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The *Janus* Protein Tyrosine Kinase Family and Its Role in Cytokine Signaling

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I. Introduction

Hematopoiesis is regulated through the interaction of a variety of cytokines with their cognate receptors. A number of the hematopoietic cytokines utilize receptors of a novel family of receptors termed the cytokine receptor superfamily (Bazan, 1989, 1990a,b, 1991). This receptor family is characterized by structural motifs in the extracellular, ligand-binding domains including four positionally conserved cysteines and a WSXWS motif. Structurally, these receptors are also related to the receptors for the interferons (IFN) and it has been proposed that they all evolved from a common progenitor. In contrast to the extracellular domains, the cytoplasmic domains of the cytokine receptor superfamily members contain only very limited sequence homology that is found in the membrane proximal region and often referred to as the box1 and box2 motifs. Importantly, the cytokine receptor superfamily members lack catalytic domains that might indicate the mechanisms by which they transduce ligand binding to cellular responses.

The cellular consequences of ligand binding are diverse and involve the induction of gene transcription as well as a diverse set of functional responses often associated with promoting cell cycling or differentiation. Moreover, a variety of cytokines have quite different effects dependent on the cell lineage and/or the stage of differentiation. The mechanisms by which cytokine receptors transmit the signal from ligand binding to the activation of specific cellular responses are largely unknown. Indeed, the structures of the members of the cytokine receptor superfamily provide little insights into potential pathways of signal transduction. Over the past several years, however, evidence began to accumulate to suggest that their ability to couple ligand binding to the induction of tyrosine phosphorylation was critical for function and raised the possibility that this function was mediated by cytoplasmic protein tyrosine kinases with which the receptors associated. Based on this hypothesis numerous studies focused on the role of members of the src family of kinases in cytokine signaling based on their demonstrated role in signaling through the T cell receptor and evidence of a role in IL-2 signaling (Taniguchi and Minami, 1993). However, over the

past 3 years, it has become apparent that a once relatively obscure family of cytoplasmic protein tyrosine kinases plays a common and central role in cytokine signaling. Also during this period, numerous studies of receptor structure have functionally dissected cytokine responses relative to the role of various signaling pathways. This chapter focuses on the origins, structure, and role of the *Janus* protein tyrosine kinases in cytokine signaling and attempts to integrate the rapidly emerging information relating the signaling pathways to which the receptors, through the *Janus* kinases (Jaks), are coupled.

II. Origins and Structure of the *Janus* Kinases

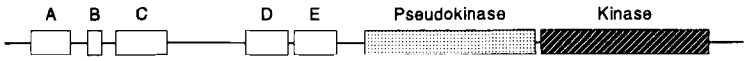
The Jaks were initially identified by polymerase chain reaction (PCR) and low stringency hybridization methods to identify novel kinases. The first full-length sequence for a Jak was obtained by screening a T cell library with a c-fms restriction fragment containing the tyrosine kinase catalytic domain (Firmbach-Kraft *et al.*, 1990). Full-length Jak1 and Jak2 cDNAs were subsequently obtained using probes generated by PCR amplification of kinase catalytic domains (Wilks *et al.*, 1991; Wilks, 1989, 1991). The term *Janus* refers to an ancient Roman god of gates and doorways. Alternatively, Jak has often been used as an acronym for Just Another Kinase, to reflect its origin during a time when a variety of novel protein tyrosine kinases were being detected by PCR approaches. Jak3 is the newest member of the family characterized. Similar to Jak1 and Jak2, Jak3 was initially identified in PCR experiments with breast cancer cells (Cance *et al.*, 1993) and, more recently, rat hippocampal neurons (Sanchez *et al.*, 1994). The PCR fragment from breast cancer cells was used to obtain full-length cDNA clones for murine Jak3 from a B cell library (Witthuhn *et al.*, 1994). Similarly, PCR approaches led to the identification and cloning of rat Jak3 from mesangial cells (Takahashi and Shirasawa, 1994), murine Jak3 from murine myeloid cells (Rane and Reddy, 1994), and human Jak3 from natural killer cells (Kawamura *et al.*, 1994).

Jak1, Jak2, and Tyk2 are widely expressed and encoded by transcripts of 5.4 kb, two transcripts of 5.3 and 5.0 kb and a 4.4-kb transcript, respectively. The structure and significance of the two Jak2 transcripts are not currently known. In contrast, the 4.0-kb Jak3 transcript is much more restricted in its pattern of expression. It is generally not detected in fibroblasts but is expressed in myeloid cells and lymphoid cells. In a myeloid cell line that is able to terminally differentiate to granulocytes in response to G-CSF, expression of Jak3 is strikingly increased (Rane and Reddy, 1994). Interestingly, in these studies, the cDNA that was obtained from cells induced to differentiate contained two stretches of additional amino

acids of 147 and 28 residues. Whether this alternatively spliced form of Jak3 occurs at significant levels, gives rise to altered proteins, and is specific for differentiating cells is yet to be determined. Interestingly, Jak3 levels also increase dramatically in activated T cells and monocytes (Kawamura *et al.*, 1994).

Although initially identified in mammalian species, a Jak homolog has been identified in *Drosophila* (Binari and Perrimon, 1994) as the gene associated with *hopscotch* (*hop*) mutations. Within the carboxyl kinase domain the *Drosophila* gene is 39% identical to Jak1/Jak2 or Tyk2. Within the kinase-like domain the identity with Jak1/Jak2 or Tyk2 is 27, 24, or 21%, while the amino terminal region has identities of 19, 23, and 20%, respectively. The maternal *hop* product is required for the proper levels of expression of particular stripes of pair-rule genes. In contrast, the *Drosophila Tumorous-lethal* gene is a dominant mutation of the *hopscotch* locus (Hanratty and Dearolf, 1993). This mutation causes the abnormal proliferation and differentiation of the larval hematopoietic system (fly leukemia), leading to late larval/pupal lethality.

The chromosomal locations of the murine and human Jaks have been determined (Fig. 1). In humans, the genes for *JAK1*, *JAK2*, *JAK3*, and *TYK2* have been reported to be at chromosome bands 1p31.3 (Pritchard *et al.*, 1992), 9p24 (Pritchard *et al.*, 1992), and 19p13.2 (Firmbach-Kraft *et al.*, 1990). The murine Jaks have been genetically mapped using interspe-



Kinase	Size	Identity	Chromosomal Position	Expression
Tyk2	140 kDa	36%	19p13.2	Ubiquitous
Jak1	135 kDa	36%	1p31.3	Ubiquitous
Jak2	130 kDa	47%	10p23-p24	Ubiquitous
Jak3	120 kDa	-	19p13	Myeloid/Lymphoid

FIG. 1. Properties of the *Janus* protein tyrosine kinases. The overall organization of the structure of the Jaks is indicated including the kinase catalytic domain, the psuedokinase domain, and the Jak homology domains which are indicated by the boxes A–E. Some of the properties of the known family members are also indicated. The percentage identity is presented as relative to Jak3. The chromosomal locations are those for the human genes which were either directly determined or are based on the location of the murine genes as indicated in the text.

cific hybrids (O. Silvennoinen, N. Jenkins, N. Copeland, and J. N. Ihle, manuscript in preparation). In mice, the *Jak1* gene is very tightly linked to *Pgm2* on chromosome 4 which corresponds to human chromosome band 1p22.1, consistent with the human mapping data. However, the murine *Jak2* gene is genetically linked to *Fas* on chromosome 19 which corresponds to human 10q23–q24.1 and not to 9p24 as reported. The basis for this discrepancy is not known but may have arisen from using the murine cDNA to localize the human gene in the initial studies. Lastly, the murine *Jak3* gene is located distal of *JunD* in the middle of chromosome 8. This region has homology with human chromosome 19p13 which localizes the gene near the *TYK2* gene.

From the initial characterizations it was clear that the Jaks were quite structurally unique from all other cytoplasmic protein tyrosine kinases characterized. The most striking structural features are the lack of any src homology 2 (SH2) or 3 (SH3) domains and the presence of two domains with the characteristic subdomains found in kinase catalytic domains. As illustrated in Fig. 1, the two kinase domains are located in the carboxyl region of the proteins which vary in size (Jak3, 120 kDa; Jak2, 130 kDa; Jak1, 135 kDa; Tyk2, 140 kDa). The more carboxyl kinase domain contains all the motifs associated with protein tyrosine kinases (Hanks *et al.*, 1988) and thus is predicted to have catalytic activity. The second domain contains kinase motifs but several of these lack residues that have been found to be essential for catalytic activity. Thus, the function of this domain is not known. The sequences amino terminal to these kinase domains bear no homology to previously defined protein motifs. However, as shown in Fig. 1, there are readily identifiable regions of homology among the four Jak family members. Of particular note are the regions indicated by the homology blocks D and E which contain long stretches of identity among the family members. The regions identified as homology blocks A, B, and C are less similar. The significance of these Jak homology domains is only now being investigated but it may be reasonably assumed that they determine the protein/protein interactions governing the function of Jaks in the different cytokine receptor signal transduction pathways.

III. *Janus* Kinases Couple Cytokine Binding to Induction of Tyrosine Phosphorylation

Studies over the past years of a variety of cytokines have demonstrated that the cytokine receptors couple ligand binding to the induction of protein tyrosine phosphorylation, a response that is critical for receptor function. Over the past several years a variety of kinases have been implicated, although consistent and compelling evidence has accumulated indicating

that the Jaks play a common and central role in cytokine signaling. The role of various kinases in signaling has generally involved the demonstration that cytokine binding induces the tyrosine phosphorylation and activation of the kinase activity of candidate kinases. In particular, it has been reported that c-fes is tyrosine phosphorylated and activated following Epo, GM-CSF, or IL-3 stimulation (Hanazono *et al.*, 1993a,b). In addition, it was demonstrated that GM-CSF can induce the physical association between c-fes and the β_c-chain of the receptor. The domains required for the association have not been identified. It should be noted, however, that other studies were unable to observe tyrosine phosphorylation or activation of c-fes in the responses to Epo (Witthuhn *et al.*, 1993) or IL-3 (Quelle *et al.*, 1994).

Several src family cytoplasmic protein kinases have been implicated in cytokine signaling. Initially, lck was implicated in the response to IL-2 and subsequently lyn and fyn (Taniguchi and Minami, 1993). Ligand stimulation induces activation of their catalytic activity in immunoprecipitates and association occurs with an acidic domain in the IL-2 receptor β-chain. Importantly, deletion of the acidic domain uncouples ligand binding from the activation of lck but does not affect mitogenesis, implying that the activation of the src-related kinases may be required for cellular responses unrelated to proliferation. Lyn has also been implicated in IL-3 signaling (Torigoe *et al.*, 1992) although there was no evidence for lyn involvement in the response to Epo in another study (Witthuhn *et al.*, 1993). More recently, the src-related kinase, hck, has been shown to be activated by stimulation of embryonic stem cells with LIF (Ernst *et al.*, 1994). Moreover, hck physically associates with gp130, the affinity converter and signal transducing component of the LIF receptor. The domains required for association and activation have not been identified and consequently the specific biological responses that can be potentially ascribed to hck are not know. Similarly, recent studies (Corey *et al.*, 1994) found that G-CSF activates lyn and syk and these kinases associate with the G-CSF receptor. The domains involved have not been identified.

Recent studies also implicated Tec and Btk in cytokine signaling. Tec and Btk belong to a family of protein tyrosine kinases that is highly related to the src family, which also includes Itk and Bmx (Tamagnone *et al.*, 1994). Mutations in Btk are responsible for the chromosome X-linked agammaglobulinemia (Rawlings *et al.*, 1993; Thomas *et al.*, 1993), indicating its critical role in B cell signaling. Tec and Btk have been found to be tyrosine phosphorylated and activated by IL-6 (Matsuda *et al.*, 1995) and Tec by IL-3 (Mano *et al.*, 1995; Matsuda *et al.*, 1995). However, other studies failed to detect activation of Tec in the response to IL-3 but did detect association of Tec with the c-kit receptor and its activation following

binding of stem cell factor (Tang *et al.*, 1994). The domains of Tec required for association with c-kit occur in the amino terminal region and include a proline-rich domain. The domains required for the association of Tec or Bkt with gp130 or the IL-3 receptor chains have not been identified.

Although several kinase families have been implicated in cytokine signaling, the most consistent family involved is the Jak family. The potential involvement of the Jaks in cytokine signaling has resulted from studies which have examined the ability of various cytokines to induce Jak tyrosine phosphorylation, active catalytic activity, and associate with the cytokine receptors. A summary of the results, indicating which Jaks are implicated in signaling by various cytokines, is shown in Table I. The first series of studies demonstrated that Jak2, but not other Jaks, is specifically tyrosine phosphorylated and kinetically activated in the responses to Epo (Witthuhn *et al.*, 1993), growth hormone (Artgetsinger *et al.*, 1993), or IL-3 (Silvennoinen *et al.*, 1993c). Subsequently, it was demonstrated that Jak1, Jak2, and to some extent, Tyk2, depending on the cell types, are activated in the responses to IL-6, OSM, LIF, and CNTF (Stahl *et al.*, 1994; Narazaki *et al.*, 1994). IL-12, which utilizes a receptor that contains a subunit that

TABLE I
JAKS IN CYTOKINE SIGNALING

Cytokine	Jak activated	Reference
IL-2/IL-4/IL-7/IL-9/IL-15	Jak1, Jak3	Witthuhn *et al.* (1994), Zeng *et al.* (1994), Johnston *et al.* (1994), Kirken *et al.* (1994), Tanaka *et al.* (1994a), Yin *et al.* (1994)
Epo	Jak2	Witthuhn *et al.* (1993), He *et al.* (1994), Miura *et al.* (1994c)
IL-3/GM-CSF/IL-5	Jak1, Jak2	Silvennoinen *et al.* (1993c), Quelle *et al.* (1994), Lutticken *et al.* (1994)
IL-6/CNTF/LIF	Jak1, Jak2, Tyk2	Stahl *et al.* (1994), Narazaki *et al.* (1994)
IL-12	Jak2, Tyk2	Bacon *et al.* (1994)
TPO	Jak2	Drachman *et al.* (1995)
Growth hormone	Jak2	Artgetsinger *et al.* (1993)
Prolactin	Jak2	Campbell *et al.* (1994), Rui *et al.* (1994), Dusanter-Fourt *et al.* (1994), DaSilva *et al.* (1994)
G-CSF	Jak1, Jak2	Nicholson *et al.* (1994), Shimoda *et al.* (1994)
IFN-α/β	Jak1, Tyk2	Muller *et al.* (1993), Velazquez *et al.* (1992)
IFN-γ	Jak1, Jak2	Muller *et al.* (1993), Watling *et al.* (1993)

has structural similarity to gp130 and G-CSF, uniquely induces the tyrosine phosphorylation and activation of Jak2 and Tyk2 (Bacon *et al.*, 1994). Like growth hormone and Epo, prolactin has been shown to primarily activate Jak2 (Campbell *et al.*, 1994; Rui *et al.*, 1994; Dusanter-Fourt *et al.*, 1994). G-CSF was initially reported to activate Jak1(Nicholson *et al.*, 1994), but more recent studies have shown that Jak2 is also activated (Shimoda *et al.*, 1994). In our experience, G-CSF strongly activates Jak2 and, to a much lesser extent, Jak1. Recently, studies implicated Jak1 and Jak3 in signaling through receptors that utilize the IL-2 γ_c-chain (Witthuhn *et al.*, 1994; Johnston *et al.*, 1994). As above, IL-2, IL-4, IL-7, IL-9, and IL-15 were found to rapidly induce the tyrosine phosphorylation and activation of Jak3 and, to a more variable extent, Jak1 (Witthuhn *et al.*, 1994; Zeng *et al.*, 1994; Johnston *et al.*, 1994; Kirken *et al.*, 1994; Tanaka *et al.*, 1994a; Yin *et al.*, 1994).

Perhaps the most intriguing example illustrating a role for Jak family members in cytokine signaling is in the responses to IFN-α/β and IFN-γ (Velazquez *et al.*, 1992; Watling *et al.*, 1993; Muller *et al.*, 1993). The involvement of Jak family members was established through the use of a series of mutants that were selected for their inability to respond to IFN-α/β or IFN-γ (McKendry *et al.*, 1991; John *et al.*, 1991; Pellegrini *et al.*, 1989). The U1 mutant fails to respond to IFN-α/β while retaining the ability to respond to IFN-γ. Using expression cloning, a gene was identified that restored the IFN response, which, when sequenced, was found to be Tyk2 (Velazquez *et al.*, 1992). The γ mutants lack IFN-γ responsiveness but retain the ability to respond to IFN-α/β. The IFN-γ responsiveness can be restored by transfecting the cells with Jak2, but not Jak1 or Tyk2, expression constructs (Watling *et al.*, 1993). Thus, the initial genetic evidence demonstrated that Jak2 is required for an IFN-γ response and Tyk2 is required for an IFN-α/β response. Consistent with this evidence, tyrosine phosphorylation and activation of Jak2 has been observed in the response to IFN-γ (Silvennoinen *et al.*, 1993a; Shuai *et al.*, 1993b).

The complexity of the system became more evident from studies with another mutant, U4 (Muller *et al.*, 1993). This mutant is unable to respond to either IFN-α/β or IFN-γ, has a truncated Jak1 transcript, and lacks serologically detectable Jak1. Responsiveness to either IFN is restored by introducing Jak1, thus demonstrating that both Jak1 and Tyk2 are essential for an IFN-α/β response, while Jak1 and Jak2 are essential for an IFN-γ response. The requirement for two kinases suggested the existence of a kinase cascade. This possibility is excluded because of the observation that, while IFN-γ stimulation of parental cells induces the tyrosine phosphorylation of both Jak1 and Jak2, no tyrosine phosphorylation of Jak1 is seen in the γ1 mutant. Conversely, in the U4 mutant lacking functional Jak1,

no Jak2 tyrosine phosphorylation occurs. Reconstitution of either mutant results in the tyrosine phosphorylation of both kinases in response to IFN-γ. Thus, both must be functionally present for activation to occur. The situation is identical in the case of IFN-α/β; both Jak1 and Tyk2 must be present for tyrosine phosphorylation of either.

The basis for the interdependence of two Jaks is not known; however, the current data suggest that the ligand-induced receptor complex may require Jak heterodimers. In particular, the receptors for IFN-γ and IFN-α consist of at least two chains (Soh *et al.*, 1994; Novick *et al.*, 1994). Thus, it can be proposed that individual receptor chains bind Jak1, Jak2, or Tyk2. Consistent with this, the newly cloned β-chain of the IFN-α/β receptor binds Jak1 (Novick *et al.*, 1994) and Tyk2 associates with the previously cloned α-chain (Colamonici *et al.*, 1994). In the case of IFN-γ, coimmunoprecipitation studies indicated that Jak1 is associated with the first chain to be cloned (α-chain) and that, following ligand binding, Jak2 becomes associated with the complex (Igarashi *et al.*, 1994). Irrespective, the current hypothesis suggests that ligand binding causes receptor aggregation and may bring Jaks into a heterodimeric complex. Whether the absolute requirement for two Jaks is unique to the IFN receptor systems or extends to other receptor systems in which two Jaks are activated is not currently known.

The potential interdependence of Jaks in the response to IL-6 has been examined with the use of the IFN signaling mutant cell lines lacking Jak1, Jak2, or Tyk2 (Guschin *et al.*, 1995). Unlike IFN signaling, the absence of one Jak does not affect the activation of the other Jaks in the response to IL-6. Thus, the absence of Jak1 has no detectable effect on the level of phosphorylation or activation of Jak2 or Tyk2. Similarly, the absence of Jak2 does not affect the levels of activation of Jak1 or Tyk2. These results are consistent with the concept that each of the Jaks can associate with gp130 and be recruited into an activation complex. However, the Jaks are not functionally equivalent since gp130 and Stat1/Stat3 phosphorylations occur in cell lines lacking Jak2 or Tyk2 but are dramatically reduced in cell lines lacking Jak1. This may reflect the unique ability of Jak1 to associate with gp130 in a manner that allows phosphorylation or may reflect a unique substrate specificity. The latter is unlikely since coexpression of gp130 and Jaks in insect cells with baculovirus expression constructs has failed to detect substrate specificity. Although the above results might suggest that Jak2 and Tyk2 are superfluous to IL-6 signaling, expression of a kinase-negative Jak2 in Jak2-deficient cells can suppress phosphorylation of Jak1 as well as phosphorylation of gp130 and the Stats. These data suggest that Jak2, or Tyk2, may be required to phosphorylate and activate Jak1 which is uniquely capable of phosphorylating gp130. The expression of a Jak2

kinase-negative mutant in Jak2-negative cells functions by competing with Tyk2 participation in the complex for Jak1 activation. These results provide the first indication that an independency of the Jaks may exist for activation.

IV. The Role of Receptor Dimerization/Oligomerization in Activation of *Janus* Kinases

The mechanisms by which ligand binding activates Jaks are not known, although, based on a variety of observations, it can be hypothesized that receptor dimerization/oligomerization results in the juxtapositioning of Jaks, either homodimerically or heterodimerically (Fig. 2). When juxtapositioned, the opportunity exists for phosphorylation at the autophosphorylation site resulting in increased catalytic activity. The activated Jak may then recruit, through phosphorylation, other juxtapositioned Jaks and thereby propagate the activation signal. Perhaps the most evidence of an essential role for receptor oligomerization has come from studies with the Epo receptor. Several studies demonstrated the ability of Epo to rapidly induce dimerization/oligomerization (Watowich *et al.*, 1994; Miura and Ihle, 1993). Dimerization/oligomerization also occurs in response to a viral glycoprotein, gp55, which is functionally equivalent to Epo in inducing Jak2 activation and mitogenesis (Li *et al.*, 1990; Showers *et al.*, 1992). More strikingly,

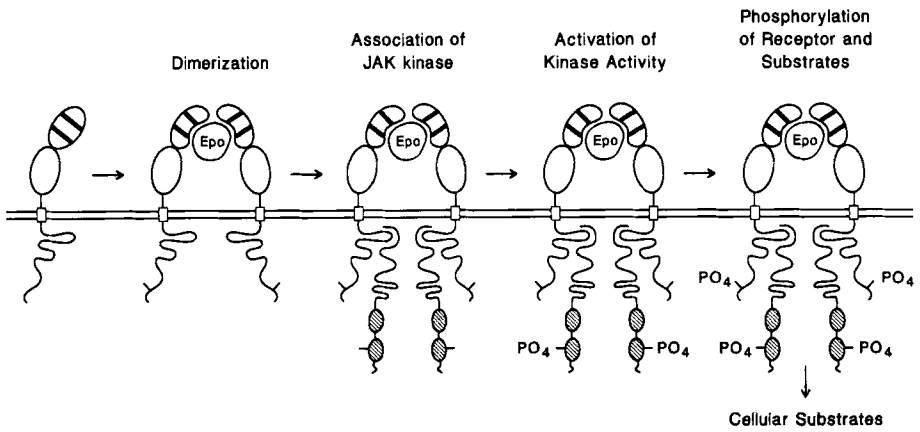


FIG. 2. Model for the activation of Jak2 in the response to erythropoietin. As detailed in the text, the primary function of erythropoietin is hypothesized to be the induced dimerization/oligomerization of the Epo receptor. This results in the recruitment and/or stabilization of association of Jak2 with the receptor and the opportunity for transphosphorylation at sites within the activation loop of the kinase domain. Once activated Jak2 then phosphorylates both the receptor and the cellular substrates.

an Arg → Cys mutation in the extracellular domain has been shown to result in the ligand-independent dimerization/oligomerization of receptors and constitutive, ligand-independent activation of Jak2 and mitogenesis (Watowich *et al.*, 1992). Lastly, chimeric receptors have been made that combine the extracellular domain of the EGF receptor with the cytoplasmic domain of the Epo receptor. EGF induces dimerization/oligomerization of the chimerics resulting in the activation of Jak2 and induction of mitogenesis (Maruyamam *et al.*, 1994).

Similar to the studies with the Epo receptor, a chimeric receptor approach has been used to demonstrate that the aggregation of the β_c-chain of the IL-3/GM-CSF/IL-5 receptors is essential for signal transduction. In this case chimerics were made in which the cytoplasmic domain of the ligand-binding α-chain was replaced with the cytoplasmic domain of the β_c-chain (Eder *et al.*, 1994; Jubinsky *et al.*, 1993). Thus, ligand binding would be anticipated to bring, homodimerically, β_c-chains in proximity. These chimerics are mitogenically active. The converse chimeric, namely replacing the cytoplasmic domain of the β_c-chain with the cytoplasmic domain of the α-chain, does not allow a mitogenic response. Thus, it is hypothesized that the role of the ligand-binding α-chain is to associate with and cause the aggregation of the β_c subunit.

Recent studies (Nelson *et al.*, 1994; Watowich *et al.*, 1994) with chimeric receptors containing the cytoplasmic domains of the IL-2 receptor chains similarly support a role for IL-2-induced dimerization in signaling. Interestingly, a chimeric receptor containing the cytoplasmic domain of the β-chain and the extracellular domain of c-kit is able to support stem cell factor-induced growth in myeloid but not T cells. In T cells, chimeric receptors containing the cytoplasmic domains of both the β- and the γ-chains are required. The possible consequences of homodimerization of β cytoplasmic domains relative to the heterodimerization of β and γ on the association and activation of Jak1 and Jak3 are only currently being examined.

In summary, a common theme is emerging regarding the activation of Jaks following cytokine binding in which the Jaks associate with one of the subunits and ligand binding results in the dimerization/oligomerization of the binding subunit, often with an increase in Jak binding affinity, and the activation of Jak activity by transphosphorylation at sites within the activation loop of the kinase domain. In the cases of Epo, growth hormone, prolactin, and G-CSF, a single receptor chain is involved. In the cases of IL-3, GM-CSF, and IL-5, a ligand-binding chain is required for aggregation of a Jak2 signaling chain. Similarly, IL-6 requires a ligand-binding chain to aggregate gp130 with which the Jaks associate. Unlike IL-3, GM-CSF, and IL-5, the IL-6 receptor subfamily does not require the cytoplasmic

domain of the ligand-binding chain for association with the signal transducing chain.

Although the previous examples evoke a relatively simple model for Jak activation, the results with the IFNs suggest that more complicated models may also be involved. In particular, the absolute requirement for two distinct Jaks may reflect the unique properties of the receptors relative to positioning of the Jaks. The other alternative is that certain Jaks may be very ineffective in cross-phosphorylations in the inactive state, while other Jaks are quite effective at activation, perhaps specifically other Jaks, in the unphosphorylated activated state. For example, unphosphorylated Jak2 may have a very low rate of "autophosphorylation," while unphosphorylated Jak1 may be very efficient at phosphorylating and activating Jak2. Thus, if Jak1 is recruited to the complex, activation of Jak2 would occur more rapidly than if only Jak2 is present in the complex. As noted previously, this model would explain the observation that a dominant negative Jak2 can suppress the IL-6 response although the downstream phosphorylations appear to uniquely require Jak1. This type of model would be very similar to the TGF-β receptor (Attisano *et al.*, 1994) in which TGF-β binds to a constitutively active type II serine/threonine kinase receptor. This complex then associates with the nonligand-binding type I serine/threonine kinase receptor and the type II receptor phosphorylates and activates the type I receptor. The type I receptors uniquely have the ability to phosphorylate and activate downstream signaling.

V. Association of Jaks with Cytokine Receptors Occurs through the Membrane Proximal Domains

The importance of the membrane proximal domains, containing the box1/box2 motifs, in mitogenic activity has been established in all the receptors examined. Therefore, it was anticipated that this region might be required for the association of the kinases required for signal transduction. The initial studies with the Epo receptor demonstrated that mutations or deletions in the membrane proximal region which abolished mitogenesis also eliminated Jak2 activation. Moreover, association of Jak2 with the Epo receptor both as fusion proteins (Witthuhn *et al.*, 1993) and *in vivo* (Miura *et al.*, 1994c) requires the membrane proximal region. Similarly, recent studies demonstrated that the association of Jak2 with the prolactin receptor occurs through the membrane proximal region (DaSilva *et al.*, 1992). The box1/box2 region has also been implicated in the association and activation of Jak1 and Jak2 with gp130 (Narazaki *et al.*, 1994). Specifically truncations outside of the region do not alter the ability of ligand binding to induce tyrosine phosphorylation or activation, while deletions into the box1/box2

region inactivate the receptor and Jak activation. Mutations of the conserved Pro-X-Pro in the box1 motif similarly inactivate the receptor and Jak activation (Murakami *et al.*, 1991). Other studies indicated that the truncations that affect the extended box2 subdomain, while eliminating mitogenesis, do not affect activation of Jak2 (He *et al.*, 1994). The basis for the differences in the results is not known but may be related to either the cell types or, more likely, to the levels of expression. With high levels of expression, sufficient sites for association may exist and allow Jak2 activation. However, these truncations may eliminate downstream signaling events that are critical for mitogenesis.

Recent studies addressed the association of Jaks with the IL-2 receptor chains (Miyazaki *et al.*, 1994; Russell *et al.*, 1994). Using fusion proteins it was found that Jak1 specifically associates with the β-chain and that this association requires the membrane proximal, serine-rich domain. In contrast, Jak3 specifically associates with the γ_c-chain and any truncations of the cytoplasmic domain dramatically affect this association. Using a monoclonal antibody that can immunoprecipitate the β-chain independent of the γ_c-chain following IL-2 binding indicated that, following IL-2 binding, Jak3 may also associate with the β-chain. The importance of the γ_c-chain in signaling is emphasized by the association of X-linked severe combined immunodeficiency with genetically acquired mutations and deletions of the γ_c-chain (Noguchi *et al.*, 1993). Based on the above studies, the deletions would be expected to eliminate Jak3 association. Moreover, a mutation of the γ_c-chain, which is associated with a more mild form of combined immunodeficiency, was also shown to affect Jak3 association.

It was recently reported (Tanaka *et al.*, 1994a) that Jak2 is activated in the response to IL-2 and that it associates with the γ_c-chain. It was noted, however, that the protein detected in the experiments consistently migrated more rapidly than the major immunoprecipitable Jak2. Thus, it was possible that a related Jak was being detected. This is due to the rather unexpected ability of the murine Jak2 antiserum used to cross-react with the human Jak3 but not the murine Jak3 (B. Witthuhn and J. N. Ihle, unpublished data).

VI. Activation of the ras Signaling Pathway by Cytokine Receptors

Transformation in a variety of cell lineages involves alterations in components of the ras signaling pathway, a complex pathway that is responsible for transducing extracellular signals into activation of transcription among other events. Over the past several years a number of biochemical events associated with the pathway have been elucidated and the reader is referred to excellent reviews dealing with the details (Schlessinger, 1993; Avruch

et al., 1994; Marshall, 1995). The activation of the pathway starts with receptor phosphorylation and recruitment of either SHC or GRB2 through their SH2 domains. SHC is subsequently phosphorylated and recruits GRB2 to the receptor complex. GRB2 then recruits the ras exchange factor SOS which mediates the conversion from GDP–ras to GTP–ras which results in the recruitment of raf-1 to the membrane and its activation. Activated raf-1 then phosphorylates MAPK/ERK kinase which in turn phosphorylates and activates the mitogen-activated protein kinases (MAPKs). The MAPKs are then responsible for the phosphorylation of a variety of effector molecules including transcription factors, other ser/thr kinases, and cytoskeletal proteins.

A variety of hematopoietic growth factors have been shown to activate the ras pathway including inducing SHC tyrosine phosphorylation (Cutler *et al.*, 1994; Damen *et al.*, 1993), inducing increases in GTP–ras (Satoh *et al.*, 1991, 1992), activation of raf-1 (Carroll *et al.*, 1991; Turner *et al.*, 1991; Carroll *et al.*, 1990), or activation of MAPKs (Pignata *et al.*, 1994; Winston and Bertics, 1992; Welham *et al.*, 1992; Miura *et al.*, 1994; Welham *et al.*, 1994b). The mechanisms of activation and the role of various domains within the receptors have been examined for the receptors for IL-3/GM-CSF, IL-2, and Epo. In the case of the Epo receptor (Fig. 3), a membrane

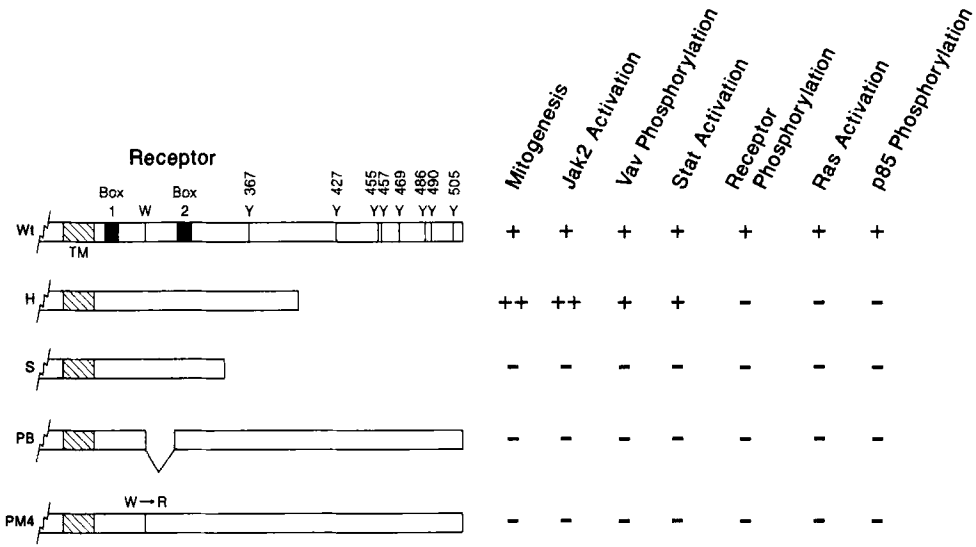


FIG. 3. Functional domains of the cytoplasmic region of the erythropoietin receptor. The effects of mutations, deletions, or carboxyl truncations of the erythropoietin receptor are indicated. The details and references for the results are provided in the text.

distal region of the cytoplasmic domain of the receptor is critical for SHC phosphorylation and activation of MAPKs (Miura *et al.*, 1994) similar to membrane distal regions of the IL-3/GM-CSF β_c-chain (Sato *et al.*, 1993) and of the IL-2 receptor β-chain (Satoh *et al.*, 1992). In each of these cases, this domain is not required for mitogenesis indicating that activation of the ras pathway is not essential. Importantly, the membrane distal region in each of these receptors contains the primary sites of tyrosine phosphorylation. Therefore, it is hypothesized that the SHC is recruited to the receptor complex through association of its SH2 domain with specific sites of tyrosine phosphorylation. Once recruited to the complex it is available as a substrate for phosphorylation by either Jaks or other receptor-associated kinases. In addition to the membrane distal region, mutations or deletions of the membrane proximal region, which affect Jak association and activation, also eliminate SHC phosphorylation and activation of the ras pathway. This observation suggests that either the Jaks are responsible for SHC phosphorylation or that they are required to activate the kinases that associate with the more distal regions of the receptor.

As noted previously, only the membrane proximal region of the IL-3, Epo, and IL-2 receptors are required for mitogenesis, indicating that activation of the ras pathway is not necessary for cytokine-induced cell cycle progression. This is consistent with the observation that introduction of activated ras does not affect the cytokine dependence of IL-3-dependent cells (Rein *et al.*, 1985; Nair *et al.*, 1989). However, in primary cells, activated ras causes immortalization, indicating an effect on cell proliferation (Rein *et al.*, 1985). In the case of the GM-CSF receptor, recent studies demonstrated that the membrane distal region is required to suppress apoptosis under certain conditions (Kinoshita *et al.*, 1995). The potential role of activation of the ras pathway for this effect was indicated by the ability of an activated *ras* allele to complement the receptor mutants. These results support the important conclusion that cytokines, through independent signaling pathways, control DNA synthesis and prevention of apoptosis.

The potential effects of ras have recently been indicated in studies with T cells expressing the Epo receptor (Yamaura *et al.*, 1994). These studies were based on the observation that T cells expressing the Epo receptor do not mitogenically respond to Epo. Introduction of v-src, v-fes, v-ki-ras, or v-raf into parental cells does not affect their dependence on IL-2. In cells transfected with the Epo receptor, only the cells expressing v-ki-ras were found to proliferate in a growth factor-dependent manner to Epo. The basis for the complementation relative to the activation of other signaling events was not examined.

VII. Cytokines Induce Increases in Phosphatidylinositol (PI)-3 Kinase Activity

A number of cytokines have been shown to induce increases in PI-3 kinase activity (Gold *et al.*, 1994; Damen *et al.*, 1995; Miura *et al.*, 1994b; Merida *et al.*, 1991; Corey *et al.*, 1993; He *et al.*, 1993). The mechanism of activation is proposed to result from phosphorylation of a p85 subunit which contains two SH2 domains and functions as an adaptor molecule that targets the catalytic 110-kDa subunit to the activated receptor complex (Otsu *et al.*, 1991; Escobedo *et al.*, 1991; Hiles *et al.*, 1992). Where examined, the phosphorylation of p85 requires the membrane distal region of cytokine receptors. Consistent with this, recent studies have shown that mutation of the most carboxyl terminal tyrosine (Y^{505}; Fig. 3) of the Epo receptor eliminates the p85 phosphorylation and activation of EpoR-associated PI-3 kinase activity (Damen *et al.*, 1995). Therefore, it is hypothesized that, in general, receptor phosphorylations create docking sites for p85 and recruite it to the receptor complex. As with SHC it has not been determined whether Jaks or other receptor-associated kinases mediate p85 phosphorylation. Importantly, mutation of the carboxyl terminal tyrosine, while eliminating PI-3 kinase activation, has no detectable effect on proliferation or tyrosine phosphorylation events.

VIII. Insulin-Receptor Substrate 1 (IRS-1) and 4PS: Signal Transducers Phosphorylated in Response to Cytokines

Studies seeking to characterize the substrates of tyrosine phosphorylation for the insulin receptor initially lead to the identification and ultimate cloning of the IRS-1 (Myers *et al.*, 1994). IRS-1 is a 185-kDa phosphoprotein that contains an amino terminal pleckstrin homology domain followed by a region that has no homology to known proteins but which contains 21 potential tyrosine phosphorylation sites. Many of the tyrosines are in motifs that would be expected to bind the SH2 domains of signaling proteins including p85, SHC, and GRB2. A potential role for IRS-1 initially came from studies of the response of cells to IL-4. IL-4 was of particular interest since initial studies indicated that, although mitogenic, it did not activate the ras pathway (Satoh *et al.*, 1991) and functionally activated signaling events that were distinct from other cytokines, such as IL-3 (Wang *et al.*, 1992), including a novel substrate of tyrosine phosphorylation termed 4PS. In a variant of an IL-3-dependent cell line which uniquely had lost the ability to proliferate to IL-4, 4PS phosphorylation was also lost. The IL-4 responsiveness of these cells could be restored by introducing IRS-1 (Wang *et al.*, 1993) indicating that 4PS might be functionally related to IRS-1 and that it was required for proliferation. The subsequent cloning of 4PS has confirmed this hypothesis.

As indicated previously, the IL-4 receptor consists of a ligand-binding α-chain which associates with the γ-chain, initially identified in the IL-2 receptor. Recent studies (Keegan *et al.*, 1994) identified a region within the IL-4 receptor α-chain that is required for the association of 4PS with the receptor and its phosphorylation. Curiously, this region has homology with the domain within the insulin receptor that is required for IRS-1 association. Importantly, mutations of this region affect the mitogenic function of the receptor. Therefore, it is hypothesized that the IL-4 receptor α-chain constitutively associates with 4PS. Ligand binding induces 4PS tyrosine phosphorylation, which may be mediated by Jaks or other receptor associated kinases. Following phosphorylation, 4PS provides docking sites for SH2-containing proteins which function either directly within the receptor complex or following phosphorylation.

Although the possibility of a general role in cytokine signaling has not been examined, it should be noted that most cytokines do not induce the detectable tyrosine phosphorylation of a substrate of the size of 4PS as noted in the initial studies. However, recent studies demonstrated growth hormone stimulation of IRS-1 phosphorylation (Souza *et al.*, 1994). Therefore, as specific reagents for 4PS become available, it will be important to further explore the possibility.

IX. Vav: A Common Substrate of Cytokine-Induced Tyrosine Phosphorylation

The *Vav* gene was initially identified in studies to clone human tumor genes by an expression approach (Katzav *et al.*, 1989, 1992). The gene product encodes a 95-kDa protein that contains an SH2 domain, two SH3 domains, as well as other structural motifs including a helix–loop–helix and leucine zipper-like domains, a zinc finger-like domain, and regions with homology to GDP–GTP exchange factors. The *Vav* gene is primarily expressed in hematopoietic cells and recent studies, using an antisense approach, suggested that Vav is critical for hematopoiesis (Wulf *et al.*, 1993). Vav is transiently tyrosine phosphorylated during activation of the T cell receptor complex (Margolis *et al.*, 1992; Bustelo *et al.*, 1992), by IL-3 in mast cells (Margolis *et al.*, 1992), in B cells by engagement of the immunoglobulin receptors (Bustelo and Barbacid, 1992), by IFN-α (Platanias and Sweet, 1994), and by stem cell factor (Alai *et al.*, 1992). Recent studies (Miura *et al.*, 1994a) also demonstrated the tyrosine phosphorylation of Vav by Epo and the receptor membrane proximal region is necessary and sufficient for this function (Fig. 3). Recent studies (Matsuguchi *et al.*, 1995) further indicated that Vav may directly associate with the phosphorylated Jaks, rather than being recruited into the receptor complex through a physical association with the receptor chains.

X. Signal Transducers and Activators of Transcription (Stats): A Novel Family of Transcription Factors Identified in Interferon Signaling

Studies dealing with the biochemical events in IFN-induced gene transcription identified a novel family of transcriptional factors (Fu, 1992; Schindler *et al.*, 1992a,b; Fu *et al.*, 1992; Veals *et al.*, 1992; Shuai *et al.*, 1992, 1993a). In particular, IFN-α/β induces the formation of a transcription complex, termed ISGF3, which binds to the interferon stimulated response element and activates transcription. The ISGF3 complex consists of a 48-kDa DNA-binding component, a p91/84-kDa protein, and a p113-kDa protein. The p91 and p84 proteins are alternatively spliced variants of the same gene. The formation of the complex and its migration to the nucleus requires the tyrosine phosphorylation of the p91/84- and p113-kDa proteins. Stimulation of cells with IFN-γ results in the tyrosine phosphorylation of p91 but not of the p113. The phosphorylated form of p91 is rapidly translocated to the nucleus and binds, independent of p113 or p48, to a γ-activation sequence (GAS) which is associated with genes that are transcriptionally activated by IFN-γ.

The p48, DNA-binding component of the ISGF3 complex was cloned (Veals *et al.*, 1992) and was found to be a member of a family of DNA proteins that include IRF-1, IRF-2, and ICSBP (Harada *et al.*, 1989). The functional roles of some of the members of this family have been explored by targeted gene disruption. The genes for both IRF-1 and IRF-2 have been deleted and found to have very subtle effects. This was somewhat surprising since initial studies suggested the possibility that IRF-1 was a tumor suppressor gene (Harada *et al.*, 1993) and the gene responsible for the $5q^-$ myelodysplasia syndrome (Willman *et al.*, 1993). Despite this, mice deficient in IRF-1 do not have an increased incidence of myeloid malignancies but do lack the ability to respond in very subtle ways to certain pathogens (Matsuyama *et al.*, 1993; Kimura *et al.*, 1994) and this may reflect the requirement for IRF-1 for NO synthase expression in macrophages (Kamijo *et al.*, 1994). Recently, fibroblasts from mice that are deficient in IRF-1 have been found to have an increased sensitivity to transformation by ras (Tanaka *et al.*, 1994b). The basis for this is not currently known and it is unclear why this phenotype does not have a more significant consequence *in vivo*.

The amino acid sequences of p91 and p113 clearly indicated that the two were highly related and defined a novel family of transcriptional factors that has been termed the signal transducers and activators of transcription (Stat) family. Thus, p91 is Stat1α and p84 is Stat1β, while p113 is Stat2. As noted below, the number of Stat proteins has been rapidly increasing with the recent cloning of Stat3 (Zhong *et al.*, 1994a,b; Akira *et al.*, 1994),

Stat4 (Zhong *et al.*, 1994a; Yamamoto *et al.*, 1994), Stat5 (Wakao *et al.*, 1994; Miu *et al.*, 1995; Azam *et al.*, 1995), and Stat6 (Hou *et al.*, 1994; Quelle *et al.*, 1995). The general structure of the Stat proteins is indicted in Fig. 4. They vary in size from 734 to 851 amino acids with the principle size differences occurring at the carboxyl terminus. The most highly conserved region is the SH2 domain which is localized in the carboxyl half of the protein. Within the SH2 domain, the central GTFLLRFS(E/D)S is completely conserved in all the known Stat proteins and is virtually identical to the core SH2 domain of src. SH2 domains are involved in the binding of phosphotyrosine residues and the Arg residue in this core in src directly binds phosphotyrosine (Waksman *et al.*, 1993). As noted, this critical residue is conserved in all Stat proteins.

In addition to the SH2 domain there is a region that has some similarity with the SH3, a domain that has been associated with binding of proline-rich motifs (Cichetti *et al.*, 1992; Koch *et al.*, 1991). This region is much less conserved among the Stat proteins than is the SH2 domain and whether it plays a functional role comparable to that of the SH3 domain of src is questionable. In particular, several of the conserved residues that have been shown to form the proline-binding pocket are not present (Yu *et al.*, 1994). The Stat proteins lack a readily identifiable known motif associated with DNA binding. However, there exist highly conserved blocks of homology within the amino terminal region of the proteins. Since all the known Stats bind similar sequences, it is likely that one or more of these blocks of homology are involved in DNA binding. In this regard there is a VTEE

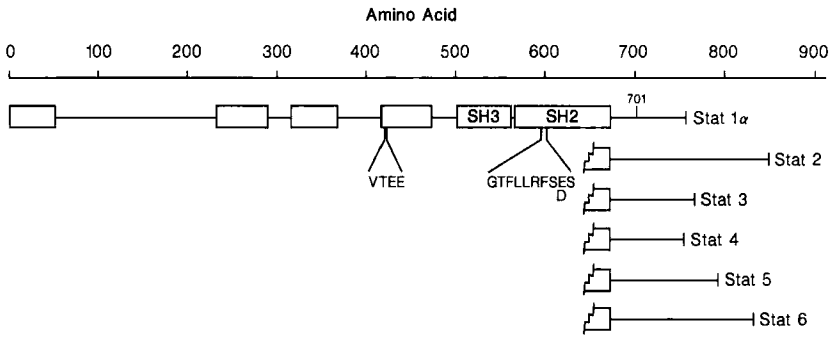


FIG. 4. Structure of Stat family of transcription factors. A comparison of the lengths and organization of the Stat proteins is shown. The amino terminal blocks indicate regions in which there is detectable identity among the six Stats. The positions of the SH2- and SH3-like domains are also indicated. The position of the very highly conserved VTEE sequence is indicated and the highly conserved phosphotyrosine binding core region of the SH2 domain is indicated.

sequence that occurs in a conserved region immediately amino terminal of the SH3-like domain which is completely conserved among the Stats. It can be speculated that this region contributes to DNA binding. This is consistent with data using chimerics Stat which allow the functional localization of DNA binding activity (Horvath *et al.*, 1995).

The activation of Stat proteins requires protein tyrosine phosphorylation and dimerization. Dimerization is hypothesized to occur through the phosphorylation site of one molecule and the SH2 domain of a second. Following IFN-γ stimulation, Stat1 becomes tyrosine phosphorylated on a single site (Y^{701}) (Shuai *et al.*, 1992, 1993a) which is immediately after the SH2 domain. Mutation of this tyrosine residue inactivates Stat1. The nonphosphorylated form of Stat1 exists as a monomer, whereas the phosphorylated form exists as a dimer (Shuai *et al.*, 1994). Dimer formation is inhibited by mutations within either the SH2 domain or the tyrosine phosphorylation site. Consistent with this, phosphopeptides corresponding to the phosphorylation site can dissociate Stat1 dimers. Dimer formation has been shown to be required for DNA binding activity and presumably is also required for migration to the nucleus. It is assumed that all the Stat family members will be regulated in a comparable manner. The high degree of similarity of many of the family members also suggests that heterodimerization will occur and the heterodimerization of Stat1 and Stat3 has been observed in the responses to IL-6. The extent to which heterodimerization alters function has not yet been addressed.

It has been hypothesized that Stat1 is a substrate for a Jak. Only recently has this been demonstrated (Quelle *et al.*, 1995b). In particular, Stat1 produced in insect cells can be phosphorylated by coinfection of cells with expression constructs for Jak1, Jak2, or Tyk2. Phosphorylation occurs on a single tryptic fragment that contains Y^{701} and is associated with the acquisition of DNA binding activity. Consistent with this, each of the Jaks can phosphorylate a peptide containing Y^{701}. Of particular importance is the ability of all three Jaks to correctly phosphorylate and activate Stat1. This result suggests that the specificity of Stat phosphorylation that occurs in cytokine responses is not due to the specificity of Jak activation. Rather, it can be hypothesized that the receptor complex determines which of the Stat proteins will be accessible.

XI. Stats: A Common Theme in Cytokine Signaling

Although Stat1 and Stat2 were initially identified and characterized within the context of the interferon system, a variety of recent studies support the hypothesis that Stat family members may play a very general role in cytokine signaling. Recent studies identified a Stat1-related

protein, termed acute phase reactive protein, that is rapidly tyrosine phosphorylated in response to IL-6 and is coimmunoprecipitated with gp130 and Jak1 (Lutticken *et al.*, 1994). The protein responsible was purified and sequenced, and cDNA clones were obtained and found to be highly related to Stat1 (Akira *et al.*, 1994). Consistent with this, the same gene was cloned by low stringency screening of a cDNA library (Zhong *et al.*, 1994a). Because of the similarity to the Stat family, the term Stat3 ws proposed.

Stat4 was independently cloned in two laboratories by either PCR approaches (Yamamoto *et al.*, 1994) or low stringency hybridization (Zhong *et al.*, 1994a). Stat4 is 52% identical to Stat1 and is genetically very tightly linked to Stat1 on mouse chromosome 1. Unlike the other Stats, however, no ligands were initially identified that induced the tyrosine phosphorylation or activation of Stat4 DNA binding activity. However, expression studies were used to demonstrate that Stat4 is a functional member of the Stat family (Yamamoto *et al.*, 1994). In particular, when Stat4 is expressed in COS cells using expression constructs the protein is produced but is not tyrosine phosphorylated nor does it have DNA binding activity. However, if Stat4 is coexpressed with either Jak1 or Jak2, it is tyrosine phosphorylated and acquires the ability to bind to the GAS sequence of the *IRF-1* gene. Interestingly, there was no apparent specificity among the Jaks for recognition and activation of Stat4.

Using the previously mentioned coexpression conditions, extensive mutagenesis of Stat4 has been performed to define the regions that are required for association with and activation by Jaks. Using this approach, two tyrosine (Y^{693} and Y^{675}) were found to be critical for DNA binding. More strikingly, mutation of the SH2 domain ($R^{598}T$) completely eliminated the ability to associate with and be phosphorylated by Jak2. This result suggests the possibility that the SH2 domain is necessary to recruite Stat4 to the kinase, perhaps through a site of tyrosine phosphorylation. Interestingly, if the kinase domain of Jak2 is used, the SH2 mutant could be tyrosine phosphorylated and a region immediately upstream of the kinase domain was essential for determining whether the mutant was phosphorylated or not. This region may contain a regulatory domain that normally controls accessibility of substrates to the catalytic domain.

Unlike other Stats, Stat4 expression is highly restricted and is limited to myeloid cells. Within the myeloid lineages, Stat4 expression is further restricted to early myeloid cells and its expression is turned off during erythroid and granulocytic differentiation. The only other site of expression is in developing spermatogonia. This is of considerable interest since Stat1 genetically colocalizes with the *juvenile spermatogonial depletion* (*jsd*) gene (Yamamoto *et al.*, 1994). Mice homozygous for the *jsd* allele fail to

produce spermatocytes and fail to express Stat4 in the testes. However, the gene is not physically disrupted nor mutated, thus it is unlikely that Stat4 is the cause of *jsd*, but rather the lack of expression reflects its expression only in more differentiated spermatocytes.

Although initially an orphan Stat, recent studies have shown that Stat4 is specifically phosphorylated in the response of various T cells to IL-12 (Jacobson *et al.*, 1994; Quelle *et al.*, 1995). This cytokine utilizes a receptor chain that has similarity to gp130 and G-CSF and a second chain which is yet to be cloned (Chua *et al.*, 1994). Interestingly, relative to the role of receptor tyrosine phosphorylation in Stat recruitment, the cloned human chain contains no tyrosine residues in the cytoplasmic domain. As noted previously, IL-12 activates both Jak2 and Tyk2 and it is hypothesized that one or both of these kinases is responsible for Stat4 phosphorylation *in vivo*.

Prolactin had been shown to rapidly induce the appearance of a DNA binding activity, termed mammary gland factor (MGF), for a region of the β-casein gene promoter that was critical for inducibility. MGF was purified from sheep mammary tissue and the gene cloned and found to encode a protein that was structurally related to Stat1 (Wakao *et al.*, 1994) and is now referred to as Stat5. *In vitro*-translated Stat5 could be phosphorylated by purified Jak2 but not the src kinases fyn, lyn, or lck (Gouilleux *et al.*, 1994). Similar to Stat1, a single carboxyl tyrosine (Y^{694}) was required for the activation of DNA binding activity by mutagenesis.

Stat5 has also been implicated in the signaling pathways utilized by other cytokines. In one study, oligonucleotide affinity columns were used to isolate an IL-3-induced DNA binding activity for a GAS element (Azam *et al.*, 1995). Sequencing of peptides demonstrated that two highly related proteins had been purified, both of which were nearly identical to the sheep Stat5 protein. Cloning of cDNAs confirmed that the factor was the murine homolog of the sheep *Stat5* gene and that two highly related genes existed. Similarly, the involvement of Stat5 in IL-3 signaling was suggested with antiserum against the sheep Stat5 which leads to the cloning of murine homologs (Miu *et al.*, 1995). In addition to IL-3, Stat5a and Stat5b are inducibly tyrosine phosphorylated by Epo and IL-2.

Stat6 was initially identified through efforts to characterize the Stat-like, GAS-binding proteins that were induced by IL-4. Purification was achieved with affinity columns containing a GAS element and sequencing of tryptic peptides was used to obtain sequence for cloning. The predicted amino acid sequence of the cDNA clearly indicated it was a member of the Stat family and was most related to Stat5 and more distantly to Stat1–4. Although initially termed IL-4 Stat, its involvement in other signaling pathways suggests that the terminology of Stat6 is more appropriate. Stat6 was

independently cloned by homology searches of a database of expressed sequences (Quelle *et al.*, 1995). Development of antiserum against Stat6 confirmed its role in the response to IL-4 and demonstrated that Stat6 is also activated in the response to IL-3.

The existence of multiple Stat proteins that are substrates in various cytokine signaling pathways may help to explain the pleiotropic responses that have been observed with many cytokines (Table II). In particular, it can be hypothesized that the response to a particular cytokine may be dependent on the Stat proteins that are present. Thus, in an early myeloid cell, Epo may induce the phosphorylation of a Stat whose expression is lost with continued differentiation, much like the pattern of expression of Stat4. As the cells commit to the erythroid lineage, a new Stat gene may be expressed and become the target for Epo signaling. The biologically distinct effects of Epo in these settings may be directly related to functional differences between the Stats activated.

XII. Mechanisms of Stat Recruitment to Cytokine Receptor Complexes

The mechanisms involved in the complex series of events that are required for Stat biological activation are relatively unknown. The first requirement is the recruitment of the appropriate Stat to the receptor complex. Since the Jaks lack specificity for the various Stats, the ability of individual receptor complexes to recruit specific Stats to the Jaks provides an important element for cytokine specificity. The means for specificity in recruitment can be envisioned to rely on the SH2 domains of Stats to

TABLE II
PROTEINS IN CYTOKINE SIGNALING

Cytokine	Stat protein	Reference
IL-4	Stat6	Schindler *et al.* (1994), Kotanides and Reich (1993)
IL-2	Stat5	Quelle *et al.* (1995)
Epo	Stat5	Wakao *et al.* (1995)
IL-3/GM-CSF/IL-5	Stat5a, Stat5b, Stat6	Quelle *et al.* (1995); Mui *et al.* (1995); Azam *et al.* (1995)
Growth hormone	Stat5	Wood *et al.* (1995)
Prolactin	Stat5	Wakao *et al.* (1994)
IL-6	Stat1, Stat3	Akira *et al.* (1994), Zhong *et al.* (1994a)
G-CSF	Stat3	Tian *et al.* (1995)
IFN-α/β	Stat1, Stat2	Darnell *et al.* (1994)
IFN-γ	Stat1	Darnell *et al.* (1994)
IL-12	Stat4, Stat3	Jacobson *et al.* (1994)

recognize unique docking sites comparable to other signaling proteins. Support for this hypothesis has been obtained for some but not all receptor systems examined. First, recent studies indicate that the specificity for Stat activation resides in the SH2 domain. In particular, IFN-γ induces the tyrosine phosphorylation of Stat1 but not Stat2. However, when the SH2 domain of Stat1 is substituted for the SH2 domain of Stat2, IFN-γ will induce its tyrosine phosphorylation (Heim *et al.*, 1995).

The specificity for recruitment of Stat1 to the IFN-γ receptor complex is speculated to require a carboxyl terminal tyrosine on the receptor α-chain. This tyrosine has been shown to be phosphorylated and to be required for Stat1 phosphorylation (Greenlund *et al.*, 1994). The ability of phosphopeptides from the IL-4 receptor α-chain to compete for dimer formation of Stat6 has also been used to support the hypothesis that Stat6 is recruited to the receptor complex through its SH2 domain (Hou *et al.*, 1994). Support for this has been obtained with receptor mutants with which it was shown that carboxyl truncations, which remove the potential docking sites, uncouple IL-4 binding to induction of Stat6 phosphorylation (Quelle *et al.*, 1995). Similarly, it has been shown that recruitment of Stat3 to the IL-6 receptor complex requires specific tyrosines on gp130 (Stahl *et al.*, 1995). Indeed, the ability of gp130 to recruit Stat3 could be conferred to a different receptor simply by attaching the relevant sequences to another receptor.

In contrast to the previous examples, a role for receptor tyrosines in Stat recruitment has not been found with some receptors. In particular, the membrane proximal region of the cytoplasmic domain of the Epo receptor is necessary and sufficient for Stat5 phosphorylation (Fig. 3). Within this region there is a single tyrosine which is not phosphorylated *in vivo* and which when mutated has no affect on Stat5 phosphorylation. Similarly, deletions of the common β-signaling chain of the IL-3/GM-CSF/IL-5 receptors, which remove the *in vivo* tyrosine phosphorylation sites, do not affect ligand-induced Stat5 phosphorylation. Therefore, either other modes exist for recruitment of these Stats to the receptor complexes or the Epo and IL-3 receptors contain additional chains, which have yet to be cloned, which provide docking sites.

XIII. Stat Proteins Regulate a Variety of Genes in Hematopoietic Cells

As noted previously, the Stat proteins were initially identified as the transcription factors that are responsible for the induction of a variety of genes by IFNs and which are thought to contribute to the antiviral state induced by IFNs. The identification of novel Stat proteins that are activated by a wide variety of cytokines has greatly expanded the number and

types of genes that are regulated through GAS-like sequences. In particular, more than 10 variants of the GAS sequence fitting the consensus TTNCNNNAA have been identified as control elements in the promoters of different cytokine response genes. Different Stat homo- and heterodimers show very different affinities for the individual GAS elements in band shift assays; different Stats forming complexes with different subsets of GAS elements. It is likely that these differences, together with cell type-specific differences in Stat expression, play a role in determining the specificity of the response to various cytokines.

One of the variations of the GAS sequence which has been of considerable interest is the sis-inducible element (SIE) found in the c-*fos* gene (GTTCCCGTCAAT or a high-affinity variant TTTCCCGTAAA) (Wagner *et al.*, 1990). This element can be bound by Stat1 (Sadowski *et al.*, 1993) and Stat3 (Zhong *et al.*, 1994b). In addition the novel Stat-related proteins that are induced in the response to Epo and IL-3 can bind SIE (W. Thierfelder and J. N. Ihle, unpublished data). The role that the Stats play in cytokine-regulated expression of the c-*fos* gene has not been explored in detail. However, carboxyl truncations of the Epo receptor that retain mitogenic activity, activate Jak2, activate GAS binding activity, but which do not activate the ras pathway (Miura *et al.*, 1994; Witthuhn *et al.*, 1993), retain Epo inducibility of c-*fos* (Miura *et al.*, 1993).

Another GAS element of considerable interest is that associated with the expression of the *IRF-1* gene. The *IRF-1* gene was initially identified as a DNA-binding protein that was suspected to be involved in the production of IFN-β (Miyamoto *et al.*, 1988) although other studies have been unable to establish a role for IRF-1 in IFN expression (Pine *et al.*, 1990; Ruffner *et al.*, 1993). It has also been identified as a G-CSF-induced gene in late granulocytic differentiation (Abdollahi *et al.*, 1991) and an immediate–early gene in prolactin-induced proliferation of Nb2 T cells (Yu-Lee *et al.*, 1990). A proposed biological function for *IRF-1* came from studies which suggested that it was the tumor suppressor gene associated with the $5q^-$ syndrome in human myelodysplastic disease (Willman *et al.*, 1993) and that IRF-1 could suppress the oncogenic activity of the related *IRF-2* gene product (Harada *et al.*, 1993). However, recent studies have questioned the role of the *IRF-1* gene in $5q^-$ based on the identification of cases in which the gene is not deleted (Boultwood *et al.*, 1993). Moreover, disruption of the gene in mice does not lead to increased susceptibility to hematopoietic or nonhematopoietic tumors (Matsuyama *et al.*, 1993). A role for the production of nitric oxide synthase has been established by gene disruption (Kamijo *et al.*, 1994) and recently it has been shown that mice deficient in IRF-1 do not express the *Gbp* gene in response to IFN-γ (Briken *et al.*, 1995). This gene is an unconventional GTPase that converts

GTP to GMP and was initially identified by its ability to bind immobilized guanine nucleotides.

