 1678–1685.

Puri, R. N., Zhou, F., Hu, C.-J., and Colman, R. W. (1991). High molecular weight kininogen inhibits thrombin-induced platelet aggregation and cleavage of aggregin by inhibiting binding of thrombin the platelets. *Blood* **77,** 500–507.

Que, B. G., and Davie, E. W. (1986). Characterization of a cDNA coding for human factor XII (Hageman factor). *Biochemistry* **8,** 1525–1528.

Ratnoff, O. D., and Crum, J. D. (1964). Activation of Hageman factor by solutions of ellagic acid. *J. Lab. Clin. Med.* **63,** 359–377.

Ratnoff, O. D., and Saito, H. (1979). Amidolytic properties of single chain activated Hageman factor. *Proc. Natl. Acad. Sci. USA* **76,** 1461–1463.

Reddigari, S., and Kaplan, A. P. (1989a). Monoclonal antibody to human high molecular weight kininogen recognizes its prekallikrein binding site and inhibits its coagulant activity. *Blood* **74,** 695–702.

Reddigari, S., and Kaplan, A. P. (1989b). Quantification of human high molecular weight kininogen by immunoblotting with a monoclonal anti-light chain antibody. *J. Immunol. Methods* **119,** 19–25.

Reddigari, S. R., and Kaplan, A. P. (1988). Cleavage of high molecular weight kininogen by purified kallikreins and upon contact activation of plasma. *Blood* **71,** 1334–1340.

Reddigari, S. R., *et al.* (1993a). Human high molecular weight kininogen binds to human umbilical vein endothelial cells via its heavy and light chains. *Blood* **81,** 1306–1311.

Reddigari, S. R., Shibayama, Y., Brunnee, T., and Kaplan, A. P. (1993b). Human Hageman factor (FXII) and high molecular weight kininogen compete for the same binding site on human umbilical vein endothelial cells. *J. Biol. Chem.* **268,** 11982–11987.

Regoli, D., and Barabe, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.* **32,** 1–46.

Revak, S. D., Cochrane, C. G., Johnston, A. R., and Hugli, T. E. (1974). Structural changes accompanying enzymatic activation of human Hageman factor. *J. Clin. Invest.* **54,** 619–627.

Revak, S. D., Cochrane, C. G., and Griffin, J. H. (1977). The binding and cleavage characteristics of human Hageman factor during contact activation: A comparison of normal plasma with plasma deficient in factor XI, prekallikrein or high molecular weight kininogen. *J. Clin. Invest.* **58,** 1167–1175.

Robinson, J. A., Klodynicky, M. L., Loeb, H. S., Recic, M. R., and Gunnar, R. M. (1975). Endotoxin, prekallikrein, complement, and systemic vascular resistances. Sequential measurement in man. *Am. J. Med.* **59,** 61–67.

Roeise, O., Bouma, B. N., Stadaas, J. O., and Aasen, A. O. (1988). Dose dependence of endotoxin-induced activation of the plasma contact system: An in vitro study. *Circ. Shock* **26,** 419–430.

Rosing, J., Tans, G., and Griffin, J. H. (1985). Surface-dependent activation of human factor XII by kallikrein, and its light chain. *Eur. J. Biochem.* **151,** 531–538.

Saito, H., Ratnoff, O. D., and Donaldson, V. H. (1974). Defective activation of clotting, fibrinolytic and permeability-enhancing systems in human Fletcher trait. *Circ. Res.* **34,** 641–651.

Schapira, M., Despland, E., Scott, C. F., Boxer, L. A., and Colman, R. W. (1982a). Purified human plasma kallikrein aggregates human blood neutrophils. *J. Clin. Invest.* **69,** 1199.

Schapira, M., Scott, C. F., and Colman, R. W. (1982b). Contribution of plasma protease inhibitors to the inactivation of kallikrein in plasma. *J. Clin. Invest.* **69,** 462–468.

Schiffman, S., Mannhalter, C., and Tynerk, D. (1980). Human high molecular weight kininogen. Effects of cleavage by kallikrein on protein structure and procoagulant activity. *J. Biol. Chem.* **255,** 6433–6438.

Schmaier, A. H., *et al.* (1983). High molecular weight kininogen: A secreted platelet protein. *J. Clin. Invest.* **71,** 1477.

Schmaier, A. H., *et al.* (1984). The effects of high molecular weight kininogen on surface-adsorbed fibrinogen. *Thromb. Res.* **33,** 51–57.

Schmaier, A. H., *et al.* (1986a). High molecular weight kininogen is an inhibitor of platelet calpain. *J. Clin. Invest.* **77,** 1565–1573.

Schmaier, A. H., Smith, P. M., Pardon, A. D., White, J. G., and Colman, R. W. (1986b). High molecular weight kininogen: Localization in the unstimulated and activated platelet and activation by a platelet calpain(s). *Blood* **67,** 119.

Schmaier, A. H., Kuo, A., Lundberg, D., Murray, S., and Cines, D. B. (1988). The expression of high molecular weight kininogen on human umbilical vein endothelial cells. *J. Biol. Chem.* **263,** 16327–16333.

Schmidt, V., Vitale, E., and Bahou, W. (1996). Genomic cloning and characterization of the human thrombin receptor gene: Structural similarity to the proteinase activated receptor-2 (PAR-2) gene. *J. Biol. Chem.* **271,** 9307.

Schreiber, A. D., Kaplan, A. P., and Austen, K. F. (1973). Inhibition by CI INH of Hageman factor fragment activation of coagulation, fibrinolysis, and kinin generation. *J. Clin. Invest.* **52,** 1402–1409.

Scott, C. F., Schapira, M., James, H. L., Cohen, A. B., and Colman, R. W. (1982). Inactivation of factor Xla by plasma protease inhibitors. Predominant role of $\alpha_1$protease inhibitor and protective effect of high molecular weight kininogen. *J. Clin. Invest.* **69,** 844–852.

Scully, M. F., Weerasinghe, K., and Kakhar, V. V. (1980). Inhibition of contact activation by platelet factor 4. *Thromb. Res.* **20,** 461.

Sheikh, I. A., and Kaplan, A. P. (1986a). Studies of the digestion of bradykinin, lysylbradykinin and des-arg9 bradykinin by angiotensin converting enzyme. *Biochem. Pharmacol.* **35,** 1951–1956.

Sheikh, I. A., and Kaplan, A. P. (1986b). Studies of the digestion of bradykinin, lysylbradykinin and kinin degradation products by carboxypeptidases A, B and N. *Biochem. Pharmacol.* **35,** 1957–1963.

Sheikh, I. A., and Kaplan, A. P. (1989). The mechanism of digestion of bradykinin and lysylbradykinin (kallidin) in human serum: The role of carboxy-peptidase, angiotensin converting enzyme, and determination of final degradation products. *Biochem. Pharmacol.* **38,** 993–1000.

Shibayama, Y., Brunnee, T., and Kaplan, A. P. (1994). Activation of human Hageman factor (Factor XII) in the presence of zinc and phosphate ions. *J. Med. Biol. Res.* **27,** 1817.

Shibayama, Y., Konishi, J.-E., Nakamura, T., Suehiro, S., and Kaplan, A. P. (1997). Activation of the plasma kinin forming cascade by aggregated Alzheimer A β amyloid protein. *American Soc. Clin. Invest.*, in press. [abstract]

Silverberg, M., and Diehl, S. V. (1987a). The activation of the contact system of human plasma by polysaccharide sulfates. *Ann. N.Y. Acad. Sci.* **516,** 268–279.

Silverberg, M., and Diehl, S. V. (1987b). The autoactivation of factor XII (Hageman factor) induced by low M_r heparin and dextran sulphate. *Biochem. J.* **248,** 715–720.

Silverberg, M., and Kaplan, A. P. (1982). Enzymatic activities of activated and zymogen forms of human Hageman factor (factor XII). *Blood* **60,** 64–70.

Silverberg, M., Dunn, J. T., Garen, L., and Kaplan, A. P. (1980a). Autoactivation of human Hageman factor: Demonstration utilizing a synthetic substrate. *J. Biol. Chem.* **255,** 7281–7286.

Silverberg, M., Nicoll, J. E., and Kaplan, A. P. (1980b). The mechanism by which the light chain of cleaved HMW-kininogen augments the activation of prekallikrein, factor XI, and Hageman factor. *Thromb. Res.* **20,** 173–189.

Sinha, D., Seaman, F. S., and Koshy, A. (1984). Blood coagulation factor XIa binds specifically to a site on activated human platelets distinct from that for factor XI. *J. Clin. Invest.* **73,** 1550.

Smith, A. M. J., Webb, C., and Holford, J. (1995). Signal transduction pathways for B1 and B2 bradykinin receptors in bovine pulmonary artery endothelial cells. *Mol. Pharmacol.* **47,** 525.

Smith, D., Gilbert, M., and Owen, W. G. (1985). Tissue plasminogen activator release in vivo in response to vasoactive agents. *Blood* **66,** 835–839.

Soter, N. A., Austen, K. F., and Gigli, I. (1975). Inhibition by ε-aminocaproic acid of the activation of the first component of the complement system. *J. Immunol.* **114,** 928–932.

Stead, N., Kaplan, A. P., and Rosenberg, R. D. (1976). Inhibition of activated factor XII by antithrombin–heparin cofactor. *J. Biol. Chem.* **251,** 6481–6488.

Stewart, J. M., *et al.* (1996). A new generation of bradykinin antagonists. *Immunopharmacology* **33,** 51–60.

Strang, C. J., *et al.* (1988). Angioedema induced by a peptide derived from complement component C2. *J. Exp. Med.* **168,** 1685–1698.

Tait, J., and Fujikawa, K. (1986). Identification of the binding site plasma prekallikrein in human high molecular weight kininogen. *J. Biol. Chem.* **261,** 15396–15401.

Tait, J. F., and Fujikawa, K. (1987). Primary structure requirements for the binding of human high molecular weight kininogen to plasma prekallikrein and factor XI. *J. Biol. Chem.* **262,** 11651–11656.

Takagaki, Y., Kitamura, N., and Nakanishi, S. (1985). Cloning and sequence analysis of cDNAs for high molecular weight and low molecular weight prekininogens. *J. Biol. Chem.* **260,** 8601–8609.

Tankersley, D. L., and Finlayson, J. S. (1984). Kinetics of activation and autoactivation of human factor XII. *Biochemistry* **23,** 273–279.

Tankersley, D. L., Fournel, M. A., and Schroeder, D. D. (1980). Kinetics of activation of prekallikrein by prekallikrein activator. *Biochemistry* **19,** 3121.

Tans, G., and Griffin, J. H. (1982). Properties of sulfatides in factor XII dependent contact activation. *Blood* **59,** 69–75.

Tans, G., Rosing, J., and Griffin, J. D. (1983). Sulfatide dependent autoactivation of human blood coagulation factor XII (Hageman factor). *J. Biol. Chem.* **258,** 8215–8222.

Thompson, R. E., Mandle, R. J., Jr., and Kaplan, A. P. (1977). Association of factor XI and high molecular weight kininogen in human plasma. *J. Clin. Invest.* **60,** 1376–1380.

Thompson, R. E., Mandle, R. J., and Kaplan, A. P. (1978). Characterization of human high molecular weight kininogen. Procoagulant activity associated with the light chain of kinin-free high molecular weight kininogen. *J. Exp. Med.* **147,** 488–499.

Thompson, R. E., Mandle, R. J., and Kaplan, A. P. (1979). Studies of the binding of prekallikrein and factor XI to high molecular weight kininogen and its light chain. *Proc. Natl. Acad. Sci. USA* **79,** 4862–4866.

Travis, J., and Salvesen, G. S. (1983). Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* **52,** 655–709.

Tuszynski, G. P., Bevacona, S. J., Schmaier, A. H., Colman, R. W., and Walsh, P. N. (1982). Factor XI antigen and activity in human platelets. *Blood* **59,** 1148.

van der Graaf, F., Koedam, J. A., and Bouma, B. N. (1983). Inactivation of kallikrein in human plasma. *J. Clin. Invest.* **71,** 149–158.

Van Iwaarden, F., deGroot, P. G., and Bouma, B. N. (1988). The binding of high molecular weight kininogen to cultured human endothelial cells. *J. Biol. Chem.* **263,** 4698–4703.

Vu, T. K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. H. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64,** 1057–1064.

Wachtfogel, Y. T., *et al.* (1983). Human plasma kallikrein releases neutrophil elastase activity during blood coagulation. *J. Clin. Invest.* **22,** 1672.

Wachtfogel, Y. T., Pixley, R. A., and Kucich, U. (1986). Purified plasma factor XIIa aggregates human neutrophils and causes degranulation. *Blood* **67,** 1731.

Wachtfogel, Y. T., *et al.* (1994). High molecular weight kininogen binds to Mac-1 on neutrophils by its heavy chain (domain 3) and its light chain (domain 5). *J. Biol. Chem.* **269,** 19307–19312.

Walsh, P. N. (1962). The effects of collagen and kaolin on the intrinsic coagulant activity of platelets. Evidence for an alternative pathway in intrinsic coagulation not requiring factor XII. *Br. J. Naematol.* **22,** 393.

Walsh, P. N., and Griffin, J. H. (1981). Contributions of human platelets to the proteolytic activation of blood coagulation factors XII and XI. *Blood* **57,** 106–118.

Wang, W., Hendriks, D. K., and Scharpé, S. S. (1994). Carboxypeptidase U, a plasma carboxypeptidase with high affinity for plasminogen. *J. Biol. Chem.* **269,** 15937–15944.

Warn-Cramer, B. J., and Bajaj, S. P. (1985). Stoichiometry of binding of high molecular weight kininogen to factor XI/XIa. *Biochem. Biophys. Res. Commun.* **133,** 417–422.

Weiss, A. S., Gallin, J. I., and Kaplan, A. P. (1974). Fletcher factor deficiency. A diminished rate of Hageman factor activation caused by absence of prekallikrein with abnormalities of coagulation, fibrinolysis, chemotactic activity, and kinin generation. *J. Clin. Invest.* **53,** 622–633.

Weiss, R., Silverberg, M., and Kaplan, A. P. (1986). The effect of C1 inhibitor upon Hageman factor autoactivation. *Blood* **68,** 239–243.

Wiggins, R. C., Bouma, B. N., Cochrane, C. G., and Griffin, J. H. (1977). Role of high molecular weight kininogen in surface-binding and activation of coagulation factor XIa and prekallikrein. *Proc. Natl. Acad. Sci. USA* **77,** 4636–4640.

Wiggins, R. C., Giglas, P. C., and Henson, P. M. (1981). Chemotactic activating generated from the fifth component of complement by plasma kallikrein of the rabbit. *J. Exp. Med.* **153,** 1391.

Wuepper, K. D. (1973). Prekallikrein deficiency in man. *J. Exp. Med.* **138,** 1345–1355.
Wuepper, K. D., Miller, D. R., and LaCombe, M. J. (1975). Flaujeac trait. Deficiency of human plasma kininogen. *J. Clin. Invest.* **56,** 1663–1672.
Wuillemin, W. A., Minnema, M., and Meijers, J. C. (1995). Inactivation of factor XIa in human plasma assessed by measuring factor XIa–protease inhibitor complexes: Major role for C1-inhibitor. *Blood* **85,** 1517.

This article was accepted for publication on February 20, 1997.

ADVANCES IN IMMUNOLOGY, VOL. 66

CD8+ Cells in Human Immunodeficiency Virus Type I Pathogenesis: Cytolytic and Noncytolytic Inhibition of Viral Replication

OTTO O. YANG and BRUCE D. WALKER

AIDS Research Center, Massachusetts General Hospital, Boston, Massachusetts 02114

I. Introduction

Since the discovery of human immunodeficiency virus type 1 (HIV-1) as the major causative agent of acquired immunodeficiency syndrome (AIDS) (1, 2) the relative contributions of viral and host factors in disease pathogenesis have remained controversial. Acute infection is marked by high levels of viremia and often symptomatic illness with depressed $CD4^+$ lymphocyte counts (3–6), but in most individuals a period of clinical latency ensues (7). During this period, $CD4^+$ lymphocyte counts normalize, and viremia is held in relative check. At later stages, viremia rises, with dropping $CD4^+$ lymphocyte counts and clinical disease progression. Immunologic factors have been proposed to play a role in establishing and maintaining the clinically asymptomatic state, but the precise correlates of immune protection have remained unclear (8).