The induction of expression of the *IRF-1* gene by IFN-γ requires a GAS-related element (TTTCCCCGAAA) in the promoter (Sims *et al.*, 1993). Interestingly, a number of cytokines induce Stat-like DNA-binding proteins that recognize the GAS element of the *IRF-1* gene although with quite different biological consequences. In the case of IL-3 or Epo, cytokine stimulation results in the rapid appearance of Stat5, an IRF-1/GAS-binding protein. However, neither cytokine induces *IRF-1* gene expression. In contrast, stimulation of myeloid cells with IFN-γ activates Stat1 which is associated with dramatic increases in *IRF-1* gene expression. With regard to proliferation, IL-3 and Epo induce mitogenesis, while IFN-γ does not, nor does IFN-γ suppress IL-3- or Epo-induced proliferation. Lastly, IL-3 or Epo marginally suppress the *IRF-1* gene induction by IFN-γ. Thus, the significance of IRF-1 to growth regulation in these cells is unclear (W. Thierfelder and J. N. Ihle, manuscript in preparation).

XIV. Negative Regulation of Cytokine Signaling by Tyrosine Phosphatases

Protein tyrosine phosphatases play a critical role in cytokine signaling as initially demonstrated by the observation that inhibitors of protein tyrosine phosphatases could partially relieve the requirements for cytokines in mitogenic responses (Tojo *et al.*, 1987). However, their importance came from studies of a myeloid-specific enzyme termed hematopoietic cell phosphatase (HCP) (Yi *et al.*, 1992), which has also been termed PTP1C (Shen *et al.*, 1991), SHP (Matthews *et al.*, 1992), and SHPTP1 (Plutzky *et al.*, 1992). HCP is a 68-kDa protein that contains a carboxyl catalytic domain and two SH2 domains in the amino terminal half of the protein. The importance of HCP was realized with the demonstration that it is the gene responsible for the *motheaten* mutation in mice and indeed the lethal form of *motheaten* represented a naturally occurring gene knockout of HCP (Shultz *et al.*, 1993; Tsui *et al.*, 1993). Homozygos *motheaten* mice die within about 3 weeks after birth (Van Zant and Shultz, 1989; Shultz, 1991) largely due to overproliferation of macrophages, particularly in the lungs. However, a number of hematopoietic lineages are affected, including the erythroid lineage in which erythropoiesis becomes relatively independent of Epo. There is also excessive proliferation of the lymphoid lineages leading to the appearance of autoimmune-like pathologies possibly by affecting the thresholds for negative selection (Cyster and Goodnow, 1995). This phenotype is consistent with the hypothesis that HCP is required to negatively regulate most lymphoid and myeloid lineages.

Evidence for the potential mechanism by which HCP affects growth came from studies of its association with various hematopoietic growth factor receptors. In particular, HCP binds to the ligand-activated, tyrosine-phosphorylated form of c-kit (Yi and Ihle, 1993), the receptor protein tyrosine kinase for stem cell factor. Binding occurs primarily through the amino terminal SH2 domains of HCP. Similarly, HCP binds through its amino terminal SH2 domain to the tyrosine-phosphorylated form of the IL-3 receptor β-chain (Yi *et al.*, 1993) and the tyrosine phosphorylated form of the Epo receptor (Yi *et al.*, 1995). Thus, it is hypothesized that following ligand binding receptor tyrosine phosphorylation occurs and confers on the receptor an HCP binding site. As a consequence, HCP is recruited to the receptor complex and is positioned to either dephosphorylate substrates of Jaks within the complex or alternatively the activation site of the Jaks and thereby downregulate the receptor complex.

Tyrosine phosphorylation of the Epo receptor requires the carboxyl region of the cytoplasmic domain, which is not required for mitogenesis (Miura *et al.*, 1991), but rather has been shown to negatively regulate the response to Epo in certain cell lines (D'Andrea *et al.*, 1991). This region also has a profound effect on the function of the receptor *in vivo*. In particular, in one form of genetically acquired erythrocytosis a mutation in the Epo receptor gene results in a 70-amino acid carboxyl truncation of the receptor (De La Chapelle *et al.*, 1993). Thus, it is hypothesized that in both cell lines and *in vivo* the inability of the Epo receptor to recruit HCP results in a more active receptor complex.

In addition to HCP, another related protein tyrosine phosphatase has been implicated in cytokine signaling. SHPTP2 is a cytoplasmic protein tyrosine kinase which, like HCP, contains an amino terminal region that consists of two SH2 domains and a carboxyl catalytic domain. Recent studies (Welham *et al.*, 1994a) have shown that IL-3 and GM-CSF induce the tyrosine phosphorylation of SHPTP2. Phosphorylation is associated with an increase in phosphatase activity and acquisition of the ability to associate with Grb2 and the p85 subunit of PI-3′ kinase. IL-6 has also been found to induce the tyrosine phosphorylation of SHPTP2 (Stahl *et al.*, 1995) and this is dependent upon a membrane proximal tyrosine which is speculated to be the docking site. The significance of the recruitment of SHPTP2 to the receptor complex is not known. The *Drosophila* homolog of SHPTP2, genetically, is a positive regulator in signaling and thus it has been speculated that SHPTP2 would similarly function in a positive manner in signal transduction. For this reason, the phenotype of mice in which the gene is disrupted will be of considerable interest relative to the phenotype of *motheaten* mice.

XV. Jaks and Stats in Other Receptor Systems

The Jaks and Stats have been generally implicated in signaling through members of the cytokine receptor superfamily. A key question is the extent to which either the Jaks or the Stats are involved in signaling through other types of receptors. Recently, a number of studies have indicated that the Stats are activated in response to ligands that utilize protein tyrosine kinase receptors, particularly in the response to epidermal growth factor (EGF). Initially, it was demonstrated that EGF induces the tyrosine phosphorylation and activation of Stat1 (Silvennoinen *et al.*, 1993b; Sadowski *et al.*, 1993; Ruff-Jamison *et al.*, 1993; Fu and Zhang, 1993) as well as the tyrosine phosphorylation and activation of Stat3 (Zhong *et al.*, 1994b). At least two mechanisms might be involved in EGF activation of Stats; namely, the activated EGF receptor may bind and directly phosphorylate the Stats or the activated EGF receptor may phosphorylate and activate a Jak which, in turn, phosphorylates the Stats. Studies have indicated that the EGF receptor can be coimmunoprecipitated with Stat1 (Fu and Zhang, 1993), although the ability of purified EGF receptor to associate with and/or activate purified Stat1 has not been examined.

Other ligands which induce Stat activation include platelet-derived growth factor (Silvennoinen *et al.*, 1993b) and colony-stimulating factor 1 (CSF-1) (Silvennoinen *et al.*, 1993b). However, we have been unable to observe CSF-1 induction of Stat1 phosphorylation or activation in fibroblasts transfected with the receptor or in a macrophage, CSF-1-dependent cell line (B. Witthuhn, W. Thierfelder, and J. N. Ihle, unpublished data). Irrespective, it will be important to continue to examine signaling through other families of receptors to establish the extent of utilization of the Jak–Stat pathways.

XVI. Summary and Conclusions

During the past 2 years, research from quite divergent areas has converged to provide the first insights into the mechanisms by which cytokines that utilize receptors of the cytokine receptor superfamily function. On the one hand, the obscure Jak family of cytoplasmic protein tyrosine kinases was independently implicated in IFN and hematopoietic growth factor signaling. Recent studies have expanded these initial observations to demonstrate that Jaks are critical to the functioning of all the receptors of the cytokine receptor superfamily. A variety of questions remain to be explored regarding the structure and function of Jaks and their interaction with receptors. It will also be important to pursue additional approaches to determine if the Jaks are necessary for various biological responses, particularly for mitogenic responses.

The second major area of convergence has been the demonstration that members of the Stat family of transcription factors, initially identified in IFN-regulated gene expression, are generally involved in cytokine signaling. Clearly, a number of Stat-like activities remain to be cloned and it can be anticipated that the family contains additional members. Although a variety of genes are known to be regulated by the Stats associated with IFN responses, much less is known concerning the genes regulated by the new Stats in cytokine signaling. Of particular importance is information relating to their potential contribution to mitogenic responses. From a biochemical standpoint, the Stats represent a remarkable family of proteins with regard to the ability of the modification of a single tyrosine residue to so dramatically affect cellular localization and DNA binding activity. Studies to identify the domains involved, and associated proteins that might contribute to either property, will be of considerable interest.

More generally, it can hypothesized that Jaks and Strats, if important for proliferation and differentiation, may be the targets for malignant transformation. Although none of the genes map to chromosomal breakpoints that have been implicated in transformation, gain of function mutations is a likely mechanism that needs to be explored. Similarly, the Jak–Stat pathway would appear to be an excellent target for the development of drugs that affect a variety of cytokine functions.

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X-Linked Agammaglobulinemia and Immunoglobulin Deficiency with Normal or Elevated IgM: Immunodeficiencies of B Cell Development and Differentiation

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I. Introduction

X-linked agammaglobulinemia (XLA) was first described in 1952 in a report by Bruton in which he described the absence of gammaglobulins in the serum of a young boy suffering from recurrent bacterial infections (1). He also demonstrated that administration of gammaglobulins derived from normal individuals resulted in a reduction in the frequency of the patient's bacterial infections. Plasma cells were found to be lacking in patients with XLA (2) and it was later shown that the cellular defect in XLA was the absence of mature B lymphocytes (3, 4). X-linked immunoglobulin deficiency with normal or elevated IgM (HIGMX-1), referred to as "hyper IgM," was first described in 1961 independently by Rosen (5) and by Burtin (6). Some of the patients found to have HIGMX-1 were previously thought to have XLA (5). In contrast to patients with XLA, patients with HIGMX-1 have circulating B cells which synthesize IgM and IgD but fail to switch to the production of other immunoglobulin isotypes (7, 8). HIGMX-1 patients also differ from XLA patients in their capacity to produce specific antibody titers albeit restricted to the IgM class (9). The presence of recombinations between the HIGMX-1 locus and the XLA locus indicated that HIGMX-1 is not a phenotypic variant of XLA (10). Mapping of XLA and HIGMX-1 to different regions of the X chromosome, Xq22 (11) and Xq26 (12), respectively, further confirmed that they represented two distinct immunodeficiency diseases. XLA results from a failure of B cell development, whereas a defect in B cell differentiation appears to be responsible for HIGMX-1. In 1993, the gene defect responsible for XLA (13, 14) and that responsible for HIGMX-1 (15–20) were discovered independently by several groups.

II. B Cell Development and Differentiation

B cell development is the process by which stem cells progress through a number of developmental stages culminating in the surface expression

of IgM and IgD antigen receptors (21). Rearrangement of the genes encoding the variable region of the immunoglobulin heavy-chain locus on chromosome 14 initiates with rearrangement of the diversity (D) and junctional (J) genes early in B cell development. Once the variable (V) gene has successfully rearranged to the DJ genes, μ heavy chain is transcribed and μ protein is detected in the cytoplasm of the cell giving rise to a pre-B cell. A light-chain molecule is produced after successful rearrangement of the variable region (VJ) of one of the κ or λ light-chain genes. A complete IgM molecule is then assembled and expressed on the surface of the immature B cell. Mature B cells coexpress IgM and IgD on their surface. IgD heavy-chain (δ) mRNA is generated by differential splicing of a long mRNA transcript encoding VDJ, the constant region of IgM ($C\mu$), and $C\delta$, which is located immediately downstream of $C\mu$, giving rise to two transcripts, one encoding VDJ-$C\mu$ and the other encoding VDJ-$C\delta$. At this stage, a naive mature B cell is ready to differentiate into a memory B cell or into an immunoglobulin-secreting plasma cell.

During differentiation, a B cell undergoes isotype switching of the constant region of the immunoglobulin heavy chain and affinity maturation of the variable region of immunoglobulin. Immunoglobulin isotype switching is a mechanism by which B cells express different heavy-chain isotypes (C) with the same variable V(D)J region thereby producing antibodies with different effector functions but retaining variable region specificity (22). In contrast, affinity maturation of the variable region allows B cells to produce antibodies with a higher affinity to antigen.

Immunoglobulin isotype switching results from a deletional recombination event which juxtaposes a downstream C gene to the rearranged V(D)J genes. Recombination involves switch (S) regions located 5′ of the $C\mu$ gene and corresponding S regions located immediately 5′ of each C gene. Deletional switch recombination in B cells is best understood in the case of isotype switching to IgE. IL-4, the switch factor for IgE, induces the transcription of a 1.8-kb ε germline mRNA which initiates 5′ of the $S\varepsilon$ region (23). This transcript is sterile because it is not translated into a functional protein. A mature 2.0-kb ε mRNA species is transcribed after the $C\varepsilon$ gene is juxtaposed to the rearranged V(D)J region by deletional switch recombination. Deletional switch recombination requires a contact-dependent signal from $CD4^+$ T cells (24) delivered by the ligand for CD40, CD40L (25). This signal can be replaced by engaging the B cell surface molecule CD40 with a monoclonal antibody (23, 26) or by a soluble form of CD40L (27). IgE isotype switching is inhibited by soluble CD40 (28) or by antibody to CD40L (29) demonstrating the critical role of CD40 and its ligand in IgE isotype switching. CD40 is a 50-kDa glycoprotein member of the NGF receptor/TNF receptor family (30) which includes

Fas (31), CD27 (32), and CD30 (33). The gene for CD40 is located on chromosome 20 in man (34) and on chromosome 2 in the mouse (35). CD40 is expressed on pro-B cells, pre-B cells, mature B cells (36, 37), interdigitating cells (38), follicular dendritic cells (39, 40), thymic epithelial cells (41), and some carcinomas (30, 42), and its expression can be induced in monocytes (43). CD40L is a 39-kDa type II membrane glycoprotein with homology to TNF. The CD40L gene is located on the long arm of the X chromosome at Xq26.3–q27.1 (44). CD40L expression is activation dependent and can be induced on the surface of most $CD4^+$ T cells and few $CD8^+$ T cells (45), T cells bearing $\gamma\delta$ T cell receptors (29), mature $CD4^+/CD8^-$ thymocytes (46), and mast cells (47).

Affinity maturation of the immunoglobulin variable region occurs in germinal centers (48). B cells activated by antigen and by T cells outside the follicles colonize germinal centers and begin to proliferate. Point mutations are introduced in the genes encoding the variable region and B cells expressing surface immunoglobulin with an increased affinity for antigen are selected. Survival of germinal center B cells is dependent on antigen and on T cells that deliver an antiapoptotic signal to B cells most probably via CD40L (49–51).

The timing of immunoglobulin isotype switching is not known but it is thought to precede selection of germinal center B cells (48). The stage at which B cells differentiate into immunoglobulin-secreting plasma cells or into memory B cells is not well known. Recent studies in mice showed that generation of memory B cells is dependent on the interaction of CD40 with its ligand. Blocking CD40L with a mAb (52) or with soluble CD40 (53) in the first few days of immunization resulted in an impairment of memory B cell function. However, no impairment of B cell memory was detected if soluble CD40 was administered 4 days after immunization suggesting that differentiation into memory B cells is either predetermined or occurs early in the immune response (53).

III. XLA: A Defect of B Cell Development

XLA is the prototype humoral immunodeficiency (54). Affected males present early in infancy or childhood with recurrent pyogenic infections. Physical examination is notable for the absence of tonsilar tissue and the lack of adenopathy. Serum levels of all immunoglobulin isotypes are markedly decreased if not absent and, despite repeated immunization, there is a lack of specific antibody production. Few if any mature B cells are found in the periphery and although *in vitro* studies of T cell number and function are normal, patients with XLA are prone to develop chronic enteroviral infections (55, 56) and vaccine-associated paralytic poliomyelitis

(57). The use of intravenous immunoglobulin (IVIG) replacement therapy has allowed patients to lead practically normal lives with a marked reduction in the number and severity of infections (58). However, IVIG treatment does not always protect against debilitating and often fatal chronic enteroviral infections (56).

The defect in XLA appears to affect the development of B cell precursors because, in addition to the absence of mature B cells in the peripheral blood of XLA patients, the ratio of pre-B cells to pro-B cells in the bone marrow is markedly decreased (59). X-chromosome inactivation studies in obligate carriers of the disease revealed that, in contrast to T cells and granulocytes, mature B cells exhibited exclusive use of the X chromosome bearing the normal gene (60) indicating that the defect is intrinsic to B lymphocytes.

XLA has been mapped to the middle region of the long arm of the X chromosome at Xq22 in close proximity to the probe DXS178 (11, 61, 62). The gene responsible for XLA was identified 2 years ago by two independent groups (13, 14). Vetrie *et al.* used a positional cloning strategy to isolate B cell-specific cDNAs which demonstrated restriction map alterations in 8/33 XLA families (13). The cDNA isolated encoded for a cytoplasmic tyrosine kinase which they called agammaglobulinemia tyrosine kinase (atk). Sequence analysis of atk cDNA derived from two XLA patients showed point mutations in the kinase domain. In an alternative approach, Tsukada *et al.* isolated a tyrosine kinase expressed early in B cell development which they called B cell progenitor tyrosine kinase. The gene was mapped to the X chromosome in the XLA region and B cell lines from some XLA patients were found to lack mRNA transcript for this gene (14).

The tyrosine kinase defective in XLA is now known as Bruton's tyrosine kinase (Btk). Btk is a 77-kDa protein tyrosine kinase (13, 14) with homology to other cytoplasmic tyrosine kinases including Dsrc28c (63), tecII (64), and Itk/Tsk (65, 66). This subfamily of tyrosine kinases shares with the src subfamily of tyrosine kinases the src homology 1 (SH1) kinase domain and the SH2 and SH3 domains involved in protein–protein interaction (Fig. 1). They differ from src tyrosine kinases in their lack of a carboxy terminal regulatory tyrosine residue, lack of myristylation signals, and the presence of an unusually long and unique basic amino terminal region. Btk was found to be expressed in cell lines representing all stages of B cell development, in myelomonocytic cell lines, in a macrophage cell line, in mast cell lines, and in cell lines of erythroid lineage (14, 67).

The involvement of Btk in the pathogenesis of XLA has been clearly demonstrated (13, 14). Vetrie *et al.* reported restriction pattern abnormalities in eight XLA families (13). Intragenic deletions were detected in two XLA patients and point mutations in the kinase domain in two other

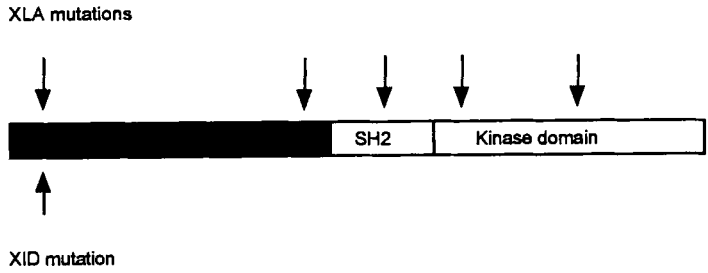

FIG. 1. A schematic representation of the Btk protein showing the functional domains and indicating the location of some of the mutations in XLA patients and in XID mice. Note that mutations in the same amino acid Arg28 are found in the XID defect in mice and in classical XLA patients.

patients. One of these mutations was expected to abolish the kinase activity of Btk, while the other mutation would affect the substrate recognition site. Tsukada *et al.* demonstrated the absent or reduced Btk mRNA, protein expression, or kinase activity in some patients with XLA (14). Since these two initial reports, several groups have identified point mutations, deletions, or insertions involving each of the Btk domains (68–73) indicting that each of these domains is important for its function. Of interest is that Btk defects have also been described in "atypical" cases of XLA patients who have low numbers of mature B cells and higher levels of serum immunoglobulins than those found in classical XLA (71, 72). Point mutations in the kinase domain and in the SH2 domain as well as deletion of half of the SH2 domain have been identified in atypical XLA patients. Because mutations in either domain also result in classical XLA, a correlation between phenotype and mutations in a specific domain cannot be made. However, these patients will be invaluable for the evaluation of the structure/function relationship of Btk.

Btk has also been implicated in murine X-linked immunodeficiency (XID) (74, 75). In contrast to XLA, CBA/N mice with the XID defect have normal numbers of B cells with decreased serum IgM and IgG_3 levels (76). However, the XID defect does resemble XLA in several respects. XID B cells are immature in phenotype and are unable to respond to T cell-independent antigens. Also, the XID defect is intrinsic to B cells because nonrandom X-chromosome inactivation is limited to B cells (77). Furthermore, the XID gene locus maps to a region of the mouse X chromosome which shares homology with the XLA locus in humans, Xq22 (78, 79). Two groups have recently demonstrated that the mouse Btk gene maps to the XID locus of the murine X chromosome and they identified a point mutation which is predicted to alter the arginine residue at position

28 to cysteine (74, 75). Of interest is that two unrelated families with XLA have been identified with a single amino acid substitution at Arg28 (69, 71). The affected patients in these families had a classical XLA phenotype rather than a phenotype similar to the XID defect in mice. This might suggest that the role of Btk in B cell development differs between mice and humans.

Btk plays an important role in B cell development as evidenced by the severe B cell deficit in XLA and in XID mice. The variety of phenotypes resulting from defects in the Btk gene suggest that Btk plays an important role at multiple stages in B cell development. However, little is known about the function of Btk. Recent studies have begun to shed some light on the role of Btk in signal transduction. The unique amino terminal domain of Btk has been shown to interact *in vitro* with the SH3 domain of members of the src subfamily of tyrosine kinases, lck, fyn, and hck (80). Cross-linking of surface IgM receptors results in tyrosine phosphorylation and activation of Btk (81–83). B cells from XID mice fail to proliferate in response to CD40 (84) suggesting that CD40-mediated B cell proliferation is mediated by Btk. Cross-linking of the high-affinity IgE receptor on mast cells induces tyrosine phosphorylation and activation of Btk (85). However, no association between Btk and the high-affinity IgE receptor was detected. It is therefore likely that Btk plays a role in signal transduction possibly at a position downstream of membrane tyrosine kinases such as members the src subfamily. Further work is necessary to determine the exact function of Btk at various stages of B cell development and in myeloid and mast cells.

IV. HIGMX-1: A Defect of B Cell Differentiation

X-linked immunodeficiency with normal or elevated IgM (HIGMX-1) is a rare genetic disorder characterized by markedly decreased or absent serum levels of IgG, IgA, and IgE immunoglobulins with normal or elevated levels of IgM (86). Since its first description in 1961 (5, 6), over 100 cases have been reported worldwide. Affected males are unduly susceptible to recurrent pyogenic infections, opportunistic infections with *Pneumocystis carinii* and with *Cryptosporidium,* autoimmune diseases, and lymphoproliferative disease (86). Patients with HIGMX-1 have normal numbers of circulating B cells that are exclusively surface IgM/IgD positive. Patients with HIGMX-1 do make specific antibodies in response to immunization but they are restricted to the IgM isotype and there is a lack of a memory response (9). Lymph node architecture is abnormal in HIGMX-1 with rare follicles and no germinal centers observed (86).

The underlying defect in HIGMX-1 appears to be an inability to undergo immunoglobulin isotype switching from IgM/IgD secretion to the produc-

tion of other immunoglobulin (Ig) isotypes, IgG, IgA, or IgE (7, 8). Immunoglobulin isotype switching in human B cells requires a contact-dependent signal from T cells (24). Therefore, HIGMX-1 could result from a defect in either T cells or B cells. Based on the observation that T cells from patients with HIGMX-1 can help normal B cells synthesize IgG in response to pokeweed mitogen, it was initially concluded that B cell dysfunction was the cause of the switch defect in HIGMX-1 (7, 8). However, pokeweed mitogen-induced IgG synthesis does not involve isotype switching (87, 88). Subsequent investigations suggested that the defect in HIGMX-1 involves T cells rather than B cells. Hendriks *et al.* reported a random pattern of X-chromosome inactivation in IgG- and IgA-secreting Epstein–Barr virus-transformed B cell lines derived from two female carriers and concluded that the defect in HIGMX-1 did not involve the immunoglobulin heavy-chain class switch mechanism (89). Mayer *et al.* demonstrated that a T cell line (Trac) derived from a patient with a Sezary-like syndrome (mycosis fungoides) was capable of inducing B cells derived from HIGMX-1 patients to secrete IgG and IgA (90). Further, using switch region and CH region DNA probes for μ, γ_4, and γ_1, the restriction pattern of DNA derived from HIGMX-1 B cells was indistinguishable from that of normal cells indicating that the pathology resides in the T cells rather than in the B cells. The latter two reports are consistent with our recent discovery that the defect in HIGMX-1 involves the gene encoding CD40L which is expressed by activated T cells (15, 16).

We discovered the role of CD40L in the pathogenesis of HIGMX-1 by studying the immunoglobulin isotype switch defect in these patients (15). We examined IL-4-dependent mechanisms of IgE synthesis and found that B cells from HIGMX-1 patients could not undergo T cell-dependent IgE synthesis but synthesized IgE in response to T cell-independent stimulation with CD40 mAb and IL-4. Furthermore, we demonstrated that HIGMX-1 B cells underwent deletional switch recombination to IgE in response to CD40 mAb and IL-4 indicating that the immunoglobulin heavy-chain recombination mechanism is intact. Next we examined the expression of CD40L by T cells from HIGMX-1 patients. In contrast to normal T cells, HIGMX-1 T cells failed to express a protein (CD40L) which binds to soluble CD40 (Fig. 2). Subsequently, we demonstrated deletions and point mutations in the CD40L cDNA in HIGMX-1 patients (16, 91, 92). Similar results were reported by four other groups. Arufo *et al.* examined CD40L expression in patients with humoral immunodeficiency and found defective expression of CD40L protein and point mutations in the CD40L cDNA in HIGMX-1 patients (17). Allen *et al.* (18), Disanto *et al.* (19), and Korthauer *et al.* (17) independently investigated CD40L expression in HIGMX-1 because the CD40L gene mapped to the

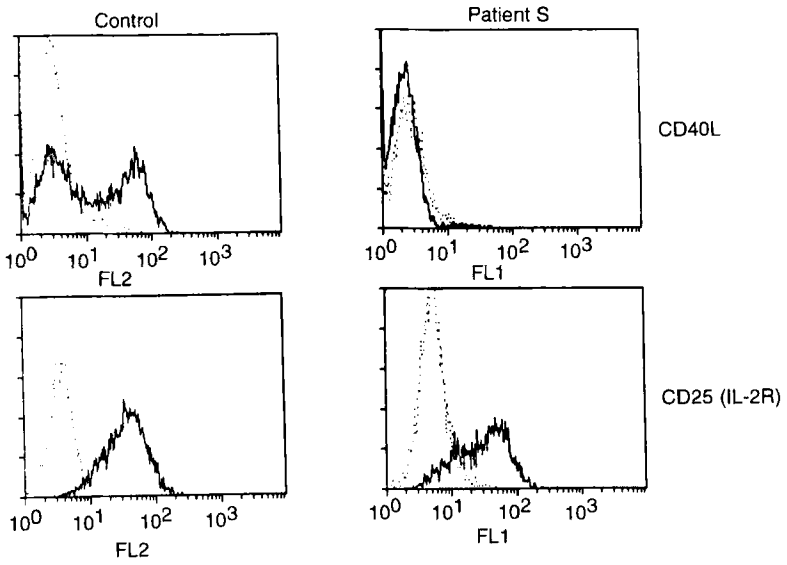

FIG. 2. Cell surface expression of CD40L in a patient with HIGMX-1 showing the lack of CD40L expression in activated T cells from the patient in contrast to CD40L expression in activated T cells from a normal individual. Both patient and control T cells expressed CD25 on activation. Continuous lines represent activated cells and dotted lines represent resting cells.

HIGMX-1 locus at Xq26. They found defective expression of CD40L protein in activated T cells from HIGMX-1 and demonstrated mutations in their CD40L cDNA.

Most patients with HIGMX-1 have been found to have a defect in the expression of CD40L as detected by binding to soluble CD40. A few of these patients were found to express a protein which was detectable by monoclonal or by polyclonal antibodies to CD40L (16, 20) but failed to bind to soluble CD40 indicating that the underlying cause of HIGMX-1 is a defect in the interaction of CD40 with its ligand. Therefore, a defect in CD40 expression or in CD40 signal transduction might also lead to a phenotype similar to that of HIGMX-1. This was confirmed by targeted disruption of the CD40 (93, 94) and of the CD40L (95, 96) genes in mice. Both CD40-deficient and CD40L-deficient mice had normal IgM levels with markedly decreased or absent IgG levels. They also failed to undergo immunoglobulin isotype switching and to develop germinal centers in response to immunization with T cell-dependent antigens. However, they did undergo IgG isotype switching to T cell-independent antigens. These results further support the role of the interaction of CD40L with CD40

in T cell-dependent immunoglobulin isotype switching. As far as human disease is concerned, Conley *et al.* recently reported that out of 17 males with X-linked Hyper IgM, 4 patients expressed CD40L that was detected by soluble CD40 (97). Sequence analysis revealed no mutations in the CD40L gene. The patients' B cells expressed CD40 but showed a defect in CD40-mediated B cell activation. Engagement of CD40 on their B cells by a mAb failed to upregulate CD23 and CD25 expression induced by IL-4. Furthermore, stimulation of their B cells with CD40 mAb and IL-4 resulted in the secretion of less than 5% of the amount of IgE synthesized by normal controls. Although the defects in these patients were not identified, this study demonstrated that the HIGMX-1 phenotype can result from a defect in CD40-dependent B cell activation. In view of the fact that B cells from CBA/N mice do not proliferate in response to CD40, it would be interesting to know whether any of the patients have a mutation in Btk and whether Btk plays a role in CD40-mediated signal transduction in B cells.

The signal transduction events that follow engagement of CD40 remain to be fully established. Cross-linking of CD40 on B cells by mAb induces the phosphorylation of multiple substrates on tyrosine residues with accompanying induction of the turnover of inositol phosphatides and activation of serine/threonine kinases (37). Because CD40 does not possess intrinsic tyrosine kinase activity, it is conceivable that it associates with other signal-transducing molecules for its effector functions. Likely candidates include transmembrane signal-transducing molecules or other cellular nonreceptor protein tyrosine kinases (PTKs) such as *src*-type protein kinases. We have recently demonstrated that cross-linking CD40 with mAb 626.1 induces phosphorylation and activation of the *src*-type PTK *lyn* (98) with no change in phosphorylation or activity of other B cell kinases *fyn, fgr,* or *lck* observed suggesting that *lyn* plays an important role in signaling induced by CD40. However, we could not demonstrate a physical association between CD40 and *lyn* suggesting that another molecule(s) might mediate activation of *lyn* via CD40. The cytoplasmic domain of CD40 has recently been shown via the yeast two-hybrid system to interact with a novel RING-finger protein (99). This protein has a N-terminal RING-finger motif found in some DNA-binding proteins such as the recombination activating gene RAG-1. The C terminal portion of this protein is homologous to the TNF receptor-associated protein domain found in two proteins. We have preliminary evidence for the association of the extracellular domain of CD40 with a transmembrane protein the identity of which remains to be established (T. Morio and R. S. Geha, unpublished observation). Both or either one of these proteins may mediate signal transduction via CD40. CD40 engagement also resulted in the phosphorylation of phospholipase

C-γ_2 and phospatidylinositol-3 (PI-3) kinase with concomitant increase in PI-3 kinase activity (98). Induction of phosphoinositide turnover by CD40 engagement is suggestive of protein kinase C (PKC) activation. Inhibitors of PTK but not PKC nor PKA inhibited CD40-mediated IgE synthesis in B cells (100) suggesting a role for PTK in CD40-mediated signal transduction. Cocross-linking of transmembrane protein tyrosine phosphatase CD45 to CD40 caused an inhibition of IgE synthesis at the level of deletional switch recombination, while IL-4-mediated ε-germline transcription was unaffected, further lending support to the involvement of PTKs in CD40-mediated signal transduction leading to immunoglobulin isotype switching (101). CD40 engagement has also been shown to activate nuclear factor κB via a PTK-dependent pathway (102).

The functional consequences of CD40 ligation on B cells have been extensively studied using mAb to CD40 and more recently using CD40L. Engagement of CD40 delivers an antiapoptotic signal to immature B cells possibly mediated by the expression of the *bcl-2* protooncogene (103, 104); however, recent studies have shown that the antiapoptotic effect of CD40 in normal and neoplastic B cells is independent of *bcl-2* induction (51). CD40 has also been shown to rescue B cells from anti-IgM-induced apoptosis (49). Anti-CD40 mAbs deliver a progression signal to resting B cells in the presence of phorbol esters, anti-CD20 mAb, or anti-IgM antibodies (42, 105, 106), synergize with IL-4 in causing B cell proliferation and in sustaining the growth of activated B cells from days to several weeks in culture (107, 108), induce homotypic B cell adhesion (109), upregulate B cell expression of the costimulatory molecule B7 (110), cause the proliferation and induction of CD23 expression in B cell precursors (111), and cause the secretion of cytokines (112–114). A very important effect of CD40 engagement is the induction of immunoglobulin class switching in B cells (115). Another approach to investigate the role of CD40L/CD40 interaction in the immune response has been to treat mice with a mAb to CD40L or with sCD40. Administration of sCD40 to mice was shown to inhibit B cell memory but not germinal center formation (53), whereas administration of CD40L mAb inhibited B cell memory and germinal center formation (52). Collagen-induced arthritis (116), acute and chronic graft vs host disease (117), and negative selection in the thymus (R. Noelle, personal communication) are also inhibited by administration of a CD40L mAb. Therefore, failure of interaction between CD40L and CD40 has more profound consequences than the inhibition of immunoglobulin isotype switching. Indeed, ligation of CD40 by its ligand is the most potent inducer of expression by B cells of the costimulatory molecules B7.1 and B7.2. Failure to express these molecules during T–B cell interaction results in failure of T cell proliferation and cytokine synthesis and induces T cell

tolerance. We have recently shown, in collaboration with Drs. G. Hollander and S. Burakoff, that B cells from $CD40^{-/-}$ mice fail to induce proliferation of allogeneic T cells. This is overcome by antibody to CD28, the counterereceptor for B7. Furthermore, *in vivo* administration of B cells from $CD40^{-/-}$ mice induced specific tolerance in the recipient T cells. GM-CSF, IFN-γ, and IL-3 have been shown to induce the expression of CD40 on monocytes (43) and interaction between CD40L and CD40 on monocytes and macrophages induces the secretion of cytokines and increases tumoricidal killing (43, 118). Defective interaction between CD40L on T cells and CD40 on monocytes may explain some of the complications of HIGMX-1 that do not appear to be related to an immunoglobulin deficiency such as opportunistic infections with *P. carinii* and *Cryptosporidium.*

In collaboration with R. Insel, we have begun to investigate the role of CD40L/CD40 in somatic mutations in B cells. B cells from HIGMX-1 patients were examined for the presence of somatic mutation in immunoglobulin gene rearrangements of the nonpolymorphic human V_{H6} gene (92). IgM and rare IgG V_{H6} productive rearrangements were isolated from HIGMX-1 peripheral blood lymphocytes, most of which had a germline V_{H6} sequence. Few (7/102 V_{H6} IgM and 1/6 V_{H6} IgG) had a mutated V_{H6} sequence with a lower frequency than rearrangements found in normal individuals but nevertheless they had characteristics and a distribution consistent with their arising by antigenic selection. These results suggest that somatic mutations may be initiated outside germinal centers in a CD40L-independent pathway.

Defective expression of CD40L has been implicated in other immunodeficiency states. Immune responses in newborn lymphocytes show a defect in isotype switching from IgM to IgG and IgA (119). We and others have demonstrated that newborn lymphocytes express decreased amounts of CD40L mRNA and protein (120–122). Decreased expression of CD40L was associated with an inability to undergo T cell-dependent IgE isotype switching *in vitro* (120). However, neonatal B cells were intrinsically capable of undergoing immunoglobulin isotype switching because they synthesized IgE in response to CD40 mAb and IL-4 (120, 122) suggesting that the defect in immunoglobulin isotype switching in the neonatal period may be related to poor expression of CD40L. Because CD40L is expressed transiently on the surface of $CD4^+$ T cells (45), we examined the effect of cyclosporin A on CD40L expression in T cells. We found that cyclosporin A inhibits CD40L expression in T cells by inhibiting the activity of calcineurin indicating that a calcineurin-dependent transcription factor(s), such as NF-AT, plays a key role in CD40L gene expression (123). We have found four functional NF-AT-like sequences in the 5′ upstream regulatory region of the mouse CD40L gene (A. Tsitsikova, E. Tsitsikov, and R. S.

Geha, manuscript in preparation) suggesting that NF-AT plays a critical role in the expression of CF40L. Cyclosporin A also inhibited IL-4-driven CD40L-dependent IgE isotype switching in PBMC but did not inhibit IgE synthesis induced by CD40 mAb and IL-4. PBMC derived from transplant patients receiving cyclosporin A failed to express CD40L on stimulation *in vitro* suggesting that patients receiving cyclosporin A may be deficient in CD40L-dependent T cell help. Farrington *et al.* showed defective expression of CD40L in some patients with common variable immunodeficiency. Most of the patients who expressed decreased amounts of CD40L also had defective expression of IL-2 suggesting that decreased expression of CD40L was secondary to a defect in T cell activation. We found defective CD40L expression secondary to a defect in T cell activation in three patients with ataxia telengiectasia and hyper IgM. T cells from all three patients had no CD40L detectable on their surface after stimulation with phorbol ester and ionophore but had a severe defect in T cell activation with lack of CD25 expression upon activation and poor proliferation to mitogens (R. Fuleihan and R. S. Geha, unpublished observations). Finally, we have found that CD40L expression is developmentally regulated. The capacity to express CD40L is acquired late in thymocyte development (46). CD40L mRNA was detectable in single-positive mature thymocytes but not in double-positive immature thymocytes. CD40L surface expression was detectable on activated $CD4^+/CD8^-$ mature thymocytes but not on activated immature thymocytes nor on activated $CD4^-/CD8^+$ mature thymocytes. The inability of immature thymocytes to express CD40L is probably related to their inability to activate NF-AT (124).

V. Conclusion and Future Prospects

XLA and HIGMX-1 are two immunodeficiency diseases resulting from defects in B cell development and differentiation. XLA is due to a defect in a cytoplasmic tyrosine kinase, Btk, that undoubtedly plays an important role in B cell development. HIGMX-1 develops as a result of a defect in the gene encoding CD40L that directs T cell-dependent B cell differentiation. Identification of the genetic basis of these diseases has lead to a better understanding of their immunopathology, will allow detection of carriers and affected fetuses for genetic counseling and *in utero* diagnosis, and will pave the way for gene therapy of XLA and HIGMX-1. In the meantime, engagement of CD40 by soluble CD40L or by a mAb might be useful in treating some aspects of HIGMX-1. Further work in animal models is needed to demonstrate efficacy and the lack of untoward side effects resulting from such therapy. Because of the role of CD40L in a variety of immune responses, blocking the interaction of CD40L with CD40 by

soluble CD40 or by a CD40L mAb might be useful in the treatment of graft rejection, graft vs host disease, or in collagen vascular diseases, such as arthritis and systemic lupus erythematosis, where, in a murine model, it has recently been shown that administration of a CD40L mAb prevented the development of lupus glomerulonephritis.

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Defective Glycosyl Phosphatidylinositol Anchor Synthesis and Paroxysmal Nocturnal Hemoglobinuria

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I. Introduction

Many cell surface proteins are bound to the membrane by means of a glycosyl phosphatidylinositol (GPI) glycolipid anchor covalently attached to the carboxy termini of the peptides. The GPI anchor is synthesized in the endoplasmic reticulum (ER) and post-translationally linked to the nascent peptides in the ER. If the peptides are not GPI anchored, they are not expressed on the cell surface due to either intracellular retention/degradation or secretion. The human disease, paroxysmal nocturnal hemoglobinuria (PNH), is caused by this mechanism. PNH is a hematopoietic stem cell disorder characterized by the presence of abnormal cells of various hematopoietic cell lineages that are deficient in the surface expression of GPI-anchored proteins. The abnormal red blood cells of patients with PNH are sensitive to the hemolytic action of complement due to the deficient surface expression of decay accelerating factor (DAF) and CD59, which are GPI-anchored complement inhibitors. Patients with PNH suffer from intravascular hemolysis upon the activation of complement and occasionally have complications such as venous thrombosis, acute leukemia, and defective hemopoiesis. The first step of GPI anchor biosynthesis is deficient in abnormal blood cells from patients with PNH. The X-linked gene *PIG-A*, which is involved in this step, has been cloned and it is somatically mutated in abnormal cells from patients with PNH. Due to its X-chromosomal location, a single inactivating mutation results in a loss of GPI anchor synthesis even in a female hematopoietic stem cell if it occurs in the active allele of the *PIG-A* gene. More than 60 patients with PNH from various countries have been analyzed and the *PIG-A* gene was responsible for PNH in all of them. The X-chromosomal location of the *PIG-A* gene would also account for this uniformity of the responsible gene among the 10 or so genes involved in GPI anchor synthesis.

II. The GPI Anchor and the GPI-Anchored Proteins

A. Background

More than 80 eukaryotic cell surface proteins are inserted into the membrane via a glycolipid termed the GPI anchor (reviewed in Ferguson

TABLE I

LIST OF SOME MAMMALIAN GPI-ANCHORED PROTEINS INCLUDING THOSE DEFICIENT IN PNH

No.	Protein	Species	Reference
1	CD14°	Human	Haziot *et al.* (1998), Simmons *et al.* (1989)
2	CD16b (FcγRIIIB)°	Human	Selvaraj *et al.* (1988)
3	CD24°	Human	Fischer *et al.* (1990), Kay *et al.* (1991)
4	CD48°	Human	Staunton *et al.* (1989)
5	CDw52 (CAMPATH-1)°	Human	Xia *et al.* (1993)
6	CD55 (Decay accelerating factor/DAF)°	Human	Davitz *et al.* (1986)
7	CD58 (LFA-3)°	Human	Dustin *et al.* (1987)
8	CD59 (MACIF, HRF20, MIRL)°	Human	Davies *et al.* (1989)
9	CD66c°	Human	van der Schoot *et al.* (1989)
10	CD66e (CEA)	Human	Hirose *et al.* (1983), Takami *et al.* (1988)
11	CD67°	Human	van der Schoot *et al.* (1990)
12	CD73 (5′-ectonucleotidase)°	Human	Taguchi and Ikezawa (1978), Low and Finean (1978)
13	CD87 (urokinase receptor)°	Human	Ploug *et al.* (1991)
14	CDw108	Human	
15	CDw109	Human	Haregewoin *et al.* (1994)
16	Acetylcholinesterase°	Human	Deeg *et al.* (1992)
17	Alkaline phosphatase°	Human	Misumi *et al.* (1988), Low and Zilversmit (1980)
18	Folate-binding protein	Human	Lacey *et al.* (1989), Luhrs *et al.* (1989)
19	JMH-bearing protein°	Human	Bobolis *et al.* (1992)
20	p50–80°	Human	van der Schoot *et al.* (1990)
21	C8-binding protein/HRF°	Human	Hänsch *et al.* (1987), Zalman *et al.* (1987)
22	Aminopeptidase P	Human	Hooper and Turner (1988)
23	Ciliary neurotrophic factor receptor	Human	Davis *et al.* (1991)

24	Cellular isoform of scrapie agent protein	Human	Stahl *et al.* (1990)
25	120-kDa peanut agglutinin-binding glycoprotein in CNS	Human	Mikol and Stefansson (1988)
26	Fibronectin receptor	Human	Symington and Symington (1990)
27	Alkaline phosphodiesterase I	Human	Nakabayashi *et al.* (1993)
28	Isoforms of TGF-β receptor	Human	Cheifetz and Massague (1991)
29	IgD	Mouse	Wienands and Reth (1992)
30	TCRβ	Mouse	Kuwabara *et al.* (1994)
31	Ly6 (Sca-1)	Mouse	Reiser *et al.* (1986)
32	Thymic shared antigen-1	Mouse	MacNeil *et al.* (1993)
33	Heat-stable antigen	Mouse	Kay *et al.* (1990)
34	B7 antigen	Mouse	Kubota *et al.* (1990)
35	Qa-2	Mouse	Robinson *et al.* (1989)
36	Thy-1	Mouse	Homans *et al.* (1988)
37	ADP-ribosyltransferase	Mouse	Zolkiewska and Moss (1993)
38	Lipoprotein lipase	Mouse	Chan *et al.* (1988)
39	F3 cell surface protein	Mouse	Durbec *et al.* (1993)
40	BST-1	Mouse	Kaisho *et al.* (1994)
41	120-kDa neural cell adhesion molecule (N-CAM)	Mouse	He *et al.* (1986)
42	ELF-1	Mouse	Cheng and Flanagan (1994)
43	Hepatoma glycoprotein	Rat	Ikehara *et al.* (1987)
44	Heparan sulfate proteoglycan	Rat	Ishihara *et al.* (1987)
45	RT-6.2	Rat	Koch *et al.* (1986)
46	Zymogen granule GP-2	Rat	LeBel and Beattie (1988)
47	Renal dipeptidase	Pig	Hooper *et al.* (1987)
48	Treharase	Rabbit	Takesue *et al.* (1986)
49	NAD^+ glycohydrolase	Rabbit	Kim *et al.* (1988)
50	Carboxypeptidase M	Dog	Deddish *et al.* (1990)

*Proteins deficient in PNH.

and Williams, 1988; Low, 1989a,b; McConville and Ferguson, 1993). The GPI-anchored proteins are abundant in protozoa (reviewed in Thomas *et al.*, 1990; Ferguson, 1994; McConville and Ferguson, 1993) and yeasts (reviewed in Herscovics *et al.*, 1993), whereas they are minor components on mammalian cells (Table I). GPI anchoring is a post-translational modification at the carboxy terminus occurring in the ER (reviewed in Low, 1989b; Low, 1989a; Cross, 1990; Tartakoff and Singh, 1992; Herscovics *et al.*, 1993; Englund, 1993). Peptides that are to be GPI anchored have, at their carboxy termini, signal peptides that are cleaved and replaced by a preassembled GPI anchor (reviewed in Ferguson and Williams, 1988; Cross, 1990; Lublin, 1992; Englund, 1993). The GPI anchor contains phosphatidylinositol (PI), the alkyl/acyl chains of which anchor the entire protein to the membrane, so they are sensitive to PI-specific phospholipases (reviewed in Cross, 1990). PI-specific phospholipase C (PI-PLC) from bacteria, such as *Bacillus cereus, B. thuringiensis,* and *Staphylococcus aureus* (Ikezawa and Taguchi, 1981; Low, 1981), releases GPI-anchored proteins from mammalian cell surfaces. Studies using this enzyme have contributed to the discovery of GPI-anchored proteins (reviewed in Low *et al.*, 1986; Cross, 1987; Low, 1987; Ferguson and Williams, 1988; Low and Saltiel, 1988) and the identification of more of them (Doering *et al.*, 1993). The GPI anchor confers unique functional properties on proteins, such as exclusive expression on the apical surface of polarized epithelial cells, signal-transducing ability, low turnover rates, and clathrin-independent endocytosis (reviewed in Lisanti *et al.*, 1990; Thomas *et al.*, 1990; Lublin, 1992; Ferguson, 1994; McConville and Ferguson, 1993).

B. Structure of the GPI-Anchored Proteins

The basic structure of the GPI-anchored proteins is presented in Fig. 1. The core backbone of the GPI anchor consists of PI, glucosamine (GlcN), three mannose (Man), and ethanolaminephosphate (EtNP) linked side by side (Ferguson *et al.*, 1988; Homans *et al.*, 1988; Roberts *et al.*, 1988b; Deeg *et al.*, 1992). The presence of GlcN is unique to the GPI anchor because most glycoconjugates contain acetylated glucosamines (GlcNAc). This is due to the deacetylation of GlcNAc during biosynthesis of the GPI anchor (see Section II, D and Fig. 2). The existence of GlcN can be used for a diagnosis that distinguishes the GPI anchor from the other glycoconjugates (Doering *et al.*, 1993). The carboxy terminus of the peptide is linked to EtNP by an amide bond to form GPI-anchored protein.

This core structure is conserved in unicellular eukaryotic cells and mammals (Ferguson *et al.*, 1988; Fankhauser *et al.*, 1993; Homans *et al.*, 1988; Deeg *et al.*, 1992), whereas various modifications have been reported. In human erythrocyte acetylcholinesterase (hAChE) and DAF, two EtNPs are bound to each of the first and second Man (Deeg *et al.*, 1992) (Fig.

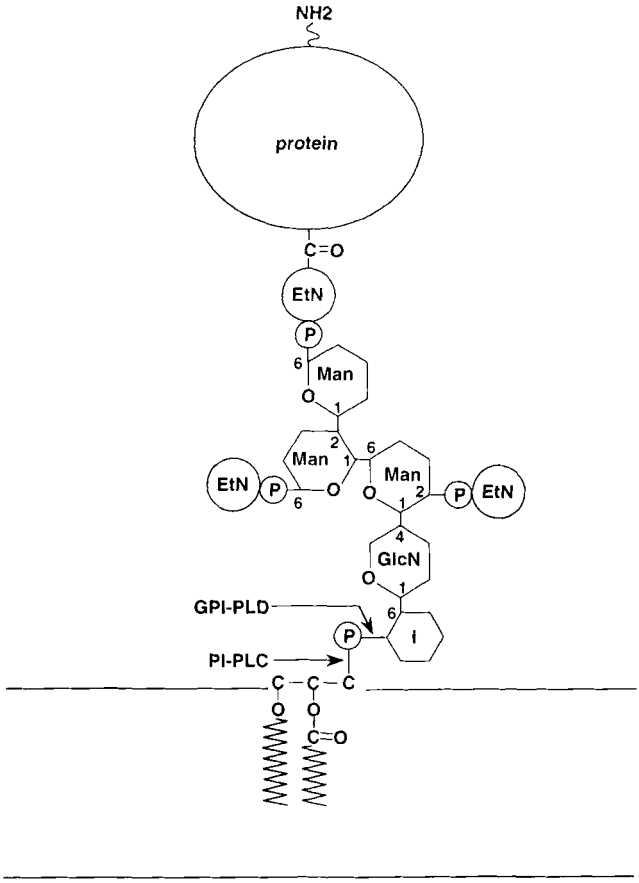

FIG. 1. Schematic representation of the structure of GPI-anchored proteins. Sites cleaved by PI-PLC and GPI-PLD are shown. EtN, ethanolamine; P, phosphate; Man, mannose; GlcN, glucosamine; I, inositol.

1). The hAChE and erythrocyte DAF also contain an additional fatty acid (palmitate) linked to the inositol ring, and this modification renders GPI anchor resistant to PI-PLC (Roberts *et al.*, 1988a; Walter *et al.*, 1990). In rat Thy-1, another Man is bound to the third Man and both EtNP and *N*-acetylgalactosamine are bound to the first Man (Homans *et al.*, 1988).

C. Structural Characteristics of Precursor Peptides That Are to Be GPI Anchored

Although the structure of the GPI anchor is well conserved, the peptides in the GPI-anchored proteins vary. From a functional perspective, there

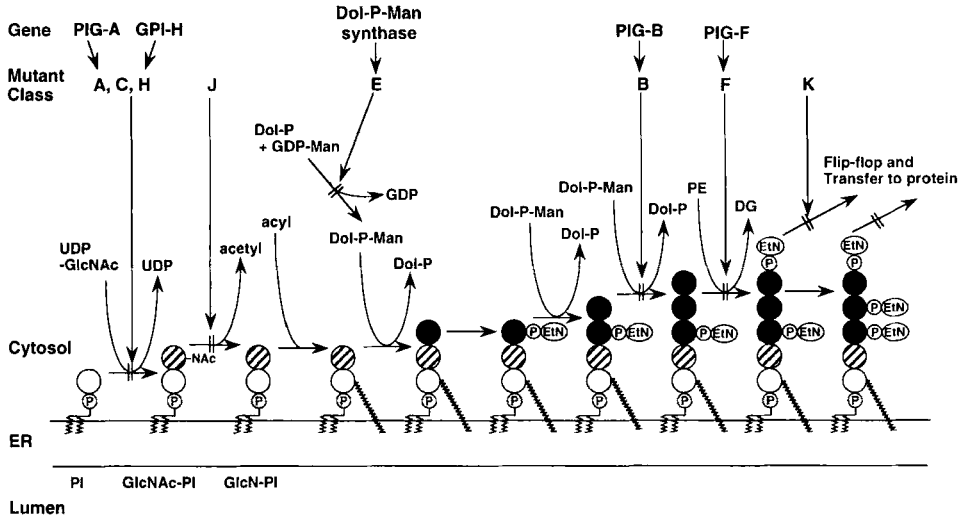

FIG. 2. Biosynthesis pathway of mammalian GPI anchor, its mutants, and genes. PI, phosphatidylinositol; GlcNAc, *N*-acetylglucosamine; GlcN, glucosamine; Dol-P-Man, dolichol-phosphate-mannose; PE, phosphatidylethanolamine; DG, diacylglycerol.

are all types, such as enzymes, receptors, complement regulatory proteins, and adhesion proteins (Table I). Precursor peptides of GPI-anchored proteins contain amino terminal signal sequences which direct them to the ER (Englund, 1993). The cleavage of amino terminal signal peptide, however, is not prerequisite for GPI anchoring (Howell *et al.*, 1994). At the carboxy terminal portion of the precursor peptide there is a sequence of 20–30 residues that directs GPI anchoring and it is removed before the addition of the GPI anchor (Fig. 3) (Boothroyd *et al.*, 1980; Tse *et al.*, 1985; Low and Kincade, 1985; Micanovic *et al.*, 1988; Hefta *et al.*, 1988; Moran *et al.*, 1991; Nuoffer *et al.*, 1991). This carboxy terminal signal sequence and a residue for GPI anchor attachment (ω site) are essential and sufficient for the peptides to be GPI anchored (Moran *et al.*, 1991). A comparison of the carboxy terminal signal sequences from a wide variety of GPI-anchored proteins revealed that they contain 15–20 residue hydrophobic domains at the extreme carboxy termini and that there is no apparent sequence similarity among these domains (Gerber *et al.*, 1992; Berger *et al.*, 1988). Five to 10 hydrophilic residues are located between the ω site and the hydrophobic domain (Gerber *et al.*, 1992).

Several investigators have examined the sequence requirement of the carboxy terminal signals around the ω site for the GPI anchor to attach (Micanovic *et al.*, 1990a; Gerber *et al.*, 1992; Moran *et al.*, 1991; Nuoffer

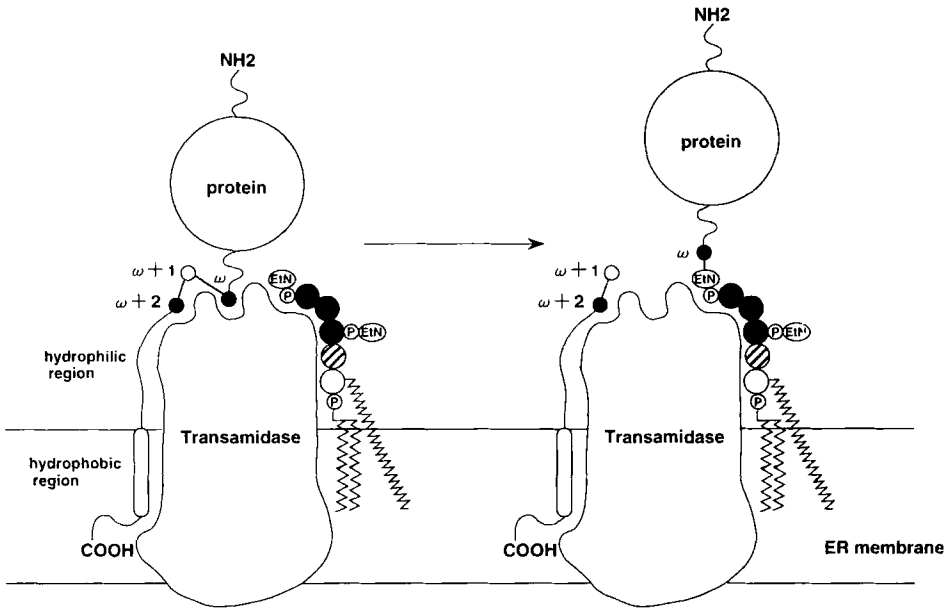

FIG. 3. Post-translational modification of the precursor peptide by the GPI anchor. The putative transmidase cleaves the carboxy terminal signal peptide between ω and ω + 1 sites and links the preassembled GPI anchor precursor to the new carboxy terminus.

et al., 1993). They found that the amino acid at the ω site should be glycine, aspartic acid, asparagine, alanine, serine, or cysteine (Micanovic *et al.*, 1990a; Nuoffer *et al.*, 1993; Antony *et al.*, 1994). However, cysteine was not allowed for DAF (Moran *et al.*, 1991). Additionally, the amino acid two residues downstream of the ω site (ω + 2 site) should also be small, but the requirement for the amino acid at the ω + 1 site was less stringent (Fig. 3) (Gerber *et al.*, 1992). Although the sequence requirements around the ω site appear to be similar among eukaryotic cells, they are not identical in protozoa and mammalian cells (Moran and Caras, 1994).

D. Biosynthesis of the GPI Anchor Precursor

Attachment of the GPI anchor to a peptide occurs very rapidly after translation; this was within 1 min for the variant surface glycoprotein (VSG) of *Trypanosoma brucei* (Bangs *et al.*, 1985). This implied that the GPI anchor is preassembled and transferred *en bloc* to the peptide. The biosynthetic pathway of the core of the GPI anchor precursor was first established for VSG (Krakow *et al.*, 1986; Menon *et al.*, 1988, 1990b; Masterson *et al.*, 1989; Mayor *et al.*, 1990a,b) and the mammalian pathway was later

shown to be similar (Hirose *et al.*, 1992b; Kamitani *et al.*, 1992). The GPI anchor precursor is assembled in the ER (Conzelmann *et al.*, 1990; Fasel *et al.*, 1989) by the sequential transfer of glycan components from their donors to PI and later intermediates. The mammalian pathway is shown in Fig. 2.

The first step of the biosynthesis is the transfer of GlcNAc to PI from UDP-GlcNAc to form the first intermediate GlcNAc-PI (Fig. 2) (Masterson *et al.*, 1989; Hirose *et al.*, 1991). αGlcNAc is linked to position 6 of inositol. Most mammalian GPI-anchored proteins contain PI with 1-alkyl, 2-acylglycerol (Roberts *et al.*, 1988b; Walter *et al.*, 1990), in contrast to VSG, which contains diacylglycerol (Masterson *et al.*, 1990; Doering *et al.*, 1994). However, neither lipid is obligatory for synthesis of the GPI anchor (Singh *et al.*, 1994). GlcNAc-PI is then deacetylated to form the second intermediate glucosaminyl-PI (GlcN-PI) (Hirose *et al.*, 1991). GTP stimulates the deacetylation by means of an unknown mechanism (Stevens, 1993). The inositol ring of a fraction of GlcN-PI is then acylated by palmitate (Mayor *et al.*, 1990a; Urakaze *et al.*, 1992). Acyl CoA serves as a donor of the acyl group in yeasts (Costello *et al.*, 1992), whereas in mammalian cells this acylation is dependent on CoA, suggesting that the acyl group is donated by other sources, such as phospholipid (Stevens and Zhang, 1994). This inositol acylation is thought to be a distinct and perhaps obligatory step in mammals and yeasts (Urakaze *et al.*, 1992; Costello *et al.*, 1992) but in trypanosomes it is regulated stage specifically. Inositol is not acylated in the blood stage, whereas it is always acylated in the insect stage (Field *et al.*, 1991, 1992).

Three Man residues are sequentially transferred from dolichol-phosphate-mannose (Dol-P-Man) (Orlean, 1990; Menon *et al.*, 1990a; Singh and Tartakoff, 1991; DeGasperi *et al.*, 1990). The linkages are Manα1–2Manα1–6Manα1–4GlcN. Before transfer of the second Man, EtNP is linked to the first at position 2 (Hirose *et al.*, 1992b; Kamitani *et al.*, 1992). The last step of core backbone synthesis is the transfer of EtNP to the third Man at position 6. The donor of this EtNP is phosphatidylethanolamine (PE) (Menon and Stevens, 1992; Menon *et al.*, 1993). Another EtNP is then added to the second Man at position 6 (Ueda *et al.*, 1993). Donors of EtNP added to the first and second Man and whether they are obligatory steps remain unknown.

A GPI anchor precursor that is transferred to the peptide has not been directly demonstrated. However, it would correspond to a glycolipid species containing three Man, EtNP on the first and third Man (also possibly on the second Man), GlcN, and acylated PI (Fig. 2).

GlcNAc-PI and GlcN-PI in sealed microsomes prepared from mouse thymoma cells and trypanosomes are sensitive to PI-PLC, so they are

exposed to the cytoplasmic face of the ER (Vidugiriene and Menon, 1993, 1994). Intermediates containing Man in sealed trypanosome microsomes are also exposed to the cytoplasmic face (Vidugiriene and Menon, 1994). A study of *Leishmania major* expressing trypanosomal GPI-specific PLC in the cytoplasm suggested that, at least up to an intermediate with two Man, the protein GPI anchor is synthesized in the cytoplasmic side of the ER (Mensa Wilmot *et al.*, 1994). Therefore, biosynthesis of the GPI anchor precursor proceeds on the cytoplasmic face of the ER (Fig. 2). Since newly formed GPI-anchored proteins are found in the luminal side, the GPI anchor precursor may flip into the lumen before addition to the carboxy terminus of the peptide (Fig. 2). The possibility that the anchor precursor is added to the peptide carboxy terminus cytoplasmically before transportation into the lumen has not been eliminated.

E. Post-translational Modification by the GPI Anchor

It is thought that a putative transamidase catalyzes removal of the carboxy terminal peptide from and transfer of the GPI anchor to the nascent precursor peptide (Bailey *et al.*, 1989; Micanovic *et al.*, 1990b; Gerber *et al.*, 1992). This step has been extensively analyzed *in vitro* using molecularly engineered placental alkaline phosphatase, a GPI-anchored protein, and microsome fraction (Kodukula *et al.*, 1991; Bailey *et al.*, 1989). This processing step requires ATP and GTP (Amthauer *et al.*, 1992). ATP is used because the chaperone protein BiP participates in this processing (Amthauer *et al.*, 1993). Removal of the carboxy terminal peptide did not occur when microsomes from GPI anchor-deficient cells were used (Kodukula *et al.*, 1992). The unprocessed precursor peptides might be degraded in the cells before reaching the Golgi apparatus (Delahunty *et al.*, 1993; Moran and Caras, 1992; Field *et al.*, 1994) or secreted (Fatemi and Tartakoff, 1986, 1988). These results suggested that the putative transamidase requires the complete GPI anchor precursor for processing precursor peptides.