Evidence for the potential role of $CD8^+$ lymphocytes in the immunopathogenesis of HIV-1 infection was first reported in 1986 (9) with the observation that peripheral blood mononuclear cells (PBMCs) from asymptomatic infected individuals produced virus upon removal of $CD8^+$ cells. Furthermore, repletion experiments demonstrated that these $CD8^+$ cells suppressed HIV-1 replication in a dose-dependent manner. Because this inhibition could be mediated by soluble factors derived from $CD8^+$ cells and the effect did not appear to be major histocompatibility complex (MHC) restricted, it was concluded that the effector cells were not cytotoxic T lymphocytes (CTLs). These initial experiments demonstrated the potential role of the cellular immune response in HIV-1 infection and have been the cornerstone of functional investigations of the inhibitory activity of $CD8^+$ lymphocytes from infected individuals.

Another major focus in the study of $CD8^+$ cells in HIV-1 immunopathogenesis has been virus-specific CTLs. In 1987, it was demonstrated that some infected individuals have extremely high levels of *in vivo* activated HIV-1-specific CTLs (10, 11). The magnitude of the response in some HIV-1-infected persons was unprecedented, in that antiviral cytolytic activity could be detected in freshly isolated PBMC without the need for

0065-2776/97 $25.00

in vitro stimulation and expansion (11, 12). Numerous laboratories have contributed to detailed studies regarding the frequencies and specificities of $CD8^+$ cytolytic cells directed against HIV-1, and emerging data indicate a role for these cells as an antiviral host defense.

The study of $CD8^+$ lymphocytes in HIV-1 infection has continued along these two major lines of investigation. The relationship of the functional antiviral properties and lytic abilities of these cells continues to be an area of controversy, as does their role in HIV-1 pathogenesis. New studies suggest that these two effector functions are at least in part mediated by the same population of $CD8^+$ cells, and that they may play a pivotal role in the pathogenesis of HIV-1 infection. This review will focus on recent advances in our understanding of both cytolytic and noncytolytic activities of $CD8^+$ cells from infected persons, and will discuss the areas in particular need of further investigation.

II. Evidence for an Antiviral Effect by $CD8^+$ Lymphocytes in HIV-1-Infected Individuals

Since the initial observations that $CD8^+$ lymphocytes are capable of inhibiting viral replication (9) and lysing target cells expressing HIV-1 antigens (11, 13), a large body of knowledge has been generated concerning the presence of these activities in HIV-1-infected individuals. The role of these cells in the immunopathogenesis of HIV-1 infection has become increasingly evident.

A. Antiviral Activity of $CD8^+$ Cells from HIV-1-Infected Individuals

Numerous laboratories have confirmed the initial findings of C. Walker and J. Levy that $CD8^+$ cells from infected persons are able to suppress HIV-1 replication. Although assay systems vary, most studies have been performed by coculturing $CD8^+$ cells with infected $CD4^+$ cells and monitoring viral replication by supernatant reverse transcriptase (RT) activity or HIV-1 p24 antigen concentration [reviewed in Refs. (14) and (15)]. A decrease in viral replication of at least 90% is often defined as a positive suppressive response in these coculture experiments. Assays have been performed either with acutely infected $CD4^+$ cells from seronegative individuals or from endogenously infected $CD4^+$ cells from infected individuals. Acutely infected $CD4^+$ cells from seronegative persons have been more commonly employed as target cells because the viral input inoculum can be readily controlled. Other studies have employed $CD8^+$ cell-depleted PBMC from HIV-1-infected individuals, to which autologous $CD8^+$ cells are added. Although the latter system has the advantage of being entirely

autologous and assessing inhibition of autologous virus, low $CD4^+$ cell yield in advanced disease often limits its use (14). Suppressive effects of $CD8^+$ cells have also been demonstrated in SIV-infected rhesus monkeys (16), SIV-infected African green monkeys (198), and HIV-infected chimpanzees (199, 200). Recently, studies of viral suppression by these cells have been extended to an *in vitro* model designed to mimic the cellular interactions occurring in the lymphoid microenvironment, with the use of dendritic cell–$CD4^+$ cell cocultures (17).

A number of features of $CD8^+$ cell-mediated HIV-1 suppression have now been confirmed. Although the inhibitory effect can be mediated by a soluble factor produced by $CD8^+$ cells, cell-cell contact is required for optimal activity (18). Ratios of added polyclonal $CD8^+$ cells to $CD4^+$ cells as low as 0.05 : 1 can mediate viral suppression, depending on the assay conditions and the cells used (19–21). The observed antiviral effect is inversely correlated to disease status (18, 20). In healthy asymptomatic individuals, low ratios of added $CD8^+$ cells (often 0.25 per $CD4^+$ cell) inhibit viral replication, as opposed to individuals with AIDS, in which ratios as high as 2 or more are often required. Furthermore, this antiviral response has been found to decrease in infected individuals followed over time, correlating to the development of disease (22). Infected individuals with a long term nonprogressing course appear to have preserved $CD8^+$ cell antiviral activity, suggesting a role for these cells in maintaining clinical latency (23). The relationship between changes in $CD8^+$ cell-mediated inhibitory activity and other changes in immune parameters with disease progression is less clear.

Additional studies have also demonstrated the antiviral effects of these cells. In acutely infected individuals, the ability of $CD8^+$ cells to mediate antiviral inhibition develops prior to the development of virus-specific neutralizing antibodies and is temporally correlated to the decline of viremia (24). In addition, certain cohorts of highly exposed yet uninfected individuals have also been identified to have $CD8^+$ cells which inhibit HIV-1 replication (19, 25). One study has suggested that uninfected children of seropositive mothers may have detectable antiviral $CD8^+$ cell-mediated activity (25). Interpretation of these results, however, is tempered by the fact that $CD8^+$ cells from seronegative individuals in general may have this HIV-1 suppressive activity under some assay conditions (17, 26, 27).

B. HIV-1-Specific Lytic Activity of $CD8^+$ Cells from Infected Individuals

A second focus of the investigation of $CD8^+$ cells in HIV-1-infected individuals has been the virus-specific direct cytolytic activity of these cells. Both murine and human studies of various viral infections indicate a role

for virus-specific CTLs in containing virus replication (28–34). CTLs are a subset of $CD8^+$ cells that specifically lyse target cells presenting a viral peptide epitope (typically 6–12 amino acids) in the context of a restricting class I HLA antigen (35–38). Viral proteins expressed by infected cells are processed intracytoplasmically by the proteosome complex and transported to the endoplasmic reticulum where they bind nascent class I MHC molecules for recognition by CTLs [reviewed in Ref. (39)]. Typically, each MHC molecule can bind only epitopes containing limited amino acids at particular anchor positions (usually the second and ninth positions of the peptide, which bind to the B and F pockets of the class I molecule). Such constraints have led to the identification of specific motifs for class I molecules, which can be used to predict CTL epitopes (35). Recognition of target cells by CTLs is therefore a specific process restricted by the epitope and MHC molecule. This specificity is conferred by interactions of the CTL T cell receptor (TCR) with the complex of epitope/MHC/β2-microglobulin on the infected cell surface (40, 41).

HIV-1-specific, $CD8^+$ CTLs have been investigated in great detail in the decade since they were first described [reviewed in Refs. (42)–(44)]. Many asymptomatic infected individuals have been shown to have vigorous and broadly directed antiviral cytolytic activity. Most of the data have been obtained by examining the ability of cells from infected individuals to lyse autologous immortalized B lymphoblastoid target cell lines that express HIV-1 proteins. These cell lines are chromium labeled, allowing an indirect measure of cytolysis by measuring chromium release after incubation with PBMC or CTL clones. Using recombinant vaccinia viruses containing structural and regulatory HIV-1 proteins, bulk PBMCs from many infected individuals have been found to recognize a variety of viral proteins including Gag, Pol, Env, Nef, Vif, Tat, and Rev (42, 44).

A number of studies have examined the ability of freshly isolated PBMCs from infected persons to lyse target cells expressing HIV-1 proteins. Degrees of lysis approaching 80% have been detected in some asymptomatic persons, without the need for *in vitro* stimulation (45, 46). Such high levels of lysis suggest very high frequencies of activated CTLs in PBMC. In addition to the *in vivo* activated cells, CTL precursors (CTLp) are also often detected with high frequency (47, 48). Analysis of these protein-specific responses has suggested that cytolytic activity is most commonly directed at epitopes in Gag, followed by Env and RT epitopes (49, 50).

Further characterization of the cytolytic response has been accomplished by cloning CTLs at limiting dilution [reviewed in Refs. (42), (43), 51), and (52)]. The use of synthetic peptides to sensitize target cells for lysis has led to the precise mapping of optimal CTL epitopes and their restricting class I alleles. More than 80 such epitopes have been described thus far,

which are found in both structural and nonstructural proteins. A compilation of such epitopes and restricting alleles [adapted from Ref. (51)] is shown in Table I. Some individuals have been noted to have extremely vigorous and broadly directed antiviral responses, with up to 14 separate epitopes recognized by a single person (53). In studies of chronically infected persons in which CTLp frequencies to defined epitopes have been evaluated, frequencies of up to 1 in 500 $CD8^+$ cells have been observed (46, 54). Healthy individuals may have virus-specific CTL clones recognizing single epitopes at frequencies of up to 5% of all circulating $CD8^+$ cells, as determined by TCR sequence analysis of bulk PBMCs (55; S. Kalams and B. Walker, unpublished observations).

As with the HIV-1 suppressive activity of polyclonal $CD8^+$ cells from infected individuals, several clinical correlations have been identified regarding HIV-1-specific CTL function. Antiviral CTL activity is more readily detectable in asymptomatic individuals than in those with advanced disease and declines with disease progression, as determined by quantitative analysis of functional antiviral CTL precursors (56, 57). Persons with long-term nonprogressing infection appear to have more vigorous and broadly directed CTL responses than progressing patients (58–61), although studies in some cohorts have not identified such a correlation (62) and a mathematical model of CTLs in HIV-1 infection has suggested that a narrowly directed response may be optimal (63). Studies in laboratory workers infected with HIV-1 IIIB suggest a broadening of the CTL response over time (64). However, studies examining the precise relationship between the magnitude and breadth of the CTL response with the magnitude of the viral load are still lacking.

The immunoprotective role of CTLs is suggested by studies of acute HIV-1 infection and in exposed but uninfected individuals. The development of the antiviral cytolytic response in primary infection precedes the production of neutralizing antibodies and correlates to the decline of viremia (65, 66). In elegant studies in a person with primary infection, a narrowly directed CTL response was shown to be associated with the rapid development of nonrecognized sequences within the targeted CTL epitope, providing further evidence for an antiviral role of CTLs (67). Other studies have used $V\beta$ repertoire analysis during primary infection to infer that a more broadly directed initial CTL response correlates to a better clinical outcome (68, 69). HIV-1-specific CTL activity has also been identified in various uninfected individuals including health care workers with percutaneous exposures (70), seronegative sexual partners of infected individuals (71), and commercial sex workers in epidemic areas (72). Whether these CTL responses in exposed uninfected persons represent abortive infection or exposure to noninfectious virus remains controversial.

TABLE I
BEST DEFINED HIV CTL EPITOPES

HLA	Protein	AA	Sequence	Reference
HLA-A2	p17	77–85	SLYNTVATL	45, 170, 171
	RT	346–354	VIYQYMDDL	172
	RT	476–484	ILKEPVHGV	173, 174
	gp41	818–827	SLLNATDIAV	175
	Nef	136–145	PLTFGWCYKL	176; U. Maier and B. Autran (PC)
	Nef	180–189	VLEWRFDSRL	176; U. Maier and B. Autran (PC)
	Nef	190–198	AFHHVAREL	176; U. Maier and B. Autran (PC)
HLA-A3.1	p17	18–26	KIRLRPGGK	59
	p17	20–28	RLRPGGKK	B. Culmann, D. Lewinsohn, S. Riddell (PC)
	p17	20–29	RLRPGGKKKY	B. Wilkes, D. Ruhl (PC)
	RT	325–333	AIFQSSMTK	Threlkeld (submitted for publication)
	gp120	37–46	TVYYGVPVWK	178
	gp41	775–785	RLRDLLLIVTR	179
	Nef	73–82	QVPLRPMTYK	108, 180
HLA-A11	p17	84–92	TLYCVHQRI	E. Harrer (PC)
	p24	349–359	ACQGVGGPGGHK	181
	RT	325–333	AIFQSSMTK	182, 183; S. Threlkeld (submitted for publication)
	RT	508–517	IYQEPFKNLK	B. Culman (PC)
	Nef	73–82	PLRPMTYK	180
	Nef	84–92	AVDLSHFLK	180
HLA-A19	RT	71–79	ITLWQRPLV	S. Rowland-Jones (PC)
HLA-A24	p17	28–36	KYKLKHIVW	D. Lewinsohn (PC)
	gp120	53–62	LFCASDAKAY	184, 185
	gp41	591–598	YLKDQQLL	145
HLA-A25	p24	145–155	QAISPRTLNAW	I. Kurane, K. West (PC)
	p24	203–212	ETINEEAAEW	186, 187
HLA-A26	p24	167–175	EVIPMFSAL	P. Goulder (in press)
	RT	593–603	ETFYVDGAANR	B. Wilkes, D. Ruhl (PC)
HLA-A28	RT	71–79	ITLWQRPLV	S. Rowland-Jones (PC)
	RT	85–93	DTVLEEMNL	S. Rowland-Jones (PC)
HLA-A29	pg120	376–384	FNCGGEFFY	C. Wilson (in press)
HLA-A31	gp41	775–785	RLRDLLLIVTR	157, 188
HLA-A32	RT	559–568	PIQKETWETW	59
	gp120	419–427	RIKQIINMW	59
HLA-B7	p24	148–156	SPRTLNAWV	D. Lewinsohn (PC)
	p24	179–187	ATPQDLNTM	B. Wilkes, R. Ruhl (PC)
	gp120	303–312	RPNNNTRKSI	J. Safrit, R. Koup (PC)
	gp41	843–851	IPRRIROGL	J. Wilkes (PC)

(*continues*)

TABLE I—*Continued*

HLA	Protein	AA	Sequence	Reference
	Nef	68–77	FPVTPQVPLR	176; U. Maier and B. Autran (PC)
	Nef	128–137	TPGPGVRYPL	176, 189; U. Maier and B. Autran (PC)
HLA-B8	p17	24–31	GGKKKYKL	190
	p17	74–82	ELRSLYNTV	P. Goulder (submitted for publication)
	gp120	2–10	RVKEKYQHL	181
	gp41	591–598	YLKDQQLL	146, 185
	Nef	13–20	WPTVRERM	P. Goulder (submitted for publication)
	Nef	90–97	FLKEKGGL	189; P. Goulder (PC)
HLA-B14	p24	183–191	DLNTMLNTV	146, 191
	p24	298–306	DRFYKTLRA	59
	gp41	589–597	ERYLKDQQL	146
HLA-B15	gp120	375–383	SFNCGGEFF	192
HLA-B18	Nef	135–143	YPLTFGWCY	180, 189
HLA-B27	p17	18–27	KIRLRPGGKK	D. Lewinsohn (PC)
	p17	19–27	IRLRPGGKK	D. Lewinsohn (PC)
	p24	263–272	KRWIILGLNK	49, 193
	gp41	590–597	RYLKDQQL	185
	gp41	791–799	GRRGWEALKY	184; J. Lieberman (PC)
	Nef	73–82	QVPLRPMTYK	B. Culmann (PC)
HLA-2705	Nef	105–114	RRQDILDLWI	P. Goulder (submitted for publication)
	Nef	134–141	RYPLTFGW	B. Culmann (PC)
	Gag	260–269	RRWIQLGLQK	S. Rowland-Jones (PC)
HLA-B35	p17	36–44	WASRELERF	P. Goulder (submitted for publication)
	p17	124–132	NSSKVSQNY	72
	p24	254–262	PPIPVGDIY	72
	p24	262–270	TVLDVGDAY	B. Wilkes, D. Ruhl (PC)
	RT	273–282	VPLDEDFRKY	181, 194
	RT	328–336	NPDIVIYQY	181, 194
	RT	342–350	HPDIVIYQY	190
	gp120	42–52	VPVWKEATTTL	B. Wilkes, D. Ruhl (PC)
	gp41	611–619	TAVPWNASW	178
	Nef	74–81	VPLRPMTY	180, 189
	Gag	245–253	NPVPVGNIY	190
HLA-B37	Nef	120–128	YFPDWQNYT	180
HLA-B39	p24	193–201	GHQAAMQML	I. Kurane, K. West (PC)
HLA-B42	p17	20–29	RLRPGGKKKY	B. Wilkes, D. Ruhl (PC)
	RT	438–446	YPGIKVRQL	B. Wilkes, D. Ruhl (PC)
HLA-B45	RT	591–600	GAETFYVDGA	B. Wilkes, D. Ruhl (PC)
HLA-B51	p24	325–333	NANPDCKTI	B. Wilkes, D. Ruhl (PC)

(*continues*)

TABLE I—*Continued*

HLA	Protein	AA	Sequence	Reference
	RT	295–302	TAFTIPSI	181
	gp41	557–565	RAIEAQQHL	181
HLA-B52	p24	275–282	RMYSPTSI	B. Wilkes, D. Ruhl (PC)
HLA-B53	HIV-2 Gag	173–181	TPYDINQML	195
HLA-B55	pg120	42–51	VPVWKEATTT	185
HLA-B57	p24	147–155	ISPRTLNAW	45, 201
	p24	140–149	TSTLQEQIGW	201
	p24	162–172	KAFSPEVIPMF	201
	p24	240–249	TSTLQEQIGW	201
	p24	311–319	QASQEVKNW	201
	p24	311–319	QASQDVKNW	201
	p24	116–125	HTQGYFPDWQ	180
	Nef	120–128	YFPDWQNYT	180
	Nef			
HLA-B58	p24	140–149	TSTLQEQIGW	201
HLA-Bw62	p17	20–29	RLRPGGKKKY	201
	p24	268–277	LGLNKIVRMY	45; B. Wilkes, D. Ruhl (PC)
	RT	415–426	LVGKLNWASQIY	R. Johnson (PC)
	RT	476–485	ILKEPVHGVY	45; R. Johnson (PC)
	Nef	84–91	AVDLSHFL	189
	Nef	117–127	TQGYFPDWQNY	B. Culmann (PC)
HLA-Cw01.02	p24	168–175	VIPMFSAL	P. Goulder (submitted for publication)
HLA-Cw4	gp120	380–388	SFNCGGEFF	192, 196

Note. PC, personal communication.

III. Mechanisms of Viral Inhibition by CD8$^+$ Cells

Despite the growing body of data regarding HIV-1-specific cytolysis and the viral suppression by CD8$^+$ cells from infected individuals, the relationship of these two activities remains controversial. The first report to document viral inhibition by CD8$^+$ cells (9) also described the ability of these cells to exert inhibition across a semipermeable membrane and proposed that viral suppression did not involve cytolysis. However, similar studies in rhesus macaques and humans indicated that CD8$^+$ cells inhibited AIDS virus replication in autologous cells better than in allogeneic cells and had phenotypic characteristics of CTLs (16, 73, 74). Although some soluble inhibitory factors and their mechanisms of action have been well characterized, the role of cytolysis in CD8$^+$ cell-mediated suppression is becoming increasingly clear. The recent demonstration that HIV-1-specific CTLs produce soluble antiviral factors (75) has revealed the probable overlap of these two effector mechanisms.

A. Soluble Suppressive Factors

The ability of $CD8^+$ cells from infected persons to inhibit HIV-1 replication across a semipermeable membrane suggests that these cells can act independently of contact with the infected target cells (18). Although HIV-1-specific CTLs produce a variety of cytokines, including TNF-α, TNF-β, IFNγ, and GM-CSF (76, 77), antibody blocking studies indicate that these do not account for the observed inhibitory activities of these cells (78). Three major classes of soluble inhibitory factors have been proposed to be important. Chemokines, a subclass of cytokines with chemotactic properties, have arisen as the best defined inhibitory substances and act to block viral entry [reviewed in Ref. (79)]. $CD8^+$ cell-derived antiviral factor (termed "CAF"), a yet undefined substance or group of substances that inhibit HIV-1 replication at the level of viral transcription [reviewed in Ref. (14) and 15)], has been identified by a number of investigators but still eludes precise characterization. Additionally, IL-16 has been suggested to have antiviral activity via yet undefined mechanisms (80). Several of these factors have been functionally divided by means of their activity in different experimental systems.

1. Chemokines

The antiviral effect of chemokines was first demonstrated by studying HTLV I-transformed $CD8^+$ cells that produced soluble substances inhibitory for HIV-1 replication (81). More detailed analysis of supernatant fluids from these cells revealed the presence of the β chemokines MIP-1α, MIP-1β, and RANTES, which suppressed replication of monocytotropic (M-tropic) isolates but not laboratory-adapted strains. Neutralizing antibodies against all three chemokines were required to block the inhibitory activity of supernatant from these $CD8^+$ cells. It is now known that these chemokines block viral entry by interacting with the chemokine receptor CCR-5 (82–86). This receptor has been demonstrated to be a necessary coreceptor with CD4 for the binding and entry of M-tropic strains of HIV-1 but not T lymphotropic (T-tropic), laboratory-adapted strains. MIP-1α, MIP-1β, and RANTES appear to block CCR-5 and prevent its function as a viral coreceptor via binding to the V3 loop of the viral envelope (83, 87, 88). The postreceptor effects of chemokine binding to CCR5, which are mediated by G proteins, do not seem to play a role in this inhibitory effect, indicating that these chemokines act by competing directly for receptor binding (89).

T-tropic strains of HIV-1 do not utilize CCR-5 as a coreceptor and are therefore insensitive to the MIP/RANTES chemokines (82, 84). The ability of $CD8^+$ cell-derived soluble factors to inhibit T-tropic strains of HIV-1

(14, 75, 90–92) has suggested the existence of other suppressive substances. The coreceptor used by T-tropic strains has now been identified, but whether $CD8^+$ cell-derived factors can block this coreceptor is less clear. The receptor for T cell tropic viruses is CXCR-4 (93), a 7-span transmembrane receptor formerly known as the orphan chemokine receptor-like molecule LESTR. This coreceptor has been shown to be necessary for the entry of several laboratory-adapted, T-tropic strains of HIV-1 and has been subsequently identified as an α-chemokine receptor. Recent work has suggested that stroma-derived factor (SDF-1α and SDF-1β) may be a ligand for CXCR-4 and block entry of T-tropic viral strains (94, 95) analogous to the ability of MIP/RANTES β-chemokines to block entry of M-tropic strains via g-CCR-5. Higher concentrations of SDF (up to 1000 ng/ml as opposed to less than 100 ng/ml for MIP/RANTES) are necessary to achieve inhibition and CXCR-4 receptor-mediated calcium flux (94, 95) raising the possibility that other ligands may exist. It will be important to define the antiviral role of these ligands as they become identified.

It is likely that HIV-1 may be able to utilize other coreceptors as well, and that the ligands of these receptors may also serve as inhibitory factors. At least one viral strain, 89.6, has been shown to utilize not only CCR-5 and CXCR-4 but also CCR-2b and CCR-3 as coreceptors for cellular entry (85). Furthermore, the $CD4^+$ cell line PM1, which expresses both CXCR-4 and CCR-5 (84, 96), does not support replication of all viral isolates that can be grown in PBMCs (81), suggesting that the coreceptor requirements for different isolates may be quite different.

2. *$CD8^+$ Cell-Derived Antiviral Factor*

Another reported class of $CD8^+$ cell-derived inhibitory factor(s) is CAF [reviewed in Refs. (14) and (15)]. The antiviral activity of this substance has been investigated in supernatant fluids from mitogen-stimulated $CD8^+$ cells of HIV-1-infected individuals (19, 26), but the precise identity of CAF remains undefined. Factors from these cells have been shown to have variable inhibitory activity across a wide spectrum of M-tropic and T-tropic HIV-1 strains as well as HIV-2 (21, 26, 97), suggesting the possible existence of a factor that is independent of antigen or coreceptor specificity (14). A novel mechanism has been proposed for the activity of CAF. Molecular studies of acutely infected $CD4^+$ cells inhibited by supernatant fluids from $CD8^+$ cells have demonstrated that these target cells appear to have reduced levels of viral RNA and proteins, suggesting that CAF acts by downregulating viral expression in infected cells (98, 99). Further studies utilizing chloramphenicol acetyl transferase or luciferase reporter genes linked to the HIV-1 long terminal repeat (LTR) have confirmed this

finding and suggest that CAF acts by suppressing LTR-driven expression of viral proteins (98–101).

The precise identity of CAF has remained elusive, however. Analysis of $CD8^+$ cell supernatants for known cytokines has failed to yield any single candidate for CAF, and blocking antibodies to various cytokines have failed to neutralize the activity of this factor (78, 90). CAF does appear to have some defining physical and chemical properties, such as stability at pH as low as 2, relative resistance to trypsin, and stability at temperatures as high as 56°C for 30 min or 100°C for 10 min (14). Size exclusion studies have demonstrated that CAF may be a protein on the order of 30 kDa in size (14). Bulk $CD8^+$ cells and $CD8^+$ cell clones appear to produce similar concentrations of CAF on a per cell basis (19, 97); in general, a 1:4 dilution is the lowest concentration of cell-free supernatant fluid that yields detectable antiviral activity (at least 50% inhibition of HIV-1 replication). This has limited the ability to purify and identify the factor or factors responsible for CAF activity.

3. *Interleukin-16*

Another factor suggested to mediate antiviral activity is interleukin-16 (IL-16) (80). This cytokine was originally described as a substance which can downregulate transcription of the CD4 molecule (102). One group has reported that IL-16 appears to suppress HIV-1 and SIV replication *in vitro* by an as yet undetermined mechanism (80), but this result remains unconfirmed and has not been reproducible in another system (103). The role of IL-16 as a possible mediator of antiviral suppression awaits further documentation.

B. HIV-1-Specific Cytolysis

In addition to mediating antiviral effects via release of soluble mediators, such as interferons and chemokines, CD8+ cells from HIV-infected persons are known to exert direct lysis of cells expressing HIV-1 proteins. This lytic activity has long been considered to be mediated by a population of cells that are distinct from those mediating the soluble inhibition. The lytic potential of $CD8^+$ cells has been well documented using autologous EBV-transformed B cells expressing recombinant viral proteins, which have demonstrated the existence of a class I-restricted CTL response (42, 43, 52). Data concerning the interaction of these CTLs with infected cells, however, have been relatively lacking. Studies of bulk $CD8^+$ cell-mediated inhibitory activity have found that viral suppression across a semipermeable membrane or by cell-free supernatants is less efficient than when contact with target infected cells is allowed (18), raising the question of a role for cytolysis. Furthermore, studies of $CD8^+$ cell activity in SIV infection sug-

gest that inhibition is class I restricted and involves cells with phenotypic characteristics of CTLs (16, 74). Recent studies have begun to address the functional aspects of CTLs and their abilities to lyse infected cells and suppress HIV-1 replication.

1. Kinetics of Lysis of Acutely Infected CD4$^+$ Cells Versus Viral Replication

The ability of HIV-1-specific CTLs to lyse infected cells has been investigated with highly HIV-1-permissive CD4$^+$ transformed cell lines (104). The use of these cell lines, which can be synchronously infected by HIV-1, has allowed a detailed comparison of the kinetics of viral replication versus the kinetics of susceptibility to cytolysis by CTL clones of differing specificities. The infected cells, which attain >98% intracellular p24 antigen positivity within 4 days, are recognized and lysed by virus-specific CTLs as soon as the cells demonstrate intracellular p24 antigen production. The lytic ability of these CTLs is highly efficient; the Gag- and Env-specific clones reach peak-level lysis comparable to that of epitope peptide-labeled positive controls. The RT-specific CTLs, however, are not as efficient, reaching a peak approximately 50% that of controls. This finding is consistent with a prior study that suggested that the RT epitope studied here is presented at far lower levels than a Gag epitope on infected cell surfaces (approximately 14 versus 200 copies per cell, respectively) (105), such that epitope density may be a limiting factor.

Analysis of virion production by the infected cell culture in the absence of CTLs suggests that the kinetics of viral replication lags behind the kinetics of susceptibility to lysis by about 12–24 hr. This suggests that HIV-1-specific CTLs may lyse acutely infected cells early in the virion life cycle, potentially before the production of infectious virions. This phenomenon has been demonstrated for other viruses as well (106, 107). Furthermore, these data indicate the possibility that CTLs of different specificities may vary in their ability to recognize infected cells. Additional study is required to determine whether recognition by CTLs specific for other proteins such as Nef, which is presumed to be an early viral protein (108), might be kinetically different from CTLs of other specificities.

2. HIV-1 Suppressive Activities and Cytolysis

As discussed previously, functional data regarding the specific antiviral potential of class I-restricted HIV-1-specific CTLs have been relatively lacking, despite the vast body of literature regarding the antiviral activity of CD8$^+$ cells in general. A dual function for CD8$^+$ cells in mediating both cytolysis and soluble inhibition has been suggested in some studies (103, 109), but the potential relationship between CTLs and CD8$^+$ cell-mediated

suppression has remained controversial. Recently, however, the role of HIV-1-specific CTLs in viral suppression has been defined in experiments analogous to the functional assays previously used to study viral suppression by bulk $CD8^+$ cells (75). HIV-1 replication in $CD4^+$ cells transfected with the appropriate restricting MHC class I molecule is heavily suppressed in the presence of virus-specific CTLs, whereas nontransfected $CD4^+$ cells are not inhibited (Fig. 1). Under certain experimental conditions, CTL clones are able to clear all detectable infectious virus, representing greater than 10^6-fold inhibition. HLA-restricted and epitope-specific triggering of CTLs results in antiviral suppression by two mechanisms: cytolytic and noncytolytic (75, 104). Analysis of supernatants from specifically stimulated CTLs revealed production of MIP/RANTES chemokines as well as other soluble inhibitory factors, the latter of which could not be neutralized completely by anti-MIP/RANTES antibodies. These inhibitory factors are released in an antigen-specific, HLA-restricted manner and act without HLA restriction once secreted.

The dominant role for cytolysis in CTL-mediated antiviral suppression has been demonstrated by mixing infected HLA-mismatched cells with a

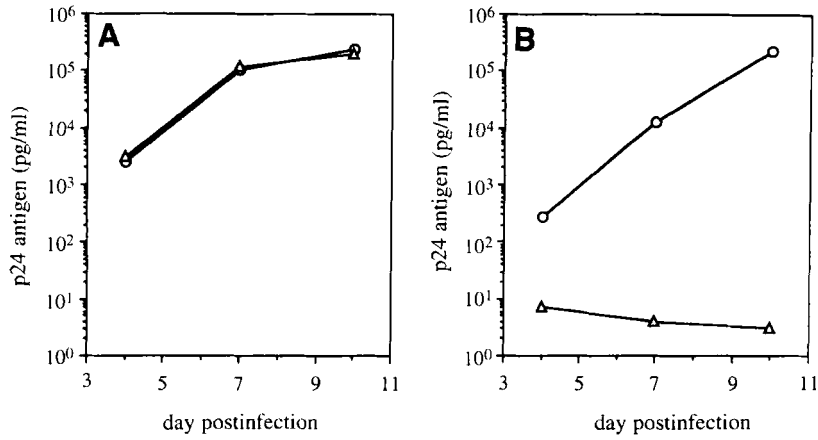


FIG. 1. The role of HLA class I antigen expression in $CD8^+$ cell-mediated antiviral activity. (A). H9 cells, which do not express HLA B14, were acutely infected with HIV-1 IIIB and incubated in the presence or absence of an HIV-1-specific, HLA B14-restricted CTL clone. Viral replication, as assessed by serial p24 antigen measurements, was similar in both cultures. (B). H9 cells transfected with HLA B14 (H9-B14) were infected and cultured as in A with or without the B14-restricted CTL clone. The clone suppressed viral replication by several orders of magnitude in the HLA-matched cells. ○, without B14-restricted CTL clone; △, with B14-restricted CTL clone.

CTL clone. Under these conditions, one sees minimal inhibition of HIV-1 replication because there is no triggering of cytolysis or production of soluble factors. However, the further addition of infected HLA-matched cells to these cultures results in triggering of release of soluble inhibitory factors by the CTLs (see Section IV). Under such conditions, viral production by the HLA-mismatched cells is reduced by less than 100-fold, indicating that the soluble factors released by the clone in contact with the HLA-matched cells were less than 100-fold inhibitory on the HLA-mismatched cells. In contrast, the HLA-matched cells alone are completely suppressed by the clone (by greater than 10^5-fold), indicating the greater potency of inhibition in the presence of the cytolytic mechanism. Further evidence for the role of cytolysis includes the observation that Env- and Gag-specific CTLs are more efficient inhibitors of replication than RT-specific clones, in agreement with the data on lysis of infected cells (104).