F. GPI Anchor-Deficient Mutants

Many mutant cell lines that are deficient in biosynthesis or attachment of the GPI anchor have been established. A number of Thy-1-negative murine thymoma cell lines established by Hyman and colleagues have been grouped into complementation classes by somatic cell fusion analysis (Hyman, 1985; Hyman and Stallings, 1974; Trowbridge *et al.*, 1978; Hyman *et al.*, 1980; Evans *et al.*, 1987; Conzelmann *et al.*, 1988). Nine complementation classes (A–I) were identified and characterized (Hyman, 1988). Six of them (A–C, E, F, and H) were deficient in biosynthesis of the GPI anchor (Fig. 2) (Conzelmann *et al.*, 1986, 1988, Fatemi and Tartakoff, 1986, 1988; Lemansky *et al.*, 1991). Two more complementation classes,

J and K, have been characterized using DAF- and CD59-negative K562 erythroleukemic cell lines (Fig. 2) (Hirose *et al.*, 1992a; Mohney *et al.*, 1994). JY5, a mutant of human B-lymphoblastoid cell line JY (Hollander *et al.*, 1988), was assigned to class A (Miyata *et al.*, 1993). The LM cell line, which is derived from murine fibroblastic L cells, and LM-TK$^-$, a thymidine kinase-negative variant of LM, are GPI deficient and belong to class H (Singh *et al.*, 1991). These mutant cell lines have been useful for understanding mammalian GPI anchor synthesis and for identifying genes involved in the biosynthesis pathway (Kinoshita and Takeda, 1994b).

Biochemical defects in the mutants of eight complementation classes were determined by analyzing GPI anchor biosynthesis. Cells belonging to classes A, C, and H do not synthesize the first intermediate GlcNAc-PI (Fig. 2) (Stevens and Raetz, 1991; Sugiyama *et al.*, 1991; Hirose *et al.*, 1992a). The existence of three complementation classes indicates that three genes are necessary for transfer of GlcNAc from UDP-GlcNAc to PI. A similar situation seems to be true for yeast GPI anchor synthesis (Leidich *et al.*, 1994). Although two of the three genes have been cloned and characterized, exactly how three gene products participate in this reaction remains unclear (see below).

Class J mutant cells synthesize GlcNAc-PI but do not convert it to GlcN-PI (Fig. 2) (Mohney *et al.*, 1994). Therefore, deacetylation of GlcNAc is defective in the class J mutant.

A defect in class E cells was identified before the discovery of the GPI anchor (Chapman *et al.*, 1980). An immature type of dolichol-linked precursor of the *N*-linked oligosaccharides accumulated in class E cells due to the defective biosynthesis of Dol-P-Man (Chapman *et al.*, 1980). Analysis of GPI anchor biosynthesis in class E cells showed that GPI intermediates containing Man are not synthesized (Sugiyama *et al.*, 1991; Singh and Tartakoff, 1991b). Therefore, class E cells are defective in the synthesis of a Man donor from GDP-Man and Dol-P (Fig. 2). A mutant T cell hybridoma that does not synthesize Dol-P-Man and probably also belongs to class E does not synthesize any GPI intermediates containing Man (DeGasperi *et al.*, 1990). These mutants accumulate GlcN-PI in which PI is acylated (Urakaze *et al.*, 1992; Puoti and Conzelman, 1993).

Class B mutant cells accumulate a GPI intermediate containing two Man (Fig. 2) (Sugiyama *et al.*, 1991; Puoti *et al.*, 1991). This intermediate probably has an acylated PI and EtNP on the first Man (Puoti *et al.*, 1991; Hirose *et al.*, 1992b). Therefore, the defect in class B is at the third Man transfer which is mediated by an α-1,2-mannosyltransferase.

Class F mutant cells accumulate an intermediate containing three Man (Fig. 2) Sugiyama *et al.*, 1991; Puoti and Conzelman, 1993). This intermediate has an acylated PI and EtNP on the first Man but not on the second

and third Man (Sugiyama *et al.*, 1991; Hirose *et al.*, 1992b; Puoti and Conzelman, 1993). Since addition of EtNP on the third Man preceeds that on the second Man in the wild-type cells, the defect in the class F cells is in the transfer of EtNP from PE to the third Man (Fig. 2). Transfer of EtNP to the first Man must be mediated by a different enzyme.

Class K mutant cells accumulate two glycolipid species that are putative GPI anchor precursors (Fig. 2) (Mohney *et al.*, 1994). They have three Man, GlcN, acylated PI, and EtNP on the third Man with an extra EtNP either on the first Man or on both the first and the second Man (Mohney *et al.*, 1994). Therefore, the class K defect resides in a step that precedes, or is associated with, transfer of the GPI anchor to the peptide (Mohney *et al.*, 1994).

G. Genes Involved in the GPI Anchor Synthesis

No enzymes or regulatory proteins for GPI anchor synthesis have been purified, so enzymology of the pathway has not progressed. It would not be practical to purify those proteins from mammalian cells because their levels would be very low. The chemical structure and the biosynthesis pathway of the GPI anchor were first elucidated in trypanosomes due mainly to an abundance of the GPI-anchored proteins and high biosynthetic activity. Those of the mammals were later elucidated. In contrast, several mammalian genes involved in the pathway have been cloned by expression cloning based upon the complementation of mutant cells.

1. Human Class A Gene Termed PIG-A

A cDNA that restores surface expression of GPI-anchored proteins on class A mutants was cloned using JY5 cells as a recipient for a cDNA library (Miyata *et al.*, 1993). The gene was termed *PIG-A* for phosphatidylinositol glycan class A (Miyata *et al.*, 1993). The *PIG-A* cDNA contained 4568 bp and an open reading frame of 1452 bp that encodes a predicted protein of 484 amino acids with 85 bp 5′ and 3031 bp 3′ untranslated regions (Miyata *et al.*, 1993). There is no apparent amino terminal signal peptide sequence. Near the carboxy terminus there is a hydrophobic sequence of 27 residues that may act as a transmembrane domain (Fig. 4). As the GPI anchor is synthesized in the ER, this hydropathy profile predicts that the PIG-A protein binds to the ER membrane with its big amino terminal portion in the cytoplasm and its small carboxy terminal portion in the lumen of the ER.

PIG-A cDNA restored the synthesis of GlcNAc-PI in class A cells (Miyata *et al.*, 1993). A data base search demonstrated a short region of significant homology (39% similarity in 93 amino acid residues) between PIG-A and a bacterial GlcNAc transferase termed RfaK that is involved in synthesis


A
-85
ACTGGCGGCCATGGAACTCACCGGTAATAGAGGACACATCTCTTAACTGGGTTGCTCTAAGAACTGATGTCTAAACCGTCTCAGC
1
ATGGCCTGTAGAGGAGGAGCTGGGAATGGCCACCGTGCCTCAGCTACACTCTCTCGGGTTAGCCCTGGAAGTCTTTACACATGTAGAACCCGTACCCATAATATATGCATGGTATCTGAC
MetAlaCysArgGlyGlyAlaGlyAsnGlyHisArgAlaSerAlaThrLeuSerArgValSerProGlySerLeuTyrThrCysArgThrArgThrHisAsnIleCysMetValSerAsp
121
TTTTTCTACCCAAATATGGGAGGCGTGGAAAGCCACATTTACCAGCTCTCTCAGTGCCTGATTGAAAGAGGGCATAAGGTTATAATTGTCACCCATGCTTATGGAAATCGAAAAGGCATC
PhePheTyrProAsnMetGlyGlyValGluSerHisIleTyrGlnLeuSerGlnCysLeuIleGluArgGlyHisLysValIleIleValThrHisAlaTyrGlyAsnArgLysGlyIle
241
CGTTACCTCACCAGTGGCCTCAAAGTCTATTACTTGCCTCTGAAAGTCATGTACAACCAGTCTACAGCCACGACCCTCTTTCACAGTCTGCCATTGCTCAGGTACATATTTGTTCGGGAG
ArgTyrLeuThrSerGlyLeuLysValTyrTyrLeuProLeuLysValMetTyrAsnGlnSerThrAlaThrThrLeuPheHisSerLeuProLeuLeuArgTyrIlePheValArgGlu
361
AGAGTCACGATAATCCATTCACATAGTTCTTTTTCTGCTATGGCCCATGATGCTCTCTTCCACGCCAAGACAATGGGGCTTCAGACAGTCTTCACGGACCATTCCCTTTTTGGATTTGCT
ArgValThrIleIleHisSerHisSerSerPheSerAlaMetAlaHisAspAlaLeuPheHisAlaLysThrMetGlyLeuGlnThrValPheThrAspHisSerLeuPheGlyPheAla
481
GATGTCAGCTCGGTGCTTACAAACAAGCTTCTAACCGTGTCTCTTTGTGATACAAACCACATCATTTGTGTGTCTTATACTAGTAAGGAAAATACTGTACTAAGAGCAGCACTGAATCCT
AspValSerSerValLeuThrAsnLysLeuLeuThrValSerLeuCysAspThrAsnHisIleIleCysValSerTyrThrSerLysGluAsnThrValLeuArgAlaAlaLeuAsnPro
601
GAAATAGTGTCCGTCATTCCTAATGCTGTAGATCCTACTGACTTCACTCCAGACCCATTTAGAAGGCATGATAGTATAACTATTGTTGTTGTCAGCAGACTTGTTTACAGAAAAGGGATC
GluIleValSerValIleProAsnAlaValAspProThrAspPheThrProAspProPheArgArgHisAspSerIleThrIleValValValSerArgLeuValTyrArgLysGlyIle
721
GATTTGCTTAGTGGTATAATACCTGAACTCTGTCAGAAATATCCAGATTTAAATTTCATAATTGGAGGAGAGGGACCAAAGAGAATCATTTTGGAAGAAGTTCGGGAAAGATACCAGCTG
AspLeuLeuSerGlyIleIleProGluLeuCysGlnLysTyrProAspLeuAsnPheIleIleGlyGlyGluGlyProLysArgIleIleLeuGluGluValArgGluArgTyrGlnLeu
841
CATGACAGGGTGCGTCTTTTGGGAGCTTTAGAACACAAGGATGTTAGAAATGTCTTAGTTCAAGGACATATTTTTCTGAATACCTCCCTTACTGAAGCATTCTGCATGGCGATCGTGGAA
HisAspArgValArgLeuLeuGlyAlaLeuGluHisLysAspValArgAsnValLeuValGlnGlyHisIlePheLeuAsnThrSerLeuThrGluAlaPheCysMetAlaIleValGlu
961
GCAGCCAGTTGTGGTTTACAGGTTGTAAGTACCAGAGTTGGTGGAATTCCTGAGGTGCTTCCAGAAAACCTTATTATTTTATGTGAGCCTTCAGTAAAATCTTTGTGTGAAGGATTGGAA
AlaAlaSerCysGlyLeuGlnValValSerThrArgValGlyGlyIleProGluValLeuProGluAsnLeuIleIleLeuCysGluProSerValLysSerLeuCysGluGlyLeuGlu
1081
AAGGCTATTTTCCAACTGAAGTCAGGGACATTGCCAGCTCCAGAAAACATCCATAACATAGTAAAGACTTTCTACACCTGGAGGAATGTTGCAGAAAGAACTGAAAAGGTATATGACCGG
LysAlaIlePheGlnLeuLysSerGlyThrLeuProAlaProGluAsnIleHisAsnIleValLysThrPheTyrThrTrpArgAsnValAlaGluArgThrGluLysValTyrAspArg
1201
GTATCAGTGGAAGCTGTGTTGCCAATGGACAAACGACTGGACAGACTTATTTCTCACTGCGGCCCAGTAACAGGCTACATCTTTGCTTTGTTGGCAGTTTTCAACTTCCTCTTCCTCATT
ValSerValGluAlaValLeuProMetAspLysArgLeuAspArgLeuIleSerHisCysGlyProValThrGlyTyrIlePheAlaLeuLeuAlaValPheAsnPheLeuPheLeuIle
1321
TTCTTGAGATGGATGACTCCAGATTCTATCATTGATGTTGCAATAGATGCCACTGGGCCACGGGGTGCCTGGACTAATAACTATTCTCACAGTAAAAGAGGGGGTGAGAATAATGAGATA
PheLeuArgTrpMetThrProAspSerIleIleAspValAlaIleAspAlaThrGlyProArgGlyAlaTrpThrAsnAsnTyrSerHisSerLysArgGlyGlyGluAsnAsnGluIle

FIG. 4. Nucleotide and deduced amino acid sequence of PIG-A and its hydropathy plot [from Miyata *et al.* (1993)].

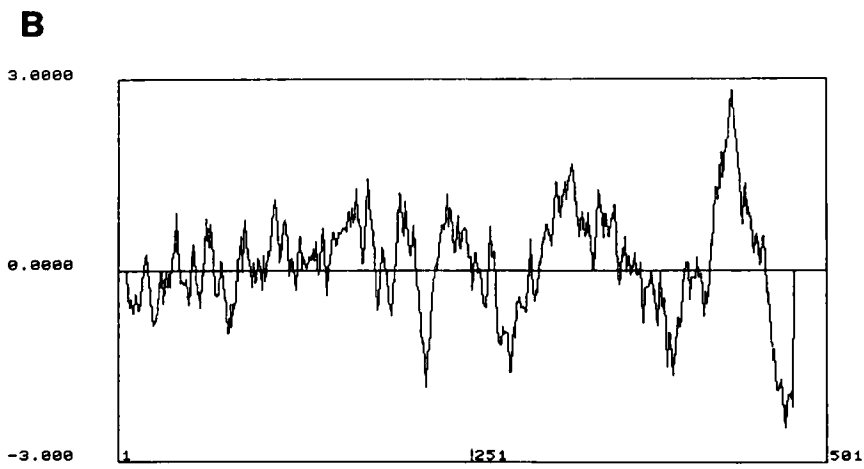

FIG. 4. *(Continued)*

of lipopolysaccharide (Kawagoe *et al.*, 1994; Bessler *et al.*, 1994a). RfaK is a member of a big family of glycosyltransferases whose members share this homologous region (MacLachlan *et al.*, 1991; Parker *et al.*, 1992). This region is within the amino terminal, presumably the cytoplasmic portion of PIG-A. Taken together with a report that the early intermediates are cytoplasmically oriented (Vidugiriene and Menon, 1993), the predicted topology of PIG-A protein is in accordance with the notion that it directly participates in the transfer of GlcNAc to PI.

Using *PIG-A* cDNA as a probe, *PIG-A* genomic clones have been isolated and characterized (Iida *et al.*, 1994; Bessler *et al.*, 1994a). The *PIG-A* gene contains six exons and spans at least 17 kbp (Fig. 5) (Iida *et al.*, 1994; Bessler *et al.*, 1994a). Exon 1 (23 bp) encodes the 5′ untranslated region and exon 2 (777 bp) encodes the rest of it and about half of the protein. Exons 3 and 4 (133 bp each) and exon 5 (207 bp) encode parts of the protein and exon 6 (2316 bp) encodes the rest of the protein and the 3′ untranslated region. A region of 583 bp upstream of exon 1 had promoter activity (Iida *et al.*, 1994). There is no TATA-like sequence in this region but there are four CAAT boxes, two AP-2 sequences, and a CRE sequence (Iida *et al.*, 1994). By means of fluorescence *in situ* hybridization using genomic *PIG-A* clone as a probe, the *PIG-A* gene was localized to the short arm of X chromosome at Xp22.1 (Table II) (Takeda *et al.*, 1993; Bessler *et al.*, 1994a).

Analyses of *PIG-A* transcripts have demonstrated that the original cDNA of 4568 nt was full length (Iida *et al.*, 1994) and that there are two shorter

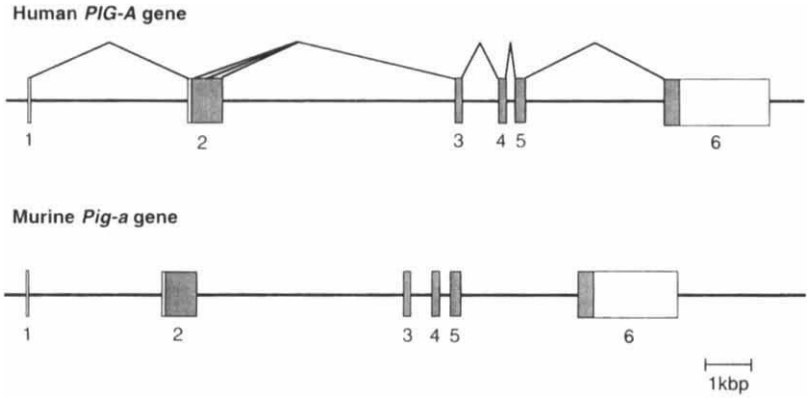

FIG. 5. Structures of the human *PIG-A* and mouse *Pig-a* genes. Boxes indicate exons. Shaded areas are coding regions. Lines connecting exons indicate introns. Splicing patterns of human *PIG-A* are indicated by thin lines.

products of alternative splicing (Miyata *et al.*, 1994; Bessler *et al.*, 1994d). One is 374 nt shorter due to splicing of the 3′ portion of exon 2 spanning from nt 342 to 715. The other is 658 nt shorter due to splicing of a bigger portion of exon 2 that spans from nt 58 to 715 (Fig. 5) (Miyata *et al.*, 1994; Bessler *et al.*, 1994d). At nt 342 and 58 there are potential 5′ splice sites, which are probably responsible for this alternative splicing. The truncated PIG-A peptides encoded by these shorter transcripts should be nonfunctional because the cDNA clones corresponding to them failed to restore the surface expression of CD59 when transfected into the JY5 cell line (Miyata *et al.*, 1994).

TABLE II
CHROMOSOMAL LOCATION OF GPI SYNTHESIS GENES

Gene	Location
Human	
PIG-A	Xp22.1
PIG-F	2p16–p21
GPI-H	14q?
PIG-B	15q21–q22
Mouse	
Pig-a	XF3–F4
Pig-f	17E4–E5
Gpi-h	12

An intronless pseudogene of *PIG-A* was found and termed ψ*PIG-A* (Bessler *et al.*, 1994a). This pseudogene extends over 3376 bp with 91% nucleotide identity to *PIG-A* and was mapped to chromosome 12 at 12q21 (Bessler *et al.*, 1994a).

2. *Mouse Class A Gene Termed Pig-a*

The mouse homologue of *PIG-A* was cloned using *PIG-A* cDNA as a probe and termed *Pig-a* (Kawagoe *et al.*, 1994). *Pig-a* is very similar to *PIG-A*, consisting of six exons spanning 16 kbp (Fig. 5). The predicted Pig-a protein is of 485 amino acids and it has 88% amino acid identity to PIG-A protein (Kawagoe *et al.*, 1994). *Pig-a* cDNA complemented both human and mouse class A mutants and vice versa (Kawagoe *et al.*, 1994; Miyata *et al.*, 1993), indicating that they are functionally interchangeable. *Pig-a* is also X-linked and mapped to X-F3/4 which is homologous to human Xp22.1 (Table II) (Kawagoe *et al.*, 1994; Ware *et al.*, 1994a). This is consistent with the predicted location (Hyman *et al.*, 1980) and accounts for the high frequency with which class A mutant cells are isolated.

3. *Yeast Homologue of PIG-A, SPT14*

A gene of *S. cerevisiae* termed *SPT14* has been isolated as a suppressor of the histidine auxotroph caused by a Ty insertion mutation (Fassler *et al.*, 1991). SPT14 has high similarity with PIG-A (45% amino acid identity) and must be a yeast homologue of PIG-A (Kawagoe *et al.*, 1994; Bessler *et al.*, 1994a). *SPT14* is an essential gene (Fassler *et al.*, 1991). Another yeast gene, *GPI1*, which is also involved in the first step of GPI anchor synthesis is also essential, indicating that the GPI anchor is essential for this unicellular eukaryote (Leidich *et al.*, 1994).

4. *Human Class H Gene Termed GPI-H*

A human cDNA that complements the GPI-anchoring defect of murine LM-TK$^-$ cells was isolated and termed *GPI-H* cDNA (Kamitani *et al.*, 1993). The predicted class H gene product is of 188 amino acids. There is no apparent amino terminal signal sequence and, although there are several hydrophobic regions, it is unclear whether there is a transmembrane domain (Kamitani *et al.*, 1993). GPI-H has no significant homology to any of the known glycosyltransferases and other genes. As described previously, classes A, C, and H share a defect in GlcNAc-PI synthesis, indicating that three genes are involved in the first step of the pathway. Among them PIG-A seems to directly participate in the reaction. Whether GPI-H directly participates in the reaction is unknown. The class C gene has not been cloned.

Using human *GPI-H* cDNA as a probe, the murine homologous gene *Pigh* has been mapped to chromosome 12 between *D12Nyu3* and *D12Nyu4* (Ware *et al.*, 1994a). This predicts that the human *GPI-H* gene would be on the long arm of chromosome 14 (Table II) (Ware *et al.*, 1994a).

5. *Human Class B Gene Termed PIG-B*

A human cDNA that restores the defect of class B murine thymoma cells has been isolated and termed *PIG-B* cDNA (Kinoshita *et al.*, 1994). The cDNA of 1929 bp encodes a predicted PIG-B protein of 554 amino acids. *PIG-B* cDNA restored transfer of the third Man in class B cells. Whether PIG-B protein is a Man transferase is yet to be determined. A genomic *PIG-B* clone has been isolated and used as a probe to determine the chromosomal location of *PIG-B*. It was mapped to the long arm of chromosome 15 at 15q21–q22 (Table II) (K. Ohishi, unpublished data).

6. *Human Class F Gene Termed PIG-F*

A 1-kbp human cDNA that restores surface Thy-1 expression on class F murine thymoma cells has been isolated and termed *PIG-F* cDNA (Inoue *et al.*, 1993). The cDNA encodes a predicted protein of 219 amino acids. There is no apparent amino terminal signal peptide sequence. The protein is very hydrophobic, suggesting that most of it is embedded in the membrane (Inoue *et al.*, 1993). The class F mutant cells have a defect in the EtNP transfer from PE to the third Man (Fig. 2). Therefore, PIG-F protein may be an EtNP transferase, but a direct demonstration of its enzymatic activity is required to confirm this notion. The hydrophobic nature of the protein is consistent with PE being a donor of EtNP in this enzymatic reaction.

Using *PIG-F* cDNA as a probe, genomic *PIG-F* clones have been obtained and analyzed (Ohishi *et al.*, 1995). The *PIG-F* gene consists of six exons spanning about 40 kbp with exons 2–6 encoding the protein. *PIG-F* was mapped to the short arm of chromosome 2 at 2p16–p21 (Table II). An intronless pseudogene of *PIG-F* termed ψ*PIG-F* was also identified and mapped to 5q35 by fluorescence *in situ* hybridization (Ohishi *et al.*, 1995).

Using *PIG-F* cDNA as a probe, mouse homologue of *PIG-F*, termed *Pig-f* has been mapped to chromosome 17 close to *Mrp-rs2* locus (Ware *et al.*, 1994a). This region is homologous with the short arm of human chromosome 2, consistent with the above result. We mapped *Pig-f* to 17E4–E5 (Table II) (Ohishi, K., unpublished data).

7. *Dol-P-Man Synthase Gene*

A gene for Dol-P-Man synthase was cloned from yeast *S. cerevisiae* and termed *DPM1* (Orlean *et al.*, 1988). This gene encodes a predicted protein

of 267 residues (Orlean *et al.*, 1988). *DPM1* expressed in Dol-P-Man synthase-deficient murine cells complemented Dol-P-Man and GPI anchor synthesis defects and surface Thy-1 expression (DeGasperi *et al.*, 1990). Its expression in Dol-P-Man synthase-deficient Chinese hamster ovary cells complemented defective Dol-P-Man synthesis and N-linked oligosaccharides (Beck *et al.*, 1990). The mammalian Dol-P-Man synthase gene has not been isolated.

III. Paroxysmal Nocturnal Hemoglobinuria (PNH)

A. PNH, A Hematopoietic Stem Cell Disorder with the Major Symptom Being Hemolytic Anemia

PNH is an anemia caused by complement-mediated hemolysis. Blood from patients with PNH contains erythrocytes that are abnormally sensitive to complement. Constant, low-level activation of the complement alternative pathway, which is presumably elevated during sleep, and heightened activation of the classical and alternative pathways during or after infection would cause intravascular lysis of those abnormal erythrocytes and lead to hemoglobinuria. Hence, the disease is called paroxysmal nocturnal hemoglobinuria. The condition was initially noted by Gull in 1866 and later described by Strubing in 1882.

PNH is an acquired, uncommon but not rare disease, occurring at a rate of 1 in 100,000–500,000 (Rotoli and Luzzatto, 1989). Its frequency seems to vary geographically, being more common in Asia than in Europe and North America (Dameshek, 1967). It occurs equally in both sexes in Europe, North America, and Japan (Sirchia and Lewis, 1975) but three to five times more frequently in males in China and Thailand (Kruatrachue *et al.*, 1978; Le *et al.*, 1990). It is a disease of middle age (Sirchia and Lewis, 1975) but it can also occur in children (Ware *et al.*, 1991).

It is a hematopoietic stem cell disorder because not only erythrocytes, but also all other hematopoietic lineage cells show abnormalities in complement interaction and others (see below and Yomtovian *et al.*, 1993). Patients with PNH have other clinical problems, such as venous thrombosis (a leading cause of death in European and American patients), development of acute myelogenous leukemia, and hematopoietic deficiencies in addition to hemolysis (reviewed in Rosse *et al.*, 1985; Rotoli and Luzzatto, 1989; Rosse, 1992). These problems may be related to the abnormal hematopoietic stem cells.

When erythrocytes are incubated with human serum that has been adjusted to pH 6.4, the alternative pathway of complement is activated on the cell surface. Erythrocytes from normal individuals are resistant to this manipulation, whereas a fraction of those from patients with PNH is

hemolyzed (Hijmans van den Bergh, 1911; Ham and Dingle, 1939). This is called Ham's test or the acidified serum lysis test and it is used to diagnose PNH. The sucrose hemolysis and sugar water tests which also detect erythrocytes that are abnormally sensitive to complement are also used for diagnostic purposes (Hartmann and Jenkins, 1966).

When erythrocytes are sensitized with antibody and incubated with increasing amounts of human serum as a source of complement, the classical pathway is activated on the cell surface. Erythrocytes from normal individuals resist the action of complement and are lysed only with very high concentrations of it. In contrast, erythrocytes from patients with PNH become lysed at low concentrations and usually show bimodal titration curves, indicating that a fraction of erythrocytes is abnormally sensitive to complement and that the remainder has normal sensitivity (Rosse and Dacie, 1966a,b). Abnormally sensitive cells are lysed with a 25-fold lower concentration of complement (Rosse and Dacie, 1966b). This test reveals the presence of two discrete populations of erythrocytes in patients with PNH and is referred to as the complement lysis sensitivity test (CLS test). It can also reveal a cell population of intermediate sensitivity in some patients. This population is lysed with a 5-fold lower concentration of complement (Rosse, 1973). The very and moderately sensitive populations are called type III and type II PNH cells, respectively, and the cells with normal sensitivity are called type I PNH cells (Rosse *et al.*, 1974).

B. Deficiencies in Complement Regulatory Proteins, CD59 and DAF, in PNH

1. Self–Nonself Discrimination in the Complement System

Complement plays an important role in eliminating microorganisms and immune complexes. Its three major functions are targeting them to cells bearing complement receptors, recruiting phagocytic cells to the area where complement is being activated, and the destruction of target membranes (Kinoshita, 1991). Activation of complement should be focused on the target surfaces because these effector functions mediated by C3 and C5 convertases and membrane-attack complexes (MAC) are harmful to the host cells. However, active fragments of complement proteins or their complexes, such as C4b, C3b, and C5b–7, inherently bind to host cell surfaces and lead to host cell damage. This danger is circumvented by widely distributed membrane-bound complement regulatory proteins that inhibit C3 and C5 convertases and MAC on host cell surfaces.

There are three complement regulatory proteins that primarily protect host cells from the action of complement. DAF (CD55) (Nicholson-Weller *et al.*, 1981; Medof *et al.*, 1984) and membrane cofactor protein (MCP or

CD46) (Seya *et al.*, 1986) inhibit C3 and C5 convertases (reviewed in Lublin and Atkinson, 1989 and Liszewski *et al.*, 1991), and CD59 (Davies *et al.*, 1989) inhibits MAC (reviewed in Holguin and Parker, 1992; Walsh *et al.*, 1992; Meri, 1994). CD59 is also called MACIF (Sugita *et al.*, 1988, 1989a,b), HRF20 (Okada *et al.*, 1989a,b), MIRL (Holguin *et al.*, 1989a), Protectin (Meri *et al.*, 1990), MEM-43 (Stefanova *et al.*, 1989), or H19 (Groux *et al.*, 1989). All of them are expressed on a wide variety of cells in contact with complement (Lublin and Atkinson, 1989; Liszewski *et al.*, 1991; Holguin and Parker, 1992; Walsh *et al.*, 1992). Erythrocytes express DAF and CD59 but not MCP. All other hematopoietic cells, platelets, and vascular endothelial cells express all three. DAF (Davitz *et al.*, 1986; Medof *et al.*, 1986; Walter *et al.*, 1990) and CD59 (Okada *et al.*, 1989a; Davies *et al.*, 1989; Holguin *et al.*, 1990; Sugita *et al.*, 1989b) are GPI-anchored proteins and MCP is a conventional transmembrane protein (Liszewski *et al.*, 1991). Complement fragments and their complexes bound to the host cell surfaces do not usually result in cell damage due to these complement regulatory proteins. In contrast, those bound to the surface of microorganisms where such complement regulatory proteins are not expressed are more stable and accomplish their effector functions.

2. *Deficiencies in Complement Regulatory Proteins, CD59 and DAF, on Erythrocytes*

Abnormal red cells from patients with PNH are sensitive to the action of complement because they cannot destabilize C3 convertases (Pangburn *et al.*, 1983b; Parker *et al.*, 1985; Pangburn and Walter, 1987) and they are also sensitive to reactive lysis, which involves C5–C9 (Packman *et al.*, 1979; Rosenfeld *et al.*, 1985, 1986; Parker *et al.*, 1985; Hu *et al.*, 1985). The molecular basis of the former phenotype of the abnormal red cells was clarified by the discovery of a DAF deficiency on these red cells (Pangburn *et al.*, 1983a; Nicholson-Weller *et al.*, 1983; Rosse, 1991). The latter phenotype proved to be due to a lack of CD59 (Holguin *et al.*, 1989a,b; Okada *et al.*, 1989a; Rosse, 1991).

Purified DAF spontaneously reincorporates into the erythrocyte membrane and expresses function (Medof *et al.*, 1984). The reincorporation of DAF into red cells from patients with PNH reversed their defective regulation of C3 convertases but not their sensitivity to reactive lysis and partially restored their resistance to classical pathway-mediated lysis, showing that deficient DAF expression is causally related to lytic abnormalities of erythrocytes from patients with PNH (Medof *et al.*, 1985, 1987). Purified CD59 also spontaneously reincorporated into the membrane of erythrocytes from patients with PNH and thereby inhibited reactive lysis, demonstrating that

a CD59 deficiency is also causally related to their lytic abnormality (Holguin *et al.*, 1989a).

As described previously, erythrocytes from patients with PNH consist of those sensitive and resistant to complement. A fluorescence-activated cell sorter (FACS) analysis of patient erythrocytes using anti-DAF antibodies has demonstrated DAF-deficient and -sufficient cell populations (Kinoshita *et al.*, 1985; Nicholson-Weller *et al.*, 1985). Upon incubation with acidified serum, DAF-deficient cells were selectively lysed, indicating that the cells sensitive to complement are DAF deficient (Kinoshita *et al.*, 1985). DAF-deficient erythrocytes are also deficient in CD59 (Holguin *et al.*, 1989b; Shichishima *et al.*, 1991). Most patients have erythrocytes that totally lack DAF and CD59. Some patients have erythrocytes expressing low levels of DAF and CD59. These variations were correlated to the complement sensitivity of the cells (Shichishima *et al.*, 1991, 1993b; Rosse *et al.*, 1991). Type III cells were completely deficient in CD59 and completely or almost completely deficient in DAF, whereas type II cells were partially deficient in both or completely deficient in DAF with a low level of CD59 (Shichishima *et al.*, 1991; Rosse *et al.*, 1991). Therefore, two-color FACS analysis for DAF and CD59 is an excellent measure of complement sensitivity of red cells from patients with PNH (Shichishima *et al.*, 1993b). Erythrocytes with normal resistance (type I) but with a subnormal level of DAF have been found in many patients (Medof *et al.*, 1987; Ninomiya *et al.*, 1988). Whether they are abnormal or normal cells suffering from an event that causes a decrease of DAF is unknown.

3. *Relative Importance of DAF and CD59 in Protecting Erythrocytes from Lysis by Complement*

CD59 seems to be more important than DAF in protecting erythrocytes from lysis mediated by serum. As described previously, sensitivity to the CLS test of abnormal erythrocytes from patients with PNH correlated well with the extent of the CD59 deficiency (Shichishima *et al.*, 1993b). Reincorporation of DAF to DAF- and CD59-negative erythrocytes from patients with PNH partially restored their resistance to acidified serum, whereas reincorporation of CD59 completely restored it (Wilcox *et al.*, 1991). A patient with a genetic deficiency of CD59 due to homozygous mutant CD59 gene had hemolytic anemia similar to that of PNH (Yamashina *et al.*, 1990; Motoyama *et al.*, 1992). This patient had a normal level of DAF expression (Ono *et al.*, 1990; Taguchi *et al.*, 1990; Yamashina *et al.*, 1990). Reincorporation of CD59 into erythrocytes from this patient completely restored resistance (Okada *et al.*, 1989c). In contrast, patients with a genetic deficiency of DAF have no hemolytic disease (Telen *et al.*,

1988; Merry *et al.*, 1989; Lublin *et al.*, 1994). Thus, CD59 is essential for protecting erythrocytes *in vivo* and DAF is important when CD59 is absent.

4. *Deficiencies in DAF and CD59 on Other Blood Cells*

DAF and CD59 are expressed on all blood cells. DAF-deficient populations have been found in all hematopoietic lineages, namely DAF-deficient granulocytes, monocytes, platelets, and T, B, and NK lymphocytes have been found in blood samples from patients with PNH (Kinoshita *et al.*, 1985; Nicholson-Weller *et al.*, 1985; Ueda *et al.*, 1989; Schubert *et al.*, 1990; Devine *et al.*, 1987b). A CD59-deficient population has also been found in other hematopoietic lineages (Schubert *et al.*, 1990; Mahoney *et al.*, 1992; Hillmen *et al.*, 1993b; Terstappen *et al.*, 1993). A DAF deficiency has been identified in hematopoietic progenitor cells, such as CFU-E, BFU-E, CFU-GM, and CFU-mix (Kanamaru *et al.*, 1988; Okuda *et al.*, 1990a,b). That megakaryocytic (Dessypris *et al.*, 1988), myeloid (Dessypris *et al.*, 1983), and erythroid (Dessypris *et al.*, 1983; Shichishima *et al.*, 1989) progenitors contain cells sensitive to complement indicates DAF and/or CD59 deficiency. There is no evidence that DAF and CD59 deficiency exists on nonhematopoietic cells such as vascular endothelial cells. Therefore, an abnormality causes DAF and CD59 deficiencies in multipotential hematopoietic stem cells. Indeed, a major fraction of the $CD34^+$,$CD38^-$ population that contains most of the multipotential hematopoietic stem cells (Terstappen *et al.*, 1991) is DAF and CD59 deficient in patients with PNH (Terstappen *et al.*, 1993).

Granulocytes and monocytes usually contain high percentages of deficient cells (Plesner *et al.*, 1990; van der Schoot *et al.*, 1990). Lymphocytes usually contain a low percentage of deficient cells (van der Schoot *et al.*, 1990; Nagakura *et al.*, 1993; Hillmen *et al.*, 1993b). Since abnormal erythrocytes are selectively lysed by complement, the percentage of deficient erythrocytes greatly decreases during hemolytic attack and sometimes the Ham test becomes negative (Plesner *et al.*, 1990). Granulocytes are more resistant to complement due to the expression of MCP and other mechanisms such as the release of membrane blebs containing MAC. Although the latter mechanism is also deficient in affected PNH cells (Whitlow *et al.*, 1993), they still express MCP so that the percentage of deficient granulocytes does not decrease during hemolytic attack. Thus, FACS analysis of granulocytes as well as erythrocytes would be important for diagnosis (Plesner *et al.*, 1990; van der Schoot *et al.*, 1990).

C. Defective GPI Anchor Synthesis—Biochemical Basis of PNH

Deficiencies in acetylcholinesterase on red cells (Auditore *et al.*, 1960; Kunstling and Rossi, 1969) and alkaline phosphatase on neutrophils (Beck

and Valentine, 1951; Lewis and Dacie, 1965) were known for a long time before the demonstration of a DAF deficiency. DAF-deficient red cells are also acetylcholinesterase deficient (Medof *et al.*, 1987; Chow *et al.*, 1986) and DAF-deficient neutrophils are alkaline phosphatase deficient (Burroughs *et al.*, 1988). Later, these enzymes (Low and Zilversmit, 1980; Roberts and Rosenberry, 1986; Haas *et al.*, 1986) and DAF (Davitz *et al.*, 1986; Medof *et al.*, 1986) were found to be GPI anchored to the membrane. Northern analyses of transcripts of DAF (Stafford *et al.*, 1988) and alkaline phosphatase (Rambaldi *et al.*, 1989) and Southern analysis of the DAF gene (Stafford *et al.*, 1988) in the affected neutrophils from patients with PNH did not show an abnormality. DAF peptide is synthesized in the affected neutrophils (Carothers *et al.*, 1990). These observations led to the notion that the basis of the deficiency of multiple surface proteins must be either deficient biosynthesis of the GPI anchor or a failure in its attachment (Carothers *et al.*, 1990). Consistent with this notion, deficiencies of many other GPI-anchored proteins have been demonstrated (Selvaraj *et al.*, 1987,1988; Huizinga *et al.*, 1988; Haziot *et al.*, 1988; Simmons *et al.*, 1989; Zalman *et al.*, 1987; Hansch *et al.*, 1987; Ramalho Pinto, 1987; Blaas *et al.*, 1988; Schubert *et al.*, 1990; Ploug *et al.*, 1992; Nagakura *et al.*, 1993; van der Schoot *et al.*, 1990; Bobolis *et al.*, 1992). To date at least 17 proteins are known to be deficient in various blood cell types (Table I).

Analyses of GPI anchor synthesis in the affected neutrophils have demonstrated that precursor GPI anchor is indeed not synthesized (Hirose *et al.*, 1992c; Mahoney *et al.*, 1992). Since neutrophils from patients contain a mixture of affected and normal cells, the analyses were performed with samples from patients with over 90% affected cells or samples enriched in affected cells by affinity chromatography on Sepharose coupled with anti-DAF antibodies. The faulty reaction step(s) was localized after affected lymphocyte cell lines were established from patients with PNH (Ueda *et al.*, 1992; Hillmen *et al.*, 1993b; Schubert *et al.*, 1993). Studies of these cell lines demonstrated that the first step of GPI anchor synthesis is defective (Armstrong *et al.*, 1992; Takahashi *et al.*, 1993; Hillmen *et al.*, 1993c; Hidaka *et al.*, 1993; Schubert *et al.*, 1993); that is, lysates of the affected cells could not synthesize GlcNAc-PI. This was later confirmed using affected neutrophils (Norris *et al.*, 1994). Results of an initial study with neutrophils that suggested that early steps are intact (Hirose *et al.*, 1991) were probably due to admixture of normal neutrophils (Takahashi *et al.*, 1993).

At least three genes are involved in the first reaction step (Fig. 2). To determine which one is defective in the affected PNH cell lines, they were analyzed by somatic cell hybridization. These cell lines complemented classes C and H, but not class A mutants on cell fusion (Takahashi *et al.*,

1993; Armstrong *et al.*, 1992), indicating that the gene defective in these PNH cells corresponds to the gene that is mutated in the class A mutants. All cell lines from six patients belonged to class A (Takahashi *et al.*, 1993; Armstrong *et al.*, 1992). This suggested that the defective step is common among most, if not all, patients. Indeed, the somatic cell hybridization analysis extended to 20 other patients using affected neutrophils demonstrated that all of them corresponded to class A (Norris *et al.*, 1994).

D. Demonstration That the *PIG-A* Gene is Responsible for PNH—Genetic Basis of PNH

The biochemical and cytogenetic analyses described previously indicated that the gene responsible for PNH is the *PIG-A* gene. Transfection of *PIG-A* cDNA into the affected cell lines from two patients restored the surface expression of CD59 and DAF as expected (Takeda *et al.*, 1993). Northern blotting demonstrated that the two affected cell lines from one patient (TK-1$^-$ and T-14$^-$ cell lines) had very low levels of *PIG-A* mRNA: almost completely lacking in TK-14 and about 10% of normal level in TK-1$^-$ (Takeda *et al.*, 1993). In the SS-1$^-$ cell line, abnormally short *PIG-A* mRNA was synthesized. Analysis of this aberrant transcript by RT-PCR and sequencing demonstrated that the 207-nt exon 5 was deleted (Takeda *et al.*, 1993). Southern analysis of the *PIG-A* gene in this cell line and the wild-type cell line SS-2$^+$ established from the same patient did not reveal any abnormalities, so abnormal splicing was suspected. Analysis of PCR-amplified exon–intron junctions demonstrated a deletion of T in the 5′ splice site adjacent to exon 5. This is a somatic mutation because the wild-type cell line SS-2$^+$ did not have this mutation (Takeda *et al.*, 1993). This mutation should account for the abnormal splicing of exon 5, because in other genetic diseases, mutations in 5′ splice sites are always associated with abnormal splicing of adjacent exons (Krawczak *et al.*, 1992).

Further analysis demonstrated that a normal allele of the *PIG-A* gene was present in SS-1$^-$ cells, indicating that the mutant allele was selectively transcribed (Takeda *et al.*, 1993). This patient was female, so this was consistent with the X-chromosomal location of the *PIG-A* gene (Takeda *et al.*, 1993; Bessler *et al.*, 1994a; Ware *et al.*, 1994a). Since one of the X chromosomes is inactivated in female cells (Lyon, 1968), the mutant and normal alleles should be on the active and inactive X chromosome, respectively. The X-chromosomal location of the *PIG-A* gene accounted for the phenotypic expression of the recessive-type mutation (Hillmen *et al.*, 1993a). These results demonstrated that *PIG-A* is the responsible gene for PNH (Takeda *et al.*, 1993).

E. Clonal Origin of the Affected PNH Cells

PNH is an acquired disease affecting a fraction of hematopoietic cells. Clonality of the affected PNH erythrocytes was shown 25 years ago by

an analysis of the X-linked glucose-6-phosphate dehydrogenase (G6PD) isozymes in a female patient who was heterozygous for the isozymes (Oni *et al.*, 1970). Only the B-type G6PD was liberated from her red cells by complement-mediated lysis (derived from the affected PNH cells), whereas that liberated by hypotonic lysis (derived from total red cells) contained both types, indicating that the affected PNH red cells are of clonal origin (Oni *et al.*, 1970). Affected granulocytes are also of clonal origin, as demonstrated by analyzing X-linked gene methylation (Josten *et al.*, 1991; Bessler *et al.*, 1992; Ohashi *et al.*, 1994).

Analysis of the affected granulocytes from patient SS showed that they have the same mutation as that found in the SS-1$^-$ cell line which is a B-lymphoblastoid cell line established from the same patient (Takeda *et al.*, 1993). Therefore, the affected granulocytes and B lymphocytes were of the same clonal origin and a hematopoietic stem cell clone that gave rise to these cell types must have harbored the mutation.

F. The *PIG-A* Gene is Responsible for PNH in All Patients Characterized to Date

GPI anchor synthesis involves 10 or so genes (Fig. 2). A lack of any one of them would result in GPI anchor deficiency. Whether or not the responsible gene for PNH is always *PIG-A* was an immediate question. As described previously, cytogenetic analyses of the affected cells from some 30 patients demonstrated that they all belonged to the class A complementation group (Takahashi *et al.*, 1993; Armstrong *et al.*, 1992; Norris *et al.*, 1994), indicating that *PIG-A* is always responsible. This was proven by analyses of *PIG-A* at the DNA and/or RNA levels in affected cells from many patients (Miyata *et al.*, 1994; Bessler *et al.*, 1994b,c,d; Ware *et al.*, 1994b; Yamada *et al.*, 1995). Analyses of the affected neutrophils from 18 Japanese patients demonstrated abnormalities of *PIG-A* in all of them: 3 had abnormal splicing, 1 lacked mRNA, and the rest had a mutation within the coding region (Miyata *et al.*, 1994; Yamada *et al.*, 1995). Analyses of the affected B cell lines from 9 European patients (Bessler *et al.*, 1994b,c,d) and of the affected neutrophils from 4 American (Ware *et al.*, 1994b) and 10 European (M. Bessler and L. Luzzatto, unpublished results) patients demonstrated a somatic mutation of *PIG-A* in all of them. Neutrophils from all of 14 Thai patients also contained somatic *PIG-A* mutations (P. Pramonjago *et al.*, 1995). Therefore, *PIG-A* is responsible for PNH in all patients from various countries.

The simplest and most likely explanation for this uniformity of the responsible gene is that only *PIG-A* is X-linked and all other genes are autosomal. The three other genes characterized to date are indeed autosomal (Table II). For an autosomal gene to cause a GPI anchor deficiency, inactivating mutations would have to occur in both alleles. However, the

likelihood of this would be extremely rare. For the X-linked *PIG-A*, one inactivating mutation results in a GPI anchor deficiency in males as well as females.

G. Somatic Mutations of the *PIG-A* Gene

1. Analysis of Somatic Mutations in the PIG-A Gene

a. Cell Type. The responsible mutation can be identified in DNA from any cell in patients with inherited genetic diseases because all somatic cells are genetically equal with a few exceptions, such as immunoglobulin and T cell receptor genes. RNA, however, can only be analyzed in cells that express a target gene. In contrast, somatic mutations of the *PIG-A* gene occur in the hematopoietic stem cell clone(s) and, hence, only a fraction of hematopoietic cells bear the mutation. Since the proportion of mutant cells is relevant to mutation analysis, the cell type is important for analysis. If cell lines bearing the PNH phenotype are available, they are of course very useful. However, they are not always available. Granulocytes have proven to be the best cell type for mutation analysis because they usually contain a higher percentage of the mutant cells than lymphocytes (Plesner *et al.*, 1990; van der Schoot *et al.*, 1990) and because large numbers of cells are available. In some patients, most of their granulocytes are mutants, so simultaneous analysis of mononuclear cells is helpful to show that the mutation found in the granulocytes is somatic rather than inherited.

b. Processing of Blood Samples. Granulocytes and mononuclear cells are prepared from heparinized blood. The percentages of CD59- and DAF-deficient cells are determined by FACS analysis using small proportions of those cells. DNA is prepared from each cell population. When a blood sample is fresh enough (within 24 hr of withdrawing blood), RNA should also be prepared because the mRNA can provide information on the consequences of mutation, such as abnormal splicing and decreased levels of mRNA. In addition, an exonic mutation of the *PIG-A* gene in female cells is easier to identify using RNA than DNA because only the mutant allele is transcribed due to its X-chromosomal location.

c. Analysis of PIG-A mRNA (Miyata et al., *1994; Yamada* et al., *1995).* The coding region of the *PIG-A* mRNA was amplified by RT-PCR. Agarose gel electrophoresis (Fig. 6) showed that samples from normal individuals contain three bands (lanes N1 and N2). The top band of 1500 bp corresponds to the full-length coding region. The 1250- and 850-bp bands are products of alternative splicing as described previously (Fig. 5). Some patients had abnormally short products (patient Nos. 16, 1, and 2 shown in lanes 16, 1, and 2, respectively). Regions missing from

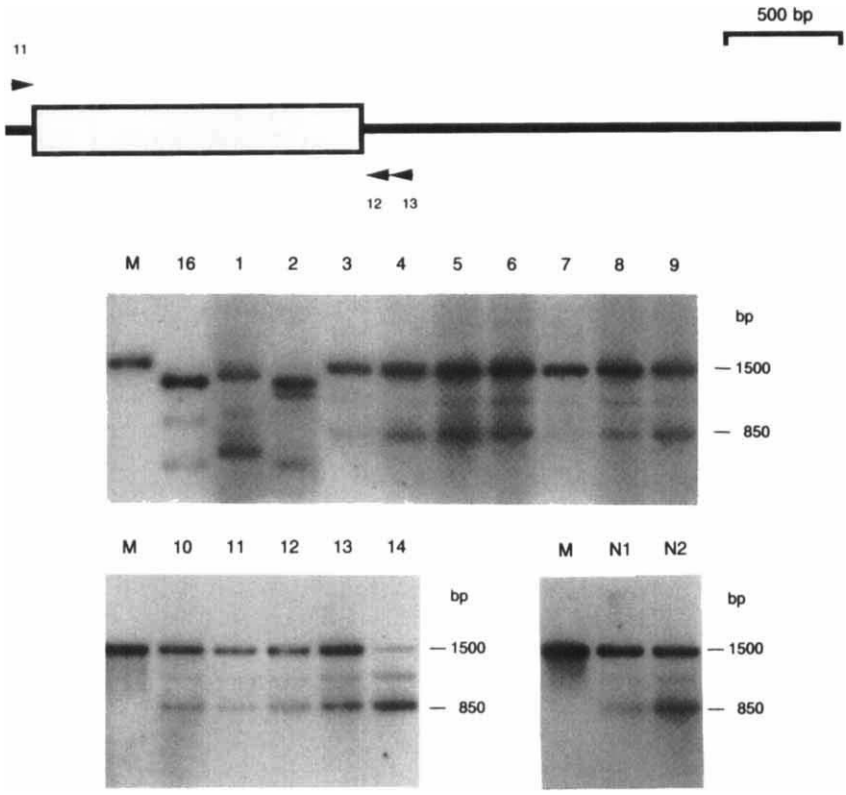

FIG. 6. RT-PCR analysis of *PIG-A* mRNA of granulocytes from patients with PNH. *PIG-A* cDNA is schematically shown at the top. The box indicates the coding region. To analyze *PIG-A* mRNA, a region containing the coding region was reverse transcribed using total RNA from granulocytes as the template and primer 13, then amplified by PCR with primers 11 and 12. The products were resolved by agarose gel electrophoresis as shown. Lanes N1 and N2, samples from normal individuals; lanes 1–14 and 16, samples from patients J1–J14 and J16, respectively (see Table III for mutations); lane M, amplification products from authentic *PIG-A* cDNA [from Miyata *et al.* (1994)].

these bands that were determined by their removal from the gel, cloning, and nucleotide sequencing corresponded to exons. The mutations causing these abnormal transcripts were determined by analyzing the DNA (see below). Many patients (lanes 3–13) seemed to have normal profiles. However, the apparently normal bands were a mixture of normal cDNA and mutant cDNA bearing a small mutation (Miyata *et al.*, 1994). A typical approach to mutations is that a region containing a mutation is first deter-

mined by either heteroduplex or single-strand conformation polymorphism analysis. For heteroduplex analysis, the coding region was amplified in seven overlapping regions (Yamada *et al.*, 1995). Samples from patients with more than 80% affected granulocytes were mixed with normal samples prepared in the same manner and analyzed. Those from patients with less than 80% affected granulocytes were analyzed without mixing. A fragment bearing the mutation was cloned and sequenced (Yamada *et al.*, 1995). If mutations are undetectable by either means, cDNA of the entire coding region can be cloned into a mammalian expression vector and transfected into PIG-A-deficient class A mutant cells to assess functional activity (Miyata *et al.*, 1994). Nonfunctional mutant clones identified in this manner are then sequenced to determine the mutation (Miyata *et al.*, 1994). Mutations found in mRNA are confirmed by analyzing the DNA.

d. Analysis of PIG-A DNA. The coding sequence is contained in five exons (Fig. 5). If a mutation is determined in RNA, the region containing it is amplified by PCR and the mutation is confirmed by restriction enzyme digestion, heteroduplex analysis, or sequencing. If a mutation is unknown, regions containing the coding sequence and splice sites are amplified by PCR (Iida *et al.*, 1994) and screened for the mutation by heteroduplex analysis. Female samples can be analyzed directly because even 100% of the affected granulocytes contain normal allele. Male samples from patients with more than 80% affected granulocytes are analyzed after mixing with normal samples. If a region bearing a mutation is identified, the fragment is cloned and the clones are sequenced.

2. *Nature of Somatic Mutations in the PIG-A Gene Found in Patients with PNH*

Sixty-two somatic mutations have been identified in patients with PNH (Table III). Except for one 4-kb deletion found in the British patient HH22, all of them are small mutations. They are heterogeneous and widely distributed in the coding regions and splice sites (Fig. 7), indicating that they occurred at random sites. Therefore, there is no mutation cluster region or mutation hot spot within the *PIG-A* gene.

Of 62 mutations, there are 21 single base deletions, 20 single base substitutions, and 7 single base insertions. Fourteen others include six examples of 2–10 base deletions, one 4-kb deletion, three examples of 2–5 base insertions, and four examples of the substitution of 2–6 bases with 1. Therefore, only 1 or 2 bases are involved in most mutations (56/62). Large deletions or gross rearrangements involving the *PIG-A* gene have not been found. These events may be rare in the hematopoietic stem cells of patients with PNH, or a big deletion might have critical effects on

TABLE III
SOMATIC MUTATIONS OF PIG-A FOUND IN PATIENTS WITH PNH

			Nucleotide Change		Consequence				
No.	Patient	Sex	Nucleotides		Codons			Location	Reference
1	J1	F	Intron 3,3′ splice site A to G	Base substitution		Abnormal splicing	Exon 4 deletion	Japan	Yamada *et al.* (1995)
2	J2	M	Intron 5,5′ splice site G to A	Base substitution		Abnormal splicing	Exon 5 deletion	Japan	Yamada *et al.* (1995)
3	J3	F	383 A to G	Base substitution	128	Missense	His to Arg	Japan	Yamada *et al.* (1995)
4	J4	F	298 C to T	Base substitution	100	Nonsense	Gln to stop	Japan	Yamada *et al.* (1995)
5	J5	M	1309 C (CTC to TC)	Single base deletion	437	Frameshift		Japan	Yamada *et al.* (1995)
6	J6	M	572 A	Single base deletion	191	Frameshift		Japan	Yamada *et al.* (1995)
7	J7	F	383 A to G	Base substitution	128	Missense	His to Arg	Japan	Yamada *et al.* (1995)
8	J8	M	1291 T to A	Base substitution	431	Nonsense	Leu to stop	Japan	Yamada *et al.* (1995)
9	J9	F	246 A (TAC to TAAC)	Single base insertion	82	Frameshift		Japan	Miyata *et al.* (1994)
10	J11	M	408 T	Single base deletion	136	Frameshift		Japan	Miyata *et al.* (1994)
11	J12	M	936 A	Single base deletion	312	Frameshift		Japan	Yamada *et al.* (1995)
12	J14	M	336 GC to T	Deletion/substitution	112/113	Frameshift		Japan	Yamada *et al.* (1995)
13	J16 (SS)	F	Intron 5,5′ splice site T	Single base deletion		Abnormal splicing	Exon 5 deletion	Japan	Takeda *et al.* (1993)
14	J17	F	269 T (TA to TTA)	Single base insertion	90	Frameshift		Japan	Yamada *et al.* (1995)
15	J18	F	Intron 4,5′ splice site G to A	Base substitution		Abnormal splicing	Exon 4 deletion	Japan	Yamada *et al.* (1995)
16	J18	F	104 T to A	Base substitution	35	Missense	Ile to Lys	Japan	Yamada *et al.* (1995)
17	J19(TK)	M	987 T	Single base insertion	329	Frameshift		Japan	[a]
18	J19(TK)	M	338 T to C	Base substitution	113	Missense	Leu to Pro	Japan	[a]
19	J20	F	715 G	Single base deletion	239	Frameshift		Japan	[a]
20	J21	F	1195 GACCGGGTAT	Multiple base deletion	399–402	Frameshift		Japan	[a]
21	T1	M	1220 T	Single base deletion	407	Frameshift		Thailand	Pramoonjago *et al.* (1995)
22	T2	M	1234 C	Single base deletion	412	Frameshift		Thailand	Pramoonjago *et al.* (1995)
23	T3	M	1280 T	Single base deletion	427	Frameshift		Thailand	Pramoonjago *et al.* (1995)
24	T4	M	851 TGCGTCTTTT	Multiple base deletion	284–287	Abnormal splicing	Exon 4 deletion	Thailand	Pramoonjago *et al.* (1995)
25	T5	F	627 T	Single base deletion	209	Frameshift		Thailand	Pramoonjago *et al.* (1995)
26	T6	M	816 A	Single base deletion	272	Frameshift		Thailand	Pramoonjago *et al.* (1995)
27	T7	F	609 GT	Multiple base deletion	203	Frameshift		Thailand	Pramoonjago *et al.* (1995)
28	T8	M	743 C	Single base deletion	248	Frameshift		Thailand	Pramoonjago *et al.* (1995)
29	T9	M	451 TTC	Multiple base deletion	151		Phe deletion	Thailand	Pramoonjago *et al.* (1995)

30	T10	M	Intron 5,5′ splice site G to A	Base substitution		Abnormal splicing	Exon 5 deletion	Thailand	Pramoonjago *et al.* (1995)
31	T11	F	Intron 4,5′ splice site G to A	Base substitution		Abnormal splicing	Exon 4 deletion	Thailand	Pramoonjago *et al.* (1995)
32	T12	M	577 GTACT	Multiple base deletion	193/194	Frameshift		Thailand	Pramoonjago *et al.* (1995)
33	T13	M	404 T (GCC to GTCC)	Single base insertion	135	Frameshift		Thailand	Pramoonjago *et al.* (1995)
34	T14	M	897 T (TTA to TTTA)	Single base insertion	299	Frameshift		Thailand	Pramoonjago *et al.* (1995)
35	UPN115		889 A to G	Base substitution	297	Missense	Asn to Asp	U.S.A.	Ware *et al.* (1994)
36	UPN166		1323 CT	Multiple base deletion	441/442	Frameshift		U.S.A.	Ware *et al.* (1994)
37	UPN261		431 C	Single base deletion	144	Frameshift		U.S.A.	Ware *et al.* (1994)
38	UPN267		250 GT (ACC to GTACC)	Multiple base insertion	84	Frameshift		U.S.A.	Ware *et al.* (1994)
39	HH2	M	820 G	Single base deletion	274	Frameshift		Europe	[b]
40	HH4	M	782 A	Single base insertion	261	Frameshift		Europe	[b]
41	HH5	M	634 TAGAT	Multiple base insertion	212	Frameshift		Europe	Bessler *et al.* (1994d)
42	HH8	F	1309 C	Single base deletion	437	Frameshift		Europe	Bessler *et al.* (1994b)
43	HH8	F	336 GC to T	Deletion/substitution	112/113	Frameshift		Europe	Bessler *et al.* (1994d)
44	HH12	F	548 G to T	Base substitution	183	Missense	Cys to Phe	Europe	[b]
45	HH13	F	142 G to A	Base substitution	48	Missense	Gly to Ser	Europe	[b]
46	HH15	F	1132 C	Single base deletion	378	Frameshift		Europe	[b]
47	HH16	M	464 C to T	Base substitution	155	Missense	Ser to Phe	Europe	Bessler *et al.* (1994d)
48	HH17	M	467 T to G	Base substitution	156	Missense	Leu to Arg	Europe	Bessler *et al.* (1994c)
49	HH18	M	163 C to T	Base substitution	55	Nonsense	Gln to stop	Europe	Bessler *et al.* (1994c)
50	HH20	F	1348 AT to C	Deletion/substitution	450	Frameshift		Europe	Bessler *et al.* (1994d)
51	HH22	F	183 T	Single base deletion	61	Frameshift		Europe	Bessler *et al.* (1994b)
52	HH22	F	4-kbp Deletion	Multiple base deletion		Frameshift	Exon 3–5 deletion	Europe	Bessler *et al.* (1994b)
53	HH24	M	189 A	Single base deletion	63	Frameshift		Europe	[b]
54	HH24	M	680 C	Single base deletion	227	Frameshift		Europe	[b]
55	HH27	M	770 T	Single base deletion	257	Frameshift		Europe	[b]
56	HH33	F	118 G	Single base deletion	40	Frameshift		Europe	[b]
57	HH40	F	298 C to T	Base substitution	100	Nonsense	Gln to stop	Europe	[b]
58	HH50	F	384 TAGTTC to A	Deletion/substitution	128–130	Frameshift		Europe	[b]
59	HH50	F	387 T to A	Base substitution	129	Missense	Ser to Arg	Europe	[b]
60	HH53	F	520 GT	Multiple base insertion	174	Frameshift		Europe	[b]
61	HH54	M	Intron 4,5′ splice site G to A	Base substitution		Abnormal splicing	Exon 4 deletion	Europe	[b]
62	HH54	M	1060 A	Single base insertion	354	Frameshift		Europe	[b]

[a] J. Nishimura, unpublished data.
[b] M. Bessler and L. Luzzatto, personal communications.

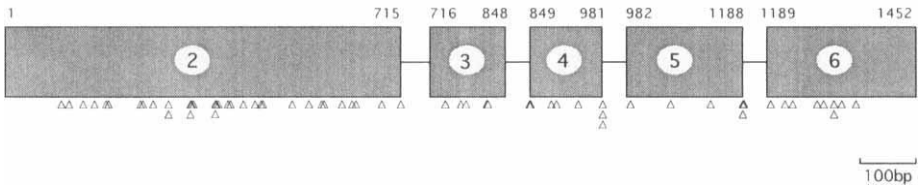

FIG. 7. Distribution of 61 somatic mutations in *PIG-A*. The sites of 61 somatic mutations of *PIG-A* are indicated. The coding regions are shown in boxes with exon numbers and introns are shown as lines connecting boxes. Nucleotide numbers are shown above the exons.

the cells since an essential gene is thought to be located near the *PIG-A* gene (Schaefer *et al.*, 1993).

Although a small somatic mutation in the *PIG-A* gene is a common characteristic of patients with PNH worldwide, the nature of the mutations seems to differ geographically. Nine of 20 mutations were base substitutions in Japan, whereas only 2 of 14 were base substitutions in Thailand. In European patients, 8 of 24 were base substitutions. The difference between Japanese and Thai patients is statistically significant ($P = 0.063$ as assessed by Fisher's exact probability test). Moreover, deletions of multiple bases are more frequent in Thailand (4 of 14) than in Japan (1 of 20). Therefore, somatic mutations occurring in the *PIG-A* gene differ regionally. These findings suggest that the mutations include those induced by different mutagens because the nature of spontaneous mutations should be common.