C. Functional Categorization of $CD8^+$ Cell-Mediated Antiviral Effects

A number of studies have begun to define further the $CD8^+$ effector cells and the conditions under which suppression is mediated, although most of these studies have not simultaneously examined the HIV-1-specific cytolytic function of these cells. Definition of the antiviral activities of $CD8^+$ cells is determined in two types of assays: those utilizing target $CD4^+$ cells that have been acutely infected with virus or cocultured with dendritic cells (DC) acutely pulsed with virus (acute assay) versus those utilizing $CD4^+$ cells that have been endogenously infected or cocultured with endogenously virus-laden DC (endogenous assay) (14, 15, 17). These approaches have yielded data suggesting that $CD8^+$ cells have at least two separate mechanisms of antiviral activity. $CD8^+$ cells from most HIV-1-infected individuals in all stages of disease have been found to have activity in the acute assay, whereas cells from seronegative individuals do not suppress viral replication in this system even at ratios as high as eight $CD8^+$ cells per $CD4^+$ cell (17, 19). This mechanism appears to be ablated by preexposure of the effector cells to gamma radiation (30 Gy), suggesting that it involves cellular activation (17). In contrast, $CD8^+$ cells from both infected and uninfected individuals are inhibitory in the endogenous assay (17, 19), and this activity is not sensitive to irradiation (17). Viral suppression by this mechanism appears to be more efficient in asymptomatic infected individuals than in those with later disease, and higher ratios of cells may be necessary to see the activity with $CD8^+$ cells from uninfected individuals (17, 19). These data have been the basis for the assertion that there exist at least two classes of inhibitory activity: one found only in infected individuals (acting in both acute and endogenous assay) and another found

in both infected and uninfected individuals (acting only in the endogenous assay).

The implications of these observations remain unclear. Both mechanisms have been presumed to involve soluble inhibitory factors because inhibition across a semipermeable membrane has been demonstrated in both assays (17, 19). Unstimulated $CD8^+$ cells have been shown to act across a membrane in the endogenous system, whereas stimulated but not unstimulated cells have transmembrane activity in the acute system (19, 103). These data do not exclude a role for HIV-1-specific CTLs which have recently been shown to produce antiviral soluble factors [see Section IV; Ref. (75)]. In addition, these studies do not address the triggering events required for elaboration of the inhibitory factors. This is a particularly important issue because $CD8^+$ cells from seronegative persons, upon nonspecific stimulation, can elaborate soluble inhibitory factors (17, 26, 27). Although inhibitory activity has been noted for various combinations of autologous and allogeneic cells in these assays, factors such as HLA matching have not been addressed, and it is therefore difficult to evaluate the effects of viral antigen-specific CTLs or alloreactive lymphocytes in these coculture systems.

IV. Roles of Lysis and Soluble Factors in Viral Suppression by $CD8^+$ Cells

Despite recent data linking CTLs and viral suppression by $CD8^+$ cells, the roles of the previously mentioned mechanisms have remained controversial. Studies of the inhibitory activity of bulk $CD8^+$ cells demonstrating their ability to act without cell contact or HLA restriction have led many investigators to conclude that the predominant mechanism is non-cytolytic and that the important effector cells are not HIV-1-specific CTLs. This finding is consistent with adoptive transfer of CTLs in a SCID animal model (110). Some have held that CTLs may be in fact deleterious due to their ability to kill $CD4^+$ lymphocytes (111, 112), and that factors such as CAF may play a dual role in directly reducing viral replication and protecting $CD4^+$ cells from CTLs by downregulating viral expression in infected cells (14). Firm evidence for this viewpoint remains lacking, however. The similarities of clinical correlations of $CD8^+$ cell antiviral activity and HIV-1-specific cytolytic activity (Sections II,A and II,B) in fact suggest that both sets of activities may be mediated by CTLs. Several points have been addressed in the debate concerning the relative importance of cytolytic- and soluble factor-mediated inhibition.

The phenotype of $CD8^+$ cells that can inhibit viral replication in cocultures has been examined in detail. Studies of bulk $CD8^+$ cells cultured in IL-2, IL-4, and IL-10 suggest that T helper 1 (Th1) cytokines (IL-2)

enhance the inhibitory properties of these cells, whereas T helper 2 (Th2) cytokines (IL-4 and IL-10) appear to repress the suppressive effect (113). This is in keeping with the hypothesis that a shift from Th1 to Th2 cytokines in infected individuals is related to clinical disease progression (114). The surface phenotype of inhibitory $CD8^+$ cells has also been addressed. The cells mediating antiviral activity appear to be activated, as demonstrated by surface expression of class II MHC antigens (14, 15), which are present on a high percentage of $CD8^+$ cells in infected individuals (115–117). It has also been suggested that the $CD8^+CD28^+$ cells appear be a subset of $CD8^+$ cells with the most antiviral activity, and that this subset of $CD8^+$ cells declines with disease progression (22). In contrast, other studies have shown that the $CD8^+CD28^-$ subset displays HIV-1-specific CTL activity in direct assays, but that *in vitro* stimulation of $CD8^+CD28^+$ cells (similar to the methods employed in assessing viral suppression by $CD8^+$ cells) results in expansion of HIV-1-specific CTL effector cells (118). These phenotypic data do not define the mechanisms involved in viral inhibition but do suggest that HIV-1-infected individuals have relatively high concentrations of activated circulating $CD8^+$ cells that mediate antiviral activities.

The role of cytolysis in the inhibitory activity of bulk $CD8^+$ cells has been controversial. Several points have been made disputing the role of HIV-1-specific CTLs. The data supporting this viewpoint have been largely indirect, and the experiments addressing this issue have been mostly conducted using bulk, polyclonal PBMCs, precluding tight control of factors such as MHC matching, mixed lymphocyte reactions, and analysis of the triggering requirements for viral inhibition.

It has been proposed that the antiviral effects of $CD8^+$ cells from infected individuals do not require cell–cell contact with target $CD4^+$ cells (14, 15). The production of soluble inhibitory factors by these cells has been demonstrated in many experiments, documenting their ability to act across a semipermeable membrane as well as through cell free supernatants. This ability to act via soluble factors does not preclude cytolysis as a mechanism, however, and it is known that CTLs produce numerous cytokines upon antigen-specific stimulation (75–77). Precedent exists for lymphocytes inhibiting other viruses by means of cytokines released upon activation, including inhibition of HBV by IFN-γ and TNF-α released from CTLs (119), as well as inhibition of measles virus by IFN-γ released from $CD4^+$ cells (120). Furthermore, HIV-1 inhibition by $CD8^+$ cells is clearly more efficient when there is direct contact with the target infected cells as compared to transmembrane or supernatant-mediated inhibition (18).

Another key point in the controversy concerning the role of cytolysis has been the assertion that viral suppression by $CD8^+$ cells is not MHC restricted. $CD8^+$ cells from infected individuals have been shown to inhibit

viral replication in MHC-mismatched allogeneic target cells (26, 121). Studies concerning this issue, however, have utilized acutely mitogen-stimulated cells, which would be activated to produce soluble inhibitory factors nonspecifically, or included CD3 cross-linking beads directly in the cocultures of the $CD8^+$ cells and the target cells. CD3 cross-linking would also provide a strong nonspecific stimulatory signal for $CD8^+$ cells, including CTLs, to produce secreted factors. In addition, viral suppression in coculture experiments has sometimes been noted to be more potent in autologous than allogenic target cells, as was noted in the original report by C. Walker *et al.* (9). Interpretation of most experiments addressing these issues is complicated by the use of polyclonal effector and target cells, in which alloreactive triggering of inhibitory factor production cannot be excluded. The observed soluble factor-mediated antiviral activity in these experiments therefore does not exclude the role for cytolysis.

The presumed reversibility of inhibition has been another issue in the debate concerning the relative roles of soluble inhibitory factors and cytolysis. In experiments in which PBMCs from infected individuals have been $CD8^+$ cell depleted and then repleted to demonstrate dose-dependent inhibition, removal of $CD8^+$ cells from the cocultures has been demonstrated to allow continued viral replication (9, 21). The mechanism of inhibition therefore has been suggested to be reversible and thus noncytolytic. The CD4:CD8 ratios appear to remain constant in these coculture experiments, which has been interpreted to reflect a lack of lysis of $CD4^+$ cells. These data are not definitive, however. The inability to demonstrate irreversibility of inhibition does not rule out an irreversible component of inhibition that is incomplete in the experimental system. Furthermore, infected individuals from whose PBMC virus could not be recovered after $CD8^+$ cell depletion, i.e., who had the most efficient control of viral replication, were necessarily excluded from the $CD8^+$ depletion/repletion experiments. The preservation of $CD4^+:CD8^+$ ratios in these experiments is also insufficient to rule out cytolysis; elimination of the low percentage of infected cells might not change the ratios significantly. These data again do not exclude a role for cytolysis in viral suppression in MHC-matched target cells.

The mechanism by which $CD8^+$ cells are triggered to release soluble factors has not been addressed in most published studies. The elucidation of this issue has been difficult because studies of soluble factor production have generally utilized mitogen activation of the cells (by PHA or anti-CD3) or use of bulk polyclonal effector cells in coculture with allogeneic target cells, with inability to exclude alloreactivity as an activating signal. Some investigators have proposed that the $CD8^+$ cellular antiviral response is not antigen specific based on findings that these cells have been found

to suppress many different strains of HIV-1, HIV-2, and SIV (14). The broad antiviral activity of $CD8^+$ cells does not exclude antigen-specific activity, however, because significant antigenic cross-reactivity exists between these viruses (51, 202). Furthermore, several groups have noted that the majority of infected individuals have cytolytic activity directed against HIV-1 proteins [reviewed in Refs. (42), (43), (51), and (52)].

Recent data have more clearly delineated a central role for antigen-specific cytolysis in HIV-1 suppression (75). CTL clones have been found to inhibit viral replication by up to 10^6-fold, with clearance of detectable virus even after removal of CTLs from the cocultures. This efficiency is almost unprecedented in the study of bulk $CD8^+$ cell-mediated inhibition in which 90% inhibition is considered a positive response in cocultures (14). CTLs are specifically triggered to lyse infected cells and produce soluble antiviral factors including β-chemokines in an antigen-specific, MHC-restricted fashion (75). The soluble factors then act without MHC restriction. Inhibition of MHC-matched infected cells is several orders of magnitude more efficient than MHC-mismatched cells by CTLs in the same coculture, suggesting that lysis plays a dominant role in viral inhibition (75, 77). Furthermore, in some cases $CD8^+$ cells have been shown to suppress viral replication in the absence of detectable soluble inhibitory factors (14). Precursor frequency analysis of particular CTL clones by means of T cell receptor sequencing in the PBMCs from infected persons indicates that individual clones can approach 5% of circulating $CD8^+$ cells (55), a ratio at which CTL clones have been found to readily inhibit viral replication *in vitro* (75). Thus, it is becoming increasingly clear that HIV-1-specific CTLs are a subset of $CD8^+$ cells that are present in sufficient concentrations to suppress viral replication and produce soluble inhibitory factors when triggered by viral antigens.

The ability of CTLs to suppress HIV-1 replication by cytolytic and noncytolytic mechanisms is consistent with observed antiviral effects of bulk $CD8^+$ cells. It is unclear whether they account for all suppressive activity and whether a noncytolytic subset of antiviral $CD8^+$ cells plays a significant role. Soluble antiviral factors in supernatant fluids of bulk and clonal $CD8^+$ cells are not reproducibly detectable at dilutions greater than 1:4 (14), suggesting that a large percentage of these cells produce these factors at modest concentrations. HIV-1-specific CTLs have been shown to produce comparable amounts of soluble factors (75), and CTLs of other specificities appear to produce these substances as well (75), suggesting that these factors are not novel to HIV-1 infection.

V. Mechanisms of HIV-1 Persistence

Despite the vigorous cytolytic and noncytolytic mechanisms of $CD8^+$ cells to inhibit HIV-1 replication, most infected individuals continue to

have progressive disease. During the clinically asymptomatic phase of infection, viremia generally is detectable in most individuals, at a stable level with a very gradual rise over time (122). During this time, a quasi-semistate of viremia is established, reflecting a remarkably active balance of viral replication and clearance (123–125). Virion production is rapid, estimated to approach 10^{10} particles per day. The minimum duration of the HIV-1 life cycle is approximately 1.2 days, and the average time from release of one virion until it infects a new cell and goes on to release a subsequent generation of virus particles is 2.6 days (124). The level of viremia during this asymptomatic period is predictive of the rate of clinical progression (126). With increased understanding of how $CD8^+$ cells suppress virion production, several potential factors may explain the ultimate failure of immune control and progression to AIDS.

The rapidity of viral replication by infected cells has been cited as a potential factor that may render the immune response ineffectual (127). A mathematical model of primary infection has suggested that the initial decline in viremia could be simply due to consumption of the available $CD4^+$ cells that support viral replication and entirely independent of any antiviral immune response (128). This model does not explain the correlations of immune responses to exposure without infection and clinical disease progression, and it may fail to consider the rapid expansion of $CD4^+$ cells during primary infection. Another argument that the immune response is ineffectual has centered on the cytopathicity of HIV-1 (111, 127). Because this virus is generally considered to be cytopathic, it has been proposed that immune clearance of infected $CD4^+$ cells might not provide any significant advantage because these cells would be cleared due to viral infection even in the absence of an immune response. However, recent data suggest that HIV-1-specific CTLs may in fact lyse infected cells before virion production and are capable of clearing detectable virus *in vitro* (75) (see Section IV).

A. Escape from Control by Soluble Inhibitory Factors

The ability of HIV-1 to escape soluble factors is poorly understood. The demonstration that soluble factors can inhibit diverse strains of HIV-1, HIV-2, and SIV suggests immune escape may not be significant against this antiviral mechanism (21). Soluble antiviral activity by the $CD8^+$ cells of infected individuals has been shown to decline with disease progression (22), indicating that a decrease in production of these factors may have a role. Rapid clinical disease progression has been correlated to a switch from non-syncytia-inducing (NSI) to syncytia-inducing (SI) viral isolates *in vivo* (129, 130), and the relevance of this change to immune suppression is unclear. Most NSI strains of virus are M-tropic viruses which utilize the coreceptor CCR-5, whereas SI strains generally are believed to be T-

tropic viruses that utilize CXCR-4 (79). The chemokine ligands for these coreceptors are different, and this switch in viral phenotype could result in an altered control by soluble factors that competitively antagonize cellular infection. Alternatively, this could reflect differences in immune clearance of infected cells; most individuals have NSI strains of HIV-1 even if initially exposed to an SI variant (131). Another possibility could involve the coreceptors present in infected individuals. A subset of highly exposed but uninfected individuals has been found to be homozygous for deletion mutants of CCR-5 (132), and one study suggests that heterozygotes for defective CCR-5 might have a slower clinical course than normal individuals (133). The difference was slight, and further studies, including studies that address the relative expression of CCR-5 and CXCR-4, will be necessary to confirm this finding.

B. Escape from CTLs

The elucidation of mechanisms of CTL activity against infected cells suggests the potential for several means of escape. Clearance of infected cells requires several conditions. A virus-specific immune response must be generated and maintained, with immunologic help. The infected cells must be accessible to CTLs and express viral antigens and surface molecules that are required for binding and lysis. Perturbation of any step in this process may lead to nonrecognition of infected cells and escape from cell-mediated immune control of viral replication.

The HIV-1-specific cytolytic response is initially established during acute infection, during which CTLs and virus-specific $CD4^+$ helper cells respond to the initial viremia (65, 66). In most individuals, the $CD8^+$ CTL response is maintained during the clinically asymptomatic phase of infection and declines late in disease with increasing viremia (56, 57). The reason for this loss of antiviral cytolytic activity is unclear. HIV-1-specific $CD4^+$ helper cells are absent in most infected individuals as measured by antigen-induced proliferation assays, in contrast to preserved responses against other antigens such as tetanus (134). The virus-specific helper response is presumably severely attenuated or lost early during primary infection due to the increased susceptibility of activated $CD4^+$ lymphocytes to infection. Thus, helper cells responding to HIV-1 antigens would be expected to be preferentially infected. This lack of HIV-1-specific help for CTLs may have a role in the decline of virus-specific cytolysis over time. Small numbers of patients with long-term nonprogressing HIV-1 infection and undetectable viral loads have been found to have vigorous virus-specific helper cell activity (E. Rosenberg and B. Walker, unpublished data), further supporting this possibility. In addition, cooperativity between helper and cytotoxic lymphocytes has been shown to be important in murine viral infection

(135), and virus-specific helper cell function has been shown to be necessary for maintenance of CTLs in chronic viral infections (136). Another potential mechanism of CTL depletion is clonal exhaustion. Continual viral replication and CTL activation may eventually lead to depletion of HIV-1-specific CTLs. This concept has precedent in the murine lymphocytic choriomeningitis infection model (137), and recent studies of telomer length in $CD8^+$ cells suggest rapid turnover leading to premature senescence of these cells (138).

Another possible means of escape from CTL recognition is sequestration of virus at immune privileged sites or in cells not subject to cytolysis. Precedent exists for sites inaccessible to CTLs. In a murine model of herpes simplex virus infection, fas-mediated apoptosis has been demonstrated as a mechanism of preventing CTL infiltration in the anterior chamber of the eye (139), presumably as a mechanism to avoid inflammation in critical areas, and a similar mechanism of immune privilege has been identified in the testis (203). Sensitive areas such as the central nervous system may therefore be potential reservoirs of viral replication. Follicular dendritic cells (FDCs) in lymph nodes, in which active viral replication occurs during all stages of disease, have also been implicated as sites where HIV-1 may avoid immune recognition (140). FDCs have been suggested to trap viable virions in their extracellular matrix for prolonged periods, and the trapped virions have been demonstrated to resist the effects of neutralizing antibodies. Another proposed mechanism is the downregulation of $CD4^+$ cell surface molecules required for recognition by CTLs. Class I MHC molecule expression by $CD4^+$ cells has been reported to decrease after infection with HIV-1 (135, 141–143, 197), and one study further suggests that this effect may be mediated by Nef-induced pinocytosis (143). The degree and kinetics of class I MHC downregulation have been inconsistent between various studies, however, and a study of HIV-1-specific CTL recognition of infected immortalized cell lines found a decrease of only approximately 50% with no detectable functional significance (104). It is thus unclear to what extent class I downregulation plays a role in viral escape from CTL detection, and further studies employing primary HIV-1 isolates as well as primary PBMCs will be needed to further define the potential role of viral immunomodulatory molecules in HIV-1 pathogenesis.

Viral sequence variation is another likely means of avoiding the cellular immune response. Lack of proofreading by HIV-1 reverse transcriptase allows a high mutation rate during viral replication (144). The resultant viral variation leads to rapid emergence of viruses that are resistant to antiviral drugs and has been postulated to lead to escape from CTL recognition as well. Several means of escape due to this variation have been demonstrated *in vitro* using CTL clones of known specificities. Lysis of

target cells by CTL clones can be ablated by single amino acid changes within the target epitopes (145–148). This occurs due to lack of TCR binding to the mutant epitope–MHC complex or because alteration of an anchor residue in the epitope prevents binding and presentation by the MHC molecule (149). One group has further documented that the presence of certain epitope mutations found *in vivo* may be antagonistic to recognition of the index sequences by CTLs, (150, 151), but the frequency with which this occurs and the effect on viral load *in vivo* remains undefined. Sequence variation may also interfere with epitope processing, leading to nonrecognition by CTLs. Mutations in the flanking sequences of epitopes can lead to lack of recognition by CTLs, despite the lack of variation in the epitopes themselves (152, 153). In addition, sequence variation within epitopes can result in alterations in protein processing, such that the epitopes are no longer presented (154). A recent study suggests that Nef may also interfere with antigen processing/presentation as another potential means to escape cytolysis (204).

The relevance of sequence variation in escape from CTL recognition and in disease progression has been controversial. Although studies of potent reverse transcriptase inhibitors have shown rapid evolution of resistant virus arising due to drug selection pressure, CTL pressure leading to immune escape has not been demonstrated as readily. Numerous studies have found evidence of immune escape (63, 145–149, 155), whereas others in both human and macaque models have failed to demonstrate that effect (156). Most studies have examined cellular proviral DNA rather than plasma viral RNA, the latter of which is reflective of the actively proliferating viral pool. Furthermore, most studies have analyzed recognition of laboratory strains of virus and have not examined autologous virus.

The ability of CTLs to exert immune pressure *in vivo* has been demonstrated by a recent detailed study of primary HIV infection (67). In the patient studied, the initial detectable CTL response was entirely directed against an epitope in gp120, with rapid subsequent evolutions of sequence variation exclusively limited to the targeted epitope. This variation led to lack of recognition by the initial CTL response, with kinetics similar to those observed for emergence of resistant viruses under drug selection pressure. The analysis of plasma RNA rather than proviral DNA in this study represents a significant difference compared to other published studies and offers the first clear evidence of *in vivo* biologic significance for the HIV-specific CTL response but still does not define the role of CTLs in overall disease pathogenesis. In fact, another study of HIV-1-specific CTLs derived during acute infection failed to show evidence of sequence variation within the targeted epitopes during the first 15 weeks of follow-up (157).

VI. Potential Targets for Therapy

Although the correlates of immune protection in HIV infection remain undefined, the data suggest that $CD8^+$ cells exert antiviral effects through both cytolytic and noncytolytic mechanisms. There is thus emerging interest in strategies designed to augment such responses as a means to prevent infection or alter the disease course in infected persons. A number of approaches are being pursued based on increasing understanding of $CD8^+$ cell functions.

The mechanisms and actions of β chemokines are becoming increasingly clear and present a potential target for therapy. Although these substances play a role in allergy and inflammation [reviewed in Ref (158)], large doses of administered chemokines appear to be safe in animals, raising the possibility that administration of exogenous chemokines may be clinically feasible. MIP and RANTES are potent suppressors of viral replication *in vitro* (159), and study is needed to determine whether these chemokines may be useful when administered *in vivo.* Because their mechanism of action is by competitive blocking of coreceptors for viral entry, it may be possible to create analogs or modified chemokines that bind the coreceptors to prevent viral entry but that do not trigger the proinflammatory postreceptor effects. The amino terminus of the chemokines appears to be crucial for receptor activation but not for receptor binding, and a report has demonstrated that a N-terminus truncated RANTES appears to be capable of blocking HIV-1 cellular infection without triggering CCR-5 (160). The clinical utility of this class of substances remains to be determined.

Many approaches to immune reconstitution involve efforts to augment HIV-1-specific CTL responses. *Ex vivo* expression of HIV-1-specific CTL clones with subsequent autoinflusion has been disappointing (161) in part due to rapid *in vivo* elimination of the CTL clones, which were transduced with a foreign protein and eliminated by de novo CTL responses. Autologous fibroblasts expressing retrovirus-encoded HIV antigens have been used to augment CTL responses in infected persons with little apparent success thus far (162). Although virus-specific CTLs decline with disease progression, this approach may not correct this defect, particularly if lack of control is related to viral variation and emergence of immune escape. Lack of $CD4^+$ cell help or factors such as IL-2 production *in vivo* may also need to be addressed. Additionally, studies of $CD8^+$ cells have demonstrated that cells from individuals later in disease appear to be more prone to apoptosis (163), and thus the reinfused cells may be abnormal from the outset. It is also unclear whether $CD8^+$ cells expanded *ex vivo* distribute and traffic normally when reinfused.

Broadening of the cytolytic response is another immunotherapeutic strategy that has been considered. Infected individuals do not recognize all the

epitopes that are predicted to be possible based on their HLA alleles (42, 43, 51, 52). Of the three most commonly recognized A2-restricted epitopes in Gag and RT, for example, the majority of HLA $A2^+$ individuals have responses to an epitope in Gag, but the other two epitopes in RT are recognized in a minority (164). Peptide-based vaccines targeting nonrecognized epitopes or augmenting responses to targeted epitopes have been suggested as a means of broadening the antiviral cytolytic response. The utility of such an approach is limited by the large number of HLA types, although this difficulty may be partially overcome by choosing epitopes that are broadly cross-reactive among members of HLA superfamilies.

Another prospective method to broaden the cytolytic response is the use of novel T cell receptors. "Universal T cell receptors" (UR) are chimeric receptors containing the effector region of the TCR (ζ chain) fused to an HIV-1 envelope binding moiety: either the human CD4 molecule or the variable regions of a human immunoglobulin molecule that bind HIV-1 gp41 (165). Transduction of a UR gene into bulk $CD8^+$ cells from uninfected individuals has been shown to confer HIV-1 specificity in the lysis of infected cells (165, 166) and inhibition of viral replication (166). The kinetics of lysis and efficiency of inhibition appear to be similar to those of naturally occurring CTL clones (166). Because binding of the UR is not dependent on specific antigen processing and presentation, this approach may circumvent the issues of HLA restriction and escape.

The loss of HIV-1-specific $CD4^+$ cell help may play a key role in the eventual decline of antiviral cytolysis in infected individuals, and reconstitution of this function may be necessary as a primary means of augmenting the immune response or as an adjunct to $CD8^+$ cell-based strategies. Clinical administration of IL-2 has been studied as a means of boosting *in vivo* helper function (167). Patients who received IL-2 had substantial increases in $CD4^+$ cell counts, although the precise mechanism for this rise was unclear. The significance of this finding has yet to be determined; at least one patient developed a serious opportunistic infection after his $CD4^+$ cell count rose to near normal levels (167), raising questions as to whether the increase in counts was a true rise in cell mass or a redistribution of existing cells and whether the cells are functional. $CD4^+$ cell reconstitution may be important to provide specific helper function in infected individuals. $CD4^+$ cells from infected individuals can be grown to large numbers *in vitro* and, in the presence of antiretroviral drugs, purged of virus (168). Another reported strategy for expanding virus-free $CD4^+$ cells *in vitro* involves expansion of the cells with CD28 cross-linking beads (169). Through CCR-5 downmodulation (205) or other yet undetermined mechanisms, these cells appear to be resistant to infection by HIV-1. $CD4^+$ cells generated by either of these methods potentially could be reinfused

to replace the depleted $CD4^+$ cells in individuals with later stages of disease, although it is not known whether important specificities may have been clonally deleted and therefore missing from the expanded cells. A possible means to counteract this possibility involves the transduction of bulk $CD4^+$ cells from infected individuals with a UR T cell receptor, which might confer upon these cells virus-specific helper cell function. Analogous to transduced $CD8^+$ cells, these chimeric TCR-bearing cells would be directed to have functional specificity against HIV-1.

Because HIV-1-specific helper cell function appears to be lost early in primary infection, a strategy to prevent this process is early treatment of infected individuals with combination antiretroviral therapy. Use of aggressive combination therapies, which have been shown to decrease viremia to undetectable levels in many individuals, may in theory prevent infection of the activated HIV-1-specific $CD4^+$ cells. If the virus-specific helper cells are preserved, this potentially could prevent a major perturbation in the antiviral cytolytic response. Clinical trials are currently under way to evaluate the utility of this approach.

VII. Conclusions

A large body of evidence indicates that $CD8^+$ cells play a key role as a protective immune response against HIV-1. Although these cells have been shown to inhibit viral replication by means of soluble factors, increasing data suggest that virus-specific cytolysis is an important function of $CD8^+$ cells, and that CTLs are potent inhibitors of viral replication via cytolytic and noncytolytic mechanisms. Both functions are triggered in an antigen-specific manner, and the cytolytic mechanism of CTLs is necessary for efficient control of viral replication. The antiviral effects of $CD8^+$ cells decline with disease progression, and factors such as the lack of $CD4^+$ HIV-1-specific helper cells, viral escape mutations and inadequate CTL responses in the tissues may be central in this process. Rational design of immunotherapies for HIV-1 infection will depend on a better understanding of these mechanisms in the immunopathogenesis of infection and the reasons for the failure of the immune response to eradicate HIV infection under *in vivo* conditions.

References

1. Barre-Sinoussi, F., Chermann, J.-C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W., and Montaigner, L., (1983). Isolation of a T-lymphotrophic retrovirus from a patient at risk for acquired immunodeficiency syndrome (AIDS). *Science* **220,** 868–871.
2. Gallo, R. C., Sarin, P. S., Gelmann, E. P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V. S., Mann, D., Sidhu, G. D., Stahl, R. E., Zolla-Pazner, S., Leibowitch, J., and

Popovic, M. (1983). Isolation of a human T-cell leukemia virus in acquired immunodeficiency syndrome (AIDS). *Science* **220,** 865–867.

3. Clark, S. J., Saag, M. S., Decker, W. D., Campbell-Hill, S., Roberson, J. L., Veldkamp, P. J., Kappes, J. C., Hahn, B. H., and Shaw, G. M. (1991). High titers of cytopathic virus in patients with symptomatic primary HIV-1 infection. *N. Engl. J. Med.* **324,** 954–960.
4. Daar, E. S., Moudgil, T., Meyer, R. D., and Ho, D. D. (1991). Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* **324,** 961–964.
5. Gaines, H., Albert, J., von Sydow, M., Sonnerborg, M., Chiodi, F., Ehrnst, A., Strannegard, O., and Asjo, B. (1987). HIV antigenaemia and virus isolation from plasma during primary HIV infection. *Lancet* **1,** 1317–1318.
6. von Sydow, M., Gaines, H., Sonnerborg, A., Forsgren, M., Pehrson, P. O., and Strannegard, O. (1988). Antigen detection in primary HIV infection. *Br. J. Med.* **296,** 238–240.
7. Fauci, A. S., Pantaleo, G., Stanley, S., and Weissman, D. (1996). Immunopathogenic mechanisms of HIV infection. *Ann. Intern. Med.* **124,** 654–663. [Review].
8. Haynes, B. F., Pantaleo, G., and Fauci, A. S. (1996). Toward an understanding of the correlates of protective immunity to HIV infection. *Science* **271,** 324–328. [Review].
9. Walker, C. M., Moody, D. J., Stites, D. P., and Levy, J. A. (1986). CD8+ lymphocytes can control HIV infection *in vitro* by suppressing virus replication. *Science* **234,** 1563–1566.
10. Plata, F., Autran, B., Martins, L. P., Wain, H. S., Raphael, M., Mayaud, C., Denis, M., Guillon, J. M., and Debre, P. (1987). AIDS virus-specific cytotoxic T lymphocytes in lung disorders. *Nature* **328,** 348–351.
11. Walker, B. D., Chakrabarti, S., Moss, B., Paradis, T. J., Flynn, T., Durno, A. G., Blumberg, R. S., Kaplan, J. C., Hirsch, M. S., and Schooley, R. T. (1987). HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature* **328,** 345–348.
12. Walker, B. D., Flexner, C., Paradis, T. J., Fuller, T. C., Hirsch, M. S., Schooley, R. T., and Moss, B. (1988). HIV-1 reverse transcriptase is a target for cytotoxic T lymphocytes in infected individuals. *Science* **240,** 64–66.
13. Plata, F., Dadaglio, G., Chenciner, N., Hoffenbach, A., Wain, H. S., Michel, F., and Langlade, D. P. (1989). Cytotoxic T lymphocytes in HIV-induced disease: Implications for therapy and vaccination. *Immunodefic. Rev.* **1,** 227–246.
14. Levy, J., Mackewicz, C., and Barker, E. (1996). Controlling HIV pathogenesis: The role of the noncytotoxic anti-HIV response of CD8+ T cells. *Immunol. Today* **17,** 217–224.
15. Walker, C. M. (1993). Non-cytolytic control of HIV replication by CD8+ T cells. *Sem. Immunol.* **5,** 195–201.
16. Kannagi, M., Chalifoux, L. V., Lord, C. I., and Letvin, N. L. (1988). Suppression of simian immunodeficiency virus replication *in vitro* by CD8+ lymphocytes. *J. Immunol.* **140,** 2237–2242.
17. Barker, T. D., Weissman, D., Daucher, J. A., Roche, K. M., and Fauci, A. S. (1996). Identification of multiple and distinct CD8+ T cell suppressor activities. *J. Immunol.* **156,** 4476–4483.
18. Walker, C. M., and Levy, J. A. (1989). A diffusible lymphokine produced by CD8+ T lymphocytes suppresses HIV replication. *Immunology* **66,** 628–630.
19. Mackewicz, C., and Levy, J. A. (1992). CD8+ cell anti-HIV activity: Nonlytic suppression of virus replication. *AIDS Res. Hum. Retroviruses* **8,** 1039–1050.
20. Mackewicz, C. E., Ortega, H. W., and Levy, J. A. (1991). CD8+ cell anti-HIV activity correlates with the clinical state of the infected individual. *J. Clin. Invest.* **87,** 1462–1446.