Five mutations have been found twice and one has been found three times. The same missense mutation at nt 383 has been found in two Japanese patients, J3 and J7 (Yamada *et al.*, 1995). The same nonsense mutation at nt 298 has been found in one Japanese (J4) and one European (HH40) patient. The GC dinucleotide was replaced by T at nt 336 in one Japanese (J14) and one European (HH8) patient (Yamada *et al.*, 1995; Bessler *et al.*, 1994d). The same single base deletion (nt 1309) has also been found in one Japanese (J5) and one European (HH18) patient (Yamada *et al.*, 1995; Bessler *et al.*, 1994d). A base substitution within the 5′ splice site adjacent to exon 5 has been found twice in one Thai (T10) (P. Pramoonjago *et al.*, 1995) and one Japanese (J2) patient (Yamada *et al.*, 1995) and another base substitution within the 5′ splice site adjacent to exon 4 has been found three times in one Thai (T11) (Pramoonjago *et al.*, 1995), one Japanese (J18) (Yamada *et al.*, 1995), and one European (HH54) (M. Bessler and L. Luzzatto, personal communications) patient. Some of these mutations common to two or three patients may have been caused by the same mechanism.

Mutations have not been found in the extreme 5′ 100 bases and the extreme 3′ 60 bases in the coding region (Fig. 7). Consistent with this, the sequence of the 3′ 60 bases is not conserved in humans and mice

(Kawagoe *et al.*, 1994) and it could be deleted from *PIG-A* cDNA without a reduction in activity (N. Inoue, unpublished results). The sequence of the 5′ 100 bases is not conserved in humans and mice either (Kawagoe *et al.*, 1994); however, frameshift mutations in this region would result in a loss of function. Thus, mutations will either be found in this 5′ region upon further analysis or there might be a mechanism that rescues inactivating mutations occurring in this region.

3. *The Consequence of Somatic Mutations in the PIG-A Gene*

Thirty-nine of 62 mutations (63%) caused a frameshift, the most prevalent mutation. There are 10 missense and 4 nonsense mutations, 8 examples of abnormal splicing, and 1 example of one amino acid deletion (Table III). All missense mutations occurred at residues conserved in the mouse homologue, Pig-a (Kawagoe *et al.*, 1994). Phe151, which was deleted in one patient, is also a conserved residue (Kawagoe *et al.*, 1994). Seven missense mutations and the one amino acid deletion were associated with a complete deficiency of GPI-anchored proteins, so these residues should be essential for the function of PIG-A protein. Three missense mutations are associated with a partial deficiency (Ware *et al.*, 1994b; Bessler *et al.*, 1994c). One of them occurring at nt 338 is close to an alternative 5′ splicing site (nt 342/343) and was associated with a reduction of full-sized mRNA, suggesting that the missense mutation affected alternative splicing (J. Nishimura, unpublished data). Whether PIG-A protein coded for by the full-sized mRNA with the missense mutation has reduced activity is not known. Two other residues involved in missense mutations with a partial deficiency should be important for function of PIG-A.

There are eight abnormal splicing events that caused a deletion of one of the exons (Table III). Six mutations were within the 5′ splice sites, causing a deletion of the immediately upstream exon (Takeda *et al.*, 1993; Yamada *et al.*, 1995). One mutation was within the 3′ splice site, causing a deletion of the immediately downstream exon (Yamada *et al.*, 1995). They are typical consequences of splice site mutations (Krawczak *et al.*, 1992). In one example, a 10-bp-deletion very close to the 5′ end of exon 4 was associated with the splicing out of exon 4 (Pramoonjago *et al.*, 1995). As described previously, one missense mutation (nt 338) near the alternative 5′ splicing site (nt 342/343) was associated with a reduction of the full-sized mRNA. One frameshift mutation (replacement of GC dinucleotide with T) that has been found in two patients was at nt 336 near the same alternative splice site. This mutation was associated with the complete disappearance of the full-sized mRNA and a concomitant increase of one of the two shorter products of alternative splicing. In one analysis of an affected B-lymphoblastoid cell line, the amount of a product

of that splicing site was increased (Bessler *et al.*, 1994d), whereas in another analysis of granulocytes, the level of a shorter product that uses the upstream 5′ splice site at nt 58/59 was increased (lane 14 of Fig. 6) (Miyata *et al.*, 1994; Yamada *et al.*, 1995). Probably, these mutations close to the alternative splice site skewed the alternative splicing. Another possibility for the latter mutation is that a premature stop codon generated by the frameshift resulted in unstable mRNA (Bessler *et al.*, 1994d).

H. Monoclonal or Oligoclonal

Two independent PNH clones have been found in some patients. Two independent B-lymphoblastoid cell lines bearing the PNH phenotype have been established from 2 patients (Bessler *et al.*, 1994b). Both cell lines had a complete deficiency, but different mutations in the *PIG-A* gene, demonstrating independent clonality (patients HH8 and HH22 in Table III) (Bessler *et al.*, 1994b). In 1 patient, granulocytes contained two PNH clones, both of which had a complete deficiency and which together consisted 95% of the granulocytes (patient J18 in Table III) (Yamada *et al.*, 1995). Two different mutations have been found in granulocytes from 3 other patients (HH24, 50, and 54 in Table III). These 6 are among some 50 patients who had PNH cells with a complete deficiency, indicating that at least 10% of such patients had two PNH clones.

In another study, two independent cell lines established from the same patient (patient J19 in Table III) had different phenotypes: one had a complete deficiency of GPI-anchored proteins and the other had a partial deficiency (Ueda *et al.*, 1992; Takeda *et al.*, 1993) due to different mutations (J. Nishimura, unpublished data). It has been reported that one-fourth to one-third of patients with PNH have erythrocytes of both types II and III PNH (Shichishima *et al.*, 1991; Rosse *et al.*, 1991; Shichishima *et al.*, 1993b). It has not been directly demonstrated that they are different clones. Erythroid colonies would have to be analyzed to establish this. However, granulocytes having partial deficiency of surface CD59 expression obtained from a patient who had type II erythrocytes harbored a missense mutation in *PIG-A* (Ware *et al.*, 1994b). A B-lymphoblastoid cell line with a partial deficiency established from a patient with type II erythrocytes also had a missense mutation (Bessler *et al.*, 1994c). These results indicated that a mutation in *PIG-A* is also responsible for the partial deficiency of the GPI-anchored proteins. Mutations underlying a partial deficiency must be different from those for a complete deficiency, so it is very likely that types II and III erythrocytes found in the same patient arose from different clones. According to these finding, half, or even the majority, of patients would have two (or more) PNH clones.

I. Clonal Dominance of *PIG-A* Mutant Cells

Mutant clonal cells occupy significant fractions of the blood cells in patients with PNH. The presence of the same *PIG-A* mutation in the granulocytes and B lymphocytes indicated that the somatic mutation occurred in the multipotential hematopoietic stem cell that gave rise to these cell types (Takeda *et al.*, 1993). Although the number of hematopoietic stem cell clones that are providing progeny cells to the periphery is unknown, it would be substantially large since if the mutant stem cell provided progeny cells at a normal rate, PNH would not be clinically manifested. Dominance of the mutant clone must therefore occur during the early stage of the disease. As described previously, FACS analysis of bone marrow cells from patients with PNH demonstrated that a major fraction of the $CD34^+$,$CD38^-$ population was DAF and CD59 deficient (Terstappen *et al.*, 1993). This indicated that the mutant stem cells expressed the GPI anchor-deficient phenotype and that the clonal dominance had occurred at the hematopoietic stem cell stage.

A mutant hematopoietic stem cell therefore must have a growth or survival advantage relative to normal clones in PNH patients. Since all mutant stem cells should have the *PIG-A* mutation, it would be responsible for the clonal dominance. However, they might have another abnormality that is responsible for clonal dominance making *PIG-A* mutation not relevant. The latter seems unlikely because there are patients with two mutant clones, indicating that *PIG-A* mutants always become dominant in these patients (Bessler *et al.*, 1994b). It would be extremely unlikely that *PIG-A* mutation always coexists in multiple clones with another independently occurring event. This suggests that the *PIG-A* mutation itself endows the stem cells with this advantage.

Three possible mechanisms of clonal dominance are envisaged. One is that the stem cell bearing *PIG-A* mutation has the intrinsic ability to expand. In other words, the generation of *PIG-A* mutation alone causes PNH. Clonal expansion, however, does not seem to continue but rather to become stabilized at a particular level of occupancy. The level of occupancy varies among patients. This is true even in patients with a null mutation of *PIG-A* (Yamada *et al.*, 1995). Therefore, the extent to which a mutant clone expands is not determined solely by the mutation of *PIG-A* but is also influenced by some other factor(s). The second possibility is that mutant stem cell has not only the *PIG-A* mutation but also another mutation both of which are relevant for clonal expansion. This model is similar to the multistep mechanism of tumorigenicity. Different combinations of putative mutated genes with the *PIG-A* mutation would cause different extents of occupancy. A third possibility is that the mutant has an advantage

relative to normal clones due to a co- or preexisting condition that inhibits normal clones but has less effect on the mutant (Rotoli and Luzzatto, 1989). More inhibition would result in a higher extent of occupancy by a mutant clone and different conditions would suppress the normal and mutant clones to relatively different degrees, resulting in various levels of dominance by the mutant.

PNH is often associated with aplastic anemia (Lewis and Dacie, 1967; Dameshek, 1967; Rotoli and Luzzatto, 1989; Rosse, 1992; Young, 1992). Recent reports indicate that GPI-anchored protein-deficient blood cell population appears in patients with severe aplastic anemia at very high frequency (>10%) during long-term survival (Tichelli *et al.*, 1988; de Planque *et al.*, 1989; Najean and Hagvenauer, 1990; Marsh and Geary, 1991; Schubert *et al.*, 1994). A fraction of patients with aplastic anemia respond to immunosuppressive therapy, so immunological mechanisms, such as cell-mediated or antibody-mediated killing or inhibition of hematopoietic stem or progenitor cells, are likely to be operating in those patients (Frickhofen *et al.*, 1991). If such mechanisms are dependent on the GPI-anchored proteins in affecting hematopoiesis, for example, if target molecules of such antibodies are GPI anchored or if a GPI-anchored protein on the target cells has a supportive role in cell-mediated killing, normal cells would be more vulnerable than the mutant cells. Therefore, the high frequency of PNH clones in patients with aplastic anemia is in agreement with the notion that clonal dominance of the mutant stem cells is due to selection by some condition (Bessler *et al.*, 1994b).

J. Clinical Symptoms Other Than Hemolysis and Other Abnormalities

1. Venous Thrombosis

Thrombosis of veins is common in patients with PNH and is a major cause of death. It is more common in European and American than in Asian patients (Kruatrachue *et al.*, 1978; Le *et al.*, 1990). It occurs in hepatic, cerebral, and abdominal veins and veins in the skin (Rosse, 1992). Formation of complement C5b-9 complexes on the platelet surface causes the release of precoagulant microparticles that promote the assembly of the prothrombinase enzyme complex (Sims *et al.*, 1991). This reaction is regulated by CD59 because neutralizing anti-CD59 antibodies enhanced the reaction (Sims *et al.*, 1991). CD59-negative platelets from patients with PNH are very sensitive to C5b-9, generating 10-fold more factor Va binding sites and subsequent prothrombinase activity than normal platelets at the same input of complement components (Wiedmer *et al.*, 1993). This abnormal susceptibility of CD59-deficient platelets may contrib-

ute to the thrombotic risk (Wiedmer *et al.*, 1993). CD87, a receptor for urokinase-type plasminogen activator, is expressed on monocytes and granulocytes and plays a role in plasmin generation. CD87 is deficient in leukocytes from patients with PNH (Ploug *et al.*, 1992) and this may also be causally related to the venous thrombosis in these patients (Ploug *et al.*, 1992).

2. *Leukemia*

A few patients with PNH develop acute myelocytic leukemia (Rosse, 1992). Leukemic cells were derived from PNH clone in two patients (Devine *et al.*, 1987a; Shichishima *et al.*, 1993a). Although the second possible mechanism of clonal dominance in PNH described previously is in line with this increased tendency of leukemia, the exact reason for this to occur is not known.

3. *Other Abnormalities*

A number of abnormalities other than a deficiency of GPI-anchored proteins have been reported. When the lack of DAF on erythrocytes from patients with PNH was discovered, a deficiency of complement receptor type 1 (CD35) was also suspected. This was later shown not to be true (Roberts *et al.*, 1985). Glycophorin-α dimers on PNH cells were qualitatively abnormal, being more susceptible to trypsin. A deficiency in cell surface sialic acid was also suggested (Parker *et al.*, 1984); however, the basis for these abnormalities remains unclear.

The abnormal expression of gangliosides on affected erythrocytes from patients with PNH has been demonstrated (Nakakuma *et al.*, 1990). The relationship of this biochemical abnormality with a *PIG-A* mutation and GPI anchor-deficiency is unclear. In yeasts, ceramide synthesis is coupled to the surface expression of GPI-anchored proteins (Horvath *et al.*, 1994), which might be related to this deficiency.

IV. Perspectives and Conclusions

Progress in the molecular pathology of PNH as reviewed previously has established the following. Patients with PNH have one or more mutant hematopoietic stem cell clones that are deficient in GPI anchor synthesis due to somatic mutations in the X-linked gene, *PIG-A*. The somatic mutations of *PIG-A* are small, varied, and widely distributed in the coding regions and splice sites, indicating that they occur at random sites. Profiles of the mutations seem to be different geographically, suggesting the presence of mutagen-induced mutations. The mutant stem cells dominate bone marrow and their progeny cells dominate the peripheral blood. The

presence of large numbers of GPI anchor-deficient, complement-sensitive erythrocytes leads to hemolytic anemia. Autonomous expansion of the mutants seems unlikely, but selection by some condition(s) that preferentially suppresses normal stem cells would result in clonal dominance by the mutants. To further understand the mechanism of clonal dominance, the establishment of mice deficient in *Pig-a*, a murine homologue of *PIG-A*, would be important. Since *Pig-a* is also X-linked, a single targeting event in the embryonic stem cells causes the GPI anchor-deficient phenotype (K. Kawagoe, unpublished data). Therefore, GPI anchor-deficient hematopoietic stem cells could be obtained in the chimeric mice and should prove useful in proving that mutant hematopoietic stem cells do not autonomously expand but become dominant upon the suppression of normal stem cells.

Due to the mutant phenotype of the *Pig-a* targeted cells, namely the absence of all GPI-anchored proteins on the cell surface, the high contribution of the targeted cells to the body would have a lethal effect. To circumvent this potential problem, conditional targeting, such as use of the Cre-*loxP* system (Gu *et al.*, 1994), might be useful. This approach may also help to evaluate the overall roles of GPI-anchored proteins in various tissues because a deficiency in these proteins would be caused in a tissue-specific manner.

Identification of the gene responsible for PNH would allow new approaches to therapy. Allogenic bone marrow transplantations have been successful in several patients with PNH who had either aplastic anemia or recurrent thromboses as a complication (Szer *et al.*, 1984; Kawahara *et al.*, 1992). Autologous transplantation after the transduction of normal *PIG-A* gene into mutant hematopoietic stem cells could be an alternative by which the graft-versus-host disease seen in allogenic transplantations would be avoided. However, reestablishment of clonal dominance by any residual mutant stem cells would be a problem. As discussed previously, the clonal dominance of mutant stem cells may not be due to autonomous expansion, but to a selective condition(s). If pretransplant bone marrow conditioning is effective in eliminating such selective condition, residual mutant stem cells would not become dominant.

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The Use of Multiple Antigen Peptides in the Analysis and Induction of Protective Immune Responses against Infectious Diseases

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I. Multiple Antigen Peptides

A. Introduction

*M*ultiple *A*ntigen *P*eptides (MAPs), synthetic macromolecules containing defined B and/or T cell epitopes of one or more antigens, have provided unique immunological reagents for the analysis and induction of cellular and humoral immune responses to a wide range of infectious diseases. In contrast to the carrier-dependent immunogenicity of most linear synthetic peptides, MAPs utilize a peptide core matrix to construct a synthetic macromolecule that is immunogenic in the absence of protein carrier. Unlike peptide-carrier protein conjugates, the size, number, ratio, and relative position of T and B cell epitopes in MAPs can be defined, and thus provides the opportunity to examine the influence of these factors on immunogenicity and antigenicity of peptide vaccines. The amplification of the peptide signal provided by the multimeric structure of the MAPs has also led to the development of these reagents for the analysis of antibody/antigen and receptor/ligand interactions.

Recent studies have demonstrated the significant potential of MAP immunogens for immunoprophylaxis of infectious diseases. In experimental models, MAPs have been shown to induce protective antibody and T cell responses to rodent malaria species and the parasitic helminth, *Schistosoma mansoni*, as well as neutralizing antibody against pathogenic viruses, including HIV-1 and foot and mouth disease virus (FMDV).

The rodent malaria model provided the first demonstration of MAPs-induced protective immunity against infectious disease (Tam *et al.*, 1990). Our research over the past several years has applied MAP technology to the development of a vaccine for the human malaria *Plasmodium falciparum*. Therefore, the studies on the MAPs vaccines based on the *P. falciparum* CS protein will be reviewed in detail and compared with other experimental systems in terms of MAPs synthesis, formulation, antigenicity, immunogenicity, and efficacy. In the context of this review, the term MAP refers to branched peptides synthesized on a polylysine core, as developed by Tam

and coworkers. Although numerous synonyms exist for these constructs, such as "trees," "octopuses," and "multiple chain peptides," for consistency, these constructs are all referred to as MAPs. In addition, since many authors have used similar terminology, i.e., MAP 1 and MAP 2, wherever possible in this review, descriptive nomenclature will be used for the MAPs in order to provide information on the orientation and identity of the epitopes contained in each construct (Fig. 1).

B. Design and Synthesis of Maps

In the original work of Tam and coworkers chemical synthesis of MAPs was carried out using the solid-phase technique (Merrifield, 1963) in which synthesis is initiated on a polystyrene solid support, such as PAM or Wang resin. Both (*t*-butoxycarbonyl) (Boc) and fluorenyl-methoxy-carbonyl (Fmoc) amino acid derivatives have been used in the solid phase synthesis of MAPs. Efficient synthesis of MAPs has also been obtained using Fmoc-*t*-butylpolyamide techniques in continuous flow, as opposed to batchwise, technology (Pessi *et al.*, 1990; McLean *et al.*, 1991).

The unique element of MAPs structure is the construction of a multibranched inner core matrix based on the trifunctional amino acid lysine (Lys). By sequential propagation using the α and ε amino groups of lysine, a branched scaffolding can be constructed on which antigenic peptide

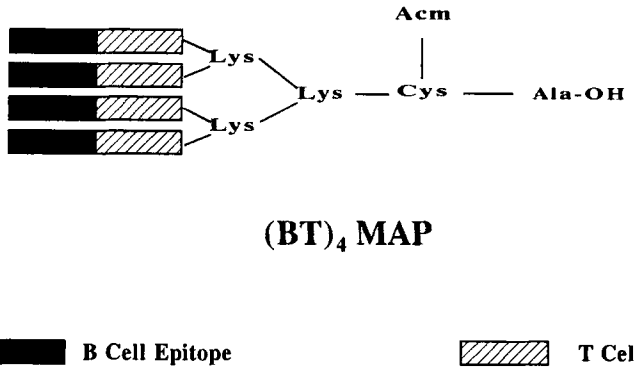

FIG. 1. Schematic diagram of $(BT)_4$ MAP-containing B and T cell epitopes to illustrate the nomenclature used throughout the text. In each MAP, the names of the B and T cell epitopes are within the parentheses and the number of branches is given as a subscript outside the parentheses. The order in which the T and B cell epitopes are written indicates their orientation relative to the polylysine core. For example, $(BT)_4$ refers to a tetrabranched MAP containing B and T cell epitopes, in which the B epitope is located at the amino terminus and the T epitope is proximal to the polylysine core. The polylysine core consists of three lysines (Lys) linked to a spacer containing a blocked (Acm) cysteine (Cys) and alanine (Ala).

sequences can be synthesized. The first level of coupling of Boc–Lys(Boc) produces a core with two reactive ends to produce a bivalent MAP; after the second level of Lys coupling, the core contains three Lys with a total of four reactive amino groups for the production of a tetravalent MAP, etc.

Attachment of the peptide epitopes to the polylysine matrix is frequently carried out by the stepwise addition of amino acids, resulting in simultaneous elongation of the peptide chain on each branch of the core. Completeness of coupling after each step is closely monitored by the ninhydrin test since the multimeric nature of the MAPs constructs amplifies any synthetic errors that may occur. Efficient synthesis to give yields of 98% or better at each stage is required in order to yield sufficient amounts of the desired target product.

By using a combination of Boc and Fmoc chemistry (Tam and Lu, 1989), or different blocking groups (Ahlborg, 1995), MAPs can be constructed that contain different antigenic sequences in each branch. An indirect approach can also be used in which the different components of the MAPs, i.e., the lysine core and/or blocks of peptides, are synthesized, analyzed, and purified separately and then covalently attached to form the macromolecular final construct (Lu *et al.*, 1991; Defoort *et al.*, 1992b; McLean *et al.*, 1992).

Following cleavage from the resin using standard techniques, i.e., hydrogen fluoride in Boc or trifluoroacetic acid in Fmoc chemistry, the completed MAP is purified by preparative reverse-phase HPLC and/or ion-exchange chromatography and the composition confirmed by amino acid analysis. The final MAP product is frequently sufficiently pure for immunological purposes, but additional purification methods can be used, such as sample displacement chromatography or capillary zone electrophoresis (Esposito *et al.*, 1993; Pessi *et al.*, 1991a). Modifications of standard purification techniques may be required to acommodate the unique chemical and physical properties of the macromolecular branched MAP structure. In situations requiring a more defined product, additional methods of analysis can be utilized, such as mass spectroscopy, circular dichroism (CD), and two-dimensional NMR spectroscopy (Esposito *et al.*, 1993; Defoort *et al.*, 1992b).

MAP technology thus makes possible the construction of complex, synthetic macromolecules of defined composition and structure. In the final product, the bulk of the MAP is composed of an outer antigenic layer, containing a high density of peptide epitopes, which surrounds a noncationic polylysine inner core. In the original studies of Tam and co-workers, MAP immunogens were shown to induce high levels of antibodies to both linear and conformational epitopes contained in the viral Hepatitis B surface antigen and in other complex proteins such as the human T cell

receptor β-chain and a guanine nucleotide-binding regulatory protein (Tam and Lu, 1989; Posnett *et al.*, 1988; Chang *et al.*, 1988).

The first demonstration that MAPs could induce a protective immune response against an infectious disease was obtained in a rodent malaria model (Tam *et al.*, 1990). The high levels of immunogenicity and protective efficacy demonstrated in these studies have led to the use of MAP antigens and immunogens to assay the immune response to various protozoan, helminthic, and viral diseases.

II. MAPS as Immunogens in Protozoan Diseases

A. Malaria

The development of drug resistance by the *Plasmodium* parasite and insecticide resistance by the *Anopheles* insect vector has focused research efforts on the development of malaria vaccines for immunoprophylaxis. Immunity to the malaria sporozoite, the stage of the parasite that initiates the infection following the bite of the infected mosquito, has been shown to be mediated by both humoral and cellular effector mechanisms (Nussenzweig and Nussenzweig, 1989; Nardin and Nussenzweig, 1993). Therefore, one component of an effective subunit malaria vaccine should induce high levels of antibody to neutralize the extracellular infective sporozoites.

Polyclonal and monoclonal antisporozoite antibodies, which can effectively neutralize parasite infectivity and passively protect naive recipients, have been shown to react with an immunodominant B cell epitope located in the repeat region of a major surface antigen, the circumsporozoite (CS) protein. The cloning of the CS gene of rodent, primate, and human malaria species has shown that the immunodominant epitope was composed of tandemly repeated sequences of 4–9 amino acids. The repetitive immunodominant B cell epitope of the CS protein has formed the basis of the first generation synthetic and recombinant vaccines tested in Phase I and II clinical trials (Herrington *et al.*, 1987; Ballou *et al.*, 1987). While immunogenic, the level of antibody, induced by these candidate vaccines was found to be suboptimal as was protection in a limited number of challenge studies. MAP technology has, therefore, been utilized to overcome the limitations of these first generation subunit vaccines in an effort to design a malaria vaccine containing parasite-derived T and B cell epitopes that would be capable of inducing high levels of protective antibody in the majority of individuals.

1. Rodent Malaria

a. Plasmodium berghei

i. T cell epitopes derived from CS protein. MAP immunogens were first shown to induce a protective immune response against sporozoite

challenge in the *P. berghei* rodent malaria model (Tam *et al.*, 1990; Chai *et al.*, 1992). A series of MAPs, based on the *P. berghei* CS protein, was constructed to contain various molar ratios of T and B cell epitopes, in different di-, tetra-, or octameric branched configurations. The immunodominant B cell epitope, represented by the sequence $(PPPPNPND)_2$ (Eichinger *et al.*, 1986), and a T helper cell epitope defined by the sequence (KQIRDSITEEWS) from the carboxy terminus of the CS protein (Romero *et al.*, 1988), were incorporated into the different MAP constructs.

The optimal immunogen was found to be $(BT)_4$, a tetrabranched MAP containing equimolar amounts of B and T cell epitopes, arranged with the B cell epitope distal to the lysine core (Figure 1). The $(BT)_4$ MAP was found to induce high titers ($>10^5$) of anti-peptide and anti-sporozoite antibodies in the sera of immunized A/J ($H\text{-}2^a$) mice, as measured by radioimmunoassay (RIA) or indirect immunofluorescence assay (IFA). The anti-sporozoite antibody levels correlated with protection against challenge, as determined by the percentage of immunized mice which did not develop infection following injection of viable sporozoites.

The genetic restriction to the *P. berghei* B cell repeat epitope is limited to the $H\text{-}2^b$ haplotype, and thus the response in the MAP-immunized A/J mice was dependent on the inclusion of a T helper epitope. No antibody response or protection was obtained in the mice which had been immunized with octameric MAPs containing only the B, or only the T, cell epitope. Covalent linkage of the epitopes was also required, as immunization with a mixture of equimolar amounts of the monoepitope MAPs failed to elicit a response.

The MAPs containing equimolar amounts of T and B epitopes linked in tandem were the most immunogenic. In the case of the $(BT)_4$ MAP, somewhat similar antibody titers could be obtained following immunization with reversely oriented MAP, i.e., $(TB)_4$. Thus, in the A/J mice, the position of the B epitope either proximal or distal to the lysine core did not greatly affect the level of antibody induced by MAPs immunization. Increasing the number of branches from four to eight also did not increase MAPs immunogenicity. Although low titers of antibody could be induced in mice immunized with MAPs alone or adsorbed to aluminum hydroxide (alum), optimal levels were obtained with Freund's adjuvant (Chai *et al.*, 1992).

MAPs containing the *P. berghei* repeat B cell epitope in combination with T cell epitopes derived from the N terminus of the CS protein were also found to induce protective immunity against sporozoite challenge (Migliorini *et al.*, 1993). MAPs were synthesized by solid-phase Fmoc strategy to contain the repeat sequence $(DPPPPNPN)_2$ in combination with one of the N-terminal T cell epitopes, either T2 corresponding to aa 20–39 (PGYGQNKSIQAQRNLNELCY) or T1 corresponding to aa 57–70

(KIYNRNTVNRLLAD). The T2 epitope induced proliferation of H-2^d mice, while the T1 epitope stimulated lymph node cells of H-$2^{d,b,\text{ or }k}$ peptide-primed mice.

BALB/c (H-2^d) mice immunized with MAP constructs containing the repeat epitope combined with either the T1 or the T2 epitope produced similar levels of anti-repeat antibodies (geometric mean titers, $3–7 \times 10^4$). Following challenge by the bite of malaria-infected mosquitoes, 62–66% of the mice immunized with MAP constructs containing these T and B cell epitopes of the *P. berghei* CS protein were protected.

Mice immunized with monoepitope tetrabranched MAPs containing the T1 (aa 57–70), but not the T2 (aa 20–39), epitope were also protected against *P. berghei* sporozoite challenge. The immunity appeared to be cell mediated since the sera of the T1 MAP-immunized mice did not react with *P. berghei* sporozoites. Protective cellular immunity mediated by $CD4^+$ T cells has, in fact, been demonstrated in rodent malaria models. $CD4^+$ T cell clones specific for malarial antigens can passively transfer protection against *P. berghei* or *P. yoelii* sporozoite challenge (Tsuji *et al.*, 1990; Renia *et al.*, 1991). The precise mechanism of immunity is unknown, but T cell-derived lymphokines, in particular IFN-γ, are potent inhibitors of the intracellular hepatic stages of the parasite (Ferreira *et al.*, 1986; Schofield *et al.*, 1987). The results in the mice immunized with the monoepitope T1 MAP demonstrate that MAP immunogens can elicit protective T cell- as well as antibody-mediated immune responses.

ii. MAPs containing T cell epitopes derived from a nonmalarial protein. T cell recognition of the central repeat domain of the CS protein of many of the malarial species, including *P. berghei*, is restricted to a single murine haplotype (for review see Nardin and Nussenzweig, 1993). One experimental approach to overcome this genetic restriction is to incorporate "universal" T helper cell epitopes, i.e., amino acid sequences recognized in the context of most, if not all, class II molecules, to provide help for anti-repeat antibody responses. Two "universal" peptides have been defined in tetanus toxoid (TT) in sequences containing aa 830–844 (QYIKANSKFIGITEL) and aa 947–967 (FNNFTVSFWLRVPKVSASHLE), represented by peptides P2 and P30, respectively (Panina-Bordignon *et al.*, 1989). The P2 and P30 synthetic peptides stimulate peripheral blood lymphocytes (PBL) of TT-sensitized individuals of most genetic backgrounds. Studies based on human tetanus-specific T cell clones have shown that the peptides are recognized in the context of both DR and DP class II molecules. The epitopes were also universal in the murine system, in that T cells from peptide-primed mice of the four strains tested (H-$2^{a,b,d\text{ or }k}$) all proliferated in response to *in vitro* challenge with the P2, as well as the P30, peptide (Valmori *et al.*, 1992).

Immunization of mice with tetrabranched MAPs containing the *P. berghei* repeat region $(DPPPPNPN)_2$, tandemly synthesized with either the P2 or the P30 T cell epitopes, induced comparable anti-repeat antibody response in CBA ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) mice (Valmori *et al.*, 1992). High antirepeat titers were obtained when either the P2 or the P30 epitopes were used to provide T cell help, reaching peak ELISA titers of 10^5 versus 10^4, respectively. The antibody response was dependent on the presence of the T helper epitope, as a MAP containing only the B cell epitope failed to induce antibody in either strain of mice.

b. Plasmodium yoelii

i. MAPs containing T epitopes of CS protein. MAPs containing T and B cell epitopes of the CS protein of the more virulent rodent malaria parasite, *P. yoelii*, have also been constructed and tested for immunogenicity and antigenicity. A T cell epitope located in the N terminus of the *P. yoelii* CS protein, termed Py1T (YNRNIVNRLLGDA), was recognized by both $H\text{-}2^d$ and $H\text{-}2^b$ peptide-immunized mice (Grillot *et al.*, 1990, 1993). Tetrabranched MAPs were constructed that contained the Py1T epitope in combination with three or four tandem repeats of the $H\text{-}2^b$ restricted *P. yoelii* repeat (QGPGAP). Mice immunized sc with MAPs emulsified in Freund's adjuvant developed anti-repeat antibody ELISA titers of 10^4. The lymph node cells of MAP-immunized mice proliferated in response to *in vitro* challenge with a MAP, or a linear peptide, containing the Py1 T cell epitope.

Protective immunity against sporozoite challenge can also be induced in mice immunized with MAPs containing B and T cell epitopes of the *P. yoelii* CS protein (Reed *et al.*, in preparation). Using a combination of Fmoc and Boc chemistry, a tetramer MAP containing B and T cell epitopes on alternating branches was synthesized. The constructs contained the *P. yoelii* repeat B cell epitope $(QGPGAP)_3$ combined with a T helper epitope from the carboxy terminus of the CS protein. Mice immunized with the *P. yoelii* MAP, in an oil-in-water vehicle containing block copolymer P1005 and detoxified Ra-LPS as adjuvant, were protected against *P. yoelii* sporozoite challenge. Sterile immunity correlated with high levels of anti-sporozoite antibody. Similarly, mice immunized with the *P. berghei* diepitope MAP containing B and T cell epitopes of the CS protein, administered in either a water-in-oil or an oil-in-water emulsion, developed high levels of sterile immunity to *P. berghei* sporozoite challenge.

MAPs constructs containing parasite-derived T helper epitopes provide T cell help for malaria-specific cytotoxic, as well as humoral, immune responses. A *P. berghei* CS-derived T cell epitope, when incorporated into a tetrameric MAP, was found to elicit T cell help for *P. yoelii* $CD8^+$ CTL

responses (Widmann *et al.*, 1992). Immunization of mice with a linear peptide representing the CTL epitope of the *P. yoelii* CS protein (SYVP-SAEQI) (Romero *et al.*, 1991) failed to induce a cytotoxic T cell response in BALB/c mice. However, when the linear peptide containing the CTL epitope was coemulsified in incomplete Freund's adjuvant with a MAP containing four copies of a T helper epitope (aa 20–39) derived from the *P. berghei* CS protein (Migliorini *et al.*, 1993), strong cytolytic responses against peptide-pulsed target cells were observed (Widmann *et al.*, 1992).

ii. MAPs containing T cell epitopes derived from a nonmalarial protein. A tetrabranched MAP containing the *P. yoelii* CS repeats $(QGP-GAP)_4$ colinearly synthesized with two universal epitopes of tetanus toxoid, P2 (aa 830–844) and P30 (aa 947–967), has been assayed for immunogenicity in different strains of mice (Wang *et al.*, 1995). The inclusion of the universal T cell epitopes derived from tetanus toxoid in the triepitope MAP resulted in the production of high levels of anti-sporozoite antibodies (10^4) in all of the murine strains. The MAP was immunogenic when formulated in different adjuvants, including Freund's, monophosphoryl lipid A, Lipofectin, nonionic block copolymer (TiterMax), liposomes, and alum.

Protection against challenge with 100 *P. yoelii* sporozoites was obtained in inbred ($H\text{-}2^{a,\ b,\ or\ d}$) and outbred (CD1) mice immunized with MAP emulsified in nonionic block copolymer (TiterMax) as adjuvant (Wang *et al.*, 1995). High levels of protection (80%) could also be obtained using the MAP in a liposome–lipid A/alum mixture, an adjuvant formulation that has been tested in humans (Fries *et al.*, 1992). Passive transfer of antibody from the MAP-immunized mice protected naive recipients against sporozoite challenge (Wang *et al.*, 1995).

2. *Human Malaria*

a. Plasmodium falciparum

The identification of the immundominant epitope within the repetitive tetramer (NANP)n of the *P. falciparum* CS protein (Nussenzweig and Nussenzweig, 1989) led to clinical trials of a vaccine based on this epitope conjugated to tetanus toxoid as a carrier, $(NANP)_3$-TT. These Phase I and II trials (Herrington *et al.*, 1987, 1990), which were the first test of a synthetic peptide vaccine against a communicable disease of man, demonstrated that the synthetic vaccine could induce an anti-sporozoite antibody response in the majority of the individuals receiving the highest dose of vaccine.

Although the overall antibody titers were suboptimal, there was a good correlation between the level of the anti-repeat antibody response and reactivity with *P. falciparum* sporozoites. When challenged by the bite of *P. falciparum*-infected mosquitoes, one of three volunteers with the highest

anti-sporozoite antibody titer was protected. The remaining two volunteers demonstrated a delayed onset of parasitemia, indicating that a large proportion of the infectious sporozoites had been neutralized. The magnitude of the antibody response was dose dependent. However, administration of larger doses of the vaccine, which consisted of 20% peptide by weight, were precluded by toxicity limitations due to the tetanus toxoid carrier. Moreover, the absence of a parasite-derived T cell epitope in the $(NANP)_3$-TT vaccine limited its ability to elicit an anamnestic response, or to prime parasite-specific T cells, in vaccine recipients.

The various shortcomings associated with the first generation of synthetic peptide–protein conjugate vaccines, namely carrier toxicity, low epitope density, possible epitopic suppression, and lack of parasite-derived T cell epitopes, can be overcome by MAP candidate vaccines. In contrast to the low epitope density of the first generation peptide–protein conjugate vaccines, greater than 90% of the total molecular weight of a MAP-based vaccine consists of peptide epitopes relevant to a specific antisporozoite immune response.

Over the past several years, we have tested various MAPs constructs, containing defined T and B cell epitopes of the *P. falciparum* CS protein, in an effort to design a synthetic vaccine that can not only induce an effective T helper cell response and high levels of antisporozoite antibodies in naive recipients, but can also elict an anamnestic response in sporozoite-primed individuals.

i. T cell epitopes derived from the P. falciparum CS protein. In our studies, the choice of *P. falciparum* T cell epitope for inclusion in the malaria vaccine was determined empirically by testing the immunogenicity, in mice, of MAP constructs containing the $(NANP)_3$ B cell epitope in combination with several different T helper epitopes identified in the repeat and nonrepeat regions of the *P. falciparum* CS protein (Fig. 2).

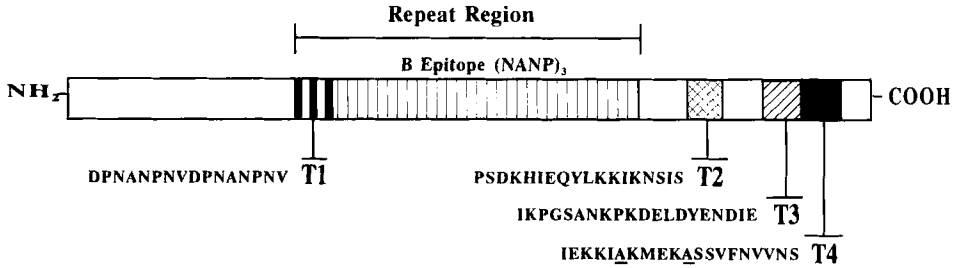

FIG. 2. Illustration of the *P. falciparum* CS protein showing the location of the B cell epitope $(NANP)_3$ and T helper cell epitopes within the repeat region (T1) and the carboxy terminus (T2–T4).

T cell epitope from the repeat region of the CS protein. The optimal immunogen revealed by vaccination of different strains of mice was a MAP construct containing the $(NANP)_3$ B cell epitope combined with a T cell epitope (T1) from the repeat region of the *P. falciparum* CS protein (Munesinghe *et al.*, 1991). The T1 epitope was originally identified by the proliferative responses of human $CD4^+$ T cell clones from a volunteer immunized with irradiated *P. falciparum* sporozoites (Nardin *et al.*, 1989). Mapping of the epitope recognized by these CD4+ T cell clones was carried out using recombinant CS proteins, which included the entire repeat region composed of a 5′ repeat region, containing three alternating NANPNVDP sequences, and a 3′ repeat region, composed of 38 repeats of the tetramer (NANP). Deletion of the 5′ repeat region from the recombinant CS proteins abrogated the proliferative T cell response, demonstrating that the T cells recognized an epitope within the alternating NVDPNANP sequences. The 5′ repeat sequence is conserved in all the P. *falciparum* strains of diverse geographical origin that have been sequenced to date (Lockyer and Schwarz, 1987; McCutchan *et al.*, 1988; Caspers *et al.*, 1989). Therefore, the inclusion of this T cell epitope should provide the potential for both priming and boosting responses to all strains of *P. falciparum.*

Synthetic peptides containing different combinations of the NANP and NVDP repeats were used to confirm the precise identity of the epitope within the 5′ repeats. The 16mer peptide DPNANPNVDPNANPNV induced optimal stimulation of the specific T cell clones and was thus chosen for incorporation into the falciparum MAP construct.

MAPs containing the T1 epitope, in combination with the B cell epitope $(NANP)_3$, using various T : B ratios and configurations, were constructed by stepwise solid-phase peptide synthesis (Fig. 3). The $(T1B)_4$ MAP, containing the T1 epitope located distal to the lysine core and the B cell epitope proximal to the core, along with additional constructs reversing the orientation, i.e., $(BT1)_4$, or varying the molar ratios of the T and B cell epitopes, were tested.

All of the MAPs constucts containing the T1 epitope were antigenic, stimulating proliferation and lymphokine secretion by human $CD4^+$ T cells specific for the T1 epitope. On a molar basis, the $(T1B)_4$ MAP construct was more efficient than the linear 16mer peptide in inducing proliferation and IFN-γ production by the human T cell clone (Table I). The response was specific for the T1 epitope; a MAP construct containing only the B cell epitope, B_4, did not stimulate the T cells.

The immunogenicity of these MAP constructs was determined in four strains of mice immunized with 50 μg of MAPs administered intraperitoneally in Freund's adjuvant. The $(T1B)_4$ construct was the most immunogenic, eliciting high titer anti-NANP antibody responses in three out of four

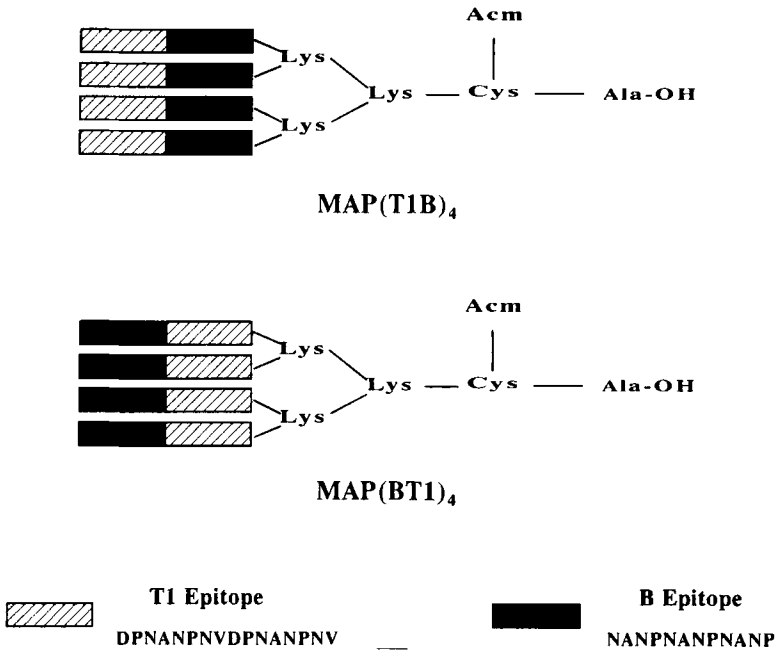

FIG. 3. *Plasmodium falciparum* CS protein MAP $(T1B)_4$ and the reverse orientation, $(BT1)_4$.

TABLE I

RECOGNITION OF *P. FALCIPARUM* REPEAT MAPS BY A $CD4^+$ T CELL CLONE DERIVED FROM A SPOROZOITE-IMMUNIZED VOLUNTEER

Antigen	M	Proliferation (SI)[a]	Interferon (U/ml)[b]
T1 16mer	10^{-8}	33.2	24.0
	10^{-9}	3.7	nd
$(T1B)_4$	10^{-8}	33.4	22.7
	10^{-9}	23.5	13.8
	10^{-10}	4.0	nd
$(B)_4$	10^{-8}	1.2	<0.1

[a] Proliferation is expressed as the stimulation index (SI) obtained following *in vitro* challenge with a 16mer linear peptide containing the T1 epitope DPNANPNVDPNANPNV, a tetrabranched diepitope MAP $(T1B)_4$ containing the T1 epitope and the B cell epitope $(NANP)_3$, or a monoepitope MAP containing only the B cell epitope $(B)_4$ using autologous irradiated PBL as antigen-presenting cells.

[b] Interferon-γ production by the T cell clone was measured in 48-hr culture supernatants by RIA and expressed as units/ml (U/ml).

strains of mice (Fig. 4A). The high responder mice, C57Bl/10 (H-2^b), gave peak antibody titers of 10^5, while intermediate responders, A/J (H-2^a) and C3H (H-2^k), had titers of 10^3 or 10^4. BALB/c (H-2^d) mice did not respond to immunization with any of the MAP constructs in this formulation.

A MAP of the reverse orientation, $(BT1)_4$, in which the B cell epitope precedes the T cell epitope, induced comparable antibody titers in mice of the high-responder strain (C57Bl/10), but was less immunogenic when tested in the intermediate-responder strains (Fig. 4B). The A/J and C3H mice required three doses of $(BT1)_4$ MAP before significant anti-repeat antibody titers could be detected. Additional constructs containing different ratios of T and B cell epitopes, i.e. one copy of the B cell epitope and four copies of the T cell epitope, or vice versa, were the least immunogenic.

The antibody response induced by $(T1B)_4$ MAP immunization was studied over a period of 6 months. As measured by both ELISA and IFA, the anti-repeat and anti-sporozoite antibodies induced by immunization with $(T1B)_4$ MAP remained at high titers for a prolonged period of time (Figure 5). Approximately 6 months after the last dose of MAP, ELISA titers of 8×10^4 and IFA titers of 10^4 were still detectable in the sera of the MAP-immunized mice.

The $(T1B)_4$ MAP construct has also been shown to be highly immunogenic in nonhuman primate hosts. Two species of Aotus monkeys, *Aotus nancymai* and *A. vociferans*, which differ in phenotype and karyotype, were immunized subcutaneously with $(T1B)_4$ MAP in Freund's (A. Moreno *et al.*, manuscript in preparation). Both species of Aotus developed similar high levels of antibody detectable by IFA and ELISA which reached titers

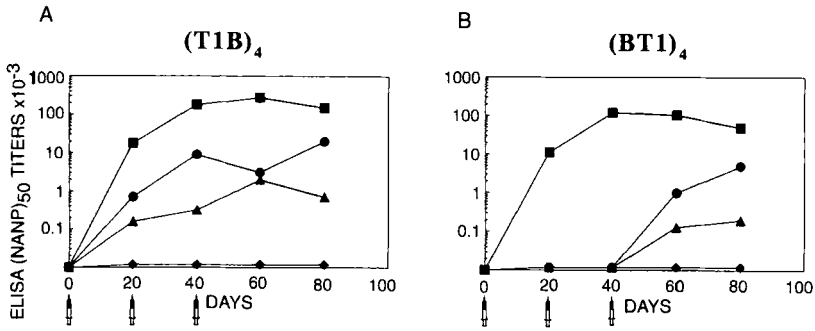

FIG. 4. Geometric mean antibody titers (GMT) as measured by $(NANP)_{50}$ ELISA in sera of mice immunized with (A) MAP $(T1B)_4$ or (B) a MAP of the reverse orientation, $(BT1)_4$. The MAPs emulsified in Freund's adjuvant were administered i.p. on Days 0, 21, and 42 to five mice of each strain: C57Bl/10 (■), A/J (●), C3H (▲), and BALB/c (◆). Adapted from Munesinghe *et al.* (1991) with permission.

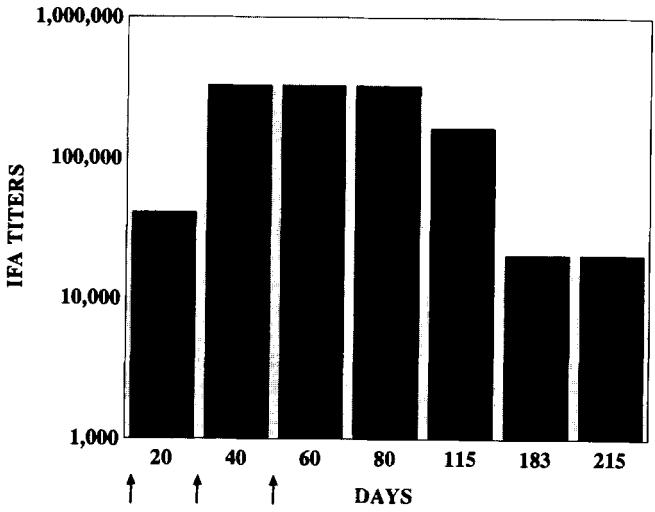

FIG. 5. Persistence of murine anti-*P. falciparum* sporozoite antibody response induced by immunization with $(T1B)_4$ MAPs in Freund's measured in pooled sera of C57Bl/6 mice by indirect immunofluorescence (IFA) using *P. falciparum* sporozoites.

$>10^5$ following three injections with $(T1B)_4$ MAP. The magnitude and specificity of the antibody responses in the MAP-immunized Aotus were similar to those observed in the MAP-immunized high-responder mice.

These high antibody titers in experimental animals had been obtained with MAPs emulsified in Freund's adjuvant, a formulation that is not acceptable for use in humans. Studies of the immunogenicity of $(T1B)_4$ MAP in alum were carried out in mice (DeOliveira *et al.*, 1994). After three immunizing doses, the anti-repeat, and more importantly the anti-sporozoite, antibody responses of the recipients of the MAP/alum formulation were comparable to those obtained with Freund's adjuvant. Peak IFA titers were 3.2×10^5 in the alum versus 1.6×10^5 in the Freund's adjuvant-immunized animals. A similar magnitude of antibody response was obtained by subcutaneous or intraperitoneal immunization.

In both primates and rodents, the $(T1B)_4$ MAP induced anti-peptide antibodies that reacted at high titers with the native CS protein on the surface of *P. falciparum* sporozoites (Munesinghe *et al.*, 1991; DeOliveira *et al.*, 1994; Moreno *et al.*, manuscript in preparation). IFA titers of over 10^6 were observed in individual sera of MAP-immunized C57Bl mice and Aotus monkeys. After reaction with viable *P. falciparum* sporozoites, the sera of the MAP-immunized C57Bl/10 mice gave positive circumsporozoite precipitation reactions (Vanderberg *et al.*, 1969) detectable at dilutions

greater than 1:800. These high levels of anti-sporozoite antibody had not been obtained by immunization with any synthetic or recombinant falciparum vaccine candidate tested to date.

The good correlation between anti-peptide antibody levels and reactivity with the native CS protein on the sporozoite surface is noteworthy since a previous drawback associated with many synthetic immunogens had been the failure of the anti-peptide antibodies to react with the native protein. It is also noteworthy that the anti-repeat antibodies induced by $(T1B)_4$ MAP immunization reacted not only with the 3′ NANP repeats, but also with the 5′ NANPNVDP repeats of the *P. falciparum* CS protein (Munesinghe *et al.*, 1991). Therefore, antibodies induced by either the B or the T cell epitope effectively reacted with the repeat region of the *P. falciparum* CS protein and contributed to a parasite-specific antibody response. The fact that each of the epitopes contained in the $(T1B)_4$ MAP could function as both B and T cell epitopes in the high-responder C57Bl mice may also have played a role in enhancing the magnitude of their immune responses.

Of particular relevance to vaccine applications, the $(T1B)_4$ MAP was found to elicit a strong anamnestic antibody response in *P. falciparum* sporozoite-primed animals. Approximately 3 months after a single iv injection of *P. falciparum* sporozoites, mice were injected with 30 μg of $(T1B)_4$ MAP/alum (Fig. 6). Anti-repeat antibody titers reached 1.9×10^5, and IFA titers 3.2×10^5, in the sporozoite-primed mice following the injection of $(T1B)_4$ MAP. Titers in naive control mice, injected only with $(T1B)_4$ MAP, were less than 10^3. Similar observations were made in an Aotus monkey, in which a preexisting low level of anti-sporozoite antibody was boosted 100-fold following a single injection of the MAP/alum formulation (A. Moreno *et al.*, manuscript in preparation).

The results obtained in experimental animals suggest that the administration of the $(T1B)_4$ MAP/alum vaccine would increase the antibody levels and resistance to reinfection of individuals living in malaria endemic areas, most of whom have low or suboptimal anti-sporozoite antibody titers. The fact that the $(T1B)_4$ MAP was immunogenic in three of four inbred mouse strains, and in two different Aotus monkey species, also supports the expectation that the $(T1B)_4$ MAP vaccine will be immunogenic in individuals of diverse genetic backgrounds. Based on these findings, Phase I and II trials of the $(T1B)_4$ MAP in HLA-typed human volunteers are planned for the near future.

T cell epitopes from the C terminus of the CS protein. The close correlation between the anti-peptide and anti-sporozoite antibody titers induced by the $(T1B)_4$ MAP was particularly noteworthy in light of the responses obtained using MAPs containing different T helper epitopes from the C terminus of the *P. falciparum* CS protein (Fig. 2). The Th2R (PSDKHIE-

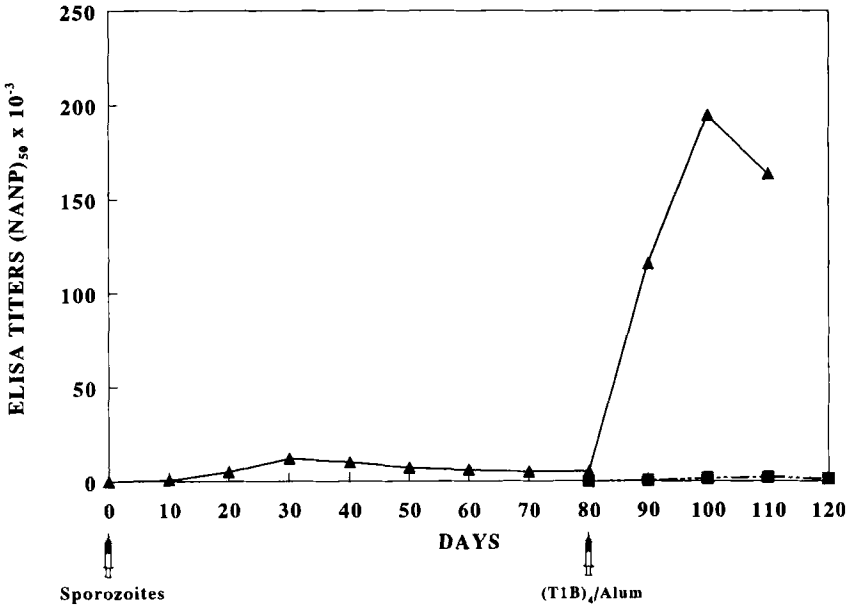

FIG. 6. $(T1B)_4$ MAP /alum elicits an anamnestic response in *P. falciparum* sporozoite-primed mice. Adapted from de Oliveira *et al.* (1994), by permission of the publishers, Butterworth-Heinemann Ltd©.

QYLKKIKNSIS) and Th3R (IKPGSANKPKDELDYENDIE) epitopes (abbreviated T2 and T3, respectively) were originally identified by Good and colleagues based on murine and human T cell responses (Good *et al.*, 1987, 1988). The CS.T3 epitope (abbreviated T4) represents the universal T helper cell epitope (IEKKIAKMEKASSVFNVVNS) of the CS protein recognized in the context of most murine and human class II molecules (Sinigaglia *et al.*, 1988). A series of MAP constructs containing the T2, T3, or T4 epitopes from the C terminus of the *P. falciparum* CS protein, combined with the $(NANP)_3$ B cell epitope, were tested in configurations in which the T epitope either preceded or followed the B epitope (Calvo-Calle *et al.*, 1993).

The MAPs containing the T helper epitopes from the C terminus of the CS protein, $(BT2)_4$, $(T3B)_4$, and $(BT4)_4$ induced very high anti-peptide antibody titers detected by ELISA using the corresponding MAPs as antigen (Table II). However, in contrast to the results obtained by immunization with the $(T1B)_4$ MAP, the anti-sporozoite antibody IFA titers of these mice were orders of magnitude lower. Thus, antipeptide ELISA titers of over 10^6 were detected in mice immunized with the $(BT4)_4$ MAP, while

TABLE II
IMMUNOGENICITY OF MAPS CONTAINING DIFFERENT T HELPER EPITOPES FROM THE *P. FALCIPARUM* CS PROTEIN

MAPS	Anti-sporozoite (IFA titer)	Anti-MAP antibody (ELISA titer)
$(\underline{T1}B)_4$	825,700	825,700
$(B\underline{T2})_4$	10,000	300,000
$(\underline{T3}B)_4$	20,000	1,200,000
$(B\underline{T4})_4$	5,200	1,200,000

an IFA titer of only 5200 was obtained when these sera were tested with *P. falciparum* sporozoites.

These findings indicate that a large proportion of the anti-peptide antibodies, induced by immunization with these MAP constructs, failed to react with the native CS protein on the parasite surface. The analysis of the fine specificity of the anti-MAP antibody response, in fact, demonstrated that significant levels of antibody specific for the T cell epitopes were induced by immunization with these MAP constructs. Monoepitope MAPs, containing only the T cell epitope, also induced high levels of anti-peptide antibody that failed to react with the sporozoite surface (Calvo-Calle *et al.*, 1993). These findings suggest that these T cell epitopes are not exposed in the native protein or that they are present in a conformation not represented by the MAP constructs.

ii. T cell epitopes from P. falciparum blood stages. In addition to T cell epitopes derived from the CS protein, T cell help may be provided by the inclusion of epitopes from other developmental stages of the malaria parasite. Immunity elicited by sporozoites does not protect against blood stage parasites, which are directly responsible for clinical disease and mortality. Thus, a malarial vaccine containing antigenic sequences from the sporozoite and blood stages of the parasite may be more efficacious. The inclusion of blood-stage epitopes may also provide additional T helper epitopes to overcome genetic restriction.

An epitope from a *P. falciparum* blood-stage antigen, Pf322, identified by a human monoclonal antibody with neutralizing activity (Ahlborg *et al.*, 1991), has been tested in combination with the 5' repeat region of the *P. falciparum* CS protein. A tetrabranched diepitope MAP containing the CS repeat $(DPNANPNV)_2$, combined with three repeats $(VTEEI)_3$ of the Pf332 blood-stage antigen, was synthesized using Fmoc chemistry (Ahlborg, 1995). In order to synthesize the two epitopes on separate branches of the MAP construct, the α and ε amino groups were orthogonally pro-

tected by Fmoc and 1-(4,4 dimethyl-2,6-dioxycyclohexylidene)ethyl respectively. Monoepitope tetramer MAPs, which contained either the CS or the Pf322 repeats, were immunogenic only in the respective responder strains, H-2^d for the $(VTEEI)_3$ epitope and H-2^b for the $(DPNANPNV)_2$. epitope. When both epitopes were combined in the MAP construct, each of the epitopes was shown to provide reciprocal T cell help resulting in the production of antibody specific for the Pf332 and the CS repeats in both H-2^b and H-2^d mice.

iii. T cell epitopes from a nonmalarial foreign protein. An alternative approach for the design of falciparum MAP vaccines that would be immunogenic in a broad spectrum of individuals is the inclusion of universal T helper cell epitopes, derived from tetanus toxoid, with the expectation that enhanced levels of antibody would be observed in TT-primed individuals (Etlinger *et al.*, 1990; Valmori *et al.*, 1992).

The immunogenicity of an octabranched triepitope MAP containing the $(NANP)_6$ B cell epitope combined with the two universal T cell epitopes P2 (aa 830–843) and P30 (aa 947–967) of tetanus toxoid, $[(NANP)_6P2P30]_8$, was compared to that of a tetrabranched diepitope MAP $[P30(NANP)_{10}]_4$ or a monoepitope MAP $[(NANP)_{10}]_4$ (Valmori *et al.*, 1992:Grillot *et al.*, 1993). Monoepitope MAPs containing more than three tandem repeats of NANP had previously been shown to elicit a response in BALB/c (H-2^d), as well as C57Bl (H-2^b), mice (Pessi *et al.*, 1991b). Immunization with either the di- or the triepitope MAPs elicited peak anti-repeat antibody titers that were an order of magnitude higher than those induced by immunization with the monoepitope MAP containing only the repeat epitope of *P. falciparum* (10^4 vs. 10^5, respectively) (Valmori *et al.*, 1992). Antibody titers induced by immunization with alum adsorbed diepitope MAPs were 10- to 50-fold lower than those obtained with Freund's adjuvant.

In humans, specific antibody and T cells present in TT-primed individuals could potentially suppress or enhance the immune response to MAPs containing TT-derived epitopes (Schutze *et al.*, 1985; Etlinger *et al.*, 1990). To examine this possibility, the di- and triepitope MAPs containing the universal P2 and P30 peptides were tested for antigenicity using human T cells and sera from tetanus toxoid positive individuals (Valmori *et al.*, 1992; Grillot *et al.*, 1993). Antibodies to the P2 or the P30 epitope were not detected in the TT- immunized individuals. It is unlikely, therefore, that epitopic suppression would be an impediment for usage of the TT–MAPs as vaccine.

In terms of human T cell responses, the di- or triepitope *P. falciparum* MAPs containing the P30 and/or P2 TT epitopes induced a proliferative response ($SI > 2$) in 9/10 TT-primed individuals. Peptide-specific TT

clones, however, varied in their capacity to recognize the T cell epitopes after incorporation into MAP constructs. While all of the P2-specific T cell clones recognized the triepitope MAP $[(NANP)_6\ P2P30]_8$, not all P30-specific clones could recognize the MAP constructs containing P30 located in either the -COOH terminus $[(NANP)_6\ P2P30]_8$ or the -NH2 terminus $[P30(NANP)_{10}]_4$.

Taken together, the studies demonstrate that MAP constructs containing the universal TT epitopes provide T cell help for antibody responses to the *P. falciparum* CS repeats. The observation that mice are protected against *P. yoelii* sporozoite challenge following immunization with MAPs containing *P. yoelii* repeats combined with these TT epitopes (Wang *et al.*, 1995) supports the use of similar MAP constructs as human vaccine candidates.

b. Plasmodium vivax

Plasmodium vivax malaria, although not as severe a disease as *P. falciparum*, has a broader geographical distribution and is responsible for greater morbidity on a worldwide basis. A limited number of Phase I trials of candidate vaccines based on the *P. vivax* CS protein have failed to identify a recombinant protein vaccine capable of inducing high levels of anti-sporozoite antibody (Gordon *et al.*, 1990; Herrington *et al.*, 1991).