21. Walker, C. M., Thomson, H. G. A., Hsueh, F. C., Erickson, A. L., Pan, L. Z., and Levy, J. A. (1991). CD8+ T cells from HIV-1-infected individuals inhibit acute infection by human and primate immunodeficiency viruses. *Cell. Immunol.* **137,** 420–428.
22. Landay, A. L., Mackewicz, C., and Levy, J. A. (1993). CD8+ T cell phenotype correlates with anti-HIV activity and clinical status in HIV-infected individuals. *Clin Immunol. Immunopathol.* **69,** 106–116.
23. Levy, J. A. (1995). *In* "Tenth International Conference on AIDS," (Y. Shiokawa and T. Kitamura, eds.). Vancouver, Canada.
24. Mackewicz, C. E., Yang, L. C., Lifson, J. D., and Levy, J. A. (1994). Non-cytolytic CD8 T-cell anti-HIV responses in primary HIV-1 infection. *Lancet* **344,** 1671–1673.
25. Levy, J. A. (1993). *AIDS* **7,** 1401–1410.
26. Brinchmann, J. E., Gaudernack, G., and Vartdal, F. (1990). CD8+ T cells inhibit HIV replication in naturally infected CD4+ T cells. Evidence for a soluble inhibitor. *J. Immunol.* **144,** 2961–2966.
27. Rosok, B., Voltersvik, P., Larsson, B.-M., Albert, J., Brinchmann, J. E., and Asjo, B. (1997). CD8+ T cells from HIV type 1-seronegative individuals suppress virus replication in acutely infected cells. *AIDS Res. Hum. Retroviruses* **13,** 79.
28. Lehmann-Grube, F., Assmann, U., Loliger, C., Moskophidis, D., and Lohler, J. (1985). Mechanism of recovery from acute virus infection. I. Role of T lymphocytes in the clearance of lymphocytic choriomeningitis virus from spleens of mice. *J. Immunol.* **134,** 608–615.
29. Lehmann-Grube, F., Moskophidis, D., and Lohler, J. (edss). (1988). "Recovery from Acute Virus Infection: Role of Cytotoxic T Lymphocytes in the Elimination of Lymphocytic Choriomeningitis Virus from Spleens of Mice." Annals of the New York Academy of Science, New York.
30. Oldstone, M. B. A. (1986). Cytoimmunotherapy for persistent virus infection reveals a unique clearance pattern from the central nervous system. *Nature* **321,** 239–243.
31. Reusser, P., Riddell, S. R., Meyers, J. D., and Greenberg, P. D. (1991). Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: Pattern of recovery and correlation with cytomegalovirus infection and disease. *Blood* **78,** 1373–1380.
32. Riddell, S. R., Watanabe, K. S., Goodrich, J. M., Li, C. R., Agha, M. E., and Greenberg, P. D. (1992). Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones [see Comments]. *Science* **257,** 238–241.
33. Sethi, K. K., Omata, Y., and Schneweis, K. E. (1983). Protection of mice from fatal herpes simplex virus type 1 infection by adoptive transfer of cloned virus-specific and H-2-restricted cytotoxic T lymphocytes. *J. Gen Virol.*
34. Yap, K. L., Ada, G. L., and McKenzie, I. F. (1978). Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature* **273,** 238–239.
35. Falk, K., Rotzschke, O., Deres, K., Metzger, J., Jung, G., and Rammensee, H. G. (1991). Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast. *J. Exp. Med.* **174,** 425–434.
36. Jardetzky, T. S., Lane, W. S., Robinson, R. A., Madden, D. R., and Wiley, D. C. (1991). Identification of self peptides bound to purified HLA-B27. *Nature* **353,** 326–329.
37. Rammensee, H. G., and Rotzschke, O. (1993). MHC molecules as peptide receptors. *Curr. Opin. Immunol.* **5,** 35–42.
38. Rotzschke, O., Falk, K., Deres, K., Schild, H., Norda, M., Metzger, J., Jung, G., and Rammensee, H. G. (1990). Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature* **348,** 252–254.

39. Eisen, H. K., Sykulev, Y., and Tsomides, T. J. (1996). Antigen-specific T cell receptors and their reactions with complexes formed by peptides with major histocompatibility proteins. *Adv. Pro. Biochem.* **149,** 1–52.
40. Garboczi, D. N., Ghosh, P., Utz, U., Fan, Q. R., Biddison, W. E., and Wiley, D. C. (1996). Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature* **384,** 134–141.
41. Garcia, K. C., Degano, M., Stanfield, R. L., Brunmark, A., Jackson, M. R., Peterson, P. A., Teyton, L., and Wilson, I. A. (1996). An $\alpha\beta$ T cell receptor structure at 2.5 A and its orientation in the TCR–MHC complex. *Science* **274,** 209–219.
42. Brander, C., and Walker, B. D. (1995). The HLA-class I restricted CTL response in HIV-1 infection: Identification of optimal epitopes. *In* "Theoretical Biology and Biophysics, 1995" (B. T. M. Korber, D. Brander, and B. D. Walker, eds.). HIV Molecular Immunology Database, Los Alamos, NM.
43. Kalams, S. A., and Walker, B. D. (1994). The cytotoxic T-lymphocyte response in HIV-1 infection. *Clin. Lab. Med.* **14,** 271–299.
44. McMichael, A. J., and Walker, B. D. (1994). Cytotoxic T lymphocyte epitopes: Implications for HIV vaccines. *AIDS* **8**(Suppl. 1), S155–S173.
45. Johnson, R. P., Trocha, A., Yang, L., Mazzara, G. P., Panicali, D. L., Buchanan, T. M., and Walker, B. D. (1991). HIV-1 gag-specific cytotoxic T lymphocytes recognize multiple highly conserved epitopes. Fine specificity of the gag-specific response defined by using unstimulated peripheral blood mononuclear cells and cloned effector cells. *J. Immunol.* **147,** 1512–1521.
46. Kalams, S. A., Johnson, R. P., Trocha, A. K., Dynan, M. J., Ngo, H. S., DAquila, R. T., Kurnick, J. T., and Walker, B. D. (1994). Longitudinal analysis of T cell receptor (TCR) gene usage by human immunodeficiency virus 1 envelope-specific cytotoxic T lymphocyte clones reveals a limited TCR repertoire. *J. Exp. Med.* **179,** 1261–1271.
47. Gotch, F. M., Nixon, D. F., Alp, N., McMichael, A. J., and Borysiewicz, L. K. (1990). High frequency of memory and effector gag specific cytotoxic T lymphocytes in HIV seropositive individuals. *Int. Immunol.* **2,** 707–712.
48. Koup, R. A., Pikora, C. A., Luzuriaga, K., Brettler, D. B., Day, E. S., Mazzara, G. P., and Sullivan, J. L. (1991). Limiting dilution analysis of cytotoxic T lymphocytes to human immunodeficiency virus gag antigens in infected persons: *In vitro* quantitation of effector cell populations with p17 and p24 specificities. *J. Exp. Med.* **174,** 1593–1600.
49. Buseyne, F., McChesney, M., Porrot, F., Kovarik, S., Guy, B., and Riviere, Y. (1993). Gag-specific cytotoxic T lymphocytes from HIV-1 infected individuals: Gag epitopes are clustered in three regions of the p24 gag protein. *J. Virol.* **67,** 694–702.
50. Riviere, Y. (1989). The cellular immune response to the human immunodeficiency virus. *Curr. Opin. Immunol.* **2,** 424–427.
51. Brander, C., and Walker, B. D. (1996). The HLA class I restricted CTL response in HIV-1 infection: Systematic identification of optimal epitopes. *In* "Theoretical Biology and Biophysics, 1996" (B. T. M. Korber, C. Brander, and B. D. Walker, eds.). HIV Molecular Immunology Database, Los Alamos, NM.
52. Johnson, R. P., and Walker, B. D. (1991). Identification of HIV-1 cytotoxic T-lymphocyte epitopes and implications for vaccine development. *Biotechnol. Ther.* **2**(1–2), 137–146.
53. Johnson, R. P., Hammond, S. A., Trocha, A., Siliciano, R. F., and Walker, B. D. (1994). Epitope specificity of MHC restricted cytotoxic T lymphocytes induced by candidate HIV-1 vaccine. *AIDS Res. Hum. Retroviruses* **10,** S73–S75.
54. Kalams, S. A., Johnson, R. P., Dynan, M. J., Hartman, K. E., Harrer, T., Harrer, E., Trocha, A. K., Blattner, W. A., Buchbinder, S. P., and Walker, B. D. (1996). T cell

receptor usage and fine specificity of human immunodeficiency virus 1-specific cytotoxic T lymphocyte clones: Analysis of quasispecies recognition reveals a dominant response directed against a minor *in vivo* variant. *J. Exp. Med.* **183,** 1–11.

55. Moss, P. A., Rowland-Jones, S. L., Frodsham, P. M., McAdam, S., Giangrande, P., McMichael, A. J., and Bell, J. I. (1995). Persistent high frequency of human immunodeficiency virus-specific cytotoxic T cells in peripheral blood of infected donors. *Proc. Natl. Acad. Sci. USA* **92,** 5773–5777.
56. Carmichael, A., Jin, X., Sissons, P., and Borysiewicz, L. (1993). Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: Differential CTL responses to HIV-1 and Epstein–Barr virus in late disease. *J. Exp. Med.* **177,** 249–256.
57. Klein, M. R., van Baalan, C. A., Holwerda, A. M., Kerkhof Garde, S. R., Bende, R. J., Keet, I. P., Eeftinck-Schaffenkerk, J. K., Osterhaus, A. D., Schuitemaker, H., Miedema F., and van Baalen, C. A. (1995). Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: A longitudinal analysis of rapid progressors and long-term asymptomatics. *J. Exp. Med.* **181,** 1356–1372.
58. Harrer, E., Harrer, T., Buchbinder, S., Mann, D. L., Feinberg, M., Yilma, T., Johnson, R. P., and Walker, B. D. (1994). HIV-1-specific cytotoxic T lymphocyte response in healthy, long-term nonprogressing seropositive persons. *AIDS Res. Hum. Retroviruses* **10,** S77–S78.
59. Harrer, T., Harrer, E., Kalams, S. A., Barbosa, P., Trocha, A., Johnson, R. P., Elbeik, T., Feinberg, M. B., Buchbinder, S. P., and Walker, B. D. (1996). Cytotoxic T lymphocytes in asymptomatic long-term nonprogressing HIV-1 infection. Breadth and specificity of the response and relation to *in vivo* viral quasispecies in a person with prolonged infection and low viral load. *J. Immunol.* **156,** 2616–2623.
60. Harrer, T., Harrer, E., Kalams, S. A., Elbeik, T., Staprans, S. I., Feinberg, M. B., Cao, Y., Ho, D. D., Yilma, T., Caliendo, A. M., Johnson, R. P., Buchbinder, S. P., and Walker, B. D. (1996). Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. *AIDS Res. Hum. Retroviruses* **12,** 585–592.
61. Rinaldo, C., Huang, X.-L., Fan, Z., Ding, M., Beltz, L., Panicali, D., Mazzara, G., Leibmann, J., Cottrill, M., and Gupta, P. (1995). High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *J. Virol.* **69,** 5838–5842.
62. Ferbas, J., Kaplan, A. H., Hausner, M. A., Hultin, L. E., Matud, J. L., Liu, Z., Panicali, D. L., Nerng-Ho, H., Detels, R., and Giorgi, J. V. (1995). Virus burden in long-term survivors of human immunodeficiency virus (HIV) infection is a determinant of anti-HIV CD8+ lymphocyte activity. *J. Infect. Dis.* **172,** 329–339.
63. Nowak, M., May, R. M., Phillips, R. E., Rowland-Jones, S., Lalloo, D. G., McAdam, S., Klenerman, P., Koeppe, B., Sigmund, K., Bangham, C. R. M., and McMichael, A. J. (1995). Antigenic oscillations and shifting immunodominance in HIV-1 infections. *Nature* **375,** 606–611.
64. Sipsas, N. (1997). *J. Cell. Immunol.*, in press.
65. Borrow P., Lewicki, H., Hahn, B. H., Shaw, G. M., Oldstone, M. B. A. (1994). Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virology* **68,** 6103–6110.
66. Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C., and Ho, D. D. (1994). Temporal association of cellular immune responses

with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* **68,** 4650–4655.

67. Borrow, P., Lewicki, H., Wei, X., Horwitz, M. S., Peffer, N., Meyers, H., Nelson, J. A., Gairin, J. E., Hahn, B. H., Oldstone, M. B. A., and Shaw, G. M. (1997). Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nature Med.* **3.**
68. Pantaleo, G., Demarest, J. F., Schacker, T., Vaccarezza, M., Cohen, O. J., Daucher, M., Graziosi, C., Schnittman, S. S., Quinn, T. C., Shaw, G. M., Perrin, L., Tambussi, G., Lazzarin, A., Sekaly, R. P., Soudeyns, H., Corey, L., and Fauci, A. S. (1997). The qualitative nature of the primary immune response to HIV infection is a prognosticator of disease progression independent of the initial level of plasma viremia. *Proc. Natl. Acad. Sci. USA* **94,** 254–258.
69. Pantaleo, G., Demarest, J. F., Soudeyns, H., Graziosi, C., Denis, F., Adelsberger, J. W., Borrow, P., Saag, M. S., Shaw, G. M., Sekaly, R. P., *et al.* (1994). Major expansion of CD8+ T cells with a predominant V beta usage during the primary immune response to HIV [see Comments]. *Nature* **370,** 463–467.
70. Pinto, L. A., Sullivan, J., Berzofsky, J. A., Clerici, M., Kessler, H. A., Landay, A. L., and Shearer, G. M. (1995). ENV-specific cytotoxic T lymphocyte responses in HIV seronegative health care workers occupationally exposed to HIV-contaminated body fluids. *J. Clin. Invest.* **96,** 867–876.
71. Langlade-Demoyen, P., Ngo-Giang-Huong, N., Ferchal, F., and Oksenhendler, E. (1993). Human immunodeficiency virus (HIV) nef-specific cytotoxic T lymphocytes in noninfected heterosexual contacts of HIV-infected patients. *J. Clin. Invest.* **93,** 1293–1297.
72. Rowland-Jones, S., Sutton, J., Ariyoski, K., Dong, T., Gotch, F., McAdam, S., Whitby, D., Sabally, S., Gallimore, A., Corrah, T., Takiguchi, M., Schultz, T., McMichael, A., and Whittle, H. (1995). HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. *Nature Med.* **1,** 59–64.
73. Kannagi, M., Masuda, T., Hattori, T., Kanoh, T., Nasu, K., Yamamoto, N., and Harada, S. (1990). Interference with human immunodeficiency virus (HIV) replication by CD8+ T cells in peripheral blood leukocytes of asymptomatic HIV carriers *in vitro*. *J. Immunol.* **64,** 3399–3406.
74. Tsubota, H., Lord, C. I., Watkins, D. I., Morimoto, C., and Letvin, N. L. (1989). A cytotoxic T lymphocyte inhibits acquired immunodeficiency syndrome virus replication in peripheral blood lymphocytes. *J. Exp. Med.* **169,** 1421–1434.
75. Yang, O. O., Kalams, S. A., Trocha, A., Cao, H., Luster, A., Johnson, R. P., and Walker, B. D. (1997). Suppression of HIV-1 replication by CD8+ cells: Evidence for HLA class I restricted triggering of cytolytic and non-cytolytic mechanisms. *J. Virol.*, **71,** 3120–3128.
76. Jassoy, C., Harrer, T., Rosenthal, T., Navia, B. A., Worth, J., Johnson, R. P., and Walker, B. D (1993). Human immunodeficiency virus type 1-specific cytotoxic T lymphocytes release gamma interferon, tumor necrosis factor alpha (TNF-alpha), and TNF-beta when they encounter their target antigens. *J. Virol.* **67,** 2844–2852.
77. Price, P., Johnson, R. P., Scadden, D. T., Jassoy, C., Rosenthal, T., Kalams, S., and Walker, B. D. (1995). Cytotoxic CD8+ T lymphocytes reactive with human immunodeficiency virus-1 produce granulocyte/macrophage colony-stimulating factor and variable amounts of interleukins 2, 3, and 4 following stimulation with the cognate epitope. *Clin. Immunol. Immunopathol.* **74,** 100–106.
78. Mackewicz, C. E., Ortega, H., and Levy, J. A. (1994). Effect of cytokines on HIV replication in CD4+ lymphocytes: Lack of identity with the CD8+ cell antiviral factor. *Cell. Immunol.* **153,** 329–343.

79. D'Souza, M. P., and Harden, V. A. (1996). Chemokines and HIV-1 second receptors. *Nature Med.* **2,** 1293–1300.
80. Baier, M., Werner, A., Bannert, N., Metzner, K., and Kurth, R. (1995). HIV suppression by interleukin-16. *Nature* **378,** 563. [Letter; comment].
81. Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1996). Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270,** 1811–1815.
82. Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., and Berger, E. A. (1996). CC CKR5: A RANTES, MIP-1 alpha, MIP-1 beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272,** 1955–1958.
83. Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., and Sodroski, J. (1996). The beta-chemokines receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85,** 1135–1148.
84. Deng, H., Lui, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R., and Landau, N. R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381,** 661–666.
85. Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G., and Doms, R. W. (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokines receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85,** 1149–1158.
86. Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P., and Paxton, W. A. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381,** 667–673.
87. Trkola, A., Dragic, T., Arthos, J., Binley, J. M., Olson, W. C., Allaway, G. P., Cheng-Mayer, C., Robinson, J., Maddon, P. J., and Moore, J. P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature* **384,** 184–187.
88. Wu, L., Gerard, N. P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardoso, A. A., Desjardin, E., Newman, W., Gerard, C., and Sodroski, J. (1996). CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokines receptor CCR-5. *Nature* **384,** 179–183.
89. Moore, J. P. (1996). Oral presentation. Tenth International AIDS Conference, Vancouver, Canada.
90. Mackewicz, C. E., Landay, A., Hollander, H., and Levy, J. A. (1994). Effect of zidovudine therapy on CD8+ T cell anti-HIV activity. *Clin. Immunol. Immunopathol.* **73,** 80–87.
91. Moore, P. S., Kingsley, L. A., Holmberg, S. D., Spira, T., Gupta, P., Hoover, D. R., Parry, J. P., Conley, L. J., Jaffe, H. W., and Chang, Y. (1996). Kaposi's sarcoma-associated herpesvirus infection prior to onset of Kaposi's sarcoma. *AIDS* **10,** 175–180.
92. Rubbert, A., Weissman, D., Combadiere, C., Pettrone, K. A., Daucher, J. A., Murphy, P. M., and Fauci, A. S. (1997). Multifactorial nature of noncytolytic CD8+ T cell-mediated suppression of HIV replication: β-chemokine-dependent and -independent effects. *AIDS Res. Hum. Retroviruses* **13,** 63.
93. Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996). HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor [see Comments]. *Science* **272,** 872–877.

94. Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T. A. (1996). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* **382,** 829–832.
95. Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Verelizier, J., Arenzana-Seisdedos, F., Schwartz, O., Heard, J., Clark-Lewis, I., Legler, D. F., Loetscher, M., Baggiolini, M., and Moser, B. (1996). The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-adapted line HIV-1. *Nature* **382,** 833–835.
96. Lusso, P., Cocchi, F., Balotta, C., Markham, P. D., Louie, A., Farci, P., Pal, R., Gallo, R. C., and Reitz, M. S., Jr. (1995). Growth of macrophage-tropic and primary human immunodeficiency virus type 1 (HIV-1) isolates in a unique CD4+ T-cell clone (PM1): Failure to downregulate CD4 and to interfere with cell-line-tropic HIV-1. *J. Virol.* **69,** 3712–3720.
97. Hsueh, F. W., Walker, C. M., Blackbourn, D. J., and Levy, J. A. (1994). Suppression of HIV replication by CD8+ cell clones derived from HIV-infected and uninfected individuals. *Cell. Immunol.* **159,** 271–279.
98. Chen, C. H., Weinhold, K. J., Bartlett, J. A., Bolognesi, D. P., and Greenberg, M. L. (1993). CD8+ T lymphocyte-mediated inhibition of HIV-1 long terminal repeat transcription: A novel antiviral mechanism. *AIDS Res. Hum. Retroviruses* **9,** 1079–1086.
99. Mackewicz, C. E., Blackbourn, D. J., and Levy, J. A. (1995). CD8+ T cells suppress human immunodeficiency virus replication by inhibiting viral transcription. *Proc. Natl. Acad. Sci. USA* **92,** 2308–2312.
100. Copeland, K. F. T., McKay, P. J., and Rosenthanl, K. L. (1995). Suppression of activation of HIV LTR by CD8+ T cells. *AIDS Res. Hum. Retroviruses* **11,** 1321–1326.
101. Powell, D. J., Bednarik, D. P., Folks, T. M., *et al.* (1993). Inhibition of cellular activation of retroviral replication by CD8+ T cells derived from non-human primates. *Clin. Exp. Immunol.* **91,** 473–481.
102. Cruikshank, W. W., Center, D. M., Nisar, N., Wu, M., Natke, B., Theodore, A. C., and Kornfeld, H. (1994). Molecular and functional analysis of a lymphocyte chemoattractant factor: Association of biologic function with CD4 expression. *Proc. Natl. Acad. Sci. USA* **91,** 5109–5113.
103. Toso, J. F., Chen, C. H., Mohr, J. R., Piglia, L., Oei, C., Ferrari, G., Greenberg, M. L., and Weinhold, K. J. (1995). Oligoclonal CD8 lymphocytes from persons with asymptomatic human immunodeficiency virus (HIV) type 1 infection inhibit HIV-1 replication. *J. Infect. Dis.* **172,** 964–973.
104. Yang, O. O., Kalams, S. A., Rosenzweig, M., Trocha, A., Koziel, M., Walker, B. D., and Johnson, R. P. (1996). Efficient lysis of HIV-1 infected cells by cytotoxic T lymphocytes. *J. Virol.* **70,** 5799–5806.
105. Tsomides, T. J., Aldovini, A., Johnson, R. P., Walker, B. D., Young, R. A., and Eisen, H. N. (1994). Naturally processed viral peptides recognized by cytotoxic T lymphocytes on cells chronically infected by human immunodeficiency virus type 1. *J. Exp. Med.* **180,** 1283–1293.
106. Anderson, J., Byrne, J. A., Schreiber, R., Patterson, S., and Oldstone, M. B. A. (1985). Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: Clearance of virus and *in vitro* properties. *J. Virol.* **53,** 552–560.
107. Zinkernagel, R. M., and Althage, A. (1977). Antiviral protection by virus-immune cytotoxic T cells. Infected target cells are lysed before infectious virus progeny is assembled. *J. Exp. Med.* **145,** 644–651.
108. Koenig, S., Fuerst, T. R., Wood, L. V., Woods, R. M., Suzich, J. A., Jones, G. M., de la Cruz, C. V. F., Davey, R. T. J., Venkatesan, S., Moss, B., *et al.* (1990). Mapping

the fine specificity of a cytolytic T cell response to HIV-1 nef protein. *J. Immunol.* **145,** 127–135.

109. Buseyne, F., Fevrier, M., Garcia, S., Gougeon, M. L., and Rivière, Y. (1996). Dual function of a human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte clone: Inhibition of HIV replication by noncytolytic mechanisms and lysis of HIV-infected CD4$^+$ cells. *Virology* **225,** 248–253.

110. van Kuyk, R., Torbett, B. E., Gulizia, R. J., Leath, S., Mosier, D. E., and Koenig, S. (1994). Cloned human CD8+ cytotoxic T lymphocytes protect human peripheral blood leukocyte–severe combined immunodeficient mice from HIV-1 infection by an HLA-unrestricted mechanism. *J. Immunol.* **153,** 4826–4833.

111. Zinkernagel, R. M. (1995). Are HIV-specific CTL responses salutary or pathogenic? *Curr. Opin. Immunol.* **7,** 462–470. [Review].

112. Zinkernagel, R. M. (1995). Immunosuppression by a noncytolytic virus via T cell mediated immunopathology. Implication for AIDS. *Adv. Exp. Med. Biol.* **374,** 165–171. [Review].

113. Barker, E., Mackewicz, C. E., and Levy, J. A. (1995). Effects of TH1 and TH2 cytokines on CD8+ cell response against human immunodeficiency virus: Implications for long-term survival. *Proc. Natl. Acad. Sci. USA* **92,** 11135–11139.

114. Clerici, M., and Shearer, G. M. (1994). The Th1–Th2 hypothesis of HIV infection: New insights. *Immunol. Today* **15,** 575–581. [Review].

115. Giorgi, J. V., and Detels, R. (1989). T-cell subset alterations in HIV-infected homosexual men: NIAID Multicenter AIDS cohort study. *Clin. Immunol. Immunopathol.* **52,** 10–18.

116. Stites, D. P., Casavant, C. H., McHugh, T. M., Moss, A. R., Beal, S. L., Zeigler, J. L., Saunders, A. M., and Warner, N. L. (1986). Flow cytometric analysis of lymphocyte phenotypes in AIDS using monoclonal antibodies and simultaneous dual immunofluorescence. *Clin. Immunol. Immunopathol.* **38,** 161–177.

117. Zeigler-Heitbrock, H. W. L., Stache, D., Schlunk, T., Gurtler, L., Schramm, W., Froschl, M., Bogner, J. R., and Rietmuller, G. (1988). Class II (DR) antigen expression on CD8+ lymphocyte subsets in acquired immunodeficiency syndrome (AIDS). *J. Clin. Immunol.* **8,** 473–478.

118. Fiorentino, S., Dalod, M., Olive, D., Guillet, J.-G., and Gomard, E. (1996). Predominant involvement of CD8+CD28- lymphocytes in human immunodeficiency virus-specific cytotoxic activity. *J. Virol.* **70,** 2022–2026.

119. Guidotti, L. G., Ishikawa, T., Hobbs, M. V., Matzke, B., Schreiber, R., and Chisari, F. V. (1996). Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity* **4,** 25–36.

120. Finke, D., Brinckmann, U. G., ter Meulen, V., and Liebert, U. G. (1995). Gamma interferon is a major mediator of antiviral defense in experimental measles virus-induced encephalitis. *J. Virol.* **69,** 5469–5474.

121. Walker, C. M., Erickson, A. L., Hsueh, F. C., and Levy, J. A. (1991). Inhibition of human immunodeficiency virus replication in acutely infected CD4+ cells by CD8+ cells involves a noncytotoxic mechanism. *J. Virol.* **65,** 5921–5927.

122. Coffin, J. M. (1995). HIV population dynamics *in vivo:* Implications for genetic variation, pathogenesis, and therapy. *Science* **267,** 483–489.

123. Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., and Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection [see Comments]. *Nature* **373,** 123–126.

124. Perelson, A. S., Neumann, A. U., Markowitz, M., Leonard, J. M., and Ho, D. D. (1996). HIV-1 dynamics *in vivo:* Virion clearance rate, infected cell life-span, and viral generation time. *Science* **271,** 1582–1586.

125. Wei, X., Ghosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H., *et al.* (1995). Viral dynamics in human immunodeficiency virus type 1 infection [see Comments]. *Nature* **373,** 117–122.
126. Mellors, J. W., Rinaldo, C. R., Jr., Gupta, P., White, R. M., Todd, J. A., and Kingsley, L. A. (1996). Prognosis in HIV-1 infection predicted by the quantity of virus in plasma [see Comments]. *Science* **272,** 1167–1170.
127. Zinkernagel, R. M., and Hengartner, H. (1995). Viral immunopathology. Introduction. Springer Sem. Immunopathol. **17,** 119–120. [Review].
128. Phillips, A. N. (1996). Reduction of HIV concentration during acute infection: Independence from a specific immune response. *Science* **271,** 497–499.
129. Tersmette, M., Gruters, R. A., de Wolf, F., de Goede, R. E., Lange, J. M., Schellekens, P. T., Goudsmit, J. Huisman, H. G., and Miedema, F. (1989). Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: Studies on sequential HIV isolates. *J. Virol.* **63,** 2118–2125.
130. Tersmette, M., Lange, J. M., de Goede, R. E., de Wolf, F., Eeftink, S. J. K., Schellekens, P. T., Coutinho, R. A., Huisman, J. G., Goudsmit, J., and Miedema, F. (1989). Association between biological properties of human immunodeficiency virus variants and risk for AIDS and AIDS mortality. *Lancet* **1,** 983–985.
131. Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A., and Ho, D. D. (1993). Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science* **261,** 1179–1181.
132. Liu, R., Paxton, W., Choe, s., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A., and Landau, N. R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance in some multiply-exposed individuals to HIV-1 infection. *Cell* **86,** 367–377.
133. Huang, Y., Paxton, W. A., Wolinsky, S. M., Neumann, A. U., Zhang, L., He, T., Kang, S., Ceradini, D., Jin, Z., Yazdanbakhsh, K., Kuntsman, K., Erikson, D., Dragen, E., Landau, N. R., Phair, J., Ho, D. D., and Koup, R. A. (1996). The role of a mutant CCR5 allele in HIV-I transmission and disease progression. *Nature Med.* **2,** 1240–1243.
134. Miedema, F., Meyaard, L., Koot, M., Klein, M. R., Roos, M. T., Groenink, M., Fouchier, R. A., Van't Wout, A. B., Tersmette, M., Schellekens, P. T., *et al.* (1994). Changing virus–host interactions in the course of HIV-1 infection. *Immunol. Rev.* **140,** 35–72.
135. Kast, W. M., Bronkhorst, A. M., de Waal, L. P., and Melief, C. J. (1986). Cooperation between cytotoxic and helper T lymphocytes is protective against lethal Sendai virus infection. *J. Exp. Med.* **164,** 723–738.
136. Matloubian, M., Conception, R. J., and Ahmed, R. (1994). CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* **68,** 8056–8063.
137. Moskophidis, D., Lechner, F., Pircher, H., and Zinkernagel, R. M. (1993). Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells [see Comments]. *Nature* **362,** 758–761.
138. Effros, R. B., Allsopp, R., Chiu, C.-P., Hausner, M. A., Hirji, K., Wang, L., Harley, C. B., Villeponteau, B., West, M. D., and Giorgi, J. V. (1996). Shortened telomeres in the expanded CD28–CD8+ cell subset in HIV disease implicate replicative senescence in HIV pathogenesis. *AIDS* **10,** F17–F22.
139. Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R., and Ferguson, T. A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* **270,** 1189–1192.

140. Heath, S. L., Tew, J. G., Szakal, A. K., and Burton, G. F. (1995). Follicular dendritic cells and human immunodeficiency virus infectivity [see Comments]. *Nature* **377,** 740–744.
141. Howcroft, T. K., Strebel, K., Martin, M. A., and Singer, D. S. (1993). Repression of MHC class I gene promoter activity by two-exon Tat of HIV. *Science* **260,** 1320–1322.
142. Scheppler, J. A., Nicholson, J. K., Swan, D. C., Ahmed, A. A., and McDougal, J. S. (1989). Downmodulation of MHC-I in a CD4+ T cell line, CEM-E5, after HIV-1 infection. *J. Immunol.* **143,** 2858–2866.
143. Schwartz, O., Marechal, V., Le Gall, S., Lemonnier, F., and Heard, J.-M. (1996). Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nature Med.* **2,** 338–342.
144. Coffin, J. M. (1990). Genetic variation in retroviruses. *In* (E. Kurstak, R. G. Marusyk, F. A. Murphy, and M. H. V. Van Regenmortel, eds.), "Virus Variability Epidemiology and Control" pp. 11–33. Plenum, New York.
145. Dai, L. C., West, K., Littaua, R., Takahashi, K., and Ennis, F. A. (1992). Mutation of human immunodeficiency virus type 1 at amino acid 585 on gp41 results in loss of killing by CD8+ A24-restricted cytotoxic T lymphocytes. *J. Virol.* **66,** 3151–3154.
146. Johnson, R. P., Trocha, A., Buchanan, T. M., and Walker, B. D. (1992). Identification of overlapping HLA class I-restricted cytotoxic T cell epitopes in a conserved region of the human immunodeficiency virus type 1 envelope glycoprotein: Definition of minimum epitopes and analysis of the effects of sequence variation. *J. Exp. Med.* **175,** 961–971.
147. Phillips, R. E., Rowland, J. S., Nixon, D. F., Gotch, F. M., Edwards, J. P., Ogunlesi, A. O., Elvin, J. G., Rothbard, J. A., Bangham, C. R., Rizza, C. R., and McMichael, A. J. (1991). Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* **354,** 453–459.
148. Takahashi, H., Merli, S., Putney, S. D., Houghten, R., Moss, B., Germain, R. N., and Berzofsky, J. A. (1989). A single amin acid interchange yields reciprocal CTL specificities for HIV-1 gp160. *Science* **246,** 118–121.
149. Couillin, I., Culmann-Penciolelli, B., Gomard, E., Choppin, J., Levy, J.-P., Guillet, J.-G., and Saragosti, S. (1994). Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the human immunodeficiency virus 1 NEF protein. *J. Exp. Med.* **180,** 1129–1134.
150. Klenerman, P., Rowland-Jones, S., McAdam, S., Edwards, J., Daenke, S., Lalloo, D., Koppe, B., Rosenberg, W., Boyd, D., Edwards, A., Giagrande, P., Phillips, R. E., and McMichael, A. J. (1994). Naturally occurring HIV-1 gag variants antagonise cytotoxic T cell activity. *Nature* 403–406.
151. Meier, U. C., Klenerman, P., Griffin, P., James, W., Koppe, B., Larder, B., McMichael, A., and Phillips, R. (1995). Cytotoxic T lymphocyte lysis inhibited by viable HIV mutants. *Science* **270,** 1360–1362.
152. Bergmann, C. C., Yao, Q., Ho, C.-K., and Buckwold, S. L. (1996). Flanking residues alter antigenicity and immunogenicity of multi-unit CTL epitopes. *J. Immunol.* **157,** 3242–3249.
153. Yellen-Shaw, A. J., and Eisenlohr, L. C. (1997). Regulation of class I-restricted epitope processing by local or distal flanking sequence. *J. Immunol.* **158,** 1727–1733.
154. Ossendorp, F., Eggers, M., Neisig, A., Ruppert, T., Groettrup, M., Sijts, A., Mengede, E., Kloetzel, P.-M., Neefjes, J., Koszinowski, U., and Melief, C. (1996). A single residue exchange within a viral CTL epitope alters proteasome-mediated degradation resulting in lack of antigen presentation. *Immunity* **5,** 115–124.
155. Goulder, P. J. R., Phillips, R. E., Colbert, R. A., McAdam, S., Ogg, G., Nowak, M. A., Giangrande, P., Luxxi, G., Morgan, B., Edwards, A., McMichael, A. J., and

Rowland-Jones, S. (1997). Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nature Med.* **3,** 212.