MAPs containing *P. vivax* repeat sequences, combined with T cell epitopes derived from either the CS protein or tetanus toxoid, have been compared for immunogenicity in mice (Herrera *et al.*, 1994). The *P. vivax* repeat peptide $(GDRADGQPA)_3$, representing one type of variant of the vivax repeats (Romero *et al.*, 1987;Herrera *et al.*, 1992), was incorporated into a tetramer MAP either alone or in combination with the CS-derived T cell epitopes identified by proliferative responses of PBL of individuals living in a malaria endemic area of Colombia (Herrera *et al.*, 1992). One peptide, P6, stimulated the cells of approximately 30% of immune individuals, while a second peptide, P25, was "promiscuous" in that it stimulated T cell proliferation in cells obtained from a majority of the immune, as well as the malaria naive, individuals. The P6 (HVGQSASRGRGENPDDEE) epitope, alone or combined with the P25 (VRRRVNAANKKPEDLTL) epitope, was synthesized in tandem with the *P. vivax* repeats in tetrabranched MAPs (Herrera *et al.*, 1994). In addition, a MAP construct combining the vivax repeat B cell epitope with the universal tetanus toxoid-derived T cell epitope, P30 (FNNFTVSFWLRVTKVSASHLE), was also synthesized.

BALB/c mice were immunized sc with MAPs containing only the B, only the T or a combination of B and T cell epitopes (Herrera *et al.*, 1994).

Despite the ability of monoepitope B MAPs to induce BALB/c T cell proliferation *in vitro*, the MAPs containing only the B cell epitope failed to induce an antibody response in these mice. When T cell help was provided by the universal TT epitope P30, anti-repeat antibody ELISA titers of 2×10^4 and reactivity with *P. vivax* sporozoites (IFA titer 1×10^3) were obtained.

In contrast, when T cell help was provided by the T cell epitopes derived from the *P. vivax* CS protein (P25 and/or P6), only low reactivity with *P. vivax* repeats or sporozoites was obtained, despite the ability of these constructs to induce high anti-MAP antibody titers (4×10^4). Therefore, as in the MAPs containing the T helper epitopes from the C terminus of the *P. falciparum* CS protein (T2, T3, and T4) (Calvo-Calle *et al.*, 1993), the T cell epitopes derived from the C terminus of *P. vivax* CS protein were highly immunogenic in the context of MAPs, but failed to induce antibody that reacted with native CS on the sporozoites.

c. Plasmodium malariae

Plasmodium malariae, the third species of human malaria, is responsible for long-term chronic infections, persisting in some cases for 30 years. While the response to linear peptides containing the repeats of *P. malariae* is genetically restricted, this restriction can be overcome when the repeats are incorporated into MAPs (Del Giudice *et al.*, 1990). The majority of inbred mouse strains immunized with an octamer MAP containing $[(NAAG)_6]_8$, but not a linear $(NAAG)_6$ peptide, developed antibody reactive with *P. malariae* repeats. Following immunization with the $[(NAAG)_6]_8$ MAP, mice of the H-2 $^{b,k, \text{ or } f}$ haplotypes were high responders, H-2 $^{d,q, \text{ or } s}$ mice were intermediate responders, and H-2^r were nonresponders. As noted in previous studies (Tam, 1988), antibody to the Lys_7 peptide core was not detected in any of the mice immunized with the octabranched MAP $[(NAAG)_6]_8$ or with a Lys_7 core peptide.

The universal response to the $[(NAAG)_6]_8$ MAP was then utilized to construct a diepitope synthetic peptide complex capable of inducing anti-repeat antibody responses to two different malarial species. The eight-branched $[(NAAG)_6]_8$ MAP was conjugated to a $(NANP)_{40}$ peptide using carbodiimide. This hybrid synthetic construct induced antibody to the *P. falciparum* CS repeats in BALB/c and C3H mice, strains that do not respond to immunization with $(NANP)_{40}$ alone, thus demonstrating the effectiveness of the (NAAG) sequence to provide T cell help for anti-(NANP) antibody production.

B. TOXOPLASMOSIS

Toxoplasmosis, a universal, highly frequent infection induced by the protozoan parasite *Toxoplasma gondii*, can lead to severe neurological

and ocular lesions in newborns of mothers infected during pregnancy. Furthermore, the incidence of toxoplasmic encephalitis in adults has increased greatly in recent years in AIDS and other immunosuppressed patients. In contrast, in immunocompetent individuals toxoplasma infections are usually inapparent and result in lifelong protective immunity against this illness. Numerous attempts are being made to develop a vaccine against toxoplasmosis based on attenuated parasites, recombinant antigens, or synthetic constructs.

A major antigen recognized by acute and convalescent human antisera is P30, a surface membrane protein of *T. gondii* tachyzoites, comprising 3–5% of the total protein of this parasite (Kasper *et al.*, 1983). P30 has been purified from parasite extracts and obtained as a recombinant protein (Burg *et al.*, 1988). P30 induces high IgG antibody titers in immunized animals, and these antibodies kill *T. gondii* tachyzoites in the presence of complement (Kasper *et al.*, 1986). P30 also induces the release of high concentrations of IFN-γ from PBL of seropositive individuals and has been shown to stimulate a specific cytotoxic T cell response in mice (Khan *et al.*, 1988a,b).

Octameric MAPs containing a peptide sequence of the amino terminal region of P30, aa 48–67, as well as the corresponding linear peptide, induced high levels of peptide specific antibodies in mice and rats (Darcy *et al.*, 1992). These sera also recognized P30 in NP-40 extracts of parasites, but failed to react with the native antigen on tachyzoites.

Despite the apparent lack of antibody reactivity with the parasite, 40% of the MAP-immunized mice resisted an otherwise lethal toxoplasma challenge. Immunization with the linear peptide, based on the same sequence, failed to confer any degree of protection. Passive transfer of T cells obtained from MAP-immunized Fischer rats to nude rats doubled the survival time of the *T. gondii*-infected recipients. While several of the T lymphocyte recipients developed detectable levels of antibody, the findings nevertheless appeared to indicate the presence of an additional antibody-independent mechanism of protection induced by MAP immunization (Darcy *et al.*, 1992).

III. MAPs as Immunogens in Helminthic Diseases

A. Schistosomiasis

Schistosomiasis is most frequently a chronic disease which affects approximately 200 million persons in South America, the Caribbean, Africa, Asia, and in the Middle East. People living in these areas are exposed to multiple reinfections, and immunity develops only gradually and is usually incomplete. These infections lead to an estimated 1 million deaths annually.

A number of studies *in vivo* and *in vitro* have shown that the larval stages (skin, lung, postlung, and preliver stage schistomulae) are the targets of immune elimination. These parasites are eliminated *in vivo* by cellular responses as well as by passive transfer of antibody (Mangold and Dean, 1986; Jwo and LoVerde, 1989; Richter *et al.*, 1993). The mechanism of resistance in mice immunized with irradiated cercariae, the infective skin stage, was shown to require functional T and B cell compartments (Sher *et al.*, 1982).

Murine monoclonal antibodies or sera of infected or vaccinated animals have been used as probes to select several immunogens as candidate vaccines. Three of these antigens were used to construct MAPs for immunization purposes. These are Sm23, an integral membrane protein (Harn *et al.*, 1985a), Sm28-GST, an excretory/secretory molecule with gluthatione transferase activity (Balloul *et al.*, 1985, 1987a), and the glycolytic enzyme triose-phosphate isomerase (TPI) (Harn *et al.*, 1985b, 1992).

B. Antigenicity and Immunogenicity of MAPs Based on Schistosome Antigens

1. MAPs Based on the Schistosome Antigen TPI

The schistosome TPI, a 28-kDa antigen, shares 50% sequence homology with the analogous human enzyme (Shoemaker *et al.*, 1992). The potential for generating autoimmunity with this particular immunogen would therefore be high if the entire molecule were to be used. However, the majority of schistosome-specific residues are clustered, so that long spans of the molecule have minimal or no homology to the human enzyme.

The immunoreactive portions of TPI were defined using tryptic fragments of full-length recombinant TPI, and the epitopes were mapped by the use of synthetic peptides (Reynolds *et al.*, 1994). T cell epitopes were defined using lymphocytes from mice immunized with irradiated cercariae, as well as Th1-type $CD4^+$ T cell clones. Two T cell epitopes were identified based on proliferative responses in immunized mice. P9 (EVCVRQLKAIANK) was recognized by CBA/J cells, and P18 (RKWFKTNAPN) was identified by cells of C57Bl/6J mice. Sera from chronically infected mice, as well as mice vaccinated with irradiated cercariae, also reacted with P9 demonstrating the presence of both B and T cell epitopes in this sequence.

The immunodominant B and T cell epitopes of TPI were constructed as tetrameric MAPs using Fmoc technology (Fig. 7). Diepitope MAPs containing $(P18P9)_4$, or the reverse orientation $(P9P18)_4$, were tested for antigenicity using murine peptide-specific T cell clones. A T cell clone specific for P9 was stimulated by MAP $(P18P9)_4$, and to a lesser extent by

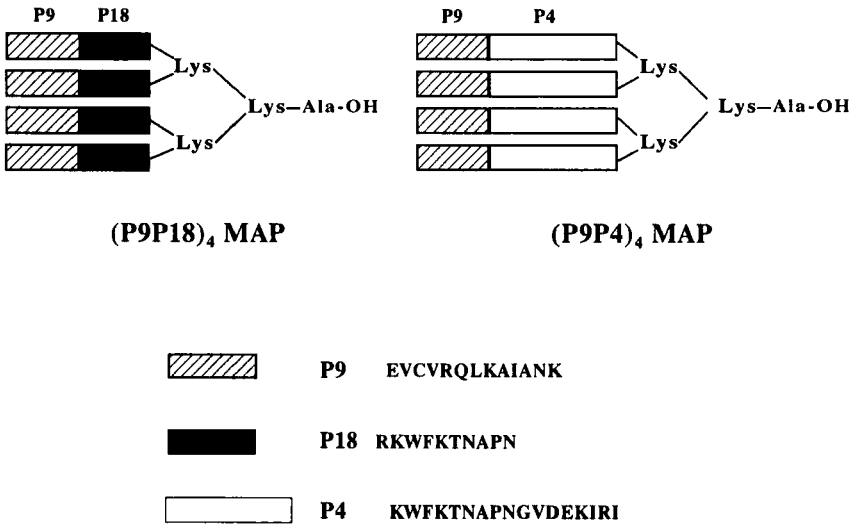

FIG. 7. Structure of MAPs containing T and B cell epitopes derived from *S. mansoni* triose phosphate isomerase (TPI).

MAP $(P9P18)_4$. Unexpectedly, the P18-specific T cell clone failed to respond to either of the MAP constructs containing the P18 sequence.

An additional MAP construct was synthesized replacing P18 by P4, a peptide which included eight additional amino acids on the C terminus, KWFKTNAPNGVDEKIRI (Reynolds *et al.*, 1994). Unlike MAPs containing the shorter amino acid sequence, the $(P9P4)_4$ MAP was highly immunoreactive with both peptide-specific T cell clones. The MAP was also highly immunogenic, inducing potent primary responses in mice and antibody reactivity with schistosome TPI (Reynolds *et al.*, 1994). Following immunization with 25 μg of $(P9P4)_4$ MAP adsorbed to alum, a significant reduction in worm burden was obtained, varying from 39 to 82% in different experiments (Harns *et al.*, 1995). The TPI MAP also elicited significant protection when it was incorporated into liposomes.

2. *MAPs Based on the Schistosome Sm23 Antigen*

MAPs were also synthesized for Sm23, an integral membrane protein of schistosomes found in all stages of the parasite in the human host. The identification of relevant B cell epitopes in this antigen was more difficult since the antibody response was directed against conformational epitopes (Oligino *et al.*, 1988; Reynolds *et al.*, 1992). Sera of chronically infected mice, or of mice vaccinated with irradiated cercariae, failed to bind to reduced native or recombinant Sm23.

Peptides spanning two hydrophilic domains of Sm23 were tested for antibody reactivity (Reynolds *et al.*, 1992). Two peptides, P12 and P14, were recognized by immune sera from two strains of inbred mice. Mercaptoethanol treatment of the peptides resulted in significant loss of antibody binding to P14, but not to P12, suggesting that P14 contained a reduction-sensitive epitope. P14 also contained a T cell epitope recognized by three different strains of mice.

The P14 epitope, which contains both B and T cell epitopes, was synthesized as a tetrameric MAP (Harn *et al.*, 1995). Mice immunized with 25 μg of $(P14)_4$ MAP in alum developed antibodies that recognized a recombinant Sm23 protein. The MAP-immunized mice had a reduction in worm burden after infection, which ranged from 36 to 75%. These results indicated that this MAP containing B and T cell epitopes of Sm23 was highly protective when administered in alum. In contrast, the Sm23 MAP was not protective when incorporated into liposomes.

3. *MAPs Based on the Schistosome Sm28-GST, the Glutathione Transferase of Schistosoma mansoni*

Sm28-GST, first isolated from *S. mansoni* adult worms, is a secretory/excretory molecule with glutathione-S-transferase activity (Balloul *et al.*, 1987a). This protein has been cloned, sequenced, and expressed in *Escherichia coli* (Balloul *et al.*, 1987b). An antiworm fecundity effect was observed in mice and baboons immunized with recombinant Sm28-GST (Boulanger *et al.*, 1991).

The use of synthetic peptides based on the Sm28-GST sequence allowed the identification of major B and T cell epitopes in mice and rats (Wolowczuk *et al.*, 1989; Auriault *et al.*, 1988, 1991). The T cell epitopes were also recognized by lymphocytes from experimentally infected baboons and naturally infected humans. A peptide containing aa 115–131 of Sm28-GST, when linked to a carrier protein (ovalbumin), induced the proliferation of T lymphocytes from *S. mansoni*-infected or -immunized animals (Wolowczuk *et al.*, 1991). Peptide aa 115–131 has also been used to detect the antibody response to *S. mansoni* of Kenyan school children (Auriault *et al.*, 1991), demonstrating the presence of both B and T cell epitopes within the sequence.

An octameric Sm28-GST MAP containing aa 115–131 was synthesized and tested for immunogenicity in rats, mice, and baboons (Wolowczuk *et al.*, 1991; Auriault *et al.*, 1991). The T lymphocytes of immunized rodents and baboons proliferated in response to challenge with the Sm28-GST MAP construct and also the native antigen in parasite extracts. The immunized baboons and rodents developed antibodies specific for the Sm28-GST, which in some instances were cytotoxic for the parasite. Furthermore,

Sm28-GST MAP-immunized rats developed an appreciable level of protection, i.e., a 40–50% reduction in worm burden (Wolowczuk *et al.*, 1991).

The antigenicity of a series of MAPs and peptide constructs, based on the 115–131 aa sequence of Sm28-GST, was compared (Marguerite *et al.*, 1992). It was found that an octamer was more effective than a dimer in binding antibodies to Sm28-GST. Both the octamer and the dimer MAPs were more effective than a glutaraldehyde-linked polymer of this sequence. Only the octameric MAP induced a slight, but reproducible, stimulation of T cells of rats immunized with recombinant Sm28-GST.

IV. MAPs as Immunogens for Viral and Bacterial Diseases

A. Viral Diseases

Concurrent with the studies in parasitic diseases, MAP technology has also been applied to the design of vaccines for human viral infections, including Hepatitis and HIV, as well as the veterinary pathogen, FMDV (Table III). Antibody reactive with the target viral protein and, in some cases, neutralizing activity has been elicited by MAP immunization, however, the absence of small animal models has precluded studies of protective efficacy of MAP-induced anti-viral immunity.

1. Hepatitis B Virus

The enhanced immunogenicity of diepitope MAPs containing both T helper and B cell epitopes was originally demonstrated using Hepatitis B surface antigen (HBsAg) as a model protein (Tam and Lu, 1989). The HBsAg amino acid sequences selected for inclusion in the diepitope MAP were defined by studies in viral carriers. The *a* determinant is the major serological reactivity in immune sera, while the conserved pre-S(2) region contains both a murine T helper epitope, as well as a protective B cell epitope (aa 14–32).Two peptides, one representing a tandem repeat of aa 140–146 of the *a* determinant of the S region, and a second peptide representing aa 12–26 of the pre-S(2) region, were tested either alone or in combination in different MAP constructs.

Diepitope MAPs were constructed using either a combination of Fmoc/tert-butyl and Boc/benzyl chemistry or by dimerization of two different monoepitope MAPs that contained Cys(Acm) at the COOH terminus. In contrast to the monoepitope octamer MAP containing only the *a* determinant, which was nonimmunogenic in rabbits, the monoepitope MAP containing the pre-(S)2 peptide elicited antibodies reactive with both MAPs and native protein. The combination of the pre-S(2) sequence with the nonimmunogenic *a* determinant in a diepitope MAP overcame the poor

TABLE III
MAPs as Immunogens in Viral Diseases

Virus	Antigen	Epitope	Animal model	Immune response elicited	Reference
Hepatitis	HBsAg	S; pre-S(2)	Rabbits	Antibodies to native protein	Tam *et al.* (1989)
FMDV	VP1	aa 141–160	Guinea pigs	Neutralizing antibodies	Francis *et al.* (1991)
HIV-1	gp120	V3 loop IIIB; MN isolates	Guinea pigs	Neutralizing antibodies	Wang *et al.* (1991)
		V3 loop IIIB; RF; MN isolates	Rabbits, guinea pigs, and mice	Neutralizing antibodies	Nardelli *et al.* (1992)
		V3 loop IIIB isolate	Guinea pigs	Neutralizing antibodies	Defoort *et al.* (1992)
			Mice	Cytokines and $CD8^+$ CTL	
		V3 loop IIIB isolate	Mice	$CD8^+$CTL	Nardelli *et al.* (1993)
		V3 loop IIIB; MN isolates	Rabbits	Neutralizing antibodies; ADCC	Vogel *et al.* (1994)
		V3 loop IIIB isolate	Mice	Mucosal and systemic immune response	Nardelli *et al.* (1994)
		C4 domain, aa 419–439	Mice	Antibodies to native protein	Kelker *et al.* (1994)

immunogenicity of the *a* determinant epitope and induced high titers of antibody reactive with both peptides as well as with the native HBsAg.

Diepitope MAPs constructed either by dimerization of monoepitope MAPs (16 branches) or by synthesis of each epitope on alternating branches of an octameric MAP construct, were equally effective in elicting antibodies reactive with the native protein. In contrast to the dimerized monoepitope MAPs, further amplification by dimerization of the heterobranched MAPs resulted in lower antibody titers against the *a* determinant in both MAPs and native protein.

Similar studies using Fmoc technology have been carried out using Herpes simplex virus (HSV) as a model antigen (McLean *et al.*, 1991, 1992). The enhanced immunogenicity of HSV-1 epitopes in octameric MAP constructs compared to linear peptide conjugated to either protein or resin carriers was demonstrated in rabbits. In additional studies, a "cassette" peptide, containing a 17mer T helper epitope derived from sperm whale myoglobin, was added to the N terminus of the octameric HSV-1 MAPs by fragment condensation. Of the six MAPs tested, five were found to contain the T helper epitope, in variable amounts, and all five of the diepitope MAPs showed enhanced immunogenicity when tested in mice.

The studies using HBsAg and HSV as model antigens demonstrated the feasiblity of constructing diepitope MAPs by various synthetic methods and provided the basis for the analysis of functional immunity induced by MAPs containing defined T and B cell epitopes of other infectious organisms.

2. *Foot and Mouth Disease Virus*

Neutralization assays have been used to demonstrate the biological activity of the antibody response induced by MAPs vaccines against viral pathogens. In FMDV, MAPs containing the major immunogenic site of VP1 antigen (aa 141–160), which contains both a B cell epitope (N terminus aa 141–149) and a T cell epitope (C terminus aa 150–160), were tested for immunogenicity in guinea pigs using di-, tri-, and octabranched constructs (Francis *et al.*, 1991). Tetrameric and octameric FMDV MAPs were more immunogenic than the dimer, which required 5- to 25-fold more antigen to induce a similar level of response. When the fine specificity of the antibodies was mapped using short synthetic peptides in an ELISA, antisera raised against tetramer and dimer, but not the octamer, MAP preferentially recognized the N-terminal peptides containing the B cell epitope. Despite the differences in the fine specificity of the anti-MAP antibody response, the neutralizing antibody levels elicited by tetrameric and octameric constructs were similar, suggesting that the tetrameric MAP structure was sufficient for an optimal response.

A comparison of the immunogenicity of the octameric FMDV MAP administered in Freund's versus alum revealed a delayed primary response in the guinea pigs immunized with alum-adsorbed MAP. However, after reinoculation, peak neutralizing titers similar to those obtained with Freund's were obtained in the MAP/alum-immunized guinea pigs.

3. *Human Immunodeficiency Virus 1*

While protective immune responses are not yet fully defined, antibody to the third hypervariable (V3) region of the HIV-1 envelop protein, gp120, can neutralize virus infectivity and mediate antibody dependent cell cyto-

toxicity (ADCC) *in vitro*. The presence of neutralizing antibodies to the V3 loop in HIV-1-positive mothers is associated with protection of the fetus from viral infection *in utero* (Devash *et al.*, 1990).

Several MAP immunogens have been designed to induce antibody specific for linear and conformational epitopes within the V3 loop of the HIV-1 gp120 protein. In one of the first studies, MAPs were constructed containing B and T cell epitopes of a principal neutralizing determinant, aa 297–329, of the V3 loop of the HIV-1 IIIB gp120 (Wang *et al.*, 1991). Immunogenicity studies in guinea pigs compared the response induced by an octameric HIV-1 MAP, in which 95% of the total mass consisted of the peptide epitope, to the response induced by a linear peptide–BSA conjugate in which the epitope comprised only 6–28% of the total mass. At 4 months after immunization with two injections of antigen, both the peptide conjugate and the MAP induced similar antibody responses that were detectable by ELISA, immunoblot, or virus neutralization. However, in contrast to the linear peptide, the neutralizing antibody titers in the MAP-immunized guinea pigs continued to increase over time until they were 100-fold higher than those obtained with the peptide conjugate and 1000-fold higher than those elicited by immunization with inactivated HIV-1 lysate. The high levels of virus neutralizing activity induced by MAP immunization were sustained over a 3.5 year period. In addition, cross-reaction with heterologous viral strains and increased binding affinity developed over time, suggesting that late-appearing conformational antibodies may be important in the later stages of the immune response. Guinea pigs immunized with a mixture of six different octamer MAPs containing the V3 loop epitope of six HIV isolates induced high levels of neutralizing antibody reactive with the two viral strains tested. MAP constructs containing the aa 297–329 epitope truncation at the amino, but not the carboxy, terminus failed to induce neutralizing activity suggesting that the relevant epitope, or conformation, was contained in the longer peptide sequence. The octamer MAPs containing the HIV V3 loop were highly immunogenic when formulated in either Freund's or alum adjuvant.

A similar MAP containing 31 amino acids of the V3 loop of the MN isolate has been synthesized by Fmoc chemistry to provide an octameric construct (Vogel *et al.*, 1994). Rabbits were immunized seven times using this MAP emulsified in RIBI, an adjuvant that contains monophosphoryl lipid A, trehalose dimycolate, and cell wall skeleton of *Mycobacterium* (MPL + TDM + CWS) (RIBI Immunochem Research, Inc.). The sera of the MAP/RIBI-immunized rabbits effectively neutralized the HIV-1 MN isolate and mediated antibody-dependent cell cytotoxicity of HIV-1 MN-infected cells. In contrast to the results observed in MAP-immunized guinea pigs (Wang *et al.*, 1991), the sera of the MAP-immunized rabbits

demonstrated minimal inhibition of the cytopathic effect of a heterologous isolate, HIV-1 IIIB (Vogel *et al.*, 1994). Differences in the HIV-1 sequences contained in the MAPs, the type of adjuvant used, and the species of animal immunized may have contributed to the different experimental results.

In an effort to broaden the response to heterologous isolates of HIV, MAPs were constructed containing a 16-aa T cell epitope derived from a conserved sequence in the carboxy terminal of gp120 combined with the V3 loop of three different isolates of HIV (Nardelli *et al.*, 1992). Preliminary experiments were carried out to assay the immunogenicity of monoepitope tetramer MAPs containing 11–24 amino acids of the HIV-1 V3 loop of IIIB, RF, or MN isolates. Nine different V3 MAPs, containing full-length or truncated sequences of the type-specific V3 epitopes, were used to immunize different species of animals. Rabbits developed high-titer anti-peptide responses following immunization with all nine MAPs constructs, while guinea pigs and mice responded to fewer of the constructs with lower titers of antibody. In mice, the longer V3 peptide sequences (>11 aa) were required to elicit antibody that reacted with the gp120 protein. Immunization with the type-specific V3 MAPs induced low levels of cross-reactivity with peptides, but not with recombinant proteins, of heterologous HIV isolates.

To further enhance the immunogenicity of the V3 loop HIV MAPs, the conserved T helper epitope from the C terminus aa 429–443 of gp120 (QIIN-MWQEVGKAMYA) (Cease *et al.*, 1987) was synthesized in equimolar ratios with the V3 loop epitopes in tetramer MAPs (Nardelli *et al.*, 1992). The inclusion of the conserved T helper epitope elicited enhanced antibody responses in mice and rabbits and induced antibody to the nonimmunogenic V3 epitopes in mice. Rabbits immunized with the diepitope MAP produced antibodies reactive with both the V3 loop as well as the T helper epitope. A correlation was observed between the type-specific ELISA titers and virus neutralization. Antisera of the MAP-immunized mice inhibited viral syncytia formation in $CD4^+$ CEM cells infected with recombinant vaccinia viruses expressing HIV gp160 of the homologous isolate.

MAPs based on the amino acid sequences derived from the CD4-binding domain of the HIV envelope protein can induce antibody responses to conformational epitopes (Kelker *et al.*, 1994). The presence of unique conformational epitopes present on MAPs, but not linear peptides, has been demonstrated using two sequences, aa 419–439 and aa 105–117, of gp120 HIV-1. The 419–439 aa sequence encodes both T and B cell epitopes and contains a conformation-dependent domain of gp120 that is known to function in binding to CD4 (Lasky *et al.*, 1987). The aa 105–117 sequence is conserved in gp120 and contains a known T helper epitope (Ahlers *et al.*, 1993).

Antisera induced by immunization with octamer MAP constructs containing the aa 419–439 sequence reacted at high titer with homologous MAPs by ELISA and with gp120 and recombinant gp160 precursor protein (Kelker *et al.*, 1994). The sera of MAP-immunized mice reacted with native, but not denatured, gp160 in ELISA and dot blot assays. In contrast, antisera raised against the linear peptides containing the aa 419–439 sequence generated high titers of anti-peptide antibody that reacted at low titers with both native and denatured recombinant protein. Similar immunogenic and antigenic properties were observed with MAPs containing the aa 105–117 sequence. The sera of mice immunized with MAPs containing the aa 419–439 sequence reacted with HIV- infected CEM-T4 cells when assayed by flow cytometry. Immunization with MAPs containing the relatively conserved aa 419–439 sequence generated antibodies that recognized gp120 of both the SF2 and the LAV-1 isolates.

Although the immunogenicity of HIV MAPs in humans remains to be tested, the fact that MAPs are recognized by human cells *in vitro* supports their potential as candidate vaccines. MAPs containing HIV-1 gp120 V3 sequences have been used as antigens in the induction of primary *in vitro* antibody responses in naive human cells (Fraisier *et al.*, 1994). Transformation of the *in vitro*-primed cells with EBV has led to the generation of neutralizing human monoclonal antibodies specific for HIV-1 gp 120. These studies also demonstrated the greater efficacy of the MAP versus linear peptide in inducing primary antibody responses *in vitro*.

While the role of $CD8^+$ cytotoxic T lymphocytes (CTL) in HIV-1 infection is not yet defined, CTL play a key role in the immune response against many viral pathogens. Linear peptides modified with a lipid moiety, tripalmitoyl-*S*-glyceryl cysteine (P3C), can elicit $CD8^+$ CTL responses in mice (Deres *et al.*, 1989).

The HIV-1 model demonstrated that P3C-modified MAPs can also generate murine $CD8^+$ cytotoxic T cells, as well as antibody and $CD4^+$ T helper responses (Defoort *et al.*, 1992a,b; Nardelli and Tam, 1993). MAPs, containing B, T helper, and cytotoxic T cell epitopes present in aa 308–331 of the HIV-1 IIIB V3 loop, were modified by the addition of the lipophilic P3C adjuvant. The synthesis of the lipopeptide MAPs was based on Fmoc strategy to avoid exposure of the secondary ester bond in P3C to the HF required for cleavage in Boc chemistry. Both the direct stepwise approach and an indirect modular approach for construction of the P3C-modified MAPs have been compared (Defoort *et al.*, 1992a,b). The P3C was linked to the side chain of a lysine spacer (Ser–Ser–Lys) at the carboxyl terminus of the MAPs in order to provide flexibilty for the P3C to serve as a lipid-anchoring moiety without interfering with the antigenic sequences at the amino terminus.

The immunogenicity of the lipopeptide MAPs, when further amplified by aggregation in micelles in aqueous solution or incorporated into liposomes, was tested in mice and guinea pigs (Defoort *et al.*, 1992a). Both the free and liposome-associated MAP–P3C induced similar levels of neutralizing antibody in guinea pigs and mice when assayed by inhibition of syncytia formation and reverse transcriptase activity of HIV IIIB-infected cells. A mixture of isolated MAPs and P3C in liposomes was not immunogenic in mice. Guinea pigs immunized with the same mixture developed antibodies that, although reactive with MAPs and gp120, lacked neutralizing activity.

The MAP–P3C induced not only CD4+ T cells, but also CD8+ T cells capable of lysing peptide coated target cells and virus-infected cells expressing the precursor gp160 (Defoort *et al.*, 1992a,b). Additional studies carried out using tetrabranched MAP–P3C, containing aa312–329 of the IIIB isolate V3 loop, demonstrated that both iv and ip *in vivo* priming with the lipid-conjugated MAPs were effective in eliciting a long-lasting CTL response in mice (Nardelli and Tam, 1993). Immunization with a mixture of two type-specific HIV MAP–P3C constructs induced both IIIB- and MN-specific $CD8^+$ CTL responses.

Oral immunization with MAP–P3C was found to induce both mucosal and systemic immunity (Nardelli *et al.*, 1994). The saliva of the MAPs-immunized mice contained IgA anti-gp120 antibodies, while their sera had significant anti-gp120 IgG titers. In addition, $CD8^+$ CTL capable of lysing peptide-pulsed target cells, or cells infected with recombinant vaccinia virus expressing the HIV-1 glycoprotein, were also detected in the spleens of the immunized mice.

B. Bacterial Diseases

MAPs have recently been applied to the development of vaccines aimed at a diverse spectrum of bacteria including those causing meningitis, dental caries, and ocular infections. In these systems, MAP immunization elicited functional antibodies that mediated complement-dependent bacterial lysis, inhibition of bacterial enzymes, and neutralization of bacterial infectivity.

1. Neisseria meningitidis

The efficacy of current vaccines based on the meningococcal capsular polysaccharides of *N. meningitidis*, the causative agent of meningococcal meningitis, is limited by strain specificity and short duration of the immune response induced by vaccination. Recent efforts at vaccine development have focused on the design of vaccines capable of inducing long-lasting bactericidal antibody responses that can lyse encapsulated *Neisseria* of different group B serotypes.

Antibodies capable of mediating complement dependent bacteriolysis recognize conformation-dependent epitopes in the apices of surface exposed hydrophilic loops of the *Neisseria* outer membrane (OM) protein. To investigate the ability of MAPs to induce antibody reactive with these conformational epitopes, a B cell epitope from the meningococcal OM protein subtype P1.16b loop, TKNTNNNLTL, was synthesized as monoepitope octamer MAP with a triglycyl spacer at the C terminus of the peptide chains to improve flexibility (Christodoulides and Heckels, 1994). Diepitope tetramer MAPs were also constructed to contain the B cell epitope synthesized in tandem with the universal tetanus toxin T cell epitope P2, represented by the sequence QYIKANSKFIGITE (Panina-Bordignon *et al.*, 1989).

The diepitope, but not the monoepitope, MAP, induced high levels of anti-peptide antibody in both rabbits and mice (Christodoulides and Heckels, 1994). More importantly, sera of the MAP-immunized animals reacted in ELISA with the meningocccal OM protein subtype P1.16b, as well as with a heterologous subtype, P1.16a. Titers induced by immunization with MAP were higher than those induced by a linear hybrid peptide containing the bacterial and TT-derived epitopes. The predominant specificity of the anti-MAP antisera was directed against conformational epitopes, as shown by the ability to react with native OM protein in ELISA, but not the denatured protein in Western blots. The antibodies induced by MAP immunization of rabbits, but not mice, were biologically active and mediated complement-dependent bactericidal killing of homologous (P1.16b) and heterologous (P1.1a) serosubtypes of meningococcus. The lack of bactericidal activity in the sera of the MAP-immunized mice was felt to be related to the use of Freund's adjuvant which induces a predominant γ-1 IgG response which does not activate complement.

2. *Streptococcus mutans*

The oral streptococci have been targeted for the development of subunit vaccines for the prevention of dental caries. Antibody capable of inhibiting a glycosyltransferase enzyme (GTFs) of *S. mutans* has been shown to reduce the accumulation of streptococci on the tooth surface and thus reduce the number of cavities. A peptide sequence containing the putative catalytic site of the GTF enzyme was synthesized as a tetramer MAP for immunogenicity studies in rodents (Smith *et al.*, 1994). The 21mer peptide sequence, DANFDSIRVDAVDNVDADLLQ, is conserved among oral streptococcal GTFs. Polyclonal antisera was raised in rats injected sc in the salivary gland region with MAPs in Freund's adjuvant, while monoclonal murine antibodies were derived from mice immunized with MAPs bound to nitrocellulose.

Murine monoclonal anti-MAP antibody, and sera of rats immunized with MAP, reacted with both *S. sobrinus* and *S. mutans* GTF antigens in ELISA and Western blots. Monoclonal antibodies derived from mice immunized with the GTF MAP were as effective as sera from mice immunized with native GTF in inhibiting bacterial enzyme activity *in vitro*.

3. *Chlamydia trachomatis*

Vaccines to prevent ocular infection due to *C. trachomatis*, based on whole cells or synthetic peptides, can only be effective if they induce very high titers of neutralizing antibody reactive with different serovars of the pathogen. Following the demonstration of a *Chlamydia* outer membrane protein (Omp) as the target of neutralizing antibody, immunodominant epitopes in the variable segments of the Omp1 have been targetted for development of synthetic peptide vaccines.

In an effort to design vaccines capable of neutralizing multiple serovars, octameric MAPs were constructed containing T and B cell epitopes derived from the Omp1 variable domain IV (VD IV) of *C. trachomatis* serovar B (Zhong *et al.*, 1993). The response to the B serovar VD IV MAP was compared to a MAP in which the B Omp1 VD IV sequence was tandemly synthesized with a 13-amino acid variable sequence (VD I) from the C serovar. The MAPs were further modified by addition of three lipidic amino acids, $\{HNCH\ [(CH_2)_{13}\ CH_3\ ]CO_3\}NH_2$, at the C terminus of the polylysine core to act as a fatty acid anchor and enhance membrane binding.

The lipid-modified octamer MAP containing the B serovar VD IV sequence was significantly more immunogenic than a lipid-modified linear peptide and induced serogroup-specific antibody responses in three of four mouse strains. When octameric lipopeptide MAP containing a combination of the B and the C serovar variable domain sequences (B Omp1 VD IV -C Omp1 VD I) was tested, high ELISA titers against the MAP immunogen were detected in four of five inbred murine strains. The anti-MAP titers were 10- to 3200-fold higher than those raised when a hybrid 30mer linear peptide was used to immunize the different strains of mice. The sera from MAP-immunized mice of four of five strains reacted not only with B and C, but also with A, serovar bacteria in ELISA and neutralized chlamydial infectivity of all three serovars *in vitro*. However, the anti-chlamydial ELISA titers were 20- to 40-fold lower, and the neutralizing titers were more than 500-fold lower, than the anti-peptide antibody titers. The discrepancy between the anti-peptide titers and reactivity with the native Omp1 on the bacteria suggests that the MAP configuration was not optimal and conformational constraints may exist in the chlamydial Omp1 protein.

V. MAPs as Antigens

In addition to their potential as immunogens for prophylaxis of infectious diseases, MAPs provide sensitive immunological reagents for immunocytochemical studies and for the measurement of antibody/antigen and receptor/ligand interactions.

In solid-phase immunoassays, MAP antigens have superior antibody-binding capacity when compared to linear peptides (Tam and Zavala, 1989; Habluetzel *et al.*, 1991; Marsden *et al.*, 1992). Unlike linear peptides, the binding of the MAPs to microtiter wells does not require conjugation to carrier protein. Therefore, the potential peptide alterations due to chemical conjugation are avoided in the MAP-based immunoassays. Sensitivity of the assay is further increased by the multimeric structure of the MAPs which amplifies the signal. Thus, the binding of polyclonal and monoclonal antibodies specific for viral antigens could be detected using 10^3- to 10^5-fold lower amounts of octamer MAPs than of the corresponding monomeric peptides (Marsden *et al.*, 1992). Introduction of a 4- or 5-amino-acid glycine spacer between the epitope and the polylysine core further increased the sensitivity of the MAP ELISA (Marsden *et al.*, 1992).

The enhanced sensitivity and specificity of the MAP-based immunoassays facilitated measurement of antibody to the *P. falciparum* and *P. malariae* CS proteins in sera of sporozoite-immunized volunteers and naturally infected individuals living in malaria endemic areas (Habluetzel *et al.*, 1991; Calvo-Calle *et al.*, 1992). The antibody responses detected by the MAP ELISA were specific for the epitopes contained in the MAPs and were inhibited by linear peptide representing the specific amino acid sequences of the malarial epitope.

The enhanced sensitivity and specificity of the MAP-based ELISA have also been utilized in the measurement of antibody responses in autoimmune diseases. An octameric MAP, containing a 25aa C terminal sequence of the small nuclear ribonucleoprotein D, proved a sensitive reagent for detecting autoantibodies in the sera of patients with systemic lupus erythematosus (Sabbatini *et al.*, 1993). A MAP-based ELISA has also been used to detect conformation-dependent anti-mitochondrial antibodies in the sera of primary biliary cirrhosis patients (Briand *et al.*, 1992b). An octameric MAP, containing a 13 aa sequence of a mitochondrial enzyme, was covalently linked with a lipoic acid cofactor to mimimic the lipoyl binding site of the target enzyme. MAP without lipoic acid was not recognized by sera of the PBC patients.

Octameric MAPs containing 31 amino acids of the V3 loop of the HIV-1 MN or IIIB isolates were used in ELISA to assay antibody responses of 27 HIV-1-infected patients (Vogel *et al.*, 1994). The high levels of ELISA

reactivity detected in the patients sera against the MN V3 loop, correlated with high levels of *in vitro* neutralizing activity, assayed by inhibition of the cytopathic effect of HIV-1 on the T cell lymphoma line MT-4. The virus-neutralizing activity of the patients sera, as well as ADCC in the presence of normal PBMC effector cells, was type specific and could be absorbed by the V3 MAP representing the MN, but not the IIIB, isolate. A critical epitope defined in the loop region, GPGRAF, was recognized only when presented as a MAP, but not as a linear peptide, suggesting the presence of a conformation-dependent epitope not present in the linear peptide.

Octameric MAPs containing conserved sequences of the CD4-binding domain of gp120 have also been used to measure conformation-dependent antibodies present in sera of mice immunized with a recombinant gp160 protein (Kelker *et al.*, 1994). Sera of these mice were shown to react in ELISA with MAPs, but not with linear peptides, representing two immunogenic HIV-1 epitopes, aa 419–439 and aa 105–117. Competition ELISA demonstrated that binding of immune sera to gp160 was inhibited by MAPs, but not by linear peptides, containing the CD4-binding domain sequences.

The multimeric structure of MAP antigens may give rise to amino acid side chain interactions that serve to constrict the conformation of the MAP constructs. In solution, synthetic peptides exist in multiple conformations, only a fraction of which may reflect the conformations of native proteins. The use of linear peptides as immunogens or antigens, therefore, frequently generates anti-peptide antibodies that do not react with native proteins and lack biological activity. The apparent ability of MAP constructs to stablize secondary structure, and therefore potential conformational epitopes, has been in one instance supported by CD and NMR spectroscopy (Esposito *et al.*, 1993).

The ability of MAPs in some, but not all cases (Briand *et al.*, 1992b), to provide superior immunogens for the induction of antibody reactive with native protein has also been utilized to generate antibody for immunocytochemical analysis of tissue distribution of proteins, such as guanine nucleotide-binding regulatory protein, G_o (Chang *et al.*, 1988). Similarly, rabbit antisera raised against a MAP containing sequences of the human lutropin hormone β-chain (hLH-β) was used as an immunochemical probe to map the three-dimensional structure of hLH and its β subunit (Troalen *et al.*, 1990). A linear peptide containing the corresponding hLH-β sequence, when conjugated to TT as a carrier protein, failed to induce antibody that recognized the native protein.

The difficulties associated with using evolutionarily conserved proteins as antigens have in some cases been overcome using MAP immunogens.

An octamer MAP construct was used to successfully produce both polyclonal and monoclonal antibody to a highly conserved protein, $p34^{cdc2}$ protein kinase, found in all eukaryotes (Kamo *et al.*, 1992). An additional advantage of MAPs is that the immunogencity of small epitopes is enhanced and therefore stretches of nonhomologous amino acids within conserved proteins can be selected for immunization.

A unique application of MAP technology has been the construction of Multimeric– Synthetic Peptide Combinatorial Library (M-SPCL) (Wallace *et al.*, 1994), which utilizes multimeric macromolecular MAP structure to enhance the sensitivity and specificity of synthetic peptide libraries. These M-SPCLs successfully identified peptide antagonists that could block cytokine receptor interactions, while peptide libraries based on linear peptides failed to identify an antagonist peptide sequence. The M-SPCLs were synthesized by the flow–polyamide method to produce an octameric lysine core on which randomized peptide sequences were assembled. The amplified peptide signal produced by the M-SPLC identified a mimotope consensus sequence which, when tested as octamer or tetramer MAP constructs, was capable of inhibiting the binding of recombinant human IL-6 receptor to IL-6 at nanomolar concentrations. The need for at least four copies of the epitope in the active MAPs constructs suggested that multiple binding sites increase the affinity of the peptide–protein interaction and/or that precise spatial arrangement of the sequences within each MAP was required for optimal binding.

VI. Conclusions

In the seven years since multiple antigen peptides were first described, a number of different MAPs have been synthesized based on sequences of biologically important proteins from infectious agents. The MAPs have been used as immunogens and as antigens, with the ultimate purpose of inducing and characterizing the protective immune responses. These constructs are diverse, and their number is still limited. Nevertheless, they make it possible to define some basic properties of MAPs and to outline the circumstances in which they may be particularly useful for immunization purposes.

The most important question in the design of MAP constructs is whether or not they will elicit immune responses directed against the native antigen. To a considerable degree this certainly is determined by the three dimensional structure of the antigen in the context of the pathogen. A successful MAP design also depends on the amino acid composition and, in some instances, the length of the peptide representing the epitope, as well as the relative position of B and T cell epitopes in relation to the lysine core.

Sequential B cell epitopes are, in general, easier to express in MAPs, but some conformational epitopes have been successfully incorporated into these constructs.

At this time, a degree of unpredictability still prevails concerning the optimal composition and configuration of MAPs which would ensure the generation of effective immune responses targeted against the native antigen in the context of the pathogen. Therefore, the optimal design of vaccines based on MAP constructs has to be determined empirically by studies in experimental models. In order to evaluate MAP efficacy it is furthermore essential to use not only peptide-based immunoassays, but also to determine reactivity with the native protein and/or biological activity.

A consistent finding has been that MAPs with multiple branches are more immunogenic and also better antigens than monomeric peptides, and in general tetrameric constructs have proved to be of equal or greater immunogenicity than octameric constructs. Experience with a larger number of constructs and diverse sequences is, however, required to determine the validity of these generalizations.

The inclusion of pathogen-derived T helper epitopes in the MAPs is highly desirable. It serves not only to elicit an anamnestic response in naturally infected individuals but, in addition, the exposure to infection can booster the vaccine-induced responses. MAPs can also elicit cytotoxic T cells when combined with a synthetic lipopeptide adjuvant.

Another example of the use of multiple epitopes in MAPs are constructs which contain polymorphic sequences, which are of critical biological importance. The immunization with MAPs containing peptides corresponding to several different HIV variants has resulted, in some cases, in the induction of antibodies which recognize and neutralize viruses of different isolates. In malaria, the incorporation of conserved T helper epitopes, derived from the CS protein of malaria parasites, has circumvented the polymorphism noted in T helper epitopes from the carboxy terminus of this protein.

A clear advantage of using defined epitopes in synthetic constructs occurs when part of the sequence of a pathogen-derived protein shares significant stretches of homology with host proteins. MAPs can then be constructed to contain short B and T cell epitope sequences, selected from nonhomologous regions of the molecule, as in the case of the MAPs based on the triose-phosphate isomerase of *S. mansoni,* discussed earlier in this chapter. A similar approach could also be useful for excluding immunosuppressive domains of antigens.

MAPs can and have been engineered to contain not only multiple epitopes, but also multiple antigens derived from different stages of the same or different pathogens, with the aim of obtaining a broader immune response, as well as potentiation of the protective effect. This multiplicity

of epitopes is also important in order to overcome the genetic restriction of recognition of individual T cell epitopes which, unless they are universal, would limit the usefulness of vaccines. Universal T helper epitopes, from unrelated antigens, such as tetanus toxoid, have also been incorporated in MAPs to broaden their recognition by individuals of diverse genetic make-up, as has been the case of MAPs based on the CS protein.

A significant recent advance has been the incorporation into the MAPs of a lipopeptide moiety which functions both as adjuvant and to enhance membrane binding. These synthetic adjuvants, different from other immunopotentiators, present the advantage of having fully defined chemical composition. The immunogenicity of MAPs containing lipidic tails can be further amplified through aggregation or by their incorporation into liposomes.

Of particular relevance to human vaccines is the fact that some MAPs, adsorbed to alum, induce levels of immunity comparable to those elicited by using Freund's. The immune response to MAPs incorporated into alum (or FCA) is quite long lived in experimental animals.

Phase I and II trials are being planned for candidate MAP vaccines for malaria, schistosomiasis, and HIV-1 infections in the near future. These first clinical trials of MAP-based vaccines will provide critical information on the immunogenicity and potential efficacy of MAPs in the immunoprophylaxis of human diseases.

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Eosinophils: Biology and Role in Disease

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I. Biology of the Eosinophil

A. Introduction

The term "eosinophile" was introduced by Ehrlich (1879) who observed that certain cells had numerous intercytoplasmic granules with an affinity for acidic dyes such as eosin. An association between the eosinophil and helminthic disease, allergy, and asthma, as well as certain cutaneous and malignant diseases was then established (Brown, 1898; Ehrlich and Lazarus, 1900). Investigators in the 1960s and 1970s were attracted by the idea that eosinophils could degrade mast cell-derived mediators of anaphylaxis suggesting that the cell might be important in ameliorating the allergic process (Goetzl *et al.*, 1975). In the mid to late 1970s the observation that eosinophils or their granule-containing proteins were toxic for helminthic parasitic larvae (Butterworth *et al.*, 1974) led to the current, widely held view that the teleological role of eosinophils is in host defense against worms. Eosinophils are also believed to be major effector cells in producing tissue damage in asthma and related allergic diseases (Gleich and Adolphson, 1986; Wardlaw and Kay, 1987). In recent years there has been a substantial increase in eosinophil research, stimulated to a large extent by the possibility that modulation of eosinophil function may prove to be effective in therapy for asthma and allergic disease. Over the years many original papers, reviews, and books devoted to the eosinophil have been published (e.g., Spry, 1988; Weller, 1991; Makino and Fukuda, 1993; Smith and Cook, 1993; Gleich and Kay, 1994; Wardlaw, 1994). Recent studies indicate that the cell may have a more complex functional role than previously appreciated. For example, eosinophils can generate large amounts of TGF-α and TGF-β raising the possibility of an important role for

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the cell in wound healing, tissue remodeling, and the development of postinflammatory fibrosis (Todd *et al.*, 1991; Wong *et al.*, 1993). Cytokine-stimulated eosinophils secrete IL-1, express HLA class II receptors, and present antigen to T cells *in vitro* suggesting they may act as important

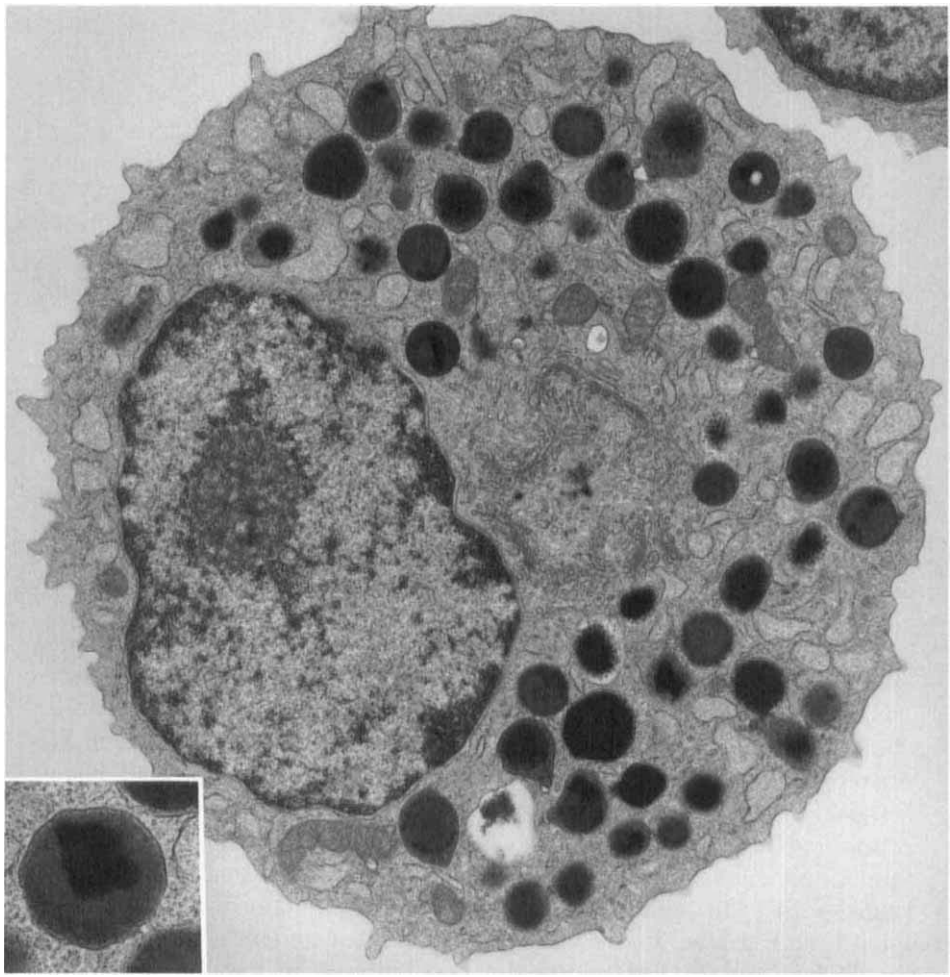


FIG. 1. Eosinophilic myelocyte in a 3 week rhIL-3 containing culture of human cord blood mononuclear cells shows an active Golgi area, large mitochondria, dilated cisterns of rough endoplasmic reticulum, and an extensive population of immature and maturing secondary granules with central dense cores (inset, higher magnification of one specific granule with core) and less dense matrix compartments. ×10,650; inset ×29,040 [Courtesy of Dr. Ann Dvorak. Reproduced with permission from Dvorak *et al.* (1991).]

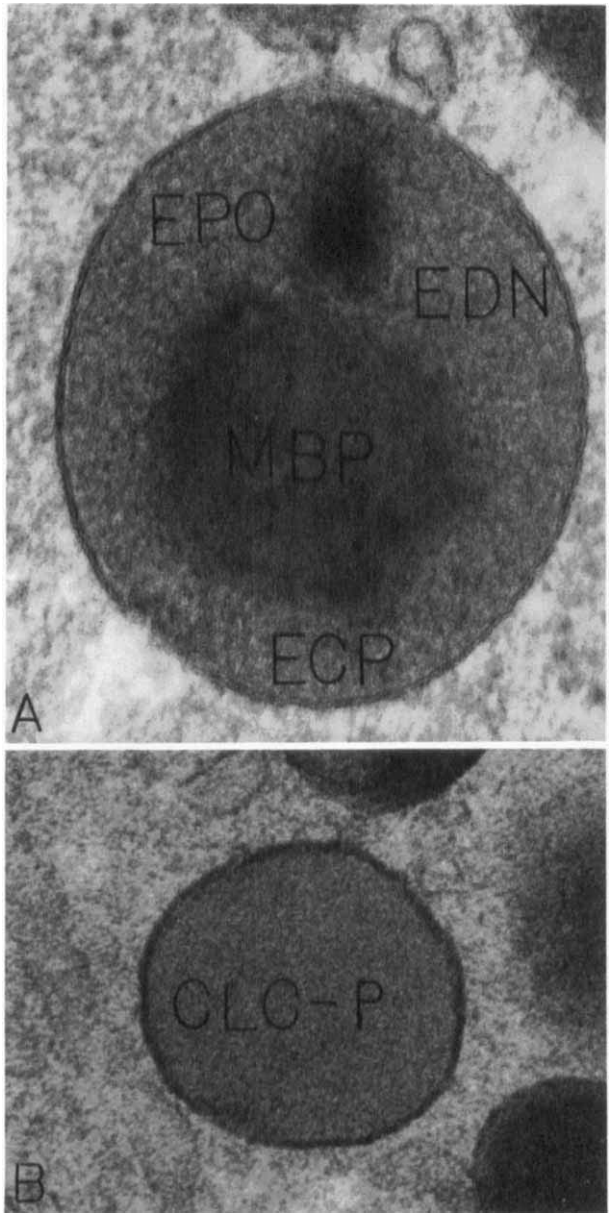
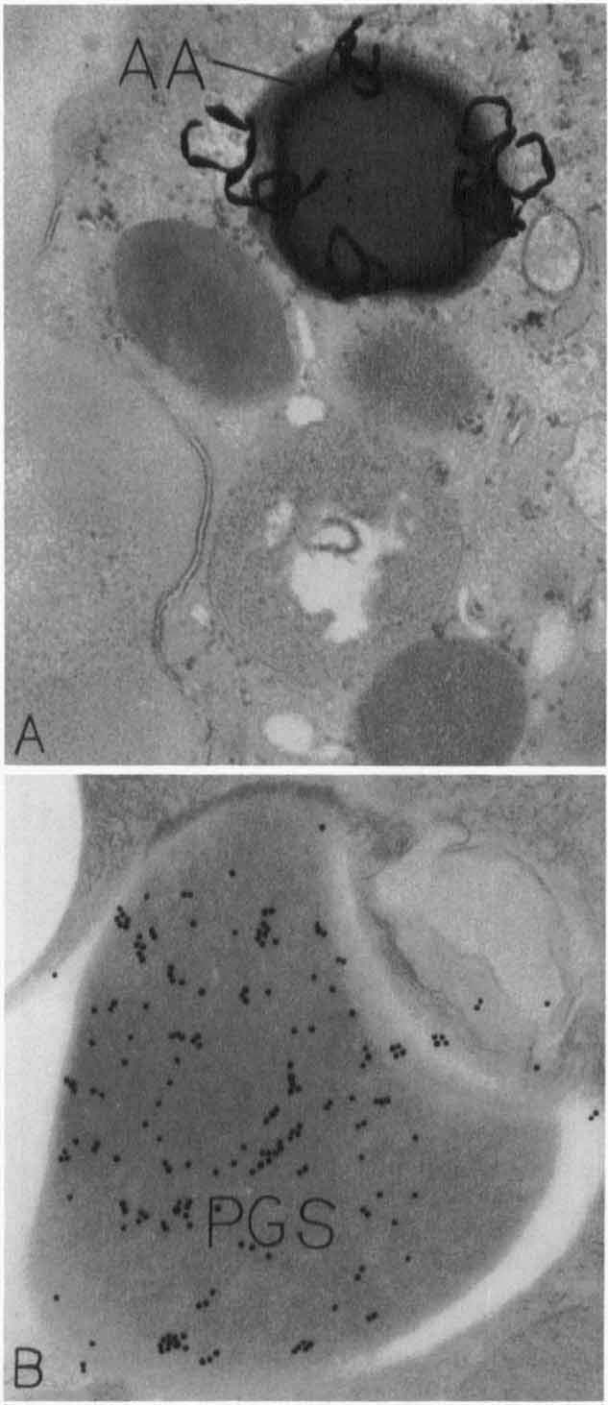


FIG. 2. Two granule populations found in mature eosinophils have different contents. The secondary (specific) granule (A) contains MBP in the dense crystalline core and EPO, ECP, and EDN in the less dense matrix. The primary granule (B) does not contain a crystalline core and is the storage location of CLC protein (CLC-P). (A) ×152,000; (B) ×88,000. [Courtesy of Dr. Ann Dvorak. Reproduced with permission from Dvorak (1994).]


AA
A
PGS
B

accessory cells in certain T-cell-mediated reactions (Weller *et al.*, 1993). There is also evidence that eosinophils slow the rate of progression of solid tumors, through tumoricidal mechanisms (Lowe *et al.*, 1981). At the same time (as discussed below) some doubt has now been cast on the host defense role of eosinophils in helminthic parasitic disease since, for example, abrogation of the eosinophil response in mice induced by infection with helminthic parasites had no appreciable effect on the course of the disease (Sher *et al.*, 1990a). Thus, in the future the eosinophil may have to be considered as pluripotential with, depending on the circumstances, distinct roles which include maintenance of normal tissue homeostasis, host defense, and production of tissue injury.

In this chapter we review recent findings on the biology of the eosinophil particularly in regard to distinctive eosinophil features. We also discuss the possible role of the eosinophil in the pathogenesis of certain diseases with which the cell is associated.

B. Morphology and Ultrastructure

1. Morphology of Normal Mature Eosinophils and Eosinophil Myelocytes (EM)

Eosinophils are nondividing, bone marrow-derived, granule-containing cells. They are approximately 8 μm in diameter. Their nucleus is bilobed but three or more lobes are not uncommon. The morphology of eosinophils has recently been reviewed in detail (Dvorak *et al.*, 1991a). One of its most characteristic features is the membrane-bound specific granules of which there are about 20 per human eosinophil (Fig. 1). These are spherical or ovoid and contain a crystalline core surrounded by a less electron-dense matrix (Sokol *et al.*, 1991) (Fig. 2). The core is composed of major basic protein (MBP) and the matrix contains the other three basic granule proteins, eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN or EPX) (Egesten *et al.*, 1986). The granules are also the site of location of several cytokines (reviewed by Moqbel *et al.*, 1994a,b). Like many other cell types eosinophils also contain lipid bodies which are the principal store of arachidonic acid

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Fig. 3. Preparations of eosinophil using autoradiography (A) and immunogold (B) show lipid bodies labeled with silver grains, indicating the presence of [^{3}H]arachidonic acid (AA), the substrate for eicosanoid synthesis (A), and labeled with 20-nm gold, indicating the presence of prostaglandin endoperoxide synthase (PGS), the rate-limiting enzyme for oxidation of arachidonic acid to produce prostaglandins (B). (A) ×30,000 [reproduced with permission from Dvorak *et al.* (1991)]; (B) ×46,000 (before reduction) [reproduced with permission from Dvorak (1994); courtesy of Dr. Ann Dvorak.]

esterified into glycerophospholipids (Weller *et al.*, 1991a). Lipid bodies are nonmembrane bound, roughly spherical, and 0.5–2 μm in diameter (Fig. 3). Special fixation techniques are required to visualize lipid bodies as they dissolve in alcohol-based stains such as Wrights and Giemsa. There are approximately five lipid bodies per normal eosinophil but their numbers are increased in activated cells. Under the electron microscope they can be seen as electron-dense bodies surrounded by cytoskeletal elements. Lipid bodies contain the enzymes cyclooxygenase (prostaglandin endoperoxidase synthase, or PGH synthase) (Dvorak, 1994) and 5-lipoxygenase (Weller and Dvorak, 1994). Thus, they contain both the substrate and the enzymes for prostaglandin and leukotriene production and may be the principal site of their synthesis in the normal eosinophil. Eosinophil primary granules are a third type of intracellular organelle. They are recognized as distinct from specific granules by the absence of a core and are of variable size, often being larger than the specific granules. They make up approximately 5% of eosinophil granules. In normal freshly isolated peripheral blood eosinophils primary granules are the sole location of Charcot–Leyden crystal protein (CLC protein) (Fig. 2), as determined by immunogold staining (Dvorak *et al.*, 1988) (Fig. 4). CLC protein is also found diffusely in the nucleus and cytoplasm in activated eosinophils. Tissue eosinophils also contain a number of small granules which stain intensely for acid phosphatase and aryl sulfatase (Parmley and Spicer, 1974; Dvorak *et al.*, 1991a) and may also contain catalase (Iozzo *et al.*, 1982). Vesicotubular structures can be seen in the eosinophil cytoplasm and have been shown to contain cytochrome b_{558} (Ginsel *et al.*, 1990). This is a component of NADPH-oxidase which is involved in superoxide production. Production of superoxide involves the fusion of the vesicotubular structures with the plasma membrane which occurs on cell activation.

The ability to culture eosinophils from cord blood mononuclear cells using cytokine growth factors has made it possible to characterize more clearly the morphology of EM as they develop into mature eosinophils (Dvorak *et al.*, 1989). EM are larger than the mature cell and contain a single nucleus with dispersed chromatin, unlike the condensed chromatin seen in the nuclei of mature eosinophils (Fig. 5). The cytoplasm contains considerably more Golgi structures, endoplasmic reticulum, and mitochondria than the mature cell in which these structures are relatively sparse. This is consistent with the more active biosynthetic capacity of the precursor cell. The eosinophil myelocyte can first be identified when specific core-containing granules appear, interspersed with a large number of immature granules. Some of these may develop into specific granules, but many are likely to be primary granules which reduce in number as the cell matures.

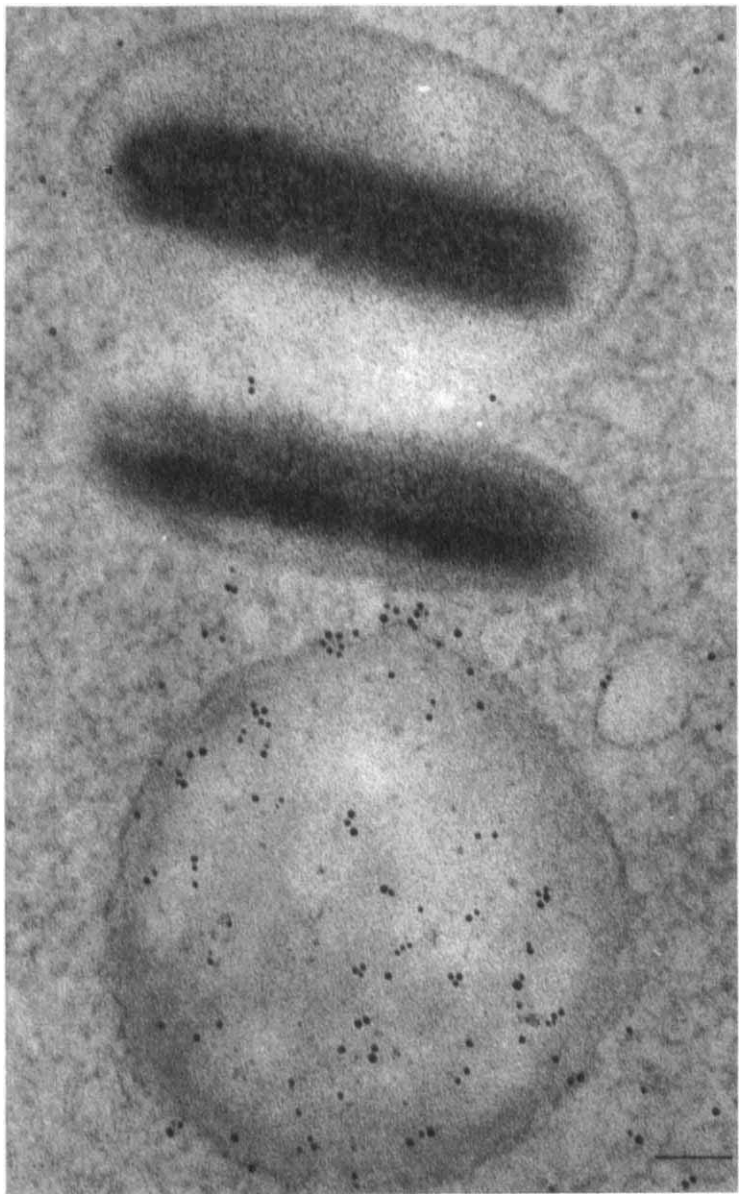

FIG. 4. Eosinophil granules in peripheral blood eosinophil of a patient with HES that is stained for CLC protein with immunogold. The large, round, coreless primary granule contains many 5-nm gold particles; the large, core-containing specific granules are not stained. ×116,000. [Courtesy of Dr. Ann Dvorak. Reproduced with permission from Dvorak *et al.* (1988).]

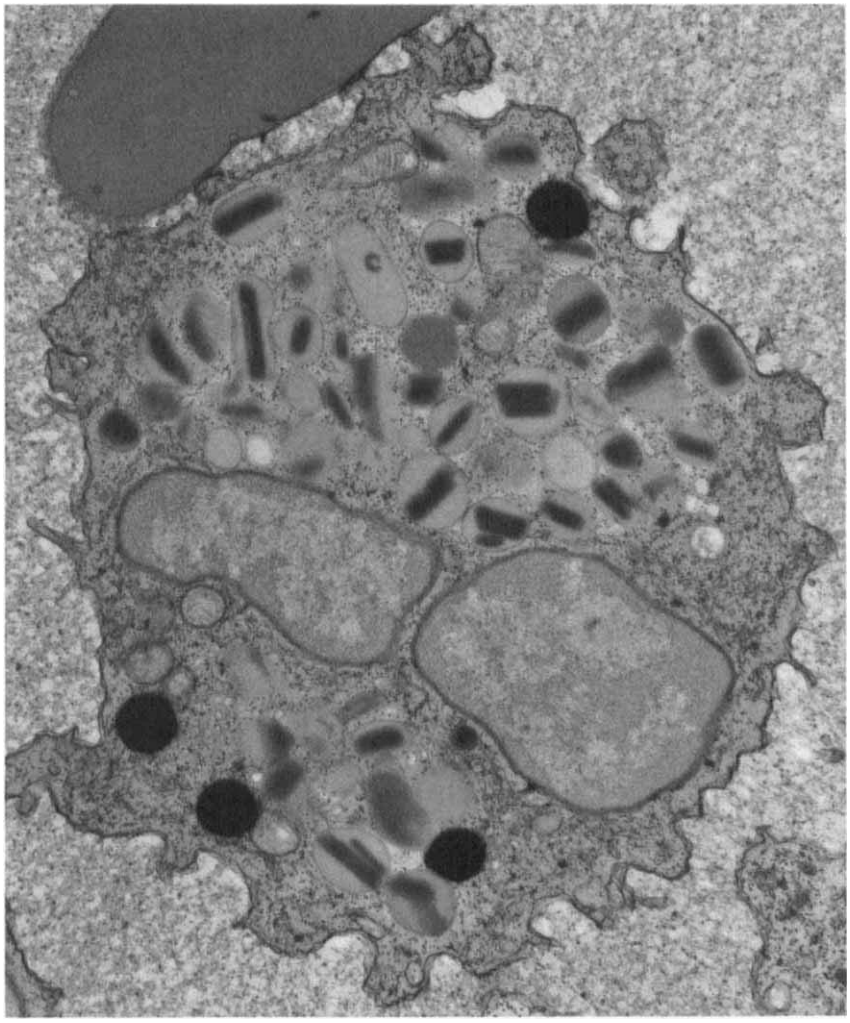

FIG. 5. Mature peripheral blood eosinophil, from a patient with HES, shows a mixture of bicompartmental secondary granules, several primary granules that do not contain dense central cores, and four osmiophilic lipid bodies. ×14,000. [Courtesy of Dr. Ann Dvorak. Reproduced with permission from Dvorak *et al.* (1991).]

2. *Morphology of Activation*

Activated eosinophils form a spectrum ranging from primed cells with only subtle differences in effector function, as observed, for example, in eosinophils from the peripheral blood of subjects with allergic disease, to

the fully activated, degranulating cells seen in biopsies of sites of florid eosinophilic inflammation. Morphological differences between normal peripheral blood eosinophils and activated cells have been studied in a number of circumstances including inflammatory bowel disease (Dvorak *et al.*, 1980) (Fig. 6), the skin and blood of patients with hypereosinophilic syndrome (Henderson *et al.*, 1988; Peters *et al.*, 1988), cytokine-activated peripheral blood eosinophils (Caulfield *et al.*, 1990), and mature cord blood-derived eosinophils (Dvorak *et al.*, 1989). Morphological markers of activation include increased numbers and size of lipid bodies and increased numbers of primary granules, small granules, and vesiculotubular structures. Smooth endoplasmic reticulum and nonmembrane-bound cyto-

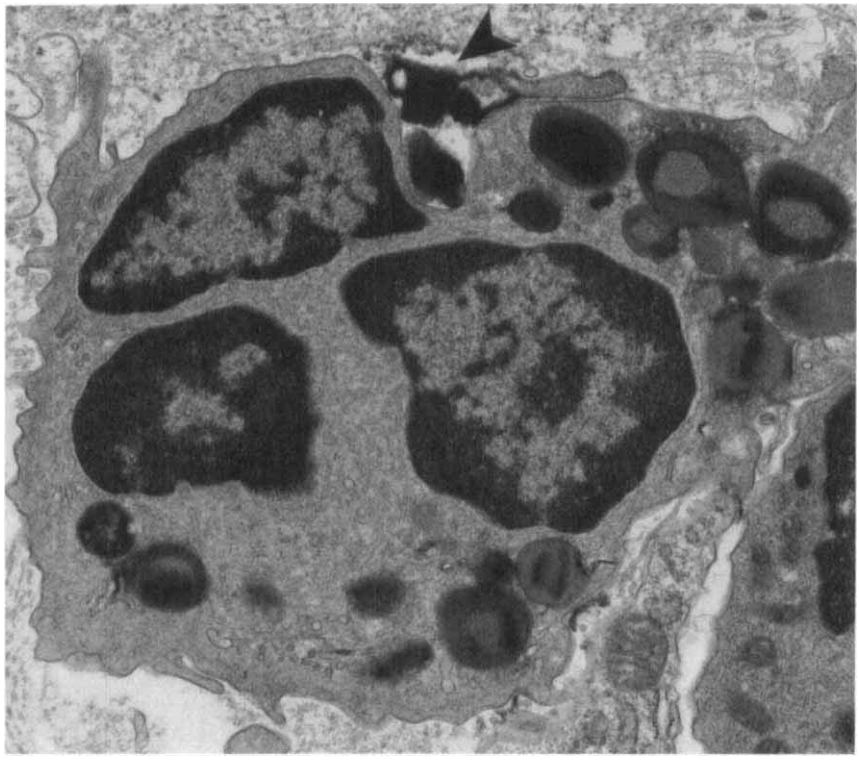

FIG. 6. Eosinophil in ileum of a patient with Crohn's disease and a biopsy culture positive for *S. aureus* shows extrusion of dense cores and matrix of specific granule (arrowhead) following fusion of granule and plasma membrane. ×21,000 [Courtesy of Dr. Ann Dvorak. Reproduced with permission from Dvorak (1991) in "Effects of Immune Cells and Inflammation on Smooth Muscle and Enteric Nerves" (W. J. Snape, and M. Collins, Eds.) pp. 139–168. CRC Press Inc., Boca Raton, FL.

plasmic crystals of CLC protein may be observed. Often, particularly in tissue eosinophils, there is a marked reduction in the numbers of specific granules which may also appear translucent as if emptied of their contents. Comparison of blood eosinophils from hypereosinophilic syndrome (HES) patients compared to normal peripheral blood eosinophils revealed that the number of granules were the same between the two cell types, but the granules in the HES eosinophils were smaller. Consistent with this difference, the HES cells contained less MBP. This was thought to be a possible explanation for the lower density of the HES cells.

3. Morphology of Degranulation

The ultrastructure of degranulating eosinophils has shed important new information on the mechanism of this central event in eosinophil biology. It appears that eosinophils can release their granule contents through three mechanisms, necrosis, secretion, and piecemeal degranulation (Fig. 7). Many eosinophils at sites of inflammation appear necrotic, with centralization of granules, loss of integrity of the granules, and plasma membrane and nuclear lysis. Release of intact or disrupted granules into the interstitium may exert toxic effects. These events have been clearly documented in the skin of patients with HES and bullous pemphigoid (Dvorak *et al.*, 1982) and are consistent with the frequent observation of massive eosinophil degranulation with few intact eosinophils seen in tissue biopsies of vigorous eosinophilic inflammation in association with many diseases. An alternative form of cell senescene is programmed cell death (or apoptosis) which appears to be a universal phenomenon characterized by cell death without loss of membrane integrity (Williams *et al.*, 1992). Eosinophils in culture undergo apoptosis, a process delayed by prior incubation by growth factor cytokines, such as IL-5, IL-3, and granulocyte macrophage colony-stimulating factor (GM-CSF) (Stern *et al.*, 1992; Yamaguchi *et al.*, 1991). Apoptotic eosinophils in culture can be identified by their smaller size, condensed nuclei, and characteristic staining with acridine orange. They also have a distinct profile of autofluorescence when examined by flow cytometry. Genomic DNA preparations from apoptotic eosinophils in agarose gels show the classic laddering pattern of degradation to oligonucleosomal fragments, which is one of the hallmarks of apoptosis (Wyllie, 1980).

The most well-characterized form of degranulation is that of cell secretion where granules fuse with the plasma membrane around the periphery of the cell with the granule matrix and core contents being extruded. Alternatively, granules can fuse intracytoplasmically into large degranulation chambers which open to the outside of the cell through degranulation pores. This is the classic form of regulated secretion well characterized in anaphylactic degranulation of mast cells and basophils (Dvorak *et al.*,

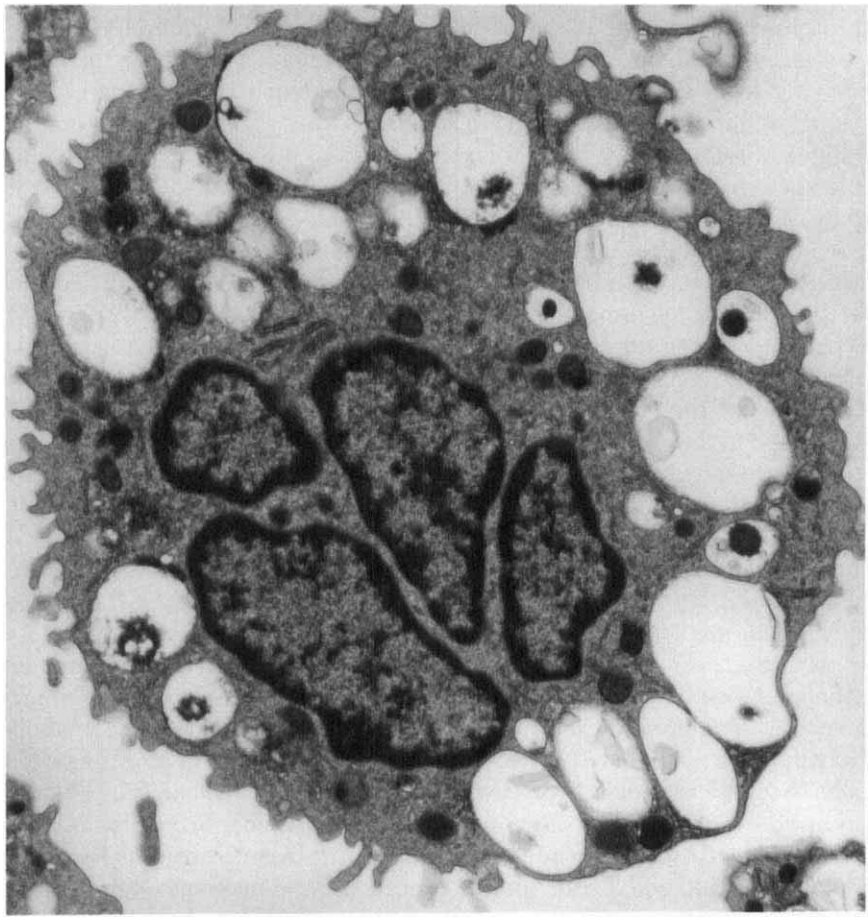

FIG. 7. Mature eosinophil grown in rhIL-5 shows polylobed granulocyte nucleus and numerous enlarged, nonfused, empty and partially empty secondary granule containers typical of piecemeal degranulation. Partially empty secondary granule containers retain some dense granule material and vesicles. ×15,500 (before reduction). [Courtesy of Dr. Ann Dvorak. Reproduced with permission from Dvorak *et al.* (1992a,b).]

1991b). This process has been shown to occur in eosinophils from patients with inflammatory bowel disease and tissue invasive infections (Dvorak *et al.*, 1993) as well as calcium ionophore-stimulated peripheral blood eosinophils (Henderson and Chi, 1985). It is unusual to see morphological evidence of this type of secretion in eosinophilic inflammation. More commonly observed are appearances consistent with a process known as piecemeal degranulation (PMD). This appears to be a process in which granule

protein-containing vesicles bud off from the secondary granule resulting in their gradual emptying. A spectrum of morphologies can be identified ranging from loss from the core compartment, partial or complete loss from the matrix compartment, to total emptying of the granule contents (Fig. 2) (Tai and Spry, 1981). This process has also been studied in cord blood-derived eosinophils using a cytochemical method to detect peroxidase (Dvorak *et al.*, 1992a,b). Vesicles containing EPO budded off from secondary granules. Other vesicles were seen in the cytoplasm or fusing with the plasma membrane. A similar process was described by Torpier *et al.* (1988), using immunogold to label MBP, in duodenal biopsy tissues of a patient with eosinophilic gastroenteritis. They localized MBP to extensive cytoplasmic vesicular and membranous formation in situations in which the cores of the granules were apparently empty. EPO or ECP was not identified in these cytoplasmic structures. It was concluded that the eosinophils were selectively secreting MBP through a PMD-like process without releasing the proteins contained in the granule matrix. This supports the hypothesis of Tomassini *et al.* (1991) who provided evidence that eosinophils selectively secrete different granule proteins depending on the particular stimulus and that the protein secreted determines in part the type of eosinophil-mediated damage which occurs in different diseases.

Activated eosinophils from a variety of circumstances display a characteristic phenotype with alterations in granule morphology and prominent endoplasmic reticulum and Golgi bodies suggestive of increased metabolic activity. The relationship between granule size and content and the generally lower density of activated eosinophils remains unclear. The granule morphology of tissue eosinophils from sites of eosinophilic inflammation indicates active mediator release through a process involving massive degranulation and cell death, granule exocytosis, or piecemeal degranulation in which granule sacs containing varying degrees of vaculolation are seen together with cytoplasmic vesicular buds containing granule proteins being transported to the cell surface. The triggers and biochemical events leading to these different patterns of secretion are still poorly understood. Apoptotic eosinophils are not often seen in tissues, possibly because of their rapid clearance by macrophages, but this is clearly a potentially important aspect of eosinophil senescence. Apoptosis is perhaps the method by which eosinophils are removed during normal tissue homeostasis.

C. Eosinophil Differentiation and Maturation

All cells of the lymphomyeloid lineage, including eosinophils, are derived from hemopoietic stem cells. This small population of cells is capable of self-renewal and differentiation into the individual lymphomyeloid lineages. The essential prerequisites of differentiation (commitment) and

the role of cytokines in regulating committed progenitors is a subject of considerable interest and debate. Till *et al.*, (1964) and Nakahata *et al.*, (1982a,b) provided evidence that the decision of stem cells to either self-renew or differentiate, and in turn, to select a lineage pathway during differentiation, is stochastic or random and thus intrinsic to the progenitor. On the other hand, the survival and expansion of progenitors appears to be controlled by a number of interacting cytokines (reviewed by Ogawa, 1994). Thus, committed eosinophil progenitors undergo their final maturation stages under the influence of interleukin-5 (Clutterbuck, 1989), whereas erythropoietin, M-CSF, and G-CSF act as late-acting factors for red cells, macrophages, and neutrophils, respectively.

The hemopoietic stem cell is a dormant primitive progenitor which requires triggering of cycling to convert to the multipotential progenitor. This is accomplished by complex interactions of a variety of early acting cytokines which include IL-6, G-CSF, IL-11, IL-12, leukemia-inhibitory factor, and Steel factor (reviewed by Ogawa, 1994). In contrast, the proliferation of the multipotential progenitor seems to be largely under the influence of IL-3, GM-CSF, and IL-4. However, these factors are only effective after the primitive progenitor has exited from the dormancy state.

It is known that all of the young lymphoid and myeloid cells, including eosinophils, are contained within the $CD34^+$ population (Clark and Kamen, 1987; Haylock *et al.*, 1992; Favre, 1990; Vellenga *et al.*, 1992). Thus, nearly all eosinophil colony-forming cells are $CD34^+$ but this marker is not expressed on the mature cell (Caux *et al.*, 1989). The CD34 molecule is a 115-kDa glycoprotein with three intracellular domains (Greaves *et al.*, 1992). It can be phosphorylated on serine residues by PKC and is not shed or downmodulated from the cell surface. Its precise function is unknown but it has a mucin-like structure and is a ligand for L-selectin (Baumheuter *et al.*, 1993).

Exposure of $CD34^+$ cells to IL-3 and GM-CSF either alone or in combination leads to progressive commitment to the myeloid lineage with IL-5 promoting the terminal maturation process (Shalit *et al.*, 1995). Interestingly, IL-5 transgenic mice, in which there is overproduction of IL-5, but not apparently GM-CSF and IL-3, have large numbers of blood and tissue eosinophils (Dent *et al.*, 1990; Tominaga *et al.*, 1991). One explanation is that *in vivo* IL-5 may also act at early differentiation steps. These apparent discrepancies between *in vivo* and *in vitro* systems need to be clarified.

One of the unresolved but intriguing issues is whether eosinophils and basophils arise from the same precursor or arise separately from the common pluripotent stem cell. Eosinophil differentiation certainly appears to be closely linked to that of the basophil (Denburg *et al.*, 1985). For example, IL-5 can act as a basophil differentiation factor for HL-60 cells and mature

basophils (Denburg *et al.*, 1991). Like eosinophils, basophils contain Charcot–Leyden crystal protein (Ackerman *et al.*, 1982) and major basic protein (Ackerman *et al.*, 1983). Eosinophil and basophil progenitors (i.e., colony-forming units) have been identified in the blood of patients with allergic rhinitis (Otsuka *et al.*, 1986) and in asthmatics during exacerbations of their disease (Gibson *et al.*, 1990, 1992). These studies suggest that terminal differentiation steps may occur in sites of allergic inflammation.

It has been suggested that gene transcription in eosinophil and basophil lineage may be regulated by the GATA-binding proteins since eosinophils expressed mRNA for GATA-1, GATA-2, and GATA-3, whereas basophils express GATA-2 and GATA-3 (Zon *et al.*, 1993).

Eosinophil myelocytes, obtained from either blood or tissue or from cultured precursor cells, contain large immature granules which are precursors of primary and secondary population. These immature granules are round and homogeneously dense (Dvorak *et al.*, 1991). Once commitment to the eosinophil lineage has been established the cell undergoes characteristic morphological changes until it is functionally mature (Walsh *et al.*, 1990a). Mature eosinophils are nondividing cells morphologically indistinguishable from peripheral blood eosinophils.

Thus, our knowledge, of the molecular basis of eosinophil differentiation remains relatively sketchy. The description of the human myeloid cell line, AML14, which can be stimulated to produce mRNA for MBP, EPO, ECP, EDN, and CLC proteins by IL-3, GM-CSF, and IL-5, indicates that this may serve as a model for studying cytokine induction of eosinophil growth and differentiation (Paul *et al.*, 1994). However, at the present time, there is little information on the genes that control eosinophil differentiation and maturation and how the level of maturation relates to phenotype, structure, and function.

D. Receptors

1. Receptors Involved in Eosinophil Adhesion and Migration

Leucocyte migration from the bone marrow to the blood and then to the tissue is one of the central events of leucocyte biology and essential to the integrity of the immune system. This is illustrated by leukocyte adhesion deficiency disease type 1 in which the inability of neutrophils to enter tissue due to defective expression of the leucocyte integrins leads to severe life-threatening infections and premature death (Anderson and Springer, 1987). A striking feature of chronic allergic inflammation is the accumulation of activated eosinophils with a relative paucity of neutrophils. As eosinophils generally constitute only a small proportion of the total blood leucocyte count this suggests a selective process of leucocyte migration.

Preferential eosinophil accumulation at the site of local tissue reactions is believed to be the result of several interrelated events. These include selective adhesion pathways, specific chemotactic factors, and enhanced survival by certain cytokines, principally IL-3, IL-5, and GM-CSF.

Stimulated by the hope that identification of selective adhesion pathways could lead to selective inhibition of migration without the risk of immunosuppression, the mechanisms involved in the tissue localization of eosinophils has been the subject of intense interest over the past 3 decades. Neutrophil migration through endothelium has been shown to be a process involving sequential steps in which the cells are initially lightly tethered to the endothelium and roll along its surface. This is followed by cell activation, mediated by a soluble chemotactic stimulus, which allows a firmer bond to develop between the leucocyte and the endothelial cell and results in successful adhesion and transmigration (Lawrence and Springer, 1991; Von Adrian *et al.*, 1991; Springer, 1994). The steps occur in series so that each is essential for transmigration to occur. Thus, selectivity can be introduced at each of the steps resulting in considerable diversity in the pattern of signals required for successful emigration at any one inflammatory site. It also suggests that migration can be modulated at each of the steps, offering a range of targets for pharmacological inhibition. While this staged migration has not been formally shown to occur with eosinophils and basophils it is assumed that the processes are similar. Rolling of eosinophils was observed *in vivo* on IL-1-stimulated microvascular endothelial cells in the rabbit mesentery, a process that was inhibited by anti-L-selectin and anti-VLA-4 (Sriramarao *et al.*, 1994).

The receptors and mediators involved in leucocyte migration have, to a large extent, been characterized (Table I). In neutrophils, a family of adhesion receptors termed the selectins mediate the initial attachment and rolling step, a number of chemotactic mediators have been implicated in the activation step, and integrins expressed on leukocytes binding to adhesion receptors belonging to the immunoglobulin superfamily are implicated in the firmer adhesion step (Bevilacqua, 1993). The paradigm of selectins mediating rolling, and integrins causing flattening and transmigration, might have to be modified in the light of the observation that VLA-4 can also mediate rolling on VCAM-1 at physiological flow rates (Berlin *et al.*, 1995). The relevance of this observation for eosinophils remains to be determined. The role of each of these families of adhesion receptors in eosinophil migration as well as the chemotactic mediators involved will be discussed in turn.

a. Selectin-Mediated Interaction

i. The selectins. The selectins are a family of three receptors consisting of L-selectin, P-selectin (formerly GMP-140), and E-selectin (formerly

TABLE I
EOSINOPHIL ADHESION RECEPTORS AND THEIR COUNTERSTRUCTURES

Eosinophil receptor	Endothelial receptor	Matrix protein
Integrin		
VLA-4 ($\alpha 4\beta 1$)	VCAM-1	Fibronectin
VLA-6 ($\alpha 6\beta 1$)		Laminin
$\alpha 4\beta 7$	MAdCAM-1, VCAM-1	Fibronectin
LFA-1	ICAM-1, ICAM-2	
Mac-1	ICAM-1	Fibrinogen
p150,95	Not known	
Immunoglobulin-like		
PECAM	PECAM	
ICAM-3 (binds LFA-1)		
Selectins		
L-selectin	GlyCAM-1, CD34, MAdCAM-1	
Carbohydrate		
PSGL-1	P-selectin	
E-selectin ligand	E-selectin	
Others		
CD44		Hyaluronate

Note. ICAM-1, intercellular cell adhesion molecule; VCAM-1, Vascular cell adhesion molecule; PECAM, platelet endothelial cell adhesion molecule; LFA-1, lymphocyte function associated receptor; PSGL-1, P-selectin glycoprotein ligand-1; MAdCAM-1, mucosal addressin cell adhesion molecule-1; GlyCAM-1, glycosylated cell adhesion molecule-1.

ELAM-1) (Rosen, 1993). E- and P-selectin are expressed on endothelium, while L-selectin is expressed by all leucocytes. P-selectin is also expressed on platelets. Whereas E-selectin expression is induced on endothelial cells as a result of cytokine-stimulated gene transcription and new protein synthesis (Bevilacqua *et al.*, 1987), P-selectin is stored in cytoplasmic Weibel–Palade bodies and translocated within minutes to the cell surface after stimulation of the endothelium by a variety of mediators including thrombin, LTC_4, and histamine (McEver *et al.*, 1989; Geng *et al.*, 1990). L-selectin is constitutively expressed and shed on cell activation (Kishimoto *et al.*, 1990). The selectins have a common structure characterized by an N terminal, calcium-dependent (C type) lectin domain which binds sugars consisting of a family of sialylated fucosylated glycosaminoglycans typified by the carbohydrate moiety sialyl Lewis X (Springer and Lasky, 1991). Adjacent to the lectin domain is an epithelial growth factor (EGF)-like domain and between two and nine (depending on the selectin) repeated units related to complement-binding proteins such as decay accelerating factor (Johnston *et al.*, 1989; Tedder *et al.*, 1989). Although the adhesion function of the selectins requires binding through sugars, the backbone

on which the sugars are presented is important in providing increased specificity and affinity of binding. Ligands for L-selectin and P-selectin have been characterized and consist of heavily O-glycosylated, mucin-like glycoprotein receptors in which large amounts of O-linked sugars are presented on a rigid protein backbone (Lasky *et al.*, 1992; Baumheuter *et al.*, 1993; Berg *et al.*, 1993; Sako *et al.*, 1993; Shimizu and Shaw, 1993). The selectin receptors and their known ligands are summarized in Table I.

ii. Eosinophil/selectin interactions. Eosinophil adhesion to cytokine-stimulated cultured human umbilical vein endothelial cells (HUVEC) can be inhibited by blocking monoclonal antibodies (mAbs) against E-selectin and L-selectin (Weller *et al.*, 1991b; Knol *et al.*, 1993). In addition, eosinophils can adhere specifically to COS cells transfected with an E-selectin cDNA (G. M. Walsh, A. J. Wardlaw, unpublished data), to tissue culture plates coated with either E- or P-selectin, and to CHO cells transfected with the P-selectin cDNA (Vadas *et al.*, 1993). Eosinophils express L-selectin in similar amounts to neutrophils and like neutrophils L-selectin is shed on eosinophil activation *in vitro*, with chemotactic mediators, and *in vivo* (Smith *et al.*, 1992). Eosinophil binding to E- and P-selectin does not appear to be affected by the state of activation of the cells. Superficially at least, eosinophils therefore appear very similar in their selectin interactions with neutrophils. There is, however, some evidence of subtle but important differences between these two cell types in this regard. Whereas neutrophils express large amounts of sialyl Lewis X eosinophils express relatively little of this sugar moiety (Bochner *et al.*, 1994). In addition, neutrophils bound much more avidly than eosinophils to purified E-selectin, whereas eosinophils may bind more avidly than neutrophils to P-selectin (Vadas *et al.*, 1993). The eosinophil P-selectin ligand appears to be a structural isoform of PSGL-1 with a higher molecular weight than neutrophil PSGL-1 (Symon *et al.*, 1995). The pattern of inhibition of eosinophil adhesion to HUVEC by a panel of L-selectin antibodies was different between eosinophils and neutrophils suggesting different functional epitopes on L-selectin among these two cell types (Knol *et al.*, 1994), although this may have been due to the weaker binding of eosinophils than neutrophils to IL-1-stimulated HUVEC under flow conditions.

Symon *et al.* (1994) attempted to study eosinophil adhesion in a more physiological setting by using the frozen-section Stamper–Woodruff (S–W) assay (Stamper and Woodruff, 1976) to investigate eosinophil adhesion to nasal polyp endothelium. Nasal polyps are usually large pieces of tissue with an abundance of eosinophils and in which the blood vessels can be readily identified. The S–W assay has been used extensively to characterize lymphocyte homing to high endothelial venules but used less often to study adhesion of other leucocytes. It involves layering the cells onto sections

of tissue and allowing adhesion to occur on a rotating platform. There was good expression on ICAM-1, E-selectin, and P-selectin with, on average, about 30% of blood vessels stained. VCAM-1 expression was either very weak or absent. Eosinophils adhered to nasal polyp endothelium in a specific manner with minimal adhesion to background structures. Adhesion was dose and temperature dependent with room temperature being optimal. Blocking monoclonal antibodies against ICAM-1, VCAM-1, E-selectin, L-selectin, VLA-4, and LFA-1 had no effect, while an antibody against Mac-1 had a significant though modest inhibitory effect. Most striking was the almost total inhibition observed with an antibody against P-selectin. Similar inhibition was seen with a IgG–P-selectin chimeric molecule that binds to the P-selectin ligand and blocks P-selectin interactions (Erbe *et al.*, 1993). As P-selectin is stored in Weibel–Palade granules within the endothelial cell it was possible that eosinophils were binding to intracellular P-selectin. However, no eosinophil binding was observed to normal skin endothelium in which P-selectin expression, determined by immunohistochemistry, was similar to that observed in nasal polyps. In addition, immunofluorescence with confocal microscopy membrane staining of P-selectin in nasal polyps compared to a granular pattern suggestive of intracellular staining in normal skin. This suggests that in allergic inflammation P-selectin is constitutively expressed on airway endothelium and is the principal receptor involved in the tethering stage of eosinophil adhesion to vascular endothelium.

iii. In vivo inhibition of eosinophil/selectin interactions. There are relatively few published studies on the role of selectins on eosinophil migration into sites of allergic inflammation. The effect of allergic inflammation in which cynomologus monkeys with a natural sensitivity to *Ascaris* antigen has been described by Gundel *et al.* (1993). Inhalation challenge of *Ascaris* antigen in these animals resulted in an airway inflammatory response and increased bronchial hyperresponsiveness (BHR). Different responses were observed depending on whether antigen challenge was single, in which case an early and sometimes dual (early and late) response was observed, or multiple over several days, in which case a marked eosinophilia and increased BHR were observed. The prechallenge state of the animal was also important in determining the response. Some animals had a prechallenge airway eosinophilia and markedly increased BHR. These animals developed a late response after a single allergen challenge which was characterized by increased numbers of airway neutrophils. Other animals had a relative paucity of eosinophils in their human bronchoalveolar lavage (BAL) prechallenge but developed a marked airway eosinophilia and increased BHR after multiple allergen challenge. The airway eosinophilia and increased BHR after multiple antigen challenge was inhibited by a

monoclonal antibody against ICAM-1 but not E-selectin (Wegner *et al.*, 1990). In contrast, the airway neutrophilia generated by a single challenge was inhibited by anti-E-selectin but not anti-ICAM-1 (Gundel *et al.*, 1991). This suggests that, in this model at least, BHR was associated with an airway eosinophilia which was mediated in part by ICAM-1, whereas the airway neutrophilia was associated with the development of a late response and mediated by E-selectin.

iv. Expression of selectins in allergic inflammation. Expression of E-selectin in allergic inflammation is variable and to some extent organ dependent. Normal skin endothelium has a low baseline expression which was increased after allergen challenge with the kinetics of upregulation broadly corresponding to the pattern observed *in vitro* with HUVEC (Kyan-Aung *et al.*, 1991). Upregulation of E-selectin was observed after allergen challenge of skin biopsy organ cultures suggesting that cells resident in the skin, possibly mast cells, were able to generate mediators, e.g., TNF-α, responsible for increased expression (Leung *et al.*, 1992). In both the upper and the lower airway a more complex pattern was observed with constitutive staining of E-selectin in the normal airway. This has made it more difficult to demonstrate increases after allergen challenge or in clinical disease. Bentley *et al.* (1993a) showed that E-selectin was increased in the airways of intrinsic asthmatics but not extrinsic asthmatics compared to nonasthmatic controls. Similar findings were reported by Montefort *et al.* (1992a). Although Bentley *et al.* (1993a) did not observe increases in E-selectin after aerosol allergen challenge, others have found upregulation after segmental allergen challenge via the fibreoptic bronchoscope (Montefort *et al.*, 1994).

Preliminary EM findings indicate expression of P-selectin on the lumenal surface of skin endothelium 1 hr after allergen challenge which dissipated by 6 hr (Murphy *et al.*, 1994). Translocation of P-selectin to the endothelial cell membrane for up to 1 hr after the injection of the neuropeptides substance P, VIP, and CGRP has also been reported (Smith *et al.*, 1993).

In summary, eosinophils could potentially utilize all three selectins. However, the evidence points toward a prominent role for P-selectin in the tethering stage of eosinophil migration in allergic inflammation. There is evidence of subtle but important differences between eosinophils and neutrophils in their selectin interactions.

b. Integrin and Immunoglobulin Family Adhesion Interactions

i. Eosinophil/integrin interactions. Eosinophils express a number of integrins that can bind to receptors of the immunoglobulin family expressed on the endothelium (Table I). Eosinophil adhesion to unstimulated HUVEC is slightly enhanced by platelet-activating factor and other eosino-

phil active inflammatory mediators. These increases were almost totally inhibited by mAbs to the leucocyte integrin Mac-1, although the endothelial cell ligand was not identified (Kimani *et al.*, 1988; Lamas *et al.*, 1988). Compared to unstimulated HUVEC eosinophil and basophil adhesion to TNF-α- or IL-1-stimulated HUVEC was markedly increased and the enhancement inhibited by mAbs against ICAM-1 and VCAM-1 on endothelium and LFA-1, Mac-1, and VLA-4 on the leucocyte membrane (Walsh *et al.*, 1991; Bochner *et al.*, 1991; Dobrina *et al.*, 1991). Eosinophil transmigration through endothelial cells using artificial models of the blood vessel wall has also been investigated. These studies demonstrated that eosinophil transmigration through HUVEC was increased by cytokine stimulation of the endothelium and that eosinophils from allergic donors showed an increased migration capacity consistent with the concept that eosinophils from subjects with allergic disease are activated (Moser *et al.*, 1992a). Similarly, *in vitro* culture of peripheral blood eosinophils from normal donors with GM-CSF, IL-3, and IL-5 increased their migration capacity. LFA-1 and Mac-1 probably mediate transmigration of eosinophils through IL-1- or TNF-α-stimulated HUVEC. Although this involves binding to ICAM-1 there are possibly additional, as yet unidentified, ligands on the endothelium (Ebisawa *et al.*, 1992). Antibodies against VLA-4 and VCAM-1 did not inhibit IL-1-stimulated transmigration. In contrast, eosinophil migration through IL-4-stimulated HUVEC was partially inhibited by anti-VLA-4 antibodies as well as antibodies against the leucocyte integrins (Moser *et al.*, 1992b). The observation that VLA-4 mediated eosinophil adhesion and transmigration through HUVEC generated considerable interest because neutrophils do not express this receptor. This could therefore be a putative eosinophil-selective adhesion pathway of relevance to allergic inflammation. This hypothesis was strengthened by the observation that IL-4 selectively induced expression of VCAM-1 on HUVEC (Thornhill *et al.*, 1991) and that, as mentioned previously, eosinophil, but not neutrophil, adhesion and transmigration through HUVEC was enhanced by IL-4 in a VLA-4-dependent manner (Schleimer *et al.*, 1992). In addition, IL-4 transgenic mice had an eosinophilic inflammation of the conjunctiva (Tepper *et al.*, 1990). On the other hand, expression of VCAM-1 in allergic inflammation is weak and the IL-4-associated eosinophilia in transgenic mice does not appear to be associated with increased VCAM-1 expression on endothelium.

Eosinophils also express $\alpha 4\beta 7$ (Erle *et al.*, 1994). The principal ligand for this receptor is the gut mucosal addressin cell adhesion molecule-1 (Berlin *et al.*, 1993). The mouse form of this receptor was recently cloned. It has an immunoglobulin-like domain closely related to VCAM-1 and ICAM-1 which presumably is the region which binds $\alpha 4\beta 7$. There is also

a region homologous to IgA1 and a serine threonine-rich mucin-like region which binds L-selectin (Briskin *et al.*, 1993).

ii. In vivo inhibition of integrin/immunoglobulin/eosinophil interactions. Animal models of allergic inflammation have provided further insight into the relative importance of integrins and their ligands in eosinophil migration. As mentioned previously, in the cynomologus monkey model anti-ICAM-1 was effective at inhibiting eosinophil migration into the airways and the development of BHR. It is of course possible that the antibody was acting indirectly on other cell types, such as mononuclear cells, rather than blocking eosinophil migration directly. In the same multiple allergen challenge model anti-Mac-1 mAb inhibited the development of BHR and reduced the levels of ECP in the BAL fluid but did not reduce the airway eosinophilia. This suggested that Mac-1 was not essential for eosinophil migration, but may be involved in triggering eosinophil activation and mediator release (Wegner *et al.*, 1993). In a sheep model of allergen challenge the anti-VLA-4 mAb HP1/2, when administered either intravenously or by inhalation, inhibited the late response to allergen challenge and the development of airway hyperresponsiveness (Abraham *et al.*, 1993). Interestingly, there was little difference between the antibody-treated animals and control animals in the recruitment of eosinophils into BAL thus dissociating the airway eosinophilia from the physiological effects of allergen challenge. One explanation suggested for this apparent discrepancy was that anti-VLA-4 treatment was inhibiting eosinophil activation and mediator release in a similar manner to that observed with the anti-Mac-1 treatment in the monkey model. In support of this hypothesis they observed that eosinophils treated *in vitro* with anti-VLA-4 mAb released less EPO after PAF stimulation than control eosinophils. Furthermore, no increase in VCAM-1 expression was seen on the airway endothelium consistent with the view that eosinophil migration was not VLA-4-dependent in this model. A different pattern has been observed in guinea pigs and mice. In guinea pigs the anti-VLA-4 mAb HP1/2 inhibited the migration of eosinophils into the skin both after injection of chemotactic factors and after passive cutaneous anaphylaxis. Expression of VCAM-1 on skin endothelium was not investigated (Weg *et al.*, 1993). Similarly, HP1/2 was able to inhibit migration of eosinophils into the airway submucosa in sensitized, ovalbumin-challenged guinea pigs. The allergen challenge-induced increase in bronchial hyperresponsiveness was also prevented as was the increase in the concentration of EPO in BAL fluid. Expression of VCAM-1 was not determined (Pretolani *et al.*, 1994a). In sensitized mice anti-VLA-4 mAb inhibited eosinophil infiltration into the trachea after ovalbumin challenge (Walker *et al.*, 1993). Anti-VCAM-1 mAb was also effective and VCAM-1 expression was strongly induced by antigen challenge. Neither the

VCAM-1 expression nor the eosinophil infiltration were IL-4 dependent. Unlike the monkey model, anti-ICAM-1 and anti-LFA-1 mAbs were ineffective at inhibiting eosinophil migration. Lastly, aerosolized anti-VLA-4 mAb inhibited allergen-induced BAL eosinophilia and BHR in rabbits sensitized to house dust mite (Metzger *et al.*, 1994). These studies point to an important role for VLA-4, Mac-1, ICAM-1, and VCAM-1 in eosinophil recruitment and activation in allergic inflammation and offer the possibility that suitably designed adhesion receptor antagonists may be therapeutically effective. They also suggest that there are important species differences in the adhesion receptors used by eosinophils to migrate into antigen-challenged airways.

iii. Expression of integrins and immunoglobulin family adhesion receptors in allergic inflammation. The expression of ICAM-1 and VCAM-1 in allergic inflammation has been investigated by a number of groups. Normal skin endothelium has a low ICAM-1 background which is increased after allergen challenge (Kyan-Aung *et al.*, 1991). VCAM-1 was weak in both normal and allergen-challenged skin. Good constitutive staining of ICAM-1 was observed in the normal human airway, with increased expression of ICAM-1 in the bronchi of intrinsic, but not extrinsic asthmatics (Bentley *et al.*, 1993a). ICAM-1, like E-selectin, was increased after segmental allergen challenge via the bronchoscope (Montefort *et al.*, 1994). Studies on endothelial VCAM-1 indicate that immunostaining is weak in the upper and lower airways in biopsies from both normal subjects and patients with rhinitis and asthma. There is evidence, however, that this is increased in seasonal allergic rhinitis and after allergen challenge. For example, in the study by Bentley *et al.* (1993a) there was a trend toward an increase in VCAM-1 with a good correlation between numbers of airway eosinophils after allergen challenge and expression of VCAM-1. This supported the view that VCAM-1 may be involved in eosinophil recruitment after allergen challenge and possibly in clinical disease. A modest increase (9 vs 26%) in the number of blood vessels staining with anti-VCAM-1 was observed after allergen challenge (by applying paper discs to the nasal mucosa) in subjects with allergic rhinitis (Naclerio *et al.*, 1994). In perennial rhinitis an increase in endothelial ICAM-1 and VCAM-1 was observed compared to normal controls, although VCAM-1 expression was relatively weak (Montefort *et al.*, 1992b). Moreover, in more severe asthmatics an increase of VCAM-1 expression in the bronchial submucosa compared with normal controls was observed (Ohkawara *et al.*, 1995). In general, therefore, there is some evidence for increases in endothelial ICAM-1 both in clinical disease and after allergen challenge although this is less marked in the airways than in the skin because of background expression in normal or unchallenged subjects. Increased ICAM-1 was

also seen on the bronchial epithelium in asthma (Bentley *et al.*, 1993a; Vignola *et al.*, 1993). Compared with normal controls VCAM-1 was slightly elevated in clinical disease associated with allergic inflammation. There is some evidence of increased expression after allergen challenge.

c. Eosinophil Adhesion to Extracellular Matrix. After migration through the endothelium the eosinophil and basophil come into contact with proteins of the extracellular matrix. This is not simply a mesh in which the leucocytes are supported, but a complex network of large fibrillar proteins that have a profound influence on cellular function, mainly through adhesive contacts with integrin receptors expressed both on the surface of resident cells, such as fibroblasts and the epithelium as well as on leucocytes migrating from the peripheral blood. Tissue eosinophils have an "activated phenotype." They express activation receptors, such as CD25 (Hartnell *et al.*, 1990) and CD69 (Hartnell *et al.*, 1993), generate mRNA for a number of cytokines that do not appear to be encoded for by normal peripheral blood eosinophils (Desreumaux *et al.*, 1992), and express the secreted form of ECP as recognized by the mAb EG2 (Azzawi *et al.*, 1990; Bentley *et al.*, 1992a,b,c). The mechanism(s) of eosinophil activation in tissues *in vivo* is not well understood. *In vitro* studies suggest that the process of migration through endothelium itself may result in eosinophil activation (Walker *et al.*, 1993). In addition, locally generated chemotactic mediators may be involved. Interaction with the extracellular matrix can result in "outside in" signaling through integrin receptors leading to eosinophil and basophil priming and mediator release (Hynes, 1992). There have been relatively few studies of such interactions. Dri *et al.* (1992) studied the production of superoxide by eosinophils resting on different surfaces including endothelial cells and a number of matrix proteins after stimulation with soluble mediators. They observed that the nature of the surface influenced the amount of superoxide produced with, for example, endothelial cells, inhibiting superoxide production and fibrinogen-priming eosinophils for enhanced superoxide generation after stimulation with fMLP. Anwar *et al.* (1994) demonstrated enhancement of calcium ionophore-stimulated leukotriene C_4 generation by eosinophils adhering to fibronectin when compared with BSA-coated surfaces and Neeley *et al.* (1994) reported that VLA-4-mediated interaction with fibronectin resulted in increased fMLP-induced eosinophil degranulation. In contrast, Kita *et al.* (1994) found that adherence to fibronectin and laminin inhibited EDN release stimulated by PAF, C5a, and IL-5, but not by PMA, indicating that the secretagogue used is important in determining the effects of matrix proteins on eosinophil degranulation. When eosinophils were cultured for several days on plasma fibronectin they had increased survival compared to eosino-

phils cultured on BSA or plastic as a result of autocrine generation of GM-CSF and IL-3. Cytokine release and survival was inhibited by anti-VLA-4 mAb (Anwar *et al.*, 1993). We have now extended these observations to show that tissue fibronectin is considerably more effective than plasma fibronectin at supporting eosinophil survival (Walsh *et al.*, 1995). This is consistent with the idea that survival is a result of triggering through α_4/β_1 (VLA-4) as tissue fibronectin contains more of the alternatively spliced 111CS region that contains the binding site for VLA-4 (Mould *et al.*, 1990). Eosinophils also adhere to laminin through $\alpha 6\beta 1$ (Georas *et al.*, 1993). In addition to being relevant to cytokine generation, the interaction between eosinophils and fibronectin may represent a homeostatic mechanism by which eosinophils survive for prolonged periods in tissue before undergoing apoptosis and removal.

d. Eosinophil Chemotaxins. For many years investigators have pursued the hypothesis that local eosinophilia is a result of the liberation of a selective eosinophil chemoattractant. Numerous candidates have been suggested but enthusiasm has not been sustained because of weak activity, lack of selectivity, or lack of evidence for their generation during the allergic process. Histamine (Clark *et al.*, 1975) and the ECF-A tetrapeptides (Goetzl *et al.*, 1975) were reported as having *in vitro* chemotactic activity but were later found to have negligible potency compared with PAF and C5a (Wardlaw *et al.*, 1986b). Dexamethasone did not inhibit PAF-induced eosinophil chemotaxis (Kurihara *et al.*, 1989). Histamine does increase cytosolic calcium in human eosinophils (Raible *et al.*, 1992) and the H1 antagonist cetirizine appears to modulate eosinophil migration both *in vitro* and *in vivo*. *In vitro* this drug inhibits eosinophil chemotaxis and adhesion to endothelium and *in vivo* it inhibits allergen-induced eosinophil migration into skin windows, but not into tissue. Some of these activities are shared by other antihistamines. The mechanism involved is unclear (Sehmi *et al.*, 1993a). As histamine challenge given to establish the histamine PC_{20} does not result in airway inflammation one can conclude that histamine is not an effective *in vivo* chemoattractant. Cyclooxygenase-derived lipid mediators have no substantive chemotactic activity for eosinophils *in vitro*. In contrast, a number of 5- and 15-lipoxygenase-derived mediators have been shown to attract the cell. These include LTB_4 and various diHETEs (Morita *et al.*, 1990; Sehmi *et al.*, 1991). LTB_4 is much more active on guinea pig than on human eosinophils and the diHETEs were only generated when exogenous arachidonic acid was present. Interestingly, inhaled LTE_4 generated an airway eosinophilia although it is not clear whether this was a direct or indirect effect (Laitinen *et al.*, 1993).

Eosinophil chemoattractants are summarized in Table II.

In recent years the chemotactic potential of cytokines, particularly growth factors and chemokines, has been explored in some detail. GM-CSF, IL-3, and IL-5 in addition to being eosinopoietic they also enhance the effector function of mature eosinophils in terms of adhesions to HUVEC, prolonged survival, mediator release and mobility (Lopez *et al.*, 1988; Wang *et al.*, 1989a; Fujisawa *et al.*, 1990; Owen *et al.*, 1990; Walsh *et al.*, 1990a,b). IL-5 and IL-3 also activate mature basophils but not neutrophils (Bischoff *et al.*, 1990; Bochner *et al.*, 1990; Hirai *et al.*, 1990; Shute, 1992; Yamaguchi *et al.*, 1992) and are therefore relatively selective. There is abundant evidence that these cytokines are generated in allergic inflammation (Walker *et al.*, 1991; Robinson *et al.*, 1992). In the Boyden chamber, IL-3, IL-5, and GM-CSF are chemotactic for eosinophils from normal individuals but less active on eosinophils from allergic subjects, possibly as a result of *in vivo* desensitization (Warringa *et al.*, 1991; Sehmi *et al.*, 1992). Although active at low concentrations, they are in general only weakly effective when compared to well-documented eosinophil chemoattractants such as PAF. They appear much more active at priming

TABLE II
EOSINOPHIL CHEMOATTRACTANTS

Type	Effectiveness (unprimed)	Comments
Lipids		
PAF	High	Nonselective
LTB_4	Low	Nonselective
LTE_4	?	Activity reported *in vivo* only
18s, 15s, diHETE	Moderate	Part of guinea pig ECF-A
Small MW peptides		
C5a	High	Nonselective
fMLP	Low	Nonselective
Cytokine growth factors		
IL-5, IL-3, GM-CSF	Low	Active only on cells from normals. Effective priming agents
IL-2, LCF	Low	Possibly mainly chemokinetic
Chemokines		
C–C family (not active neutrophils)		
RANTES	High	Active *in vivo* and *in vitro*
MIP-1α	Low/moderate (eos)	Histamine releasers
MCP-3	High	Histamine releasers
Eotaxin	High	Characterized in the guinea pig. Active *in vivo* and *in vitro*
C–X–C family		
IL-8	Low	Only active on primed cells

eosinophils *in vitro* for enhanced chemotactic responses to suboptimal concentrations of lipid and low-molecular-weight chemoattractants, such as LTB_4 and fMLP, that are otherwise weakly active on unprimed normal eosinophils. Neuropeptides, such as SP, neurokinin A, calcitonin gene-related peptide, and cholecystokinin octapeptide, had similar modulatory effects on PAF- and LTB_4-induced chemotaxis of human eosinophils (Numao and Agrawal, 1992). IL-5 and GM-CSF also markedly potentiated the transendothelial migration of eosinophilia *in vitro* (Ebisawa *et al.*, 1994). This priming effect appears to be relevant *in vivo* in that eosinophils from allergic subjects with a mild to moderate eosinophilia are far more responsive in the Boyden chamber assay to chemotactic stimuli, such as PAF, than to eosinophils from normal subjects (Warringa *et al.*, 1992). This effect is also seen *in vivo* as shown by Henocq and Vargaftig (1986) who demonstrated that subcutaneous injections of PAF induced a neutrophil infiltration in normal subjects but an eosinophil-rich infiltration in allergic subjects. It is possible that a selective priming stimulus from growth factors active on mature eosinophils combining with a nonselective but highly effective chemoattractant results in selective migration. Consistent with this distinction between a priming stimulus from the growth factors and a chemotactic stimulus from mediators, such as PAF, is the different nature of the growth factor and chemoattractant receptors. The former are heterodimeric receptors related to other members of the growth factor receptor family, whereas chemoattractant receptors tend to be G protein-linked seven transmembrane "serpentine" receptors (Miyajima *et al.*, 1992). Presumably these two types are linked to distinct signal transduction mechanisms which result in different effector functions.

Despite the relative lack of activity of IL-5 in the Boyden chamber, inhalation of IL-5 in animals and humans leads to eosinophil recruitment (Terada *et al.*, 1992; Iwama *et al.*, 1992) and antibodies against IL-5 have been effective at inhibiting eosinophil migration into the lung of allergen-challenged guinea pigs (Van Oosterhour *et al.*, 1993; Mauser *et al.*, 1993). Antibody-mediated inhibition occurs even when the anti-IL-5 antibody is given at the time of the challenge so it is not primarily working through an effect on eosinopoiesis. A possible explanation of the apparent discrepancy between the *in vivo* and *in vitro* findings is that IL-5 may be a more effective chemoattractant *in vivo* than *in vitro*. Alternatively, IL-5 priming of adhesion to vascular endothelium may be important in eosinophil transmigration after allergen challenge. A third possibility is that anti-IL-5 may be inhibiting eosinophil release from the bone marrow.

In a recent study IL-4 was shown to be chemotactic for eosinophils, but not neutrophils, from patients with atopic dermatitis (Dubois *et al.*, 1994).

Eosinophils from normal donors did not respond. These results suggest that most TH2-type cytokines may have a priming effect on eosinophils.

An exciting development in the area of eosinophil migration is the identification of relatively cell-specific chemotactic peptides termed chemokines or intercrines (reviewed by Baggiolini and Dahinden, 1994). Chemokines are 6- to 10-kDa proteins which share similar amino acid sequences. They are basic polypeptides which bind firmly to heparin and are highly effective leucocyte chemoattractants. The family shares a common four-cysteine motif and is divided into two subfamilies based on the position of the first two of the conserved cysteines. In the C–X–C subfamily the cysteines are divided by an amino acid and in the C–C family the cysteines are adjacent (Schall, 1991). The C–X–C family, typified by IL-8, is particularly active on neutrophils and monocytes, although IL-8 has chemotactic activity for primed eosinophils (Sehmi *et al.*, 1993b) and is also a basophil chemotaxin and secretagogue (Dahinden *et al.*, 1989; Collins *et al.*, 1993; Geiser *et al.*, 1993). The C–C family, typified by RANTES, in addition to being chemotactic for monocytes and $CD4^+$/$UCHLI^+$ "memory" T cells (Schall *et al.*, 1990), is composed of potent basophil chemotaxins and histamine releasers as well as being chemotactic for eosinophils (Kameyoshi *et al.*, 1992; Rot *et al.*, 1992; Bischoff *et al.*, 1993; Kuna *et al.*, 1993; Dahinden *et al.*, 1994; Jose *et al.*, 1994a,b). In addition to being active *in vitro*, RANTES has been shown to result in eosinophil accumulation in the skin of dogs after intradermal injection (Meurer *et al.*, 1993).

A recently described CC chemokine is monocyte chemotactic protein-3 (MCP-3) (Opdenakker *et al.*, 1993). MCP-3 is approximately equivalent, in terms of potency, to RANTES in eosinophil and basophil chemotaxis (Baggiolini and Dahinden, 1994; Dahinden *et al.*, 1994). The effect of MCP-3 on T cell locomotion has yet to be established. Since MCP-3 and RANTES also stimulate histamine release from human basophils and promote exocytosis of eosinophil granule proteins *in vitro* (Baggiolini and Dahinden, 1994; Rot *et al.*, 1992; Alam *et al.*, 1993), they have the potential to play a special role in allergic inflammatory reactions. Ying *et al.* (1995b) found that exposure to specific allergen induced the transcription of mRNA for MCP-3 and RANTES in the skin of human atopic subjects and that the time course of appearance of $mRNA^+$ cells for RANTES paralleled the appearance of $CD3^+$, $CD4^+$, and $CD8^+$ cells, whereas the accumulation of eosinophils followed the kinetics of cells $mRNA^+$ for MCP-3.

An alternative approach for identifying eosinophil chemoattractants is to purify and characterize activity generated in an appropriate *in vivo* model. Griffiths-Johnson *et al.* (1993) purified a specific eosinophil chemoattractant in bronchoalveolar lavage fluid from sensitized guinea pigs after challenge with aerosolized allergen. Activity in bronchoalveolar lavage fluid

was assayed using an *in vivo* system: the fluid was injected intradermally in naive guinea pigs and the accumulation of ^{111}In-eosinophil was measured. Activity was found as early as 30 min after challenge, was markedly elevated at 3 and 6 hr, and returned to baseline at 24 hr. Three-hour bronchoalveolar lavage fluid was purified in a series of HPLC steps using the skin assay system to identify activity throughout. Microsequencing of purified protein revealed a novel 73 amino acid C–C chemokine, "eotaxin" (Jose *et al.*, 1994a), which is highly potent and selective in inducing eosinophil accumulation in the skin and lung *in vivo*. Eotaxin has subsequently been cloned and its mRNA expression has been shown to be markedly elevated in challenged/sensitized guinea pig lung (Jose *et al.*, 1994b). The precise human equivalent of eotaxin has not been established; human MCP-3 is the closest in homology of the proteins recently shown to stimulate eosinophils.

Chemokines are generated by several cell types. For instance, IL-8 is produced by airway epithelial cells (Standiford *et al.*, 1990) and RANTES by eosinophils (Ying *et al.*, 1995b). RANTES has also been identified *in vivo* in human nasal polyps (Beck *et al.*, 1994).

The precise role of chemokines in regulating eosinophil migration remains to be determined; however, they fulfill the criteria of potentially important chemotactic mediators in allergic inflammation in that they are selective, effective, and active *in vivo*.

e. Summary and Conclusions. Considerable progress has been made in our understanding of the molecular mechanisms involved in eosinophil migration into sites of allergic inflammation. It appears to be a staged process (with each stage offering a level of control over the cell specificity and degree of migration). Once in the tissues eosinophils may survive for several days or weeks under the influence of locally generated cytokines; this persistence may also partly explain selective tissue accumulation of eosinophils. An understanding of the molecular mechanism involved in leucocyte migration offers the possibility of selective and effective antagonists to treat allergic disease by preventing cell migration. Results from various animal models indicate that this approach may be successful. A diagrammatic outline of some of the events involved in local eosinophil recruitment is shown in Fig. 8.

2. *Receptors for Immunoglobulins*

Eosinophils express receptors for IgG, IgA, IgD, and IgE. However, the precise molecular structure of these receptors has not yet been fully determined, and with IgE in particular there exists controvery as to the exact nature of the IgE/eosinophil interaction.

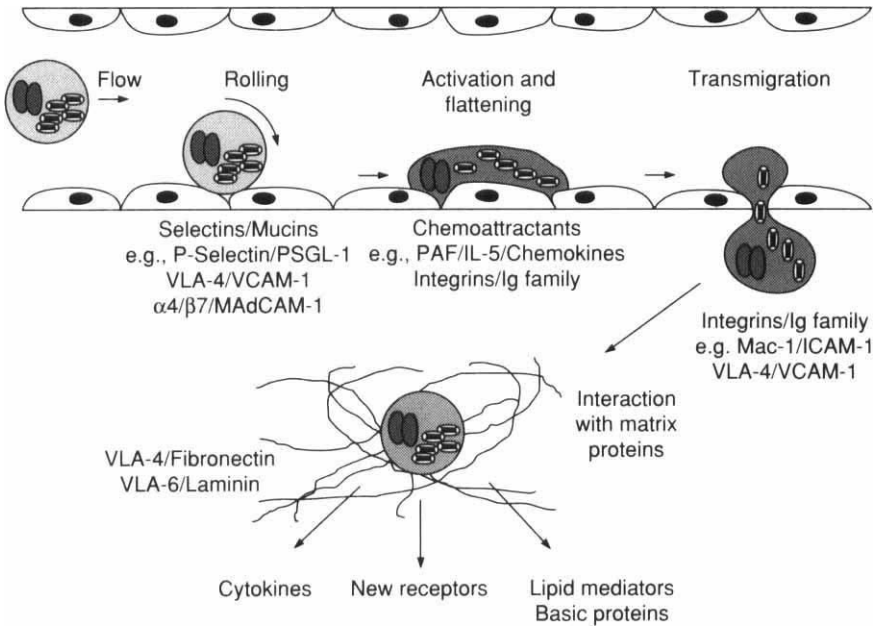

FIG. 8. Schematic diagram of the stages of eosinophil migration in relation to adhesion receptors and chemotactic mediators.

a. FcγR. Using monoclonal antibodies and functional analysis three distinct types of FcγR have been identified on human leukocytes (Unkeless, 1989; Fanger *et al.*, 1989). FcRI (CD64) is a high-affinity receptor of 72 kDa that is constitutively expressed by monocytes only. FcRIII (CD32) is a low-affinity receptor of 40 kDa and has a wide cellular distribution, including monocytes, neutrophils, eosinophils, platelets, and B cells. Only CD32 is constitutively expressed by resting eosinophils to any significant degree (Hartnell *et al.*, 1990).

FcRIII (CD16) is also a low-affinity receptor of 50–70 kDa which is constitutively expressed by neutrophils and natural killer (NK) cells. These three receptors are members of the Ig superfamily and have highly homologous extracellular domains (Kinet, 1989). The cDNA of each FcγR has been cloned (Allen and Seed, 1988; Stuart *et al.*, 1987; Simmons and Seed, 1988), revealing further complexity due to divergent transmembrane or cytoplasmic domains but conserved extracellular domains.

Two isoforms of FcRIII that are encoded by two distinct genes have been identified (Ravetch and Perussia, 1989; Scallon *et al.*, 1989). FcRIII-1 (CD16-1) is expressed by neutrophils and has a P13 glycan membrane

anchor. FcRIII-1 is shed from neutrophils on stimulation with fMLP and can be detected in human plasma (Huizinga *et al.*, 1988, 1989, 1990a). FcRIII-2 (CD16-2) is expressed by NK cells and is a transmembrane protein. Surface expression of FcRIII-2 is dependent on coexpression of FcRI-γ or CD3-ζ (Kurosaki and Ravetch, 1989; Hibbs *et al.*, 1989; Lanier *et al.*, 1991).

FcγR mediate many important cellular functions such as degranulation, respiratory burst, phagocytosis, clearance of immune complexes, and ADCC (Unkeless, 1989; Fanger *et al.*, 1989). FcRIII-1 promotes the binding of immune complexes, particularly small complexes, and mediates degranulation, whereas FcRII, in addition to these functions, is essential for the respiratory burst and phagocytosis (Tosi and Berger, 1988; Huizinga *et al.*, 1988, 1989, 1990b; Kimberley *et al.*, 1990). There is some evidence that FcRIII cooperates with FcRII to facilitate cell activation (Salmon *et al.*, 1991), whereas FcRI, FcRII, and FcRIII-2 can trigger lysis of certain target cells such as heteroantibody-coated chicken E. Recently, it has been reported that FcRII and FcRIII engagement leads to a signal transduction pathway that is pertussis toxin insensitive and, therefore, different from that which follows fMLP receptor ligation (B. Walker *et al.*, 1991; Reibman *et al.*, 1991; Brennan *et al.*, 1991).

FcRI expression is induced on neutrophils and upregulated on monocytes by long-term culture with IFN-γ (Perussia *et al.*, 1987; Shen *et al.*, 1987; Pan *et al.*, 1990). In addition, FcRIII is upregulated on neutrophils by IFN-γ, GM-CSF, and granulocyte-CSF (Buckle and Hogg, 1989) and induced on monocytes by TGFβ (Welch *et al.*, 1990). However, FcRII expression on neutrophils and monocytes appears to be unaffected by culture with cytokines. Valerius *et al.* (1990) reported that IFN-γ enhanced eosinophil FcRII-dependent ADCC and that this was accompanied by a modest increase in FcRII expression after 24 hr. Hartnell *et al.* (1992a) investigated the effect of IFN-γ, in comparison with that of IL-3, on eosinophil FcγR expression. IFN-γ had a profound effect on eosinophil surface receptors, inducing the *de novo* expression of FcRI and functionally active FcRIII and upregulating the expression of FcRII. By contrast, culture with IL-3 caused an upregulation of eosinophil FcRII expression but did not induce expression of FcRI or FcRIII. The FcRIII expressed by eosinophils after IFN-γ stimulation was functionally active, as shown by the triggering of eosinophil membrane depolarization and LTC_4 generation by an anti-CD16 mAb. Treatment of IFN-γ stimulated eosinophils with phosphatidylinositol-specific phospholipase C-reduced FcRIII expression, suggesting that, like neutrophils, eosinophils express the phosphatidylinositol glycan-linked form of this receptor. Therefore, IFN-γ-treated eosino-

phils express a functionally active, phosphatidylinositol glycan-anchored form of FcRIII.

b. FcεR. In 1994 Gounni *et al.* described high-affinity IgE receptors on eosinophils from hypereosinophilic patients. The evidence to support this claim was comprehensive and included inhibition of [^{125}I]IgE binding to human eosinophils by anti-FcεRI α-chain monoclonal antibody (15-1), surface expression of FcεRI α-chain on eosinophils by flow cytometric analysis, immunostaining of tissue eosinophils with 15-1, the demonstration of FcεRI α-, β-, and γ-chain transcripts in eosinophils, the release of EPO and ECP after stimulation of eosinophils with anti-FcεRI antibody, and the inability of eosinophil-mediated IgE-dependent cytotoxicity against schistosome targets with the anti-FcεRI α-chain antibody. However, all subjects had eosinophils in association with idiopathic hypereosinophilic syndrome, various skin diseases, and lymphomas. There were no data on high-affinity IgE receptors on eosinophils from healthy noneosinophilic donors and/or subjects with eosinophilia due to more common conditions such as atopic allergy and helminth infection.

Previous studies from these workers had found that eosinophils expressed low-affinity IgE receptors (A. Capron *et al.*, 1986; Joualt *et al.*, 1988; Capron *et al.*, 1991a,b). However, some of these data were difficult to reconcile with the additional or alternative presence of significant numbers of functional high-affinity IgE receptors on eosinophils [as reported by Gounni *et al.*, (1994)]. In particular, the eosinophil IgE receptor was found to have a low affinity ($K\varepsilon$ of 10^{-7} M^{-1}) and to correspond to CD23, i.e., the low-affinity FcεRII. Furthermore, it was said to consist of proteins of molecular weight corresponding to those of the low-affinity IgE receptor (Joualt, 1988) and to function in IgE binding and cytotoxicity to schistosomula with inhibition by monoclonal antibodies to CD23 (A. Capron *et al.*, 1986; Capron *et al.*, 1991a,b). Hence, these earlier studies demonstrated a low-affinity IgE receptor on eosinophils but did not detect evidence of significant high-affinity IgE receptor activity. Therefore, this report on high-affinity IgE receptor-mediated eosinophil function needs to be reconciled with previous reports on the relative expression and function of the low-affinity IgE receptor.

Another level of complexity has been added by the description of expression of the IgE-binding molecule Mac-2/ε BP on human eosinophils. This S-lectin-type molecule was also able to bind radiolabeled IgE and participate in IgE-dependent effector functions of eosinophils (Truong *et al.*, 1993). The interrelationship of putative Fcε receptors and Mac-2/ε BP requires further clarification especially since Mac-2 is also expressed on neutrophils, but neutrophils have no IgE function (Truong *et al.*, 1993).

c. Fcα. A number of studies have shown that eosinophils possess functional surface IgA receptors (Abu-Ghazaleh *et al.*, 1992; Capron *et al.*, 1988; Kita *et al.*, 1991a). IgA, particularly secretory IgA, is effective in stimulating eosinophil degranulation and IgA-coated Sepharose beads released EDN. This effect was enhanced by preincubation of eosinophils with GM-CSF and IL-3.

Monteiro *et al.* (1993) analyzed the expression, regulation, and biochemical nature of FcαR on eosinophils using the natural IgA ligand and anti-Fcα IM antibodies. FcαR molecules were detected on eosinophils from normal individuals after *in vitro* activation and from unstimulated eosinophils from allergic individuals. The FcαR molecules expressed by eosinophils differed from those on neutrophils and macrophages and the former had a higher content of N-linked carbohydrate moietieis.

3. *Complement Receptors*

Eosinophils express low levels of CR1 (CD35), a polymorphic single-chain glycoprotein of about 250 kDa that binds the complement fragment C3b (Hartnell *et al.*, 1992b). In contrast, CR3, which binds to C3bi, is strongly expressed. Complement receptor type 3 (CR3, Mac-1), or CD11b/CD18, is a member of the β2 integrin family of adhesion molecules. This receptor has at least two binding sites which recognize a number of ligands, such as c3bi, ICAM-1, fibrinogen, and polysaccharides, and a subpopulation of CR3 is thought to have a functional relationship with Fc receptors (reviewed by Arnaout, 1990; Diamond *et al.*, 1990). Peripheral blood eosinophils expressed significantly fewer CR3 molecules on their surface than neutrophils (Hartnell *et al.*, 1990). However, this receptor is involved in a number of important eosinophils functions, such as adhesion to endothelial cells (Walsh *et al.*, 1990a,b) and IgE- and IgG-dependent schistosomula killing (Capron *et al.*, 1987). Binding of particles via this receptor is a potent stimulus for eosinophils to undergo respiratory burst (Koenderman *et al.*, 1990) and degranulation (Zeiger and Colten, 1977).

Neutrophils undergo a rapid upregulation of CR3 expression, of at least twofold, when stimulated with fMLP (Berger *et al.*, 1984; Fearon and Collins, 1983) or platelet-activating factor (PAF) (Shalit *et al.*, 1988), presumably as a result of translocation of CR3 from intracellular pools (O'Shea *et al.*, 1985; Jones *et al.*, 1988). In addition, GM-CSF causes a rapid as well as a long-term upregulation of CR3 expression on neutrophils (Buckle and Hogg, 1989), the latter being sustained over hours by increased translation of the receptor (Neumann *et al.*, 1990). In contrast, short-term stimulation of eosinophils with LTB_4, IL-3, IL-5, and GM-CSF causes only a small increase in CR3 numbers (Walsh *et al.*, 1990a,b; Fischer *et al.*, 1986;

Lopez *et al.*, 1986), and there are few studies on the longer-term effects of cytokines on eosinophil CR3 expression (Valerius *et al.*, 1990).

Hartnell *et al.* (1992b) previously investigated the capacity of eosinophils to increase their CR3 expression in response to IL-3, IL-5, and GM-CSF and determined the requirement for protein synthesis during short-term and long-term stimulation. Culture of human eosinophils with IL-3 produced a marked dose-dependent upregulation of CR3 expression. This was dependent on protein and RNA synthesis. IL-5 and GM-CSF had a similar effect on eosinophil CR3 expression but the maximal response to IL-5 was always less than that to IL-3 or GM-CSF. Dexamethasone inhibited the IL-3-induced upregulation of CR3 expression in a dose-dependent manner, with an IC_{50} of $5 \times 10^{8}M$. This study confirms the capacity of eosinophils to modify their phenotype through *de novo* protein synthesis. This process could be inhibited by physiological concentrations of glucocorticoids, thus providing an additional mechanism for their mode of action in allergic disease.

Blom *et al.* (1994) investigated the modulatory role of GM-CSF, IL-3, and IL-5 on the interaction of human eosinophils with opsonized particles (serum-treated zymosan, STZ). Addition of STZ to eosinophils isolated from the peripheral blood of normal human donors resulted in an interaction of the STZ particles with only 15–25% of the cells. Treatment of the eosinophils with GM-CSF, IL-3, or IL-5 strongly enhanced both the rate of particle binding and the percentage of eosinophils binding STZ. The effect of the cytokines was attributed to a change in affinity of CR3 for iC3b on the STZ particles. These cytokines also strongly enhanced STZ-induced PAF synthesis. However, cytokine priming appeared to be largely independent of the synthesis of PAF.

4. Receptors for Cytokines

In addition to functional surface receptors for immunoglobulins, eosinophils also respond at picomolar concentrations to GM-CSF, IL-3, and IL-5 (Silberstein *et al.*, 1986; Owen *et al.*, 1987a; Rothenberg *et al.*, 1988, 1989; Her *et al.*, 1991) via high-affinity cytokine receptors which have been fully characterized (DiPersio *et al.*, 1988; Lopez *et al.*, 1989, 1991; Chihara *et al.*, 1990; Migita *et al.*, 1991; Ingley and Young, 1991). All of these receptors are of single high affinity for each cytokine with an average k_d value of 120 pmol for the IL-5R, 500 pmol for the IL-3R, and 50 pmol for GM-CSFR. Eosinophils and basophils, but not neutrophils, express IL-5R, while GM-CSFR is present on both eosinophils and neutrophils with a similar affinity-binding value on both. Binding studies showed that there was a common component of the receptors which may explain the cross-inhibition observed between the three cytokines (Lopez *et al.*, 1989,

1991). This was explained by the observation that these receptors are composed of heterodimers which share a common β, but distinct α-chains (Tavernier *et al.*, 1991; Kitimura *et al.*, 1991). Thus, the homologous α-chains (60–80 kDa) are specific and form a low-affinity association with their receptive cytokines, whereas the common β-chain (120–140 kDa) combines with the α-chain to form a high-affinity binding site (Miyajima *et al.*, 1992). The cross-competition observed may result from limiting numbers of β-chains which may in turn regulate the extent of eosinophil activation. As stated, IL-5, IL-3, and GM-CSF have a range of similar biological effects on human eosinophils and induce phosphorylation of a common set of proteins (Kanakura *et al.*, 1990; Linnekin and Farrar, 1990; Murata *et al.*, 1992). This suggests that the β-chain or an additional common component may be responsible for signal transduction. Woodcock *et al.* (1994) used site-directed mutagenesis and binding assays with radiolabeled GM-CSF, IL-3, and IL-5 to identify residues in the β subunit involved in affinity conversion for each ligand. They identified three residues in the common β-chain of the human GM-CSF, IL-3, and IL-5 receptors that were essential for GM-CSF and IL-5, but not IL-3, high-affinity binding which interacted with Glu21 of GM-CSF.

Most of the IL-5R mRNA encodes a soluble isoform which has inhibitory activity in an IL-5-dependent eosinophil differentiation assay (Tavernier *et al.*, 1992). The α-chain of IL-5 is encoded on chromosome 3 and occurs as two soluble isoforms (S1 and S2) produced by "normal" or no splicing, respectively. The membrane-anchored form of the IL-5R is produced by alternative splicing (Tavernier *et al.*, 1992). IL-5R α-chain mRNA is downregulated by TGFβ1 (Zanders, 1994). Since TGFβ induced eosinophil apoptosis (Alam *et al.*, 1994) this raises the possibility that soluble IL-5R acts at several levels of control including eosinophil survival, expression of the membrane-anchored IL-5R, and terminal eosinophil differentiation steps. Increased expression of mRNA IL-5R (soluble and anchored) has recently been observed in bronchial biopsies from asthmatics (Ploysongsang *et al.*, 1995). Virtually all the IL-5R mRNA was associated with eosinophils suggesting that this cell type is the major target for IL-5 effects.

The receptor for IL-5 has also been investigated in terms of eosinophil density (Chihara *et al.*, 1990). By saturation binding intact peripheral blood normodense eosinophils had an association constant of 3.7×10^{-8} M for IL-s compared with a five-fold increase observed in hypodense eosinophils. Interestingly, the numbers of IL-5 receptors (450/cell) were unchanged in both cell populations. Incubation with GM-CSF induced an elevation in IL-5 binding to normodense eosinophils to a similar level observed in unstimulated hypodense eosinophils.

Murine eosinophils have been shown to express approximately 50 high-affinity receptors with a K_d range of $4–6 \times 10^{-11}$ M and approximately 5000 low-affinity receptors with a K_d range of $0.3–2 \times 10^{-9}$ M (Barry *et al.*, 1991). Based on sequence data from the mouse receptor, the human IL-5 receptor has now been cloned and the α-chain shown to be a unique peptide having 70% homology with the mouse α-chain (Tavernier *et al.*, 1991; Murata *et al.*, 1992). The β-chain, which is identical to that of the GM-CSF receptor, has also been shown to be homologous to the mouse IL-3 receptor-like protein (Tavernier *et al.*, 1991).

In addition to the receptors for IL-3, IL-5, and GM-CSF, eosinophils also expressed functional high-affinity receptors for IL-2 which may be associated with their activation state (Rand *et al.*, 1991a). This observation was made on eosinophils obtained from hypereosinophilic syndrome patients who were shown to have an enhanced membrane expression of the CD25 subunit of the IL-2 receptor. Partially purified supernatant from the U937 cell line, but not from GM-CSF, IL-3, IL-5, IFN-γ or the lymphocyte chemotactic factor, was able to enhance CD25 expression on normal density eosinophil after 24–48 hr incubation (Rand *et al.*, 1991b). The IL-2 receptor (CD25) has been suggested to be a ligand for IL-2 in chemotaxis. IL-2 has been shown to be approximately four-fold more potent in eosinophil chemotaxis than PAF. It is interesting that coculturing eosinophils obtained from hypereosinophilic syndrome patients with GM-CSF, IL-3, or IL-5 in the presence of 3T3 fibroblasts significantly decreased the percentage of CD25 immunoreactivity in these cells. This may suggest that changes in the microenvironment of the eosinophil may cause alterations in eosinophil proteins resulting in reduced expression of IL-2, thus modulating IL-2-dependent chemotaxis.

5. *Receptors for Inflammatory Mediators*

Eosinophils possess a number of specific receptors for soluble mediators including LTB_4, PAF, C5a, and C3a. Receptors for LTB_4 on guinea pig alveolar and peritoneal eosinophils have been shown to be of both high and low affinity (Maghni *et al.*, 1991; Sehmi *et al.*, 1992a); however, this has not been confirmed for human eosinophils. Human eosinophils have been reported to express receptors of two affinities for PAF (Kroegel *et al.*, 1989; Kurihara *et al.*, 1989). Although the presence of a receptor for fMLP on eosinophils has not been demonstrated directly, there is sufficient evidence to indicate that a low-affinity binding site may be present on eosinophils which is associated with a functional response of eosinophils to high concentrations of this peptide (Yazdanbakhsh *et al.*, 1987). Koenderman *et al.* (1988) have shown that the increase in intracellular Ca^{2+} induced by fMLP was inhibited by pertussis toxin suggesting that a GTP-

binding protein may be involved in fMLP-mediated cell activation. Receptors for C5a, PAF, fMLP, and LTB_4 have been recognized as belonging to a family of seven transmembrane GTP-binding proteins (Gerard and Gerard, 1991; Honda *et al.*, 1991). C5a receptors are involved in the stimulation of human eosinophils and in inducing their chemotaxis and mediator release, possibly through the activation of phospholipase D (Minnicozzi *et al.*, 1990). Eosinophil C3a receptors are also ligands for chemotaxis, mediator release, and activation of reactive oxygen radical species production as well as intracellular calcium transport in human eosinophils (Elsner *et al.*, 1994).

6. *Other Receptors*

Eosinophils also express receptor for CD4, regardless of their density and state of activation, although eosinophils obtained from hypereosinophilic syndrome patients had an elevated level of CD4 immunoreactivity (Lucey *et al.*, 1989a). The increased expression of CD4 by hypodense eosinophils suggests that the eosinophil may provide an additional reservoir for HIV infections.

HLA-DR expression has also been described in eosinophils (Lucey *et al.*, 1989b) following culture in the presence of GM-CSF and 3T3 fibroblasts. Thus, eosinophils act as relatively weak antigen-presenting cells (Weller *et al.*, 1991c, 1993).

Nishikawa *et al.* (1992) described the expression *in vivo* of CD69 on lung eosinophils obtained from patients with eosinophilic pneumonia. Hartnell *et al.* (1993) showed that CD69 could be induced on human peripheral blood eosinophils by GM-CSF stimulation as early as 1 hr after incubation with optimal upregulation at 24 hr and sustained over 2 days of incubation *in vitro*. IL-3, IL-5, and IFN-γ, but not PAF, also induced eosinophil CD69 expression. This appeared to be protein synthesis dependent and not inhibitable by corticosteroids. Furthermore, *in vivo* expression of this receptor was also present in eosinophils from bronchoalveolar lavage fluid of mild asthmatic subjects. These two studies suggested that CD69 may be a marker of eosinophil activation by cytokines. CD69 is an early activation marker for lymphocytes (Corte *et al.*, 1981) [previously known as EA1 (Hara *et al.*, 1986), AIM (Cebrian *et al.*, 1988), and Leu 23 (Lanier *et al.*, 1988)]. CD69 expression in T cells is maximal after PMA stimulation for 3 or 4 hr *in vitro* (Hara *et al.*, 1986). CD69 is also inducible on B cells (Hara *et al.*, 1986; Risso *et al.*, 1989), NK cells (Lanier *et al.*, 1988), and some lymphoid and nonlymphoid cell lines.

Other receptors expressed on eosinophils and other leucocytes, and whose function remains ill-defined, include CD31 and CD9. The former binds to other CD31 molecules in homophilic interactions and participates

in heterophilic reactions involving proteoglycans, thus amplifying integrin-mediated adhesion of T cells (Tanaka *et al.*, 1992). CD9, on the other hand, is a 24-kDa protein found on eosinophils, platelets, and pre-B cells, but not on neutrophils. This suggests that CD9 may be a useful marker for separation of eosinophils from neutrophils in mixed population. CD9 mediates homotypic adhesion of platelets and pre-B cell lines (Masellis-Smith *et al.*, 1990) but its precise function in eosinophils is unknown.

Eosinophils express CD4 antigen on their surface and bind HIV GP120 (Lucey *et al.*, 1989a). Similarly, $CD4^+$ eosinophil precursors in human bone marrow cultured with IL-5 can be infected with HIV (Freedman *et al.*, 1991).

Eosinophils bind anti-CD25 (IL-2R) (Riedel *et al.*, 1990) and generate messenger RNA for IL-2R following culture with cytokines. The p55 chain of this receptor was not detectable on unstimulated eosinophils from normal donors. In contrast, Rank *et al.* (1991b) identified the p55, but not the p75 chain on unstimulated eosinophils. There was increased expression following cytokine stimulation. P55 (but not p75) together with mRNA for p55 was also present on the surface of low-density eosinophils from patients with the hypereosinophilic syndrome (Plumas *et al.*, 1991).

Table III summarizes the surface receptors associated with human eosinophils.

E. Eosinophil Mediators

Eosinophils have the capacity to secrete a number of potent mediators (Table IV). These include basic proteins stored in eosinophil granules, newly formed membrane-derived lipids, cytokines, various proteases, and products of oxidative metabolism including the superoxide anion and hydrogen peroxide.

TABLE III
Eosinophil Receptors Other Than Adhesion Molecules

Immunoglobulin receptors
FcαR, FcεR,[a] FcγRII (CDw32)
Mediator receptors
IL-5,[a] IL-3,[a] GM-CSF, RANTES,[a] C5a, PAF, fMLP, LTB_4
Newly expressed receptors
IL-2 (CD25), FcγRIII (CD16), CD4,[a] ICAM-1, HLA-DR, CD69[a]
Other receptors
CR1, CR3 (Mac-1), Mac-2, CD9,[a] CD45

[a]Not expressed on neutrophils.

TABLE IV
EOSINOPHIL-DERIVED MEDIATORS

Granule-associated mediators
Basic proteins
major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin, eosinophil peroxidase
Enzymes
e.g., lysophospholipase, phospholipase D, arylsulfatase, histaminase, catalase, acid phosphatase, nonspecific esterases, glycosaminoglycans, hexosaminidase
Cytokines
IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, GM-CSF; TGF-α, TGF-β, TNF-α
Chemokines
MIP-1α, RANTES
Membrane-derived mediators
Leukotriene C_4, platelet-activating factor, 15-hydroxyeicosatetraenoic acid, prostaglandin E1 and E2, thromboxane B2

1. Lipid Mediators

Eosinophil-derived lipid mediators have recently been reviewed in detail (Weller, 1993). They are principally eicosanoids and PAF.

a. Eicosanoids. Eicosanoids are oxidation products of arachidonic acid. Esterification of phospholipid-containing arachidonic acid results in release of the free fatty acid by the action of activated phospholipase A_2. Eicosanoid production occurs widely in leukocytes and cells resident in tissues although the amount and pattern of mediators generated differ between various cell types. Eicosanoid formation occurs at the cell membranes and in lipid bodies. With eosinophils exogenously added [^{3}H]arachidonic acid was found to localize principally to lipid bodies (Weller and Dvorak, 1985) as was the case with other cell types (Galli *et al.*, 1985). Arachidonic acid was incorporated mainly into phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine (Weller *et al.*, 1991a). The principal pathways for the metabolism of arachidonic acid in eosinophils are via the actions of cyclooxygenase, which produce the prostaglandins and thromboxanes, and 5- and 15-lipoxygenase, which lead to the generation of leukotrienes and the HETEs and diHETEs.

Eosinophils can generate picogram quantities of prostaglandin E_2, PGD_2, and $PGF_{2\alpha}$ (Hubscher, 1975; Parsons and Roberts, 1988), although the predominant cyclooxygenase product was thromboxane B_2 (TXB_2). After stimulation with calcium ionophore human peripheral blood eosinophils produced over 2 ng of TXB_2 per 10^6 cells (Foegh *et al.*, 1986).

5-lipoxygenase, a fully characterized enzyme (Matsumoto *et al.*, 1988), catalyzes the oxidation of arachadonic acid at the 5 position to 5S-hydroperoxyeicosatetraenoic acid (t-HPETE) to produce the unstable epoxide leukotriene A_4. The activity of 5-lipoxygenase is dependent on an 18-kDa protein called 5-lipoxygenase-activating protein which binds to arachidonic acid (Miller *et al.*, 1990). Neutrophils contain an epoxide hydrolase (LTA hydrolase) which converts LTA_4 to LTB_4; eosinophils have a specific glutathione-S-transferase which links glutathione to LTA_4 to produce the stable sulfidopeptide leukotriene LTC_4 (Samuelsson, 1983). LTC_4 is converted to LTD_4 by the action of γ-glutamyltranspeptidase and to LTE_4 by a dipeptidase. LTE_4 is the stable metabolite. Eosinophils generate relatively large amounts (up to 70 ng/10^6 cells) of the sulfidopeptide leukotriene LTC_4 after stimulation with the calcium ionophore, but only negligible amounts of LTB_4 (Weller *et al.*, 1983; Jorg *et al.*, 1982). Conversely, neutrophils produce large amounts of LTB_4, but little, if any, LTC_4. Interestingly, guinea pig eosinophils resemble human neutrophils in being able to synthesize LTB_4 but not LTC_4 (Sun *et al.*, 1989). LTC_4, LTD_4, and LTE_4 contain the activity originally known as "slow reacting substance of anaphylaxis." Sulfidopeptide leukotrienes have a number of properties relevant to asthma including smooth muscle contraction, mucous hypersecretion, and increased vascular permeability. In addition to calcium ionophore, human eosinophils can also generate LTC_4 following a more physiological stimulation such as IgC-coated Sepharose beads (Shaw *et al.*, 1985), opsonized zymosan (Brujnzeel *et al.*, 1985), or IgG–*Apergillus fumigatus* immune complexes (Cromwell *et al.*, 1988).

In general, eosinophils from asthmatics generated more LTC_4 than eosinophils from normal donors (Aizawa *et al.*, 1990; Kohi *et al.*, 1990; Taniguchi *et al.*, 1985), although there were exceptions. *In vivo* studies are complicated by different degrees of "activation," differences in density between eosinophils obtained from different sources, the catabolism of LTC_4 by simultaneously released EPO, and the balance between secreted and cell-retained LTC_4. Hodges *et al.* (1988), using exogenously added L-serine to prevent the catabolism of LTC_4 by EPO, found that, whereas normal-density eosinophils from asthmatics and normal subjects released comparable amounts of LTC_4 after stimulation with ionophore, low-density eosinophils from normal subjects released more amounts of LTC_4 than low-density eosinophils from asthmatics. *In vitro* stimulation of eosinophils with cytokines or chemoattractants consistently produced enhancement of leukotriene generation by peripheral blood eosinophils (Silberstein *et al.*, 1986), although the mechanism by which this occurs is unclear. Coculture of eosinophils with endothelial cells (Rothenberg *et al.*, 1987) or exposure to GM-CSF (Silberstein *et al.*, 1986; Owen *et al.*, 1987a; Howell *et al.*,

1989), IL-3 (Rothenberg *et al.*, 1988), IL-5 (Rothenberg *et al.*, 1989), TNF-α (Roubin *et al.*, 1987), and monocyte-derived factors (Dessein *et al.*, 1986; Fitzharris *et al.*, 1986; Elsas *et al.*, 1987, 1990) was shown to enhance LTC_4 release after incubation with calcium ionophore A23187. PAF alone can also elicit LTC_4 release (Bruijnzeel *et al.*, 1986, 1987; Tamura *et al.*, 1988).

There is good evidence that the SRS-A leukotrienes are generated in asthma and allergic inflammation. Several studies have reported increased amounts of leukotrienes in asthmatic BAL fluid (Wardlaw *et al.*, 1989; Wenzel *et al.*, 1990) and increased concentrations of LTE_4 were detected in urine from asthmatics (Christie *et al.*, 1991). In addition, sulfidopeptide leukotriene receptor antagonists and 5-LO inhibitors have shown promising results in early clinical trials (Spector *et al.*, 1994). Eosinophils, unlike neutrophils, contain large quantities of 15-lipoxygenase, a 70-kDa cytosolic enzyme homologous to 5-lipoxygenase (Sigal *et al.*, 1988a), which catalyzes the insertion of an oxygen at position 15 on arachidonic acid to form 15-hydroperoxyeicosatetranoic acid (15-HPETE) and subsequently 15-HETE (Sigal *et al.*, 1988b). Eosinophils can generate microgram quantities of 15-HETE (Turk *et al.*, 1982). This may be of importance in asthma since 15-HETE stimulated mucus production by cultured human airway (Marom *et al.*, 1983).

The dual actions of two lipoxygenase enzymes on a molecule of arachidonic acid gives rise to a further set of compounds termed the lipoxins. Eosinophils can generate lipoxin A_4 (5S,6R, 15S-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid) through the dual actions of 5- and 15-lipoxygenase (Serhan *et al.*, 1987). The activities of lipoxins in relation to eosinophilic inflammation are not entirely clear but they seem to have a general downregulatory role, for example, inhibiting neutrophil chemotactic responses to LTB_4 and fMLP (Lee *et al.*, 1989).

b. Platelet-Activating Factor. Platelet-activating factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid formed by the acetylation of its inactive precursor, lyso-PAF, through the actions of a specific acetyltransferase. Lyso-PAF is believed to be generated by the cleavage of an acyl group from the C2 position of 1-O-alkylglycero phospholipids by phospholipase A_2. In some situations the acyl group is arachidonic acid so that eicoanoids and PAF may be generated together. Biodegradation occurs as a result of the actions of an acetylhydrolase which cleaves the acetyl group to generate lyso-PAF (Snyder, 1985). Both of these enzymes are present in eosinophils (Lee *et al.*, 1982, 1984). PAF has a number of proinflammatory activities and in particular acts as a leukocyte chemoattractant and activating agent. Inhalation of PAF causes bronchoconstriction in

humans and variable increases in bronchial hyperresponsiveness (Henson, 1989). The receptor for PAF is a member of the serpentine, G protein-linked receptor family (Honda *et al.*, 1991). Eosinophils can generate substantial quantities of PAF after stimulation with calcium ionophore, zymosan, and IgG-coated Sepharose beads (Cromwell *et al.*, 1990; Burke *et al.*, 1990). Cromwell *et al.* (1990) found that eosinophils generated 25 and 2 ng/10^6 cells of PAF after stimulation with calcium ionophore and IgG-coated Sepharose beads, respectively. Much of the PAF remained cell associated. Stimulation of the eosinophils with fMLP did not augment PAF release and hypodense eosinophils from patients with a marked eosinophilia released less PAF than did normal eosinophils. This appeared to be the result of increased metabolism of the PAF by the activated cells rather than decreased generation. Release of PAF was maximal after 45 min. In general, it has been difficult to detect increased amounts of PAF in eosinophil-associated diseases, possibly because it is rapidly metabolized and is bioactive at a very local level. Similarly, PAF antagonists have so far been disappointing in clinical trials for asthma.

2. *Eosinophil Granule Proteins*

The distinctive eosinophil-specific granules contain four basic proteins. MBP, which makes up 50% of the granule protein, is found in the core of the granule where it accounts for virtually all the protein in the core (Lewis *et al.*, 1978). EPO, ECP, and EDN (also known as EPX) are found in the granule matrix (Egesten *et al.*, 1986; Peters *et al.*, 1986). The biology of these proteins has recently been reviewed in detail (Gleich *et al.*, 1995; Ackerman, 1993).

a. MBP. MBP is a single polypeptide chain of 117 amino acids, with a molecular weight of 13.801 and a pI of 10.9. It contains 17 arginine residues, which accounts for its basicity, and 9 cysteine residues, which explains its tendency to form disulfide bonds. Immunoreactive MBP can be detected in biological fluids only after it has been reduced and alkylated. The reduced form is as toxic for parasites as the native form, but is considerably less potent (O'Connell *et al.*, 1983). In addition to the mature MBP peptide, the cDNA for MBP encodes for a preprosequence which is a putative signal peptide and a 90-amino acid acidic proprotein which may neutralize MBP's toxicity as it is processed through the Golgi and transported to the granule where the prosequence is cleaved (McGrogan *et al.*, 1988; Barker *et al.*, 1988). The gene for MBP is 3.3 kb and contains six exons and five introns (Barker *et al.*, 1990). MBP can also be detected in basophil granules although basophils contain considerably less MBP than eosinophils (Ackerman *et al.*, 1983). Plasma concentrations of MBP

are elevated in the sera of pregnant women with a peak 2 or 3 weeks before parturition. Placental eosinophils are few in number and MBP has been shown by immunofluorescence to be in placental X and giant cells (Maddox *et al.*, 1984). In addition to the mature form of MBP, pro-MBP is also found in the placenta where it is bound to the placental protein PAPP-A (Oxvig *et al.*, 1993). Recent studies using *in situ* hybridization have shown that PAPP-A mRNA is detected in placental X cells and syncytiotrophoblasts, whereas MBP mRNA is localized only to placental X cells (Bonno *et al.*, 1994a,b). The function of MBP in pregnancy is obscure.

It has been known for some years that major basic protein is toxic for a number of parasites, particularly the larval forms of helminthic parasites such as schistosomulae of *Schistosoma mansoni* (reviewed by Butterworth, 1984; Gleich and Adolphson, 1986). Indeed, the hypothesis that the teleological role of the eosinophil is in host defense against parasite infection is based largely on this observation. MBP is also toxic for certain strains of *S. aureus* and *Escherichia coli* (Lehrer *et al.*, 1989). One function of MBP that has received considerable attention is its cytotoxicity for mammalian cells, particularly airway epithelial cells. Together with the observation (using immunofluorescence) that MBP was deposited in the asthmatic airway, this led to the concept that the epithelial desquamation characteristic of asthma and related allergic disorders may be due to the effects of the eosinophil granule proteins (discussed in Wardlaw and Kay, 1987; Gleich, 1990). MBP was cytotoxic toward guinea pig and human epithelium at concentrations as low as 10 μg/ml. Impaired cilial function was observed after 10 min followed by ciliostasis with exfoliation and lysis after exposure for 1–6 hr (Frigas *et al.*, 1980; Hastie *et al.*, 1987; Hisamatsu *et al.*, 1990). MBP caused bronchoconstriction and increased hyperresponsiveness to methacholine when administered to both rats and monkeys by intrathecal installation. In rats the effect was mimicked by synthetic cations, such as poly-L-arginine, and there was no histological evidence of accompanying epithelial damage (Uchida *et al.*, 1993). Thus, cationic protein-induced hyperresponsiveness appears to be dependent on charge interactions (Coyle *et al.*, 1993) but airway epithelial cell shedding is not a necessary prerequisite for this effect (Uchida *et al.*, 1993). In primates other eosinophil basic proteins were ineffective apart from a weak and transient effect of EPO suggesting that it was not simply a function of the charge of the protein (Gundel *et al.*, 1991a). In addition to having cytotoxic properties, MBP is also a secretagogue. It caused noncytotoxic release of histamine from basophils (not a property of the other granule proteins) and rat peritoneal mast cells (Zheutlin *et al.*, 1984). MBP can also cause neutrophil and macrophage activation (Moy *et al.*, 1990) and together with EPO is a strong agonist for platelet-mediator release through a mechanism

distinct from thrombin (Rohrbach *et al.*, 1990). These properties suggest the presence of a membrane receptor for MBP but no such receptor has as yet been identified. The highly basic nature of MBP is likely to be central to functional properties such as activation of the alternative pathway of complement (Weiler *et al.*, 1992). Using lipid bilayers it was shown that MBP interacted with acidic lipids causing membrane disruption and lyzosomal fusion and lysis (Abu-Ghazaleh *et al.*, 1992). However, while many of the properties of MBP can be mimicked by other highly basic proteins, including the other granule proteins, and inhibited by heparin, not all properties are shared suggesting that the charge characteristics are not the only factor involved. MBP, ECP, EPO, and EDN all caused increased microvascular transport in the hamster cheek pouch, a phenomenon independent of histamine release (Minnicozzi *et al.*, 1995).

b. ECP. Eosinophil cationic protein is a single-chain polypeptide with a pI in the region of 10.8. On molecular sizing ECP displays marked heterogeneity, probably as a result of differential glycosylation, with molecular weight bands ranging between 16,000 and 21,400. Two isoforms, ECP-1 and ECP-2, can be identified using heparin Sepharose (Gleich *et al.*, 1986). The ECP cDNA encodes for a leader sequence of 27 amino acids and a mature protein of 133 amino acids with a calculated molecular weight of 15,600 (Rosenberg *et al.*, 1989a; Barker *et al.*, 1989). The amino acid sequence is 66% homologous to EDN and 31% homologous to human pancreatic ribonuclease. ECP does have ribonuclease activity but it is 100 times less potent than that of EDN (Slifman *et al.*, 1986). The gene for both EDN and ECP is on chromosome 14q. It contains a single intron in the 5′ untranslated region and an intronless coding region (Hamann *et al.*, 1990). Monoclonal antibodies can distinguish between a form of ECP found in the granules of resting eosinophils (mAb EG1) and a secreted or extracted form of ECP (both mAbs EG1 and EG2) (Tai *et al.*, 1984). EG2 also recognizes EDN. The differences between these two forms is not known but EG2 has been routinely used to identify activated eosinophils in tissue. There is approximately 25 pg of ECP per eosinophil which is about an order of magnitude less than that of MBP. ECP is toxic for schistosomulae of *S. mansoni* and other helminthic parasites and is more potent on a molar basis then MBP (McLaren *et al.*, 1981). It is also toxic for guinea pig tracheal epithelial cells at concentrations of 100 μg/ml (Motojima *et al.*, 1989) and, like EDN, can cause a neurological syndrome termed the Gordon phenomenon when injected into the CSF of various animals including rabbits and rats (Fredens *et al.*, 1982). ECP exerted its cytotoxic effect through the formation of membrane pores (Young *et al.*, 1986).

c. EDN. EDN, also called EPX (Slifman *et al.*, 1989), is a single-chain peptide with a molecular weight on gel electrophoresis of 18.6 kDa and a calculated molecular weight from the cDNA of 15.5 kDa. Like ECP, it is a member of a ribonuclease multigene family and has marked ribonuclease activity (Rosenburg *et al.*, 1989b). EDN expression is not restricted to eosinophils, as it is found in mononuclear cells and possibly neutrophils. It is probably secreted by the liver and the EDN sequence is also identical to that of human urinary RNase (Beintema *et al.*, 1988). It is only weakly toxic for parasites or mammalian cells and its only known function, other than its ribonuclease activity, is the neurotoxicity exhibited in the Gordon phenomenon (where rabbits become progressively ataxic, followed by severe weakness and muscle wasting after injection of eosinophil extracts). ECP is more potent in some species than EDN suggesting that ribonuclease activity is not the only factor involved in the pathogenesis. Further studies have shown that the ribonuclease activity is necessary but not sufficient for the Gordon phenomenon to occur (Sorrentino *et al.*, 1992). The antitumor protein, oncanase, another member of the RNase superfamily, will also cause the Gordon phenomenon (Newton *et al.*, 1994).

d. EPO. EPO is a heme-containing protein composed of an approximately 14-kDa (light) and a 58-kDa (heavy) subunit derived from the same strand of mRNA and subsequently cleaved. The cDNA encodes for a 381-leader sequence as well as the light and heavy chains (Ten *et al.*, 1989). EPO shares a 68% amino acid identity with human neutrophil myeloperoxidase and other peroxidase enzymes and is antigenically similar. The human EPO gene is located on chromosome 17, spans 12 kb, and consists of 12 exons and 11 introns (Sakamaki *et al.*, 1989). Three protected regions of the EPO promoter have been identified and indicate a unique transcriptional feature of eosinophil gene regulation (Yamaguchi *et al.*, 1994). There is approximately 15 pg of EPO per eosinophil (Carlson *et al.*, 1985). In the presence of H_2O_2 EPO is able to oxidize halides to form reactive hypohalous acids with bromide as the preferred halide. EPO is toxic for microorganisms, parasites, respiratory epithelium, and pneumocytes. Potency is increased 10,000-fold when combined with H_2O_2 and a halide (Weiss *et al.*, 1986; Mayeno *et al.*, 1989). However, it appears that thiocyanate, a pseudohalide, at physiological concentrations is able to compete for EPO and inhibit the effects of bromide and iodide even when the halide is present in marked excess (Slungaard *et al.*, 1991). The weak oxidant hypothiocyanous acid may therefore be the major product of EPO reactions *in vivo*. Thus, the studies demonstrating the cytotoxic effects of EPO through the H_2O_2/halide system may need revision.

3. *Cytokines*

As described elsewhere, the accumulation of eosinophils into the site of the inflammatory focus in allergic-type response is believed to be regulated by a complex series of events which involve T cells and cytokines (Kay, 1991; Corrigan and Kay, 1992). Cytokines, originally considered to be derived predominantly from lymphocytes and monocytes, are now known to be elaborated by many other cell types. Although blood and tissue eosinophils are predominantly "end cells" with a presumed limited capacity to transcribe and translate new proteins, it is now clear that eosinophils can synthesize and secrete several important inflammatory and regulatory cytokines.

a. Interleukin-1α. Del Pozo *et al.* (1990) showed that murine eosinophils expressed mRNA for IL-1α as detected by *in situ* hybridization. Messenger RNA in murine peritoneal eosinophils, stimulated for 6 hr with LPS, were hybridized with a ^{35}S-labeled cDNA probe for IL-1α. IL-1α was also detected in supernatants from these stimulated cells. The ability of human eosinophils to synthesize IL-1α was examined by Weller *et al.* (1993) in cells obtained from a hypereosinophilic patient. Eosinophils contained transcripts for IL-1α mRNA both before and after stimulation of cells with phorbol esters. The expression of IL-1α protein was also detectable by immunocytochemistry. Additionally, IL-1α release was shown to be associated with the induction of cytokine-induced HLA-DR expression suggesting that eosinophils may act as antigen-presenting cells.

b. Transforming Growth Factor-α and -β. TGF-α was detected in tissue eosinophils found in abundance in close proximity to the site of oral squamous carcinoma (Wong *et al.*, 1990). The latter type of tumor is known to be associated with increases in the level of tissue eosinophils. TGF-α mRNA and the protein were colocalized to eosinophils. The observation was confirmed in peripheral blood eosinophils from hypereosinophilic syndrome patients. It is now known that eosinophils both *in vivo* and *in vitro,* have a constitutive expression of mRNA for TGF-α. This observation was confirmed in eosinophil-associated wound healing in the rabbit skin tissue (Todd *et al.*, 1991). The majority of the eosinophils, particularly during the peak period of healing, expressed TGF-α mRNA as well as protein immunoreactivity. Epithelial wound healing coincided with TGF-α mRNA disappearance and was followed by the dispersal of eosinophils from the site. This ability of eosinophils to generate TGF-α was also observed in the Syrian hamster cheek pouch mucosa. Normal hamster bone marrow eosinophils were also shown to express TGF-α mRNA and the protein (Wong *et al.*, 1993).

TGF-β1 is also a product of eosinophils from patients with blood eosinophilia (Wong *et al.*, 1991). Eosinophils synthesize and elaborate TGF-β as shown by Northern blot analysis, *in situ* hybridization, and immunocytochemistry. Using a porcine TGF-β1 cDNA probe, Ohno *et al.* (1992) examined mucosal tissue from nasal polyps and detected TGF-β1-specific mRNA. TGF-β1 was also localized in nasal tissue from a patient with allergic rhinitis but not in normal nasal mucosa. By a combination of *in situ* hybridization and specific eosinophil staining (Carbol chromotrope 2R), approximately 50% of the eosinophils which accumulated in the polyp tissue were TGF-β1 mRNA$^+$. The presence of TGF-β1 in these cells was confirmed using immunocytochemical staining. TGF-β1 has also been localized to eosinophils in nodular sclerosis-associated Hodgkin's disease (Kadin *et al.*, 1993). In this study, TGF-β1 immunoreactivity was detected mainly at the margins of sites of new collagen synthesis in the vicinity of areas rich with Hodgkin/Reed/Sternberg (H/RS) cells. Probing for TGF-β1 revealed that the major sites for TGF-β1 mRNA expression were confined to eosinophils, but not H/RS cells. Eosinophil-derived TGF-β1, with its recognized role in chronic inflammation and fibrosis, may exert its effect in tissue repair by promoting fibroblast growth and activation. Thus, by exerting their effects on extracellular matrix, including stimulation of collagen synthesis, eosinophils may contribute toward structural abnormalities observed in inflammatory conditions and asthma, such as stromal fibrosis and basement membrane thickening. Hamster eosinophils were also shown to express TGF-α and -β in association with cutaneous wound healing (Wong *et al.*, 1993).

c. GM-CSF. Using *in situ* hybridization, normal density human eosinophils (obtained by metrizamide gradient separation) were shown to transcribe and translate mRNA for GM-CSF after stimulation with either IFN-γ or the calcium ionophore A23187 (Moqbel *et al.*, 1991). GM-CSF mRNA was colocalized to the stimulated eosinophil using a combination of *in situ* hybridization and histochemistry (Carbol chromotrope 2R), or immunocytochemistry (EG2) (Moqbel *et al.*, 1991). Additionally, a concurrent study demonstrated the release of eosinophil-derived GM-CSF following stimulation of cells with ionomycin (Kita *et al.*, 1991d) and presented elegant evidence for an autocrine effect of eosinophil-derived GM-CSF on prolongation of eosinophil survival. It was also shown that ionomycin-induced (GM-CSF-dependent) enhanced eosinophil survival was blocked by cyclosporin A. This is an important observation because the beneficial effect of cyclosporin A in chronic corticosteroid-dependent asthma (Alexander *et al.*, 1992) might be related to the direct effect of the drug on eosinophil cytokine synthesis.

GM-CSF has recently been shown to be stored in association with crystalloid granules of eosinophils obtained from asthmatic subjects (Levi-Schaffer *et al.*, 1995). Following ultracentrifugation of postnuclear supernatant of eosinophil subcellular components on a 0–40% Nycodenz gradient in sucrose buffer, GM-CSF coeluted with granule proteins and enzymes (i.e., major basic protein, eosinophil cationic protein, eosinophil peroxidase, aryl sulfatase, and β-hexosaminidase), but not in fractions associated with CD9 activity (a marker for eosinophil plasma membrane). The presence of stored GM-CSF which appears to be residing in association with granules in eosinophils obtained from asthmatic subjects has important implications in activation of these cells in this disease condition whereby eosinophils may utilize endogenous GM-CSF for more effective effector function.

GM-CSF mRNA has also been detected in eosinophils, *in vivo*, in association with nasal polyposis (Ohno *et al.*, 1991), and immunoreactive GM-CSF was identified in 24-hr culture supernatant of nasal polyp tissue using a specific ELISA. By *in situ* hybridization and counterstaining with chromotrope 2R, approximately 30% of eosinophils infiltrating the polyp tissue expressed mRNA for GM-CSF. The presence of GM-CSF mRNA expression was confirmed in BAL eosinophils obtained from asthmatic subjects after endobronchial allergen challenge (Broide *et al.*, 1992). It was suggested that the expression of GM-CSF by eosinophils at allergic inflammatory foci may provide an autocrine pathway to maintain the viability and effector function of these cells.

d. Interleukin-2. IL-2 is an essential growth factor for T cells. As stated previously human eosinophils respond to IL-2 in chemotaxis and a subpopulation of eosinophils express the IL-2 receptor (CD25). We recently showed that an average of 10% of freshly isolated unstimulated blood eosinophils showed cytoplasmic staining for IL-2. Using a cell fractionation method (Levi-Schaffer *et al.*, 1995) the majority of IL-2 was shown to coelute with eosinophil granule markers, but small peaks were also detected in the cytosolic and membrane fractions. Immunogold labeling of intact eosinophils using an anti-IL-2 monoclonal antibody revealed IL-2 immunoreactivity in association with eosinophil crystalline granule cores (Levi-Schaffer *et al.*, 1995). Thus, eosinophil granule-associated IL-2 may serve as a rapidly mobilizable source of IL-2 for T cell growth and activation.

e. Interleukin-3. Ionomycin-treated eosinophils produced IL-3 at concentrations equivalent to 50% of the capacity of mononuclear cells (Kita *et al.*, 1991d). Thus, IL-3 may be an additional autocrine factor that can be produced and utilized by the eosinophil following stimulation. Of interest, two immunosuppressants, FK-506 and rapamycin, inhibited calcium

ionophore A23187-induced production of IL-3 and GM-CSF from eosinophils (Hom and Estridge, 1993). Also, rapamycin, unlike FK-506, suppressed IL-5-induced prolongation of eosinophil survival. Such immunosuppressive agents may have an important modulating role in eosinophil-associated allergic reactions.

f. Interleukin-4. Initial studies using double *in situ* hybridization and immunocytochemical staining of bronchial biopsies and BAL cells from atopic asthmatic subjects indicated that very few $EG2^+$ cells coexpressed IL-4 mRNA (Ying *et al.*, 1995a,b). However, by RT-PCR, IL-4 mRNA was detected in highly purified blood eosinophils obtained from atopic asthmatics (Moqbel *et al.*, 1995). About 30% of these cells were immunoreactive with a monoclonal anti-IL-4. IL-4 was associated with the eosinophil granule as assessed by cell fractionation and lysed cells by repeated freeze-thawing contained an average of 75 pg/ml of IL-4 per 10^6 cells. The majority of eosinophils infiltrating 6-hr allergen-induced cutaneous late-phase reactions were immunoreactive with an anti-IL-4 antibody. Thus, a subpopulation of eosinophils from allergic subjects produces IL-4. This may be important in local IgE production and other IL-4-dependent events associated with allergic tissue reactions. It may, for example, be a factor in the expression of the TH2-type phenotype characteristic of atopic disease.

g. Interleukin-5. Eosinophils infiltrating mucosa of patients with active coeliac disease were shown to express mRNA for IL-5 (Desreumaux *et al.*, 1992) and IL-5 $mRNA^+$ cells were found in diseased, but not normal, tissue and were absent after treatment with a gluten-free diet. By *in situ* hybridization, mRNA was identified in peripheral blood eosinophils from the hypereosinophilic syndrome. IL-5 mRNA expression in eosinophils was also demonstrated, *in vivo,* in bronchoalveolar lavage cells obtained from asthmatic subjects (Broide *et al.*, 1992), where individual eosinophils were shown to exhibit four different cytokine mRNA profiles. Of the eosinophils counted, 34% were $IL\text{-}5^+/GM\text{-}CSF^+$, 34% were $IL\text{-}5^+/GM\text{-}CSF^-$, 11% were $IL\text{-}5^-/GM\text{-}CSF^+$, and only 21% were $IL\text{-}5^-/GM\text{-}CSF^-$, suggesting that eosinophil IL-5 and GM-CSF expression at sites of allergic inflammation in asthma may provide a combined autocrine pathway, thus maintaining the viability and effector function of the recruited eosinophils. IL-5 mRNA expression and immunoreactivity was also observed in eosinophils from the blood and tissue of patients with eosinophilic cystitis, the hypereosinophilic syndrome (Dubucquoi *et al.*, 1994), and eosinophilic heart disease (Desereumaux *et al.*, 1993). IL-5 was released following stimulation of IgA-, IgE-, or IgG-immune complexes and was colocalized to eosinophilic granules by immunogold staining (Dubucquoi *et al.*, 1994),

suggesting that some of these cytokines reside intracellularly in association with eosinophil granules. By simultaneous *in situ* hybridization and immunocytochemistry on nasal biopsy tissue from atopic rhinitic subjects following allergen exposure it was possible to colocalize IL-5 mRNA to MBP^+ eosinophils (Ying *et al.*, 1993). However, only 5.4% of the total leukocyte population were IL-5 $mRNA^+/MBP^+$ compared with 83.2% IL-5 $mRNA^+/CD3^+$ and 18% IL-5 $mRNA^+$/$tryptase^+$.

h. Interleukin-6. Using a similar approach to that of GM-CSF, IL-6 mRNA was also found in normal density eosinophils (Hamid *et al.*, 1992), and approximately 20% of unstimulated eosinophils were shown to be IL-6 $mRNA^+$ which was elevated (55%) following stimulation with IFN-γ. Transcription of IL-6 mRNA was confirmed by Northern blot analysis. Translation of the product was detected by immunocytochemistry in which staining was also "granular" and its release in the supernatant of cultured eosinophils before and after stimulation was measured (Hamid *et al.*, 1992). As with other cytokines, eosinophil IL-6 is stored in association with the crystalloid granules (Moqbel *et al.*, 1994a,b). The capacity of blood eosinophils obtained from both normal and hypereosinophilic donors to synthesize IL-6 and its constitutive expression have been confirmed using reverse-transcriptase polymerase chain reaction (RT-PCR) and *in situ* hybridization (Melani *et al.*, 1993). In this study, neutrophil granulocytes from healthy individuals were also shown to express variable levels of IL-6 although this was rapidly downregulated following *in vitro* culture of cells.

The precise significance and biological role of eosinophil-derived IL-6 is yet to be revealed. IL-6 plays an important role in the regulation of the immune response by modulating T and B lymphocyte functions as well as priming granulocytes and endothelial cells. Whether IL-6 from eosinophils in association with allergic or helminth-induced inflammation participates in the regulation and/or modulation of the site of reaction remains unclear.

i. Interleukin-8. Highly purified eosinophils cultured *in vitro* with calcium ionophore released IL-8 into the supernatant as shown by specific ELISA (Braun *et al.*, 1993). This was inhibited by both the immunosuppressant agent cyclosporin A and by cyclohexamide, an inhibitor of protein synthesis. IL-8 mRNA was demonstrated by PCR amplification following stimulation. IL-8 immunoreactivity was detected by immunocytochemical staining with granular appearance suggesting that the cytokine resides in the cell in a stored form (Braun *et al.*, 1993). While IL-8 appears to be a very abundant eosinophil cytokine its precise role in allergic inflammation remains unknown.

j. Tumor Necrosis Factor-α. By *in situ* hybridization, 44–100% of eosinophils obtained from the peripheral blood of normal subjects or patients with hypereosinophilia were positive for TNF-α mRNA (Costa *et al.*, 1993) and the stored protein was detected by immunocytochemical staining. Eosinophils purified from atopic individuals spontaneously released TNF-α, *in vitro,* which was inhibitable by cyclohexamide pretreatment. Infiltrating eosinophils in necrotizing enterocolitis were also shown to express TNF-α mRNA. These cells may play a role in pathogenesis of intestinal necrosis (Tan *et al.*, 1993). TNF-α immunogold-positive signals were shown to be localized to the matrix compartment of the specific secondary granules of eosinophils obtained from patients with the idiopathic hypereosinophilic syndrome (Beil *et al.*, 1993).

k. Macrophage Inflammatory Protein-1α. A high percentage of blood eosinophils (39–91%) obtained from hypereosinophilic patients showed positive mRNA expression for the chemokine MIP-1α (Costa *et al.*, 1993). In contrast, eosinophils obtained from normal donors exhibited weak or undetectable expression. *In vivo,* the majority of eosinophils infiltrating nasal polyp tissue had strong expression for MIP-1α mRNA; the expression was also confirmed by Northern blot analysis.

Thus, eosinophils synthesize, store, and release a wide array of cytokines as well as basic granule-derived proteins and lipid mediators. This functional versatility has a number of implications on the potential role of the eosinophil in allergic tissue reactions. Eosinophils might act to amplify the allergic response which, in asthma, for example, would enhance tissue damage but in helminth infections could promote adaptive immunity. On the other hand, eosinophil cytokine production may be a redundant process in the spectrum of immune and inflammatory systems. Conversely, as stated eosinophils may be involved in tissue repair and remodeling by, for example, promoting collagen synthesis through release of TGF-α and -β. It is not known whether eosinophil-derived cytokines act synergistically with other eosinophil mediators, i.e., basic proteins, LTC_4, and PAF. Eosinophils have also been shown to express MHC class II molecules, elaborate IL-1α and act as antigen-presenting cells (APCs). Eosinophils might function as specialized APCs in certain situations such as helminthic infections. Although the control and regulation of eosinophilia associated with asthma and allergic disease are not yet fully understood, they are likely to be dependent on IL-3, IL-5, and GM-CSF release from T cells, mast cells, and eosinophils themselves. The recent advances in eosinophil-derived cytokines suggest that the capacity of this cell to participate in effector functions during allergic-type reactions may be greater than hitherto assumed.

The relative contribution of eosinophil-derived cytokines to the development and maintenance of inflammatory reactions associated with allergic reactions, asthma, and allied disorders remains to be determined. Although the amounts of cytokines generated by these cells appear to be less than those produced by T lymphocytes, the synthesis of these cytokines and their storage and potential autocrine and paracrine usage may have particular pathophysiological relevance.

4. Other Eosinophil-Derived Mediators

A major constituent of the human eosinophil is CLC protein. This is a 17.4-kDa hydrophobic protein which was shown to be lysophospholipase (Weller *et al.*, 1980). It comprises approximately 10% of the total cellular protein of eosinophils and basophils (Ackerman *et al.*, 1982; Weller *et al.*, 1984). The protein forms characteristic bypyramidal crystals both intracellularly and in tissues infiltrated by activated eosinophils (Dvorak *et al.*, 1990). Cloning of the CLC protein revealed a cDNA encoding for a protein of 16.4 kDa with a single N-linked glycosylation site and homology to the S-type animal lectins such as Mac-2 (Ackerman *et al.*, 1993). The gene promoter has been characterized and shown to be functional after transfection. Consensus sequences that correspond to GATA and PUI have been identified (Gomolin *et al.*, 1993). Both eosinophils and basophils expressed approximately 900 bp mRNA for CLC protein. The gene for CLC protein is on chromosome 19q (Mastrianni *et al.*, 1992). The function of CLC protein is currently unclear.

In addition, the eosinophil contains a number of other granule-stored enzymes whose role in eosinophil function has not been defined (Fig. 9) (reviewed by Spry (1988). They include acid phosphatase (large amounts of which have been isolated from eosinophils), collagenase, arylsulfatase B, histaminase, phospholipase D, catalase, nonspecific esterases, vitamin B_{12}-binding proteins, and glycosaminoglycans. Eosinophils can undergo a respiratory burst with release of superoxide ion and H_2O_2 in response to stimulation with both particulate stimuli, such as opsonized zymosan, and soluble mediators, such as LTB_4 and phorbol myristate acetate. Eosinophils can generate twice as much chemoluminescence as neutrophils and the capacity of the eosinophil to generate reactive oxygen species is increased in cells from patients with allergic rhinitis (Shult *et al.*, 1985).

F. Eosinophil Activation

1. Introduction

The deleterious pathological changes associated with tissue eosinophilia appear to be directly linked to their state of activation. Eosinophils from normal individuals circulate in a resting state in which their effector func-

tions and response to inflammatory mediators is blunted. Exposure to eosinophil-active mediators, either *in vitro* or *in vivo* as they move into sites of inflammation, results in priming in which eosinophil effector function is enhanced. Further stimulation by inflammatory mediators or pertubation of certain membrane receptors results in degranulation of stored proteins and *de novo* synthesis and secretion of mediators allowing full expression of eosinophil effector functions such as cytotoxicity. We have used the term "activation" to describe this transition of the eosinophil from a resting through a primed to a secretory state.

2. *Morphology of Eosinophil Activation*

The activated eosinophil has a distinct phenotype in terms of both morphology and receptor expression. Many of the details of these changes have been discussed in previous sections. In particular, the ultrastructure of the activated eosinophil is discussed under Section I,B on morphology and ultrastructure, and changes in receptor expression with activation are detailed under Section I,D.

a. Change in Density. One of the most well-studied features of eosinophil activation has been alterations in density. Peripheral blood eosinophils from normal individuals are dense cells which seperate out from other leucocytes in the lower bands of Percoll or Metrizamide discontinuous density gradients. These differences in density have been used as a basis for purifying eosinophils (Vadas *et al.*, 1979; Gartner, 1980). A proportion of eosinophils from individuals with elevated eosinophil counts are of lower density than eosinophils from normal subjects (Bass *et al.*, 1980a). The mechanism for this heterogeneity in density is not clear. Hypodense eosinophils appear vacuolated and contain smaller-sized granules, although they are of equal numbers to those of normal density eosinophils (Caulfield *et al.*, 1990). The presence of low-density (or hypodense) eosinophils appears to be a nonspecific phenomenon which occurs in any eosinophilic condition including allergic disease (Fukuda *et al.*, 1985). It is believed that hypodense eosinophils are more activated since they have increased oxygen consumption (Winqvist *et al.*, 1982), increased cytotoxicity toward helminthic targets (Prin *et al.*, 1983), and release more LTC_4 after physiological stimulation (Shaw *et al.*, 1985). Activation of eosinophils *in vitro* with inflammatory mediators, such as PAF, as well as long-term culture with cytokines was also associated with a decrease in eosinophil density (Rothenberg *et al.*, 1987; Owen *et al.*, 1987a; Fukuda and Makino, 1989). In contrast, there was no difference in expression of $\beta 2$ integrins and Fcγ receptors between normal and hypodense eosinophils (Hartnell *et al.*, 1990). Normal density eosinophils from patients with an eosinophilia have

enhanced function compared with eosinophils from normal individuals. It is possible that the association between hypodensity and activation is coincidental with the less-dense cells being more immature.

Tissue eosinophils appear to be hypodense. For example, eosinophils obtained from BAL of asthmatic individuals are of low density (Prin *et al.*, 1986). When peripheral blood and BAL eosinophil phenotype and function were analyzed following segmental lung allergen challenge of atopic subjects, a greater percentage of BAL eosinophils were hypodense compared with peripheral blood (Kroegel *et al.*, 1994).

3. *Factors Which Induce Eosinophil Priming and Secretion*

A number of inflammatory mediators have been shown to be active on eosinophils causing enhancement of several eosinophil functions, particularly directional migration, adhesion, and cytotoxicity. Many of these are discussed throughout this chapter, particularly under Section I,D,1,d dealing with eosinophil chemoattractants (see Table II). The eosinophil-activating effects of cytokines are detailed below.

a. Eosinophils and Cytokines. There is a clear relationship between the development and effector function of eosinophils, basophils, mast cells, and IgE production, and cytokines produced by so-called type 2 helper T cells. The concept of a subdivision of $CD4^+$ helper T cells based on patterns of their cytokine profile was first proposed by Tim Mosmann and Robert Coffman in 1987. TH2 (type 2) subsets preferentially transcribe and translate messenger RNA for IL-4 and IL-5 and predominate in blood and tissues in atopic allergy and helminthic parasitic infections. Interleukin-4 coordinates IgE production by causing IgE switching in B cells, and IL-5 together with GM-CSF and IL-3 enhance eosinophil production through terminal differentiation of the committed eosinophil precursor. The counterpart of the type 2 subsets is the TH1 (type 1) subset. Through the elaboration of IFN-γ and IL-2 (but not IL-4 and IL-5), type 1 cells regulate classical delayed-type hypersensitivity reactions and other effector functions centered around macrophage activation and T cell-mediated immunity.

Although this T cell dichotomy is firmly established in mice, there was scepticism initially as to whether there was a human counterpart of type 1 and type 2 $CD4^+$ cells. However, it was found that allergen-specific T cell clones derived from the peripheral blood of atopic donors secreted a type 2 pattern of cytokines, whereas bacterial antigen-specific clones secreted a type 1-like pattern (Parronchi *et al.*, 1991). Furthermore, skin biopsy (Kay *et al.*, 1991) and nasal biopsy (Durham *et al.*, 1992) specimens and BAL cells obtained from atopic asthmatics (Robinson *et al.*, 1992)

contained an inflammatory infiltrate rich in cells expressing mRNA encoding predominantly IL-4 and IL-5, but not IL-2 or IFN-γ. Conversely, the cells infiltrating tuberculin reactions expressed IFN-γ and IL-2, but with little IL-4 and IL-5 (Tsicopoulos *et al.*, 1992).

As described previously eosinophil differentiation, activation, and secretion are to a large extent promoted by IL-5, IL-3, and GM-CSF. These events can be inhibited by IFN-α and IFN-γ. For example, IFN-α suppressed antigen-induced eosinophilia and $CD4^+$ T cell recruitment into airway tissue in mice (Nakajima, 1994). IFN-α has also been partially successful in treatment of the hypereosinophilic syndrome (Zielinski and Lawrence, 1990).

GM-CSF, IL-3, and IL-5 activate mature eosinophils and delay programmed cell death (apoptosis) in culture, thus prolonging survival of the cell *in vitro*. These effects can be reversed by corticosteroids (Wallen *et al.*, 1991). IL-5 is not only a terminal differentiation factor but enhances integrin-dependent adhesion of eosinophils to plasma-coated glass and human microvascular endothelial cells (Walsh *et al.*, 1990a,b). These effects were not observed with neutrophils. IL-5 also enhances a number of other effector functions of the eosinophil including the generation of superoxide radical and LTC_4, *in vitro* cytotoxicity against helminthic targets, and IgA- and IgG-dependent degranulation of eosinophils. Long-term culture (7 days) was associated with at least 50% release of total granule proteins into the culture supernatants. Eosinophil-active IL-5 production from human T cells was enhanced by LTB_4, but not by the stereoisomer 5s,12s-dihydroxy-6,8,10,14-ETE (Yamaoka and Kolb, 1993), suggesting a further link between TH2-type cytokines and allergic inflammation. Taken together, these observations suggest that IL-5, as well as IL-3 and GM-CSF, exerts important proinflammatory effects on the mature human eosinophil, thus influencing their adhesion to the microvasculature, recruitment and accumulation in the inflammatory site, prolonged survival, and subsequent activation of the cell with release of their mediators.

Recombinant human IL-5 (rhIL-5) was administered repeatedly onto the nasal mucosa of individuals with Japanese cedar pollinosis outside the pollen season (Terada *et al.*, 1992). The numbers of eosinophils and epithelial cells and the amounts of eosinophil cationic protein, secretory IgA (S-IgA), and IgA in the nasal lavage fluid increased significantly after the application of rhIL-5. Responsiveness to histamine was also enhanced after the application. When S-IgA was administered onto the nasal mucosa after application of rhIL-5, the amount of ECP in the nasal lavage fluid was also significantly increased. The authors suggested that IL-5 enhanced the release of ECP from eosinophils activated by S-IgA and/or IgA and that

epithelial damage to the nasal mucosa preceded the development of nasal hyperreactivity to histamine.

IFN-γ has also been shown to enhance eosinophil cytotoxicity in long-term cultures (Valerius *et al.*, 1990). Stimulation over 24 hr resulted in maximal effect and was greater than that observed after incubation with GM-CSF, IL-3, and IL-5. Incubation of eosinophils with IFN-γ for 1 or 2 days also induced the expression of FcγRIII (CD16), a receptor not expressed on normal or activated eosinophils, *in vivo* (Hartnell *et al.*, 1992a). Short-term (1-hr) incubation with IFN-γ, however, inhibited the release of H_2O_2 following organized zymosan stimulation and decreased the numbers of eosinophils adhering to complement-coated erythrocytes. In addition, secretory IgA-dependent eosinophil degranulation was inhibited by IFN-γ after 3 hr incubation (Fujisawa *et al.*, 1990). TNF-α and IL-1 have also been shown to upregulate a number of eosinophil functions.

TNF-α has the capacity to prolong eosinophil survival *in vitro*, enhance LTC_4 synthesis, and increases *in vitro* helminthotoxicity against *S. mansoni* larvae and endothelial cells (Silberstein and David, 1986). IL-1 has also been shown to influence, both positively and negatively, the release of superoxide or granule enzymes from eosinophils, but this was shown to be dependent on the concentration and choice of the stimuli used (Pincus *et al.*, 1986). A summary of the effects of cytokines on eosinophils is shown in Table V.

4. Eosinophil Mediator Release and Cytotoxicity

a. Lipid and Cytokine Mediator Release. The ability of activated eosinophils to produce increased amounts of LTC_4 and PAF is discussed under Section I,E as is the secretion of cytokines by eosinophils.

b. Granule Protein Release. Substantive secretion of eosinophil granule proteins is observed following interaction of the cell with large opsonized targets such as a metazoan parasite or Sepharose beads. *In vitro* secretion can be triggered physiologically via IgG and IgA Fc receptors (Shaw *et al.*, 1985; Abu-Ghazaleh *et al.*, 1989). Glucocorticosteroids do not suppress immunoglobulin-induced eosinophil degranulation. However, cAMP analogues, phosphodiesterase inhibitors, and β-adrenergic agonists inhibit these processes (Kita *et al.*, 1991a). Furthermore, pretreatment of eosinophils with pertussis toxin abrogates sIgA-induced degranulation and increases in phospholipase C activity. However, pertussis toxin treatment only transiently inhibited eosinophil activation via immobilized IgG (Kita *et al.*, 1991c). These experiments indicate that eosinophils contain several pertussis-sensitive G proteins.

TABLE V
EFFECTS OF CYTOKINES ON THE EFFECTOR FUNCTION OF MATURE HUMAN EOSINOPHILS

Effect	Cytokine	Reference
Prolonging survival and viability	IL-3, IL-5, GM-CSF	Owen *et al.* (1987), Yamaguchi *et al.* (1988), Rothenberg *et al.* (1989), Tai *et al.* (1991), Stern *et al.* (1992)
Inducing expression of IL-2 receptors (CD25)	IL-3, GM-CSF	Riedel *et al.* (1990)
Inducing CD69 expression	IL-5, GM-CSF	Hartnell *et al.* (1993)
Inducing expression of CD16	IFN-γ	Hartnell *et al.* (1992)
Promoting EDN release	GM-CSF	Fujisawa *et al.* (1990)
Promoting and enhancing EDN release by sIgA- and IgG-dependent mechanisms	IL-3, IL-5, GM-CSF	Fujisawa *et al.* (1990)
Enhancing C3b-induced ECP/EDN release	IL-3, GM-CSF	Tai and Spry (1990)
Increasing ADCC cytotoxicity to Daudi lymphoma cells	IL-3, IL-5, IFN-γ	Valerius *et al.* (1990)
Enhancing helminthotoxicity *in vitro*	IL-5, GM-CSF, TNF-α	Rothenberg *et al.* (1989), Owen *et al.* (1987), Silberstein and David (1986)
Promoting cytotoxicity toward vascular endothelial cells	TNF-α	Slungaard *et al.* (1990)
Increasing cytotoxicity to opsonized yeast	GM-CSF	Lopez *et al.* (1988)
Promoting hypodense phenotype	IL-5, GM-CSF	Owen *et al.* (1987), Rothenberg *et al.* (1989)
Upregulating hyperadherence to HUVEC	IL-5	Walsh *et al.* (1990b)
Increasing binding of IL-5	GM-CSF	Chihara *et al.* (1990)
Synergizing with IL-5 in inducing ICAM-1 expression	TNF-α	Hansel *et al.* (1992)
Upregulating LTC_4 production	GM-CSF, TNF-α	Silberstein *et al.* (1986), Owen *et al.* (1987), Roubin *et al.* (1987), Howell *et al.* (1990)
Stimulating oxidative metabolism of eosinophils following adherence	TNF-α, IFN-γ	Slungaard *et al.* (1990), Dri *et al.* (1991)
Priming for PMA-induced respiratory burst	IL-5	Tagari *et al.* (1993)
Inducing GM-CSF mRNA transcription and translation	IFN-γ	Moqbel *et al.* (1991)
Inducing IL-6 mRNA transcription and translation	IFN-γ	Hamid *et al.* (1992)
Causes eosinophil apoptosis	TGF-β	Alan *et al.* 1994

As discussed previously, IgE-dependent secretion has also been described (Capron and Capron, 1987), but this and the role of other receptors triggering eosinophil secretion, particularly the CR3 and CR1 receptors, is not fully understood.

Compared with neutrophils, eosinophils stimulated by receptor-ligand independent mechanisms, such as phorbol myristate acetate or calcium ionophore (Fukuda *et al.*, 1985), release negligible amounts of their stored mediators. Similarly, PAF, a potent chemoattractant for eosinophils, induces marginal release of granule proteins (Kroegel *et al.*, 1989a). On the other hand, cytokines, particularly GM-CSF and IL-5 (Kita *et al.*, 1992), induced substantial eosinophil degranulation even in the absence of any particulate stimulus.

Eosinophils exposed to immunoglobulin- or complement-coated surfaces also released large amounts of granule proteins. For example, secretory IgA, IgG, or C3b induced the release of 15–25% of the contents of normal human eosinophils (Winqvist *et al.*, 1984; Capron *et al.*, 1988; Abu-Ghazeleh *et al.*, 1992). Furthermore, eosinophils from patients with an eosinophilia released significantly higher concentrations of their granule proteins (Carlson *et al.*, 1991, 1992; Venge, 1993) than normal cells. Priming by cytokines (e.g., IL-4) prior to exposure to a particulate stimulus enhanced the capacity of normal eosinophils to release granule proteins (Fujisawa *et al.*, 1990). Eosinophil degranulation appeared to be dependent on cell–cell contact with a critical role for $\beta 2$ integrins. For example, degranulation induced by GM-CSF or PAF was inhibited by antibodies to CD11b/CD18 (Mac-1) (Horie and Kita, 1994).

Suomalainen *et al.* (1994) presented evidence that eosinophil activation, *in vivo*, was a feature of cow's milk allergy in children. Interestingly, the level of serum ECP was significantly elevated after oral cow's milk challenge in patients with skin, but not gastrointestinal manifestations. Elevated levels of serum ECP and MPO were also detected in patients with cystic fibrosis when compared with normal nonatopic controls (Koller *et al.*, 1994).

As mentioned previously, eosinophil granule proteins may be compartmentalized to different granule populations. This is based on the observation of specific degranulation of some, but not all, of the granule proteins following stimulation with various agonists. Thus, IgE-dependent stimulation was reported to induce the release of MBP and EPO, but not ECP, while IgG-coated surfaces induced a selective release of ECP, but not EPO (Capron *et al.*, 1989; Tomassini *et al.*, 1991). IgA, on the other hand, when bound to Sepharose beads triggered the nonselective release of all granule proteins. The precise mechanisms controlling the selective release of granule proteins in response to various ligands remain unexplained.

In vivo, the most striking feature of an eosinophil-rich inflammatory reaction is the marked deposition of large amounts of eosinophil granule proteins with fully intact eosinophils being relatively small in number. Many laboratories have reported the nonselective elaboration of eosinophil-associated granule proteins in peripheral blood and body fluids (which to some extent conflicts with the concept of selective *in vitro* release). In many allergic or eosinophil-associated inflammatory diseases blood eosinophil counts correlated with concentrations of circulating eosinophil granule proteins (reviewed by Venge, 1993). Some of these granule proteins (particularly MBP and EPO) may adhere to other serum or fluid proteins and so reduce the sensitivity of the assay. Nevertheless, there is ample evidence for *in vivo* activation of eosinophils in diseases associated with eosinophilia.

Cromwell *et al.* (1991) studied G protein regulation of eosinophil exocytosis using systems which bypass the initial steps involving surface receptors and their associated G proteins. These involved permeabilization methods, e.g., bacterial cytolysin, streptolysin-O (SL-O), and patch clamping in the whole cell configuration. In cells permeabilized with SL-O, secretion was dependent on the presence of both Ca^{2+} and guanine nucleotide (Cromwell *et al.*, 1991). Secretion of *N*-acetyl-β-D-glucosaminidase correlated well with the extent of degranulation as assessed morphologically. There is no absolute requirement for ATP, but in its presence the affinities for both Ca^{2+} and GTP-γ-S were enhanced by a protein kinase-dependent mechanism. In these respects, the mechanism closely resembles that of other myeloid cell types, although there were differences, mainly relating to the extended time over which exocytosis occurs. For example, patch-clamp experiments indicated that exocytosis involved fusion of discrete granules with the plasma membrane in two temporally dissociated phases (Nüsse *et al.*, 1990). The first phase involves small granules or vesicles and was at least partly Ca^{2+} dependent. The second phase reflected sequential fusion of all the crystalloid granules as individual units with the plasma membrane and shows an absolute requirement for GTP-γ-S. Membrane reuptake then occurs in the form of small vesicles or coated pits.

Other mechanisms of eosinophil degranulation include incubation with monoclonal antibodies to human eosinophil plasma membrane antigens, calcium ionophore, or estradiol (Tchernitchin *et al.*, 1985)—causing eosinophil degranulation through an estrogen receptor.

5. *Signal Transduction*

Studies on human, guinea pig, and murine eosinophils have indicated that, following agonist stimulation, a biochemical cascade is initiated which leads to the regulation and control of granule- and membrane-derived mediator release. The interaction of agonists with a cell surface receptor

induces the activation of phospholipase C enzyme (PLC) through the action of a Pertussis toxin-sensitive G protein. Among various agonists, PAF and LTB_4 have been shown to activate PLC directly (Kroegel *et al.*, 1989, 1991). PLC catalyzes the hydrolysis of phosphatidyl inositol-4,5-bisphosphate (PtdIns-4,5-P_2) resulting in two second messenger molecules, inositol 1,4,5-trisphosphate (IP_3), and diacylglycerol (DAG). IP3 interacts with specific receptors found on intracellular stores for Ca^{2+} within the endoplasmic reticulum, which liberates Ca^{2+} through specific putative release channels. This event may be associated with a distinct, but as yet ill-defined, G protein. DAG, on the other hand, activates protein kinase C (Rabe *et al.*, 1992). For eosinophil exocytosis to occur, uptake of extracellular Ca^{2+} by the cell is supplemented by the intracellular release of Ca^{2+} via IP3. It is suggested that this pathway may require the activation of an as yet undefined eosinophil Ca^{2+}-binding protein, together with a putative, membrane-associated, eosinophil protein (G_E) (reviewed by Giembycz and Barnes, 1993). Recent studies have also implicated both phospholipase A2 (PLA2) and arachidonic acid in eosinophil activation (Aebischer *et al.*, 1993). The precise details of the pathways involved in eosinophil signal transduction leading to exocytosis are still not fully understood and much of the data available are based on studies using guinea pig cells, in which large numbers of highly purified eosinophils needed for such analytical studies can be obtained.

The role of tyrosine kinases in IL-5-mediated eosinophil signal transduction has been studied by Alam *et al.* (1995). As previously discussed the receptor for IL-5 consists of α and β subunits. Neither subunit has intrinsic enzymatic activity. Many tyrosine kinases have been shown to associate with cytokine receptors. Lyn, a member of the src tyrosine kinase family, has been detected in cells of myeloid origin. Alam *et al.* (1994) detected two species of lyn kinase (56 and 53 kDa) in eosinophils. Both of them were phosphorylated and activated within 1 min following stimulation of eosinophils with IL-5. Lyn kinase was physically associated with IL-5 β receptor as determined by coprecipitation studies.

Tyrosine kinases activate many downstream signaling molecules including a group of proteins that exchange GDP for GTP on ras. The latter exists in inactive GDP-bound and active GTP-bound forms. Alam *et al.* (1994) also studied the effect of IL-5 on the GTP-binding of ras using two different techniques. IL-5 stimulated the binding of GTP to p21 ras within 1–3 min by the GTP-overlay technique. The ras-bound GTP and GDP were also analyzed by thin-layer chromatography which confirmed the increased binding of GTP. The GTP-bound ras causes translocation of raf-1 to the membrane and its activation. Raf-1 then phosphorylates MEK kinase which subsequently activates MAP kinases. Raf-1 and MEK were

activated within 3 min after IL-5 stimulation. Both 45- and 41-kDa MW species of MEK were present and activated in eosinophils. The activity of MAP kinase in eosinophils peaked at 20 min. The optimal concentration of IL-5 for stimulation of kinases was 10^{-11} to 10^{-10} *M*, which is in accord with other biologic activities of this cytokine on eosinophils.

In another set of studies (Alam *et al.*, 1995) Jak2 was found to be physically associated with the IL-5 β receptor and was tyrosine phosphorylated within 3 min after IL-5 stimulation. Activated Jak2 underwent autophosphorylation *in situ*. Jak2 is known to phosphorylate nuclear factors of the Stat family. Electrophoretic mobility-shift assay was performed with a IFN-γ activation site (GAS) probe from the promoter region of the Ly6 gene. IL-5 induced two GAS-binding proteins in the nuclear extract from eosinophils. One of them was identified as Stat1 (p91) as evidenced by supershifting of the band in the presence of an anti-Stat1 antibody.

Thus, two major signaling pathways in eosinophils were identified. The final products of these two pathways include MAP kinases and Stat1. MAP kinases activate many cytosolic proteins including PLA2, PLC, rsk, and cytoskeleton proteins. Further, MAP kinases migrate into the nucleus and phosphorylate nuclear factors such as NFIL6, c-*jun*, c-*myc*, elk-1, TCF, and others. The activation of raf-1 leads to the generation of NFκB. PLA2 is involved in the metabolism of arachidonic acid and generation of prostaglandins and leukotrienes. PLC facilitates the production of inositol triphosphates and diacylglycerol. Thus, nuclear factors may be involved in the transcription of genes that are responsible for prolongation of survival and priming of this cell type.

II. Eosinophils and Parasites

A. Effector Role of the Eosinophil in Parasitic Helminthiases

As in allergic inflammation, the precise role of eosinophils in the immunopathological changes associated with helminthic infection remains incompletely understood and rather controversial. Increases in the numbers of tissue and peripheral eosinophils together with elevations in the levels of total and parasite-specific IgE and mastocytosis are considered hallmarks of infection with parasitic worms, especially during their tissue migratory phases. However, it is still unclear whether their presence may be a reflection of the pathology of disease or an indication of a protective immune response against the particular parasitic worm infection (Kay *et al.*, 1985; Finkleman *et al.*, 1991). Basten and Beeson (1970) originally observed that helminth-associated eosinophilia was T cell-dependent. The precise factors involved in this response are now understood more clearly due to advances in the molecular and immunobiology of cytokines (Nathan and Sporn,

1991). The identification and subsequent cloning of GM-CSF, IL-3, and particularly IL-5 has provided a rational explanation of T cell control of the eosinophilic response in terms of both eosinophilopoiesis and differentiation, as well as priming and activation of the mature cell. The question, however, remains as to why there is a selective increase in eosinophils and what is their function, both locally and systematically, in the infected host.

1. In Vitro Evidence for Antihelminthic Effector Function

Initial *in vitro* experiments using the ADCC reaction demonstrated that human peripheral blood granulocytes (particularly eosinophils) from patients infected with *S. mansoni* were able to adhere to and kill larvae of this trematode (Butterworth *et al.*, 1974, 1975, 1977; Vadas *et al.*, 1979, 1980b; Butterworth and Richardson, 1985; Butterworth and Thorne, 1993). These observations were confirmed using rat and human eosinophils against schistosome larvae (McLaren *et al.*, 1977, 1978, 1981, 1984; Ramalho-Pinto *et al.*, 1978). Similar antibody-dependent, eosinophil-mediated *in vitro* cytotoxicity was reported for a number of other helminthic parasites. The targets used in these systems included the eggs of *S. mansoni* using murine eosinophils (James and Colley, 1976, 1978; Hsu *et al.*, 1980; Feldman *et al.*, 1990) and invading larvae of *Fasciola hepatica* (Duffus and Franks, 1980) and *Mesocestoides corti* and (Cook *et al.*, 1988). Newborn larvae of *Trichinella spiralis*, nematode larvae that although less susceptible because of their thick collagen-rich cuticle, were also killed following adherence to eosinophils (Kazura and Aikawa, 1980). These studies were extended by others to demonstrate eosinophil cytotoxicity *in vitro* against microfilariae of *Onchocerca volvulus* (Greene *et al.*, 1981; Mackenzie *et al.*, 1981; Williams *et al.*, 1987) and larval stages of *Dictyocaulus viviparus* (Butterworth and Thorne, 1993), *Toxocara canis* (Fattah *et al.*, 1986; Badley *et al.*, 1987; Lombardi *et al.*, 1990), *Brugia malayi* (Sim *et al.*, 1982; Chandrashekar *et al.*, 1986), and *Necator americanus* (Desakorn *et al.*, 1987).

Eosinophils readily and firmly adhere to opsonized worms or larvae and release their granule content which can be detected by electron microscopy as thick layers of electron-dense deposits on the surface of the organism (McLaren *et al.*, 1977, 1978, 1981; Glauert *et al.*, 1978; Caulfield *et al.*, 1980a,b). Such deposition results in damage to the parasite in the vicinity of contact with the attached eosinophil. Damage was associated with the appearance of vacuoles in the synctial tegument of the larvae and was followed by detachment of the tegumental membrane leading to the exposure of the underlying muscle layers. It is suggested that tegumental membrane detachment may be mediated in part by worm-derived lysophospholipids (Golan *et al.*, 1986; Furlong and Caulfield, 1989; Caulfield and

Chiang, 1990). These morphological changes are believed to be mediated by released eosinophil granule cationic proteins including EPO, MBP, ECP, and EDN. As already mentioned these products have the capacity to damage schistosomula directly when incubated as isolated proteins with parasitic larvae at very low molar concentrations. MBP and ECP both produced ballooning in the tegument in a similar pattern to that observed with whole eosinophils (Butterworth *et al.*, 1979a,b; McLaren *et al.*, 1984). ECP was 10 times more active on a molar basis than MBP. However, because MBP is present in the granule in larger amounts, it may account for higher proportion of the toxicity observed (Ackerman *et al.*, 1985). In contrast, EPO and EDN are relatively inactive on their own in causing direct damage to the parasite (Pincus *et al.*, 1981; Ackerman *et al.*, 1985).

That eosinophil cationic proteins induce damage to schistosomula of *S. mansoni* through their basic charge was confirmed by use of a variety of synthetic polycations which all produced similar patterns of damage (Butterworth *et al.*, 1979b; Jones *et al.*, 1988) and by its inhibition by polyanions such as heparin (Young *et al.*, 1986). Purified eosinophil cationic proteins were also toxic *in vitro* for the newborn larvae of *T. spiralis* (Wassom and Gleich, 1979; Hamann *et al.*, 1987), the eggs of *S. mansoni* (Sher *et al.*, 1980; Kephart *et al.*, 1988), and larvae of both *B. malayi* and *B. pahangi* (Hamann *et al.*, 1990). On a molar basis, MBP, ECP, and EPO were approximately equipotent in terms of their cytotoxic effect. EPO action was enhanced in the presence of hydrogen peroxide and a halide. The effects of these polycations were shown to be inhibited by polyanions such as heparin (Gleich *et al.*, 1980; Venge *et al.*, 1983). These cytotoxic effects may, therefore, be due primarily to the intensely basic nature of these proteins rather than to other properties such as the ribonucleus activity of ECP (Barker *et al.*, 1989). Although eosinophils can release superoxide radicals in response to stimulation, Pincus *et al.* (1981) demonstrated that damage to schistosomula by normal eosinophils can occur under strictly nonaerobic conditions, suggesting that such oxidative metabolism is not necessary for *in vitro* killing. However, oxygen may be required for degranulation (Baskar and Pincus, 1988). Eosinoplasts (granule-containing eosinophils devoid of a nucleus) generated oxygen metabolites and synergized with ECP in helminth toxicity (Yazdanbakhsh *et al.*, 1987). Oxidative mechanisms also appear to be essential in killing of newborn larvae of *T. spiralis* by eosinophils (Bass and Szejada, 1979; Buys *et al.*, 1981, 1984), while a reduction in oxygen tension limited the capacity of intact eosinophilic granulomas or isolated granuloma cells to kill eggs of *S. mansoni in vitro* (Feldman *et al.*, 1990).

Adherence of eosinophils to both live and fixed *Schistosoma* larvae induced *de novo* synthesis and release of lipid mediators including LTC_4

(Moqbel *et al.*, 1983, 1985, 1986, 1990a,b; Tamura *et al.*, 1988). The precise role of this mediator in parasite damage is not yet known.

As already discussed the ligands that mediate killing by both normal and activated eosinophils include immunoglobulins, particularly IgG, IgA, and IgE. In addition, complement components C3b and C3bi were shown to affect adherence and killing in the absence of any immunoglobulin and this may be achieved through eosinophil adherence by CR1 and CR3, respectively (Anwar *et al.*, 1979; Moqbel *et al.*, 1983, 1985; Fischer *et al.*, 1986). Antibody-dependent killing may also be enhanced by LFA-1-associated mechanisms. Monoclonal antibodies against the α-chain of this β1 integrin partially blocked the killing of *S. mansoni* larvae (Capron *et al.*, 1987).

That eosinophils utilize the receptor for IgA (Abu-Ghazaleh *et al.*, 1989) to induce eosinophil degranulation (following incubation with either anti-IgA or IgA-coated Sepharose beads, particularly sIgA) (Fujisawa *et al.*, 1990) suggests that this ligand is an important receptor for mediator release. However, the involvement of sIgA in eosinophil-mediated cytotoxic response against parasitic helminthic targets is not yet established.

2. *In Vivo Studies*

The precise regulatory and functional roles of eosinophils in the progression of human helminthiases are unknown. Information in man is largely limited to measurements of blood and tissue eosinophilia and IgE during the migration of helminth(s) in various tissue sites (Wardlaw and Moqbel, 1992). There is some evidence of direct contact between eosinophils and adult worms during natural infections. Eosinophil-rich granulomas surrounding dead fragments of skin invading larvae of *Strongyloides ratti* were found in hyperimmune rats after challenge with infective larvae (Moqbel, 1980). Eosinophils were also found in close contact with the surface tegument of schistosomula of *S. haematobium* in the cutaneous tissue of immune monkeys. This was associated with the presence of large numbers of dead larvae in eosinophil-rich sites (Hsu *et al.*, 1974). Similar observations were made in other host/parasite systems (Butterworth and Thorne, 1993; Gleich and Adolphson, 1986). Using appropriate antibodies, eosinophil-derived toxic proteins were identified on worm targets *in vivo*. Immunofluorescent staining for MBP revealed the deposition of eosinophil-derived product onto the surface of microfilariae of *Onchocerca volvulus* in skin biopsies of patients with onchocerciasis following treatment with diethylcarbamazine (Kephart *et al.*, 1984). The presence of an eosinophilic infiltrate in association with human onchocerciasis was shown to be correlated with microfilarial production from pregnant female adult worms but not with the host's immune status (Wildenburg *et al.*, 1995). Adult *O.*

volvulus worms elicited tissue eosinophilia only if microfilarae appeared in the surrounding tissue. The levels of blood ECP were elevated in patients with filariasis suggesting the activation and degranulation of eosinophils (Spry, 1981). The rate of reinfection in African children with *S. haematobium* indicated that both IgE and eosinophils appear to influence resistance in that age group (Hagan *et al.*, 1991; Woolhouse *et al.*, 1991). Thus, much of our existing knowledge about the possible *in vivo* role of eosinophils in helminth-induced inflammation arises from studies in laboratory animals. Little is known about the sequence of events that control eosinophil-mediated responses in man.

While rat and human eosinophils have been shown to possess IgE-dependent antiparasitic effector functions both *in vivo* and *in vitro*, BAL eosinophils from lungs of mice infected with *T. canis* were shown to be devoid of receptor expression for sIgM, sIgA, and FcεRII, but were positive for sIgG1 and FcγRII (Jones *et al.*, 1994). This suggests that there is a heterogeneity in the profile of eosinophils in different host species which raises doubts concerning the role of eosinophils in helminthic disease. It has been suggested that the presence of a TH2-type response (i.e., IgE-dependent mechanisms with its associated eosinophilia) to helminthic infection may either contribute to host protection or lead to the parasite's prolonged survival. This appears to be particularly true in mouse models of parasitic helminthiases in which the presence of IFN-γ and IgG indicates they have a more prominent protective role than IL-4 and IL-5.

Studies on the release of allergic mediators from eosinophils have provided further supportive evidence for a possible eosinophil effector function of eosinophils against helminthic parasites. Immune rats undergoing systemic anaphylaxis following intravenous challenge with *N. brasiliensis* antigens released high levels of LTC_4, in both the gut mucosa and blood (Moqbel *et al.*, 1986). Similar LTC_4 release was observed in rats undergoing rapid expulsion of *T. spiralis;* a process akin to immediate-type hypersensitivity and associated with elevated numbers of eosinophils in the gut mucosa (Moqbel *et al.*, 1987b). PAF has also been shown to be generated from gut tissue during the course of a primary infection with *N. brasiliensis* when large infiltrates of eosinophils are present in the lamina propria (Moqbel *et al.*, 1989). Not only can PAF be released from the prominent eosinophil infiltrate in the gut tissue but it can also play a regulatory role as a potent chemoattractant and activator of these cells.

3. *Eosinophils, Cytokines, and Helminths*

As detailed previously, several cytokines play an important role in eosinophil pathophysiology. Many enhance the *in vitro* effector function (cytotoxicity) of human eosinophils against helminthic targets, especially IL-5, IL-

3, TNF-α, and GM-CSF (Silberstein and David, 1987). IL-5, IL-3, and GM-CSF prolong eosinophil survival in culture and enhance mediator release following appropriate stimulation. Selective release of IL-5 from cultured mononuclear cells of patients with eosinophilia was shown to be associated with filarial infection (Limaye *et al.*, 1990). This observation has provided further support to the hypothesis that IL-5 is a particularly critical component of the regulatory mechanisms involved in the induction of eosinophilia in helminth-associated inflammatory reactions. What these cells do once their numbers are increased both systemically and locally remains unclear. When mice infected with either *N. brasiliensis* or *S. mansoni* were treated with a neutralizing anti-IL-5 monoclonal antibody, eosinophilia was abolished without influencing the protective responses against the parasite (Coffman *et al.*, 1989; Sher *et al.*, 1990a,b). Similarly, Herndon and Kayes (1992) observed that depletion of eosinophils with a monoclonal anti-IL-5 antibody in mice infected with *T. spiralis* did not affect parasite burden or immunological resistance to infection. Although these observations raise considerable doubts on the putative antiparasite role of the eosinophil, it should be emphasized that the development of the protective response of the host (man or laboratory animal) to helminths involves a complex cascade of events with the eosinophil as one participant. Furthermore, eosinophils and their cytotoxic products may cause parasite damage and/or death directly or indirectly through the creation of an unfavorable local environment for enteral, and possibly parenteral, parasites. This in turn may result in protection of the host and the elimination of the worm burden. This latter function may be an element of the overall multifactorial inflammatory strategy in favor of the host immune system. However, parasites have ingenious adaptive mechanisms which can evade or manipulate the immune and inflammatory reactions of the host to favor their own survival.

4. Conclusions

The ability of the eosinophil to release large amounts of potent mediators with the potential to damage and/or kill helminthic parasites, *in vitro*, mimics many of the pathological changes characteristic of asthma and allergic disease. That eosinophils are important in host defense against parasites and that allergic disease is the price we have to pay for this protection remains an attractive hypothesis (Kay, 1976), although it is not compatible with recent studies in which parasite-induced eosinophilia was abolished without altering the host's protective immune process (and in some cases improving host defense). Therefore, the central question regarding the precise role of these inflammatory cells and whether they are causally linked with allergic disease remains open. The problem can only

be resolved by the introduction of more precise methods for specifically inhibiting eosinophil accumulation and function at sites of helminth- and allergen-induced inflammatory reactions.

III. Eosinophils and Disease

A. Eosinophilia

An isolated count of eosinophil numbers in the blood offers only a limited and sometimes misleading picture of eosinophil involvement in a particular disease. The blood eosinophil count represents the balance between the rate of eosinophil migration from the bone marrow and entry into the tissues. Once in the tissue eosinophils can survive for many days under the influence of locally generated cytokines. Eosinophils can be enumerated in the peripheral blood by "wet counts" in modified Neubauer chambers, differential counts on dried smears, or by automated cell counting. The automated counting which uses detection of eosinophil peroxidase is the most accurate method followed by counting in a cell chamber. It is preferable to record the eosinophil count in absolute numbers rather than as a percentage as the latter will depend on the total cell count. The normal eosinophil count is (generally taken as) less than 0.4×10^9/liter although a study of 765 medical students in the United States measured counts ranging from 0.015 to 0.65×10^9/liter (Krause and Boggs, 1987). It is higher in neonates (Matheson *et al.*, 1957). The eosinophil count varies with age, time of day, exercise, and environmental stimuli, particularly allergen exposure. Blood eosinophil counts undergo diurnal variation which is lowest in the morning and highest at night. This effect resulted in a greater than 40% variation in one study (Winkel *et al.*, 1981). This may be related to the reciprocal diurnal variation in cortisol levels which is highest in the morning.

The most common cause of an eosinophilia worldwide is infection with helminthic parasites which can often result in a very high count. The most common causes of an eosinophilia in industrialized countries are the atopic allergic diseases, seasonal and perennial rhinitis, atopic dermatitis, and asthma. Allergic disease generally results in only a mild increase in eosinophil counts. A moderate or high eosinophil count in asthma raises the possibility of a complication such as Churg–Strauss syndrome or allergic bronchopulmonary aspergillosis (ABPA). Apart from allergic disease and helminthic parasites, a raised eosinophil count, especially a moderate or high count, is unusual.

B. The Role of Eosinophils

Views on the role of eosinophils in health and disease have changed with time. For many years eosinophils were thought to ameliorate inflammatory

responses, now they are believed to have a tissue-damaging role (Weller and Goetzl, 1979; Gleich, 1990). Both views may be correct. As discussed previously, it has become apparent that eosinophils are the source of a range of cytokines, several of which are thought to have a homeostatic, rather than proinflammatory function. Nonetheless, there is little doubt that eosinophils can cause severe tissue damage in certain circumstances. Persistently high eosinophil counts seen with drug reactions, helminthic parasitic infections, eosinophilic leukemia, and the HES are associated with endomyocardial fibrosis, a condition which presents with heart failure and signs consistent with a restrictive cardiomyopathy. The ventricle is thickened and histologically there are areas of fibrosis, thrombus formation, and inflammation in the endomyocardium with large numbers of both intact and degranulating eosinophils. Eosinophil granule products are deposited adjacent to myocytes and *in vitro* have been shown to be toxic for cardiac myocytes. HES, a condition in which there is a high eosinophil count of unknown etiology, is associated with a number of features which could be ascribed to the toxic properties of eosinophils (Spry, 1993). Much of the work undertaken in recent years on eosinophils has been in association with allergic disease and helminthic infection. These conditions are the most important eosinophilic diseases in terms of numbers of affected individuals.

C. Eosinophils and Allergic Inflammation

1. Pathology of Asthma Deaths

The association between eosinophils, asthma, and allergic disease has been known for many years. It is well established that large numbers of eosinophils together with mononuclear cells are frequently found in and around the bronchi in patients who have died of asthma (Ellis, 1908; Dunnill, 1978; Huber and Koessler, 1992). Immunostaining of bronchial tissue from asthma deaths revealed large amounts of MBP deposited in the airway adjacent to areas of desquamated epithelium (Filley *et al.*, 1982). Although an airway eosinophilia is a consistent and often striking finding in asthma deaths, it is not universal, with case reports of childhood asthma deaths showing no evidence of airway eosinophilia (Sur *et al.*, 1993).

2. Peripheral Blood Eosinophil Counts

The presence of increased numbers of peripheral blood eosinophils in both atopic and nonatopic chronic asthma is also well established, although this elevation is not as great as that seen in other eosinophil-associated diseases and the peripheral blood eosinophil count is often normal. Horn and colleagues (1975), in a longitudinal study of 14 oral corticosteroid-dependent asthmatics being treated at a chest clinic, found that eosinophil

counts correlated with several measurements of airflow obstruction. Durham and Kay (1985) observed that the degree of bronchial hyperreactivity inversely correlated with the peripheral blood eosinophil count in patients who had a late-phase response after antigen challenge. A similar correlation was observed in a cross-sectional study of asthmatics seen at a routine chest clinic (Taylor and Luksza, 1987). Concentration of ECP in serum of asthmatics also correlated with severity of clinical disease and monitoring of ECP has been suggested as a useful adjunct to clinical assessment (Venge, 1984).

3. *Fibreoptic Bronchoscopy Studies*

A more detailed appreciation of the extent of eosinophil involvement in asthma has come from the use of fibreoptic bronchoscopy to obtain BAL fluid and endobronchial biopsies from the airways of patients with mild to moderate asthma. Over the past 10 years a large number of baseline studies have been performed on asthmatics as well as measurements after allergen challenge. This has proved to be a safe approach in experienced hands when appropriate selection procedures are used and adequate precautions taken (NHLBI Workshop Summaries, 1985).

a. Allergen Challenge. Aerosolized challenge of sensitized asthmatics with allergen results in an early phase of bronchoconstriction lasting about 1 hr and returning to baseline. In about 40% of subjects this is followed by a delayed or late-phase period of airway obstruction generally increasing up to 6 hr after challenge and lasting up to 12 hr. The early phase is thought to be due to the immediate release of bronchoconstricting mediators, especially from mast cells, and the late response to be associated with the influx of inflammatory cells thereby more closely mimicking the pathology of clinical asthma (Dolovich *et al.*, 1989; Durham, 1991). Allergen challenge has been used extensively as a model of asthma to both study pathogenesis and test the efficacy of antiasthma treatments.

De Monchy *et al.* (1985) observed an increase in the number of eosinophils in BAL fluid and an increase in the ECP/albumin ratio 6 hr after allergen challenge in late responders but not those subjects who only had a single early response. Diaz and colleagues (1989) studied 14 asthmatics 6 hr after allergen challenge, 7 of whom had a late-phase response. They found increased numbers of eosinophils and, to a lesser extent, neutrophils, but only in those subjects who had a dual response. Aalbers *et al.* (1993) observed increased numbers of eosinophils in the bronchial wash of dual compared to single responders, the response to allergen challenge having been determined 3–7 weeks before the bronchoscopy. They argued that

the increased airway eosinophils was a predictor of dual-response status rather than a consequence of it.

Metzger and co-workers (1987), using a technique of segmental challenge by instilling allergen directly into the airways through the bronchoscope, found up to 50% of the lavage cells were eosinophils 24 hr after challenge. Challenge with agents that cause occupational asthma also produced a bronchoalveolar eosinophilia. Lam *et al.* (1987) found increased numbers of eosinophils and epithelial cells 24 hr after challenge with plicatic acid in patients with red cedar wood asthma. A BAL neutrophilia was also observed after 48 hr. Fabbri and colleagues (1987) observed both an airway eosinophilia and neutrophilia in patients with a late response to challenge with toluene diisocyanate. Similar findings have been found after allergen challenge to the skin and nose (Frew and Kay, 1988; Bentley *et al.*, 1992c). A recent detailed kinetic examination of the time course of the LPR in the skin revealed that migration of granulocytes was maximal at 6 hr with much of the recruitment having occurred by the first hour. Eosinophil infiltration was still present in the skin at 96 hr (Tsicopoulos *et al.*, 1994). Despite the almost invariable finding of an eosinophilia after allergen challenge, the extent to which this is a causal relationship remains uncertain. For example, in the monkey model of asthma (Gundel *et al.*, 1993) the late response appeared to be neutrophil rather than eosinophil related and not all studies have observed a clear distinction in the BAL cellular profile between single and dual responders (Bentley *et al.*, 1993). Other reasons suggested to explain the development of the late response include the degree of mast cell responsiveness to allergen challenge and the sensitivity of the lung to an inflammatory stimulus (Machado and Stalenheim, 1990).

b. Clinical Disease. An almost invariable increase in the number of eosinophils, often in association with increased numbers of mast cells and epithelial cells, has been observed in BAL fluid and endobronchial biopsies from clinical asthmatics compared with normal controls (Godard *et al.*, 1982; Tomioka *et al.*, 1984; Flint *et al.*, 1985; Kirby *et al.*, 1987; Wardlaw *et al.*, 1988; Kelly *et al.*, 1988; Beasley *et al.*, 1989; Foresi *et al.*, 1990; and reviewed in Djukanovic *et al.* 1990). The increase in the number of eosinophils in the asthmatic airway is modest. For example, in BAL fluid this ranged between 1 and 5%, compared with 0 or 1% in normal controls (Wardlaw *et al.*, 1988). Unlike the allergen challenge studies increased numbers of airway neutrophils have not generally been observed. One of the most important observations that has emerged from these studies is that even in very mild disease requiring only occasional use of bronchodilators there is clear evidence of airway inflammation with increased numbers

of airway eosinophils and BAL epithelial cells. This has led to increased emphasis on the early use of anti-inflammatory drugs, particularly inhaled corticosteroids, in the management of asthma. Airway eosinophils in asthma are activated as determined by staining with the mAb EG2 and expression of the activation receptor CD69 (Azzawai *et al.*, 1990; Hartnell *et al.*, 1993). The importance of the state of activation of the eosinophils and the extent to which they are releasing their mediators is emphasized by several studies. For example, in the study by Wardlaw *et al.* (1988) the concentration of MBP in the BAL fluid was a better discriminator of disease activity than the BAL eosinophil count. The inhibitory effect of anti-CD18 mAbs in the development of allergen-induced bronchial hyperresponsiveness in cynomolagus monkeys was associated with a decrease in BAL ECP concentrations without any change in the eosinophil count (Wegner *et al.*, 1993). Thus, measurement of eosinophil-associated basic proteins may be a better guide to the degree of eosinophil inflammation than eosinophil numbers. In support of this Adelroth and co-workers (1990) found that, whereas inhaled corticosteroids had no effect on the number of eosinophils in BAL fluid from asthmatics, they markedly reduced the amounts of ECP in lavage fluid in association with clinical improvement.

Further support for the idea that eosinophil activation and mediator release is required for tissue damage (rather than the presence of eosinophils per se) to occur is offered by animal models. For example, IL-5 transgenic mice, which have a marked peripheral blood and tissue eosinophilia, appear to be healthy (Dent *et al.*, 1990). In addition, when systemically sensitized guinea pigs were challenged by antigen inhalation they developed a BAL eosinophilia without evidence of eosinophil activation and no increase in bronchial hyperresponsiveness. However, after intratracheal challenge with LTB_4 concentrations of MBP and EPO increased in the BAL fluid and the animals developed bronchial hyperresponsiveness (Pretolani *et al.*, 1994a).

The specificity of an airway eosinophilia has been addressed by several groups. Allen *et al.* (1990) found that BAL eosinophilia was found in only a restricted range of lung diseases of which asthma is by far the most common. The difference in airway eosinophils and neutrophils between patients with asthma and those with smoking-related airway disease was studied by Lacoste *et al.* (1993). They found that both groups of patients had a significant increase in BAL eosinophils compared with normal subjects. There was a significant increase in the concentration of BAL ECP and the number of degranulating eosinophils, but not in the total number of eosinophils, between the asthmatics and patients with smoking-related lung disease. The presence of sputum eosinophilia was noted in seven nonsmoking subjects with chronic productive cough but without evidence

of asthma. In this report the presence of a sputum eosinophilia was a marker for corticosteroid responsiveness (Gibson *et al.*, 1989). The specificity of an airway eosinophilia in relation to asthma was also examined by Azzawi *et al.* (1992) who compared the immunohistology of fatal asthma and cystic fibrosis. They found that, whereas in 15 patients with asthma there was a mean of 62.7 eosinophils per millimeter length of epithelial basement membrane in 6 patients who died of cystic fibrosis, there was 1.8 eosinophils in the other patients and in normal controls there was 1 eosinophil. A lesser, but often significant, increase in airway eosinophils is seen in atopic nonasthmatics or seasonal asthmatics out of season.

Although most studies have involved atopic asthmatics airway eosinophilia is also a marked feature of intrinsic asthma and isocyanate-induced asthma (Bentley *et al.*, 1992a,b). In addition to asthma, eosinophils are also prominent in other forms of allergic inflammation including allergic rhinitis and atopic dermatitis. Essentially, the findings in rhinitis have been similar to those in asthma with increased numbers of activated eosinophils and epithelial mast cells (Viegas *et al.*, 1987; Pipkorn *et al.*, 1988; Bentley *et al.*, 1992b). In rhinitis, however, the nasal epithelium generally appears intact. Nasal polyps contain large numbers of activated eosinophils and the syndrome of aspirin sensitivity, eosinophilia, and nasal polyposis is well recognized (Slavin, 1993). In atopic dermatitis a peripheral blood eosinophilia is a common feature and the skin lesions are characterized by marked deposition of eosinophil granule proteins, often in the absence of many intact eosinophils (Leiferman *et al.*, 1985; Bruyjnzeel-Koomen *et al.*, 1988).

An eosinophilia is therefore an almost invariable feature of allergic inflammation. However, if there was a causal relationship between the eosinophil and the pathogenesis of asthma it would be expected that fluctuations in the severity of the disease would be mirrored by fluctuations in the degree of eosinophilia. As discussed previously, there was a correlation between the peripheral blood eosinophil count and the FEV_1 in clinical disease and the development of a late-phase response after allergen challenge. In addition, most studies have found a correlation between asthma or rhinitis and the number of hypodense eosinophils in the peripheral blood (Fukuda *et al.*, 1985; Frick *et al.*, 1988, 1989). Bousquet *et al.* (1990) reported a significant correlation with increasing severity of asthma between both the airway eosinophil count and the BAL ECP concentration. In an electron microscopy study a correlation was observed in 18 asthmatics between the number of epithelial eosinophils and the opening of epithelial tight junctions which in turn was inversely correlated with the histamine PC_{20} (Ohashi *et al.*, 1992). Inhibition of an airway eosinophilia by DSCG (Diaz *et al.*, 1984) or more effective corticosteroids (Juniper *et al.*, 1990; Schleimer, 1990) was associated with an improvement in bronchial hyper-

responsiveness, symptoms, and lung function. Inhibition of migration of eosinophils into the airways of allergen-challenged nonhuman primates, using an mAb directed against the adhesion molecule ICAM-1, also inhibited the development of airway hyperresponsiveness (Wegner *et al.*, 1990). However, none of these treatments are specific to the eosinophil. Glucocorticoids, for example, probably act to a large extent through inhibition of the release of eosinophil active cytokines from T cells and monocytes (Taylor and Shaw, 1993).

For many years it was considered that eosinophil accumulation in allergic inflammation was a result of the release of mast cell-derived mediators. Doubts about this hypothesis started to emerge in the 1980s as a result of several observations. Effective inhibitors of mast cell degranulation were found to be of limited benefit in asthma (Johnson, 1980). Increases in mast cells in asthmatic airways were modest and were found in many other diseases such as sarcoidosis (Wardlaw *et al.*, 1986a,b). Glucocorticoids, though effective in asthma, were unable to inhibit human lung mast cell degranulation *in vitro* (Schleimer *et al.*, 1983). The recent observation that mast cells can generate IL-4, IL-5, and other cytokines of relevance to eosinophil functions has once more renewed interest in the mast cell as an orchestrator of the asthma process (Galli *et al.*, 1994). However, in the intervening years the T lymphocyte has emerged as important in directing eosinophil function in allergic inflammation. Activated T cells, as defined by the expression of the IL-2 receptor CD25, are found in increased numbers in the peripheral blood from patients with acute severe asthma and clinical improvement was associated with a decrease in the number of CD25-positive cells in the blood (Corrigan *et al.*, 1988). Similarly, CD25-positive T cells were found in increased numbers in BAL and endobronchial biopsies from asthma (Azzawi *et al.*, 1990) and have a Th2-like profile of cytokine mRNA expression (Robinson *et al.*, 1992).

The association between eosinophils and cytokines derived from type 2 helper cells has been discussed previously. Current evidence suggests that IL-5 plays a critical role in eosinophil-mediated tissue damage. IL-5 $mRNA^+$ cells were detectable in BAL (Robinson *et al.*, 1992) and bronchial biopsies (Hamid *et al.*, 1991) from ongoing steady-state asthmatics as well as in asthma provoked by inhalational challenge (Bentley *et al.*, 1992a,b). Chronic severe asthmatics had elevated serum concentrations of IL-5 compared to controls and levels decreased following treatment with corticosteroids (Corrigan *et al.*, 1993). In a placebo-controlled study in moderately severe asthma the numbers of IL-5 $mRNA^+$ cells in BAL also decreased after 2 weeks of treatment with prednisolone (Robinson *et al.*, 1993).

In summary, eosinophils are almost invariably present in increased numbers at sites of allergic inflammation. They are actively secreting mediators

which could cause many of the pathological features of the disease process. The numbers of eosinophils and amounts of eosinophil mediators correlate broadly with disease activity and effective treatments for asthma, particularly, glucocorticoids reduce the number of tissue and blood eosinophils. There is, therefore, very good evidence for a proinflammatory role for eosinophils in asthma and related diseases. However, the evidence still remains circumstantial. In a sense, any further debate becomes sterile until we have conclusive evidence from agents which specifically inhibit eosinophil accumulation or their products in tissue. The emergence of such antagonists from the pharmaceutical locker is keenly awaited.

D. Other Eosinophilic Diseases

1. HES

The sporadic occurrence of striking eosinophilia without apparent cause and with a predisposition to cardiac and neurologic injury was identified as a syndrome by Hardy and Anderson in 1968. Subsequent reports have referred to it as the hypereosinophilic syndrome (reviewed in Spry, 1993). HES is a rare condition with a 9 : 1 preponderance of males over females. It occurs worldwide. The cause is unknown and its course varies. The organ damage is thought to be largely a result of noxious effects of eosinophil granule contents in certain tissues, especially the heart and nervous system (Olsen and Spry, 1985; Shah *et al.*, 1990).

The clinical picture varies from asymptomatic to a severe, life-threatening multisystem disorder. The onset is often marked by anorexia, weight loss, fatigue, nausea, abdominal pain, diarrhoea, nonproductive cough, pruritic rash, and fever accompanied by night sweats. Hepatic and splenic enlargement is common. Virtually all patients have cardiac involvement (Parrillo *et al.*, 1979). The key finding is a leukocytosis with a striking eosinophilia, usually greater than 1500 eosinophils/μl (1.5×10^9/liter), occasionally approaching 10,000 eosinophils/μl (100×10^9/liter). Marrow examination shows eosinophilia with few other specific findings. Eosinophilia may be progressive, with eosinophil counts of 50,000 cells/μl (50×10^9/liter) or more occurring in over half the patients during the course of the illness. The distinction from eosinophilic leukemia, a very rare disorder, is made principally by determining the presence or absence of leukemic blast cells in the marrow (and blood). The presence of a clonal cytogenic abnormality and progressive anemia and thrombocytopenia also indicates leukemia is more likely the correct diagnosis.

Cogan *et al.* (1994) described a man with the hypereosinophilic syndrome and clonal proliferation of type 2 helper T cells. Some patients with HES have a premalignant condition with similarities to chronic myeloid leuke-

mia, whereas others have T cell tumors. It has already been shown in several patients with the hypereosinophilic syndrome that bone marrow and blood mononuclear cells express mRNA encoding IL-5 (Spry *et al.*, 1993). The report by Cogan *et al.* (1994), however, documented clonal expansion of T cells and a clonal rearrangement of the β-chain of the T cell receptor gene. This, together with the finding that large quantities of IL-5 and IL-4 were secreted, whereas IL-2 and IFN-γ were virtually undetectable, strongly suggests the clonal expansion of a type 2 helper T cell.

The disease follows a variable course, which sometimes is indolent, but often it is progressive and fatal. Although symptoms may remit and relapse, the organ damage is usually steadily progressive, with cardiac failure resulting from endomyocardial fibrosis that often involves the valve leaflets. Central nervous system dysfunction is often progressive, leading to encephalopathy, polyneuropathy, or stroke. Episodes of venous thrombosis may complicate the course. In one series, over three-fourths of the patients died after 3 years of observation despite therapy with glucocorticoids or cytotoxic agents (Parrillo *et al.*, 1978). Symptomatic or progressive disease requires therapy. Glucocorticoids and hydroxyurea have been the mainstays of treatment and have been used with apparent success in some patients. Responses to other cytostatic agents (e.g., methotrexate and cyclophosphamide) have been very infrequent. Etoposide has been used successively in a glucocorticoid- and hydroxyurea-resistant patient (Schooley *et al.*, 1981). A promising new treatment is IFN-α which appears generally well tolerated and may induce remission in patients unresponsive to other forms of treatment (Parrillo *et al.*, 1978; Moore *et al.*, 1985; Smit *et al.*, 1991). Leukapheresis (Murphy *et al.*, 1990) and marrow transplantation have also been used (Zielinski and Lawrence, 1990). Surgical replacement of severely damaged heart valves may be necessary (Ellman *et al.*, 1974; Busch *et al.*, 1991).

2. *Eosinophilia Myalgia Syndrome*

This disorder was first described in October 1989 in New Mexico (Henderson and Sage, 1973). Over 1500 cases were reported over the next 2 years, with over 30 deaths (Franchi *et al.*, 1984). The syndrome was caused by the ingestion of L-tryptophan and is thought to be the result of a contaminant, possibly 1,1′-ethylidenebis (tryptophan). There is a perivascular lymphocytic and eosinophilic infiltrate in the dermis, fascia, and skeletal muscle, with a pulmonary vasculitis and alveolitis mimicking the disease eosinophilic fascilitis. Severe myalgia and an eosinophil count greater than 1000 cells/μl (1×10^9/liter) are constant features. Arthralgias, cough, shortness of breath, dependent edema, and hair loss are very common. A

significant proportion of patients, perhaps 50%, develop paresthesias, peripheral neuropathy, and/or scleroderma-like skin changes. A high proportion of patients have symptoms and signs 1 year after onset of the disease (Keidan *et al.*, 1985; Editorial, 1989a,b). Glucocorticoid or nonsteroidal anti-inflammatory therapy has had little effect on the course of the disease, although some improvement may occur in symptoms. Cytotoxic drug therapy has also had little effect on symptoms or course (Culpepper *et al.*, 1991).

3. *Toxic Oil Syndrome*

In Spain in 1981, more than 20,000 cases of a syndrome manifested by fever, cough, dyspnoea and leukocytosis, neutrophilia, and an eosinophil count greater than 750 cells/μl (0.75×10^9/liter) were reported (Belonga *et al.*, 1990). Occasionally, the eosinophil count rose above normal only after the onset of the pulmonary symptoms. Pulmonary infiltrates were evident on X rays of the chest. Pleural effusion was common, and hypoxemia was frequent. There were over 300 deaths (about 1.5% of affected subjects). About half of the patients went on to a chronic course that mimicked the eosinophilia–myalgia syndrome, with myalgias, eosinophilia, peripheral neuritis, scleroderma-like skin lesions, hair loss, and a sicca syndrome. Most patients improved from either acute or chronic symptoms and signs but some residual nerve, muscle, or skin damage may persist indefinitely. Endothelial cell proliferation, mononuclear cell infiltrates around blood vessels (vasculitis), and perineural inflammatory infiltrates were identified histopathologically. Glucocorticoid therapy may have decreased the pulmonary symptomatology. The disease is thought to be a response to an unlabled food oil, aniline-denatured rapeseed oil, marketed as pure olive oil (Varga *et al.*, 1992).

4. *Reactive Hypereosinophilia and Neoplasms*

Exaggerated eosinophilia has been reported in association with a variety of lymphoid (Spitzer and Carson, 1973; Catovsky *et al.*, 1980; Pestell, 1982; Watanabe *et al.*, 1989; Fischel *et al.*, 1990; Meeker *et al.*, 1990; Samoszuk and Hansen, 1990; Belonga *et al.*, 1993; Kilbourne *et al.*, 1983, 1991) and solid tumors (Slungaard *et al.*, 1983; Kodama *et al.*, 1984; Reddy *et al.*, 1984; O'Shea *et al.*, 1987; Vukelja *et al.*, 1988; Samoszuk *et al.*, 1993). In these cases, the eosinophilia is thought to be an epiphenomenon, not derived from the malignant clone but rather a response to IL-5 and other cytokines elaborated by the tumor cells. The eosinophilia may precede the clinical diagnosis of the tumor but is usually manifested concomitantly. In some cases successful treatment of the tumor is associated with amelioration of the eosinophilia. Angiolymphoid hyperplasia also has been associated with eosinophilia (Balducci *et al.*, 1989; Haldane *et al.*, 1989).

5. *Pulmonary Eosinophilia*

Pulmonary eosinophilia describes a syndrome of peripheral blood eosinophilia in association with lung shadowing which is often fleeting in nature (Stefanini *et al.*, 1991). The syndrome includes allergic granulomatosis (Churg–Strauss syndrome), ABPA, simple Loefflers pulmonary eosinophilia, and chronic pulmonary eosinophilia.

Churg–Strauss syndrome is an unusual disorder of unknown etiology characterized by hypereosinophilia and systemic vasculitis in association with asthma (Nuzum and Nuzum, 1954; Frohnert, 1967; Hallam *et al.*, 1989; Sharp *et al.*, 1989; Leavitt and Fauci, 1986). Biopsy evidence showing granulomas and a vasculitis are required to make the diagnosis with certainty but often the diagnosis is appropriately made on clinical grounds and granulomas are often absent (Churg and Strauss, 1951). Patients are usually diagnosed in middle age and there is approximately equal sex incidence. Rhinitis and nasal polyposis in association with atopy are common. Gastrointestinal disease is not an uncommon occurrence and there is an overlap between Churg–Strauss syndrome and eosinophilic gastroenteritis. Asthma may precede the onset of the vasculitis by 30 years (Fauci, 1983). The severity of the asthma and the vasculitis does not correlate closely. Pulmonary infiltrates are common and have a variable appearance (Guillevin *et al.*, 1987). The prognosis of untreated Churg–Strauss syndrome is poor. Treatment is essentially the same as that for other forms of vasculitis with glucocorticoids together with azothiaprine or cyclophosphamide (Lanham *et al.*, 1984).

ABPA is an unusual complication of asthma and comprises a syndrome of pulmonary eosinophilia in association with proximal bronchiectasis (Chumbley *et al.*, 1977; Wardlaw and Geddes, 1992). The criteria for diagnosis include specific IgE and precipitating IgG antibodies against *Aspergillus fumigatus*, a peripheral blood and sputum eosinophilia, *A. fumigatus* cultured in the sputum, and bronchiectasis. Once recognized it is generally well controlled with the appropriate use of glucocorticoids, although untreated it can lead to severe lung damage. Simple pulmonary eosinophilia, Loefflers syndrome, is characterized by wheeze, eosinophilia, and fleeting lung shadows. The illness lasts less than a month and is due to the migration of parasitic larvae, usually *Ascaris*, through the lung. The similar tropical pulmonary eosinophilia is due to migration of filarial parasites. Chronic pulmonary eosinophilia is a disease of unknown etiology in which a pneumonic-like illness is associated with corticosteroid-responsive lung shadowing and pulmonary and, generally, peripheral blood eosinophilia.

6. *Eosinophilic Fasciitis*

This syndrome may occur at any age in both sexes and is characterized by stiffness, pain, and swelling at the arms, forearms, thighs, legs, hands, and feet in descending order of frequency. Malaise, fever, weakness, and weight loss also occur (Abeles *et al.*, 1979; Kent *et al.*, 1981). Eosinophilia greater than 1000 cells/μl (1×10^9/liter) is present in most patients but may be intermittent. A biopsy, usually required for the diagnosis, shows inflammation, edema, thickening, and fibrosis of the fascia. Synovial tissue may show similar changes. Aplastic anemia, isolated cytopenias, pernicious anemia, and leukemia have been associated with eosinophilic fasciitis.

E. Eosinopenia

The eosinophil count is less than 10 cells/μl (0.01×10^9/liter) in only 0.1% of patients. In virtually all such cases the eosinopenia can be ascribed to treatment such as glucocorticoids or adrenaline, both of which decrease eosinophil counts (Weingarten *et al.*, 1985; Calame *et al.*, 1986; Asperilla and Smego, 1989). In contrast, β blockers inhibit adrenaline-induced eosinopenia and can cause a rise in the eosinophil count.

There have been isolated reports of patients with absent eosinophils in the blood and marrow (Beeson and Bass, 1977; Juhlin and Michaelsson, 1977; Bass *et al.*, 1980b; Krause and Boggs, 1987). Patients without eosinophils had asthma or allergic symptoms or some other immunologic problem. In one case it occurred after drug-induced agranulocytosis, and in another case there was a serum inhibitor of eosinophil colony formation. Eosinophil peroxidase deficiency is a rare disorder that may be brought to light by automatic cell counting that measures peroxidase to count eosinophils. Eosinophil peroxidase deficiency does not have any adverse clinical consequences (Juhlin, 1987).

IV. Final Conclusions

Regrettably we are still not in a position to assign, unequivocally, a role for the eosinophil in biological and pathological processes. There is no "experiment of nature" in which this cell or its progenitors are selectively depleted and at the present time no pharmacological or biological inhibitor is available for laboratory-based or clinical studies. However, in the past few years evidence has shifted away from the cell having "anti-allergic properties" and even its role in adaptive immunity to helminth infections is being seriously questioned. Earlier studies have not been expanded and more recent experiments have cast serious doubt on the role of this cell in worm infection.

It is important to bear in mind that the eosinophil is an inflammatory cell and that the purpose of inflammation is to restore, repair, and remodel injured tissue. The role of the eosinophil in wound healing has recently received considerable attention and it seems reasonable to expect that this will be a "growth area" for eosinophil biologists. Also, intriguingly there is now some firm data to implicate the eosinophil in immunological responses against cancer cells. For many years it has been suspected that atopic individuals were less susceptible to neoplastic processes (although hard data for this are lacking). We anticipate that these recent findings on the potential of eosinophils in cancer immunity may stimulate a better understanding of the role of this cell type. It can be stated with reasonable assurity that the eosinophil probably has some homeostatic function in certain physiological situations and that this needs to be considered further. That the eosinophil is essentially a redundant cell which has survived evolutionary processes through default is an unattractive but real possibility. It is expected that with the rapid pace of technology the secrets of the eosinophil will soon be unraveled. However, we have not yet reached that point.

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In Situ Studies of the Germinal Center Reaction

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I. Introduction

New histologic and molecular genetic techniques permit the cellular dynamics of the immune response to be followed with great precision and in the context of the architecture of the lymphoid tissues. These methods have lead to a new appreciation of the importance of specialized microenvironments that direct the antigen-driven proliferation and differentiation of T and B lymphocytes. Although I shall emphasize these processes as they occur in the spleen, very similar events occur in other secondary lymphoid tissues such as the lymph nodes (Niewenhuis and Kuening, 1974) or tonsil (MacLennan, 1994).

The humoral immune response is the culmination of a complex series of cellular migrations and collaborations that define a series of steps in the pathway of antigen-driven B cell differentiation. The precise choreography provides necessary information to the responding