156. Meyerhans, A., Dadaglio, G., Vartanian, J. P., Langlade, D. P., Frank, R., Asjo, B., and Plata, F., S. W.-H. (1991). *In vivo* persistence of a HIV-1-encoded HLA-B27-restricted cytotoxic **T** lymphocyte epitope despite specific *in vitro* reactivity. *Eur. J. Immunol.* **21,** 2637–2640.
157. Safrit, J., Andrews, C. A., Tuofu, Z., Ho, D. D., and Koup, R. A. (1994). Characterization of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte clones isolated during acute seroconversion: Recognition of autologous virus sequences within a conserved immunodominant epitope. *J. Exp. Med.* **179,** 463–472.
158. Adams, D. H., and Lloyd, A. R. (1997). Chemokines: Leucocyte recruitment and activation cytokines. *Lancet* **349,** 490–495.
159. Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1996). Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270,** 1811–1815.
160. Arenzana-Selsdedos, F., Virelizier, J.-L., and Rousset, D. (1996). HIV blocked by chemokine antagonist (scientific correspondence). *Nature* **383,** 400.
161. Koenig, S., Conley, A. J., Brewah, Y. A., Jones, G. M., Leath, S., Boots, L. J., Davey, V., Pantaleo, G., Demarest, J. F., Carter, C., Wannebo, C., Yanelli, J. R., Rosenberg, S. A., and Lane, H. C. (1995). Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. *Nature Med.* **1,** 330–336.
162. Ziegner, U. H. M., Peters, G., Jolly, D. J., Mento, S. J., Galpin, J., Prussak, C. E., Barber, J. R., Hartnett, D. E., Bohart, C., Klump, W., Saijadi, N., Merchant, B., and Warner, J. F. (1995). Cytotoxic T-lymphocyte induction in asymptomatic HIV-1-infected patients immunized with Retrovector-transduced autologous fibroblasts expressing HIV-1 IIIB Env/Rev proteins. *AIDS* **9,** 43–50.
163. Lewis, D. E., Tang, D. S. N., Adu-Oppong, A., Schober, W., and Rodgers, J. R. (1994). Anergy and apoptosis in CD8+ T cells from HIV-infected persons. *J. Immunol.* **153,** 412–420.
164. Brander, C., Walker, B. D., Korber, B. T. M., Brander, C., Moore, J. P., D'Souza, P. M., Walker, B. D., Koup, R., Haynes, B. F., and Myers, G. (1996). Systematic identification of optimal HIV-1 CTL epitopes. Los Alamos National Laboratory, Los Alamos, NM, pp. IV-50–IV-60.
165. Roberts, M. R., Qin, L., Zhang, D., Smith, D. H., Tran, A.-C., Dull, T. J., Groopman, J. E., Capon, D. J., Byrn, R. A., and Finer, M. H. (1994). Targeting of human immunodeficiency virus-infected cells by CD8+ T lymphocytes armed with universal T-cell receptors. *Blood* **84,** 2878–2889.
166. Yang, O. O., Tran-Chen, A., Kalams, S. A., Johnson, R. P., Roberts, M. R., and Walker, B. D. (1997). Lysis of HIV-1-infected cells and inhibition of viral replication by universal receptor T cells. Submitted for publication.
167. Kovacs, J. A., Baseler, M., Dewar, R. J., Vogel, S., Davey, R. T., Falloon, J., Polis, M. A., Walker, R. E., Stevens, R., Salzman, N. P., Metcalf, J. A., Masur, H., and Lane, H. C. (1996). Increases in CD4 T lymphocytes with intermittent courses of interleukin-2 in patients with human immunodeficiency virus infection. A preliminary study. *N. Engl. J. Med.* **332,** 567–575.
168. Wilson, C. C., Wong, J. T., Girard, D., Merril, D. P., Dynan, M., An, D., Kalams, S. A., Johnson, R. P., Hirsch, M. S., D'Aquila, R. T., and Walker, B. D. (1995). Ex vivo expansion of CD4+ lymphocytes from HIV-1 infected persons in the presence of combination antiretroviral therapy. *J. Infect. Dis.* **172,** 88–96.

169. Levine, B. L., Mosca, J. D., Riley, J. L., Carroll, R. G., Vahey, M. T., Jagodzinski, L. L., Wagner, K. F., Mayers, D. L., Burke, D. S., Weislow, O. S., St. Louis, D. C., and June, C. H. (1996). Antiviral effect and ex vivo CD4+ T cell proliferation in HIV-positive patients as a result of CD28 costimulation. *Science* **272,** 1939–1943.
170. Parker, K. C., Bednarek, M. A., and Coligan, J. E. (1994). Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J. Immunol.* **152,** 163–175.
171. Parker, K. C., Bednarek, M. A., Hull, L. K., Utz, U., Cunningham, B., Zweerink, H. J., Biddison, W. E., and Coligan, J. E. (1992). Sequence motifs important for peptide binding to the human MHC class I molecule, HLA-A2. *J. Immunol.* **149,** 3580–3587.
172. Harrer, E., Harrer, T., Barbosa, P., Feinberg, M., Johnson, R. P., Buchbinder, S., and Walker, B. D. (1996). Recognition of the highly conserved YMDD region in the human immunodeficiency virus type 1 reverse transcriptase by HLA-A2-restricted cytotoxic T lymphocytes from an asymptomatic long-term nonprogressor. *J. Infect. Dis.* **173,** 476–479.
173. Tsomides, T. J., Walker, B. D., and Eisen, H. N. (1991). An optimal viral peptide recognized by CD8+ T cells binds very tightly to the restricting class I major histocompatibility complex protein on intact cells but not to the purified class I protein. *Proc. Natl. Acad. Sci. USA* **88,** 11276–11280.
174. Walker, B. D., Flexner, C., Birch, L. K., Fisher, L., Paradis, T. J., Aldovini, A., Young, R., Moss, B., and Schooley, R. T. (1989). Long-term culture and fine specificity of human cytotoxic T-lymphocyte clones reactive with human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **86,** 9514–9518.
175. Dupuis, M., Kundu, S. K., and Merigan, T. C. (1995). Characterization of HLA-A*0201-restricted cytotoxic T cell epitopes in conserved regions of the HIV type 1 gp160 protein. *J. Immunol.* **155,** 2232–2239.
176. Haas, G., Plikat, U., Debre, P., Lucchiari, M., Katlama, C., Dudoit, Y., Bonduelle, O., Bauer, M., Ihlenfeldt, H., Jung, G., Maier, B., Meyerhans, A., and Autran, B. (1996). Dynamics of viral variants in HIV-1 Nef and specific cytotoxic T lymphocytes *in vivo*. *J. Immunol.* **157,** 4212–4221.
177. Hadida, F., Haas, G., Zimmermann, G., Hosmalin, A., Spohn, R., Samri, A., June, G., Debre, P., and Autran, B. (1995). CTLs from lymphoid organs recognize an optimal HLA-A2 restricted and HLA-B52-restricted nonpeptide and several epitopes in the C-terminal region of HIV-1 Nef. *J. Immunol.* **154,** 4174–4186.
178. Johnson, R. P., Hammond, S. A., Trocha, A., Siliciano, R. F., and Walker, B. D. (1994). Induction of a major histocompatibility complex class I-restricted cytotoxic T-lymphocyte response to a highly conserved region of human immunodeficiency virus type 1 (HIV-1) gp120 in seronegative humans immunized with a candidate HIV-1 vaccine. *J. Virol.* **68,** 3145–3153.
179. Takahashi, K., Dai, L. C., Fuerst, T. R., Biddison, W. E., Earl, P. L., Moss, B., and Ennis, F. A. (1991). Specific lysis of human immunodeficiency virus type 1-infected cells by a HLA-A3.1-restricted CD8+ cytotoxic T-lymphocyte clone that recognizes a conserved peptide sequence within the gp41 subunit of the envelope protein. *Proc. Natl. Acad. Sci. USA* **88,** 10277–10281.
180. Culmann, B., Gomard, E., Kieny, M. P., Guy, B., Dreyfus, F., Saimot, A. G., Sereni, D., Sicard, D., and Levy, J. P. (1991). Six epitopes reacting with human cytotoxic CD8+ T cells in the central region of the HIV-1 NEF protein. *J. Immunol.* **146,** 1560–1565.
181. Sispas, N. V., Kalams, S. A., Trocha, A., He, S., Blattner, W. A., Walker, B. D., and Johnson, R. P. (1996). Identification of type-specific cytotoxic T lymphocyte responses

to homologous viral proteins in laboratory workers accidentally infected with the human immunodeficiency virus-1. *J. Clin. Invest.* **99,** 752–762.

182. Johnson, R., and Walker, B. (1994). Cytotoxic T lymphocytes in human immunodeficiency virus infection: Responses to structural proteins. *Curr. Top Micro. Immunol.* **189,** 35–63.
183. Zhang, Q. J., Gavioli, R., Klein, G., and Masucci, M. G. (1993). An HLA-A11-specific motif in nonamer peptides derived from viral and cellular proteins. *Proc. Natl. Acad. Sci. USA* **90,** 2217–2221.
184. Lieberman, J., Fabry, J. A., Kuo, M. C., Earl, P., Moss, B., and Skolnik, P. R. (1992). Cytotoxic T lymphocytes from HIV-1 seropositive individuals recognize immunodominant epitopes in Gp160 and reverse transcriptase. *J. Immunol.* **148,** 2738–2747.
185. Shankar, P., Fabry, J. A., Fong, D., and Lieberman, J. (1995). Three regions of gp160 contain overlapping CTL epitopes restricted by multiple HLA class I alleles. *J. Cell. Biochem.* **S21B,** D4 (345). [Abstract].
186. Klenerman, P., Luzzi, G., McIntyre, K., Phillips, R., and McMichael, A. (1996). Identification of a novel HLA-A25 restricted epitope in a conserved region of p24 gag (positions 71–80). *AIDS* **10,** 348–350.
187. Van Baalen, C. A., Klein, M. R., Huisman, R. C., Dings, M. E., Kerkhof Garde, S. R., Geretti, A. M., Gruters, R., Van Els, C. A., Miedema, F., and Osterhaus, A. D. (1996). Fine-specificity of cytotoxic T lymphocytes which recognize conserved epitopes of the Gag protein of human immunodeficiency virus type 1. *J. Gen. Virol.* **77,** 1659–1665.
188. Safrit, J. T., Lee, A. Y., Andrews, C. A., and Koup, R. A. (1994). A region of the third variable loop of HIV-1 gp120 is recognized by HLA-B7-restricted CTLs from two acute seroconversion patients. *J. Immunol.* **153,** 3822–3830.
189. Culmann-Penciolelli, B., Lamhamedi-Cherradi, S., Couillin, I., Guegan, N., Levy, J. P., Guillet, J. G., and Gomard, E. (1994). Identification of multirestricted immunodominant regions recognized by cytolytic T lymphocytes in the human immunodeficiency virus type 1 Nef protein [published erratum appears in *J. Virol.* **69**(1), 618, (1995). *J. Virol.* **68,** 7336–7343.
190. Rowland-Jones, S. L., Powis, S. H., Sutton, J., Mockridge, I., Gotch, F. M., Murray, N., Hill, A. B., Rosenberg, W. M., Trowsdale, J., and McMichael, A. J. (1993). An antigen processing polymorphism revealed by HLA-B8-restricted cytotoxic T lymphocytes which does not correlate with TAP gene polymorphism. *Eur. J. Immunol.* **23,** 1999–2004.
191. Nixon, D. F., and McMichael, A. F. (1988). Cytotoxic T cell recognition of HIV proteins and peptides. *AIDS* **5,** 1049–1059.
192. Wilson, C. C., Kalams, S. A., Wilkes, B. A., Ruhl, D. H., Gao, F., Hahn, B. H., Hanson, I. C., Luzuriaga, K., Wolinksy, S., Koup, R., Buchbinder, S. P., Johnson, R. P., and Walker, B. D. (1996). Overlapping epitopes in human immunodeficiency virus type 1 gp120 presented by HLA A, B, and C molecules: Effects of viral variation on cytotoxic T-lymphocyte recognition. *J. Virol.* **71,** 1256–1264.
193. Nixon, D. F., Townsend, A. R., Elvin, J. G., Rizza, C. R., Gallwey, J., and McMichael, A. J. (1988). HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. *Nature* **336,** 484–487.
194. Shiga, H., Shioda, T., Tomiyama, H., Takamiya, Y., Oka, S., Kimura, S., Yamaguchi, Y., Kojoubori, T., Rammensee, H. G., Miwa, K., and Takiguchi, M. (1996). Identification of multiple HIV-1 cytotoxic T-cell epitopes presented by human leukocyte antigen B35 molecule. *AIDS* **10,** 1075–1083.

195. Gotch, F., McAdam, S. N., Allsopp, C. E., Gallimore, A., Elvin, J., Kieny, M. P., Hill, A. V., McMichael, A. J., and Whittle, H. C. (1993). Cytotoxic T cells in HIV2 seropositive Gambians. Identification of a virus-specific MHC-restricted peptide epitope. *J. Immunol.* **151,** 3361–3369.
196. Johnson, R. P., Trocha, A., Buchanan, T. M., and Walker, B. D. (1993). Recognition of a highly conserved region of human immunodeficiency virus type 1 gp120 by an HLA-Cw4-restricted cytotoxic T-lymphocyte clone. *J. Virol.* **67,** 438–445.
197. Kerkau, T., Schmitt, L. R., Schimpl, A., and Wecker, E. (1989). Downregulation of HLA class I antigens in HIV-1-infected cells. *AIDS Res. Hum. Retroviruses* **5,** 613–620.
198. Ennen, J., Findeklee, H., Dittmar, M. T., Norley, S., Ernst, R., and Kurth, R. (1994). CD8⁺ T lymphocytes of African green monkeys secrete an immunomodulatory virus-suppressing lymphokine. *Proc. Natl. Acad. Sci. USA* **91,** 7207–7211.
199. Castro, B. A., Walker, C. M., Eichberg, J. W., and Levy, J. A. (1991). Supression of human immunodeficiency virus replication by CD8⁺ cells from infected and uninfected chimpanzees. *Cell Immunol.* **132,** 246–255.
200. Husch, B., Eibl, M. M., and Mannhalter, J. W. (1993). CD3,CD8 double-positive cells from HIV-I-infected chimpanzees show group-specific inhibition of HIV-I replication. *AIDS Res. Hum. Retroviruses* **9,** 405–413.
201. Goulder, P. J., Bunce, M., Kransa, P., McIntyre, K., Crowley, S., Morgan, B., Edwards, A., Giangrande, P., Phillips, R. E., and McMichael, A. J. (1996). Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type I infection. *AIDS Res. Hum. Retroviruses* **12,** 1691–1698.
202. Ferrari, G., Humphrey, W., McElrath, M. J., Excler, J. L., Duliege, A. M., Clements, M. L., Corey, L. C., Bolognesi, D. P., and Weinhold, K. J. (1997). Clade B-based HIV-I vaccines elicit cross-clade cytotoxic T lymphocyte reactivities in uninfected volunteers. *Proc. Natl. Acad. Sci. USA* **94,** 1396–1401.
203. Bellgrau, D., Gold, D., Selawry, H., Moore, J., Franzusoff, A., and Duke, R. C. (1995). A role for CD95 ligand in preventing graft rejection. *Nature* **377,** 630–632.
204. Collins, K., and Baltimore, D. (1997). Oral presentation, Keystone Symposia on Molecular and Cellular Biology, AIDS Pathogenesis (April 8–13).
205. Carrol, R. G., Riley, J. L., Levine, B. L., Feng, Y., Kaushal, S., Ritchey, D. W., Bernstein, W., Weislow, O. S., Brown, C. R., Berger, E. A., June, C. H., and St. Louis, D. C. (1997). Differential regulation of HIV-I fusion cofactor expression by CD28 costimulation of CD4⁺ cells. *Science* **276,** 273–276.

This article was accepted for publication on March 31, 1997.

INDEX

A

B

C

D

E

F

G

H

I

Q

R

S

T

V

CONTENTS OF RECENT VOLUMES

Volume 62

Volume 63

Volume 64

Volume 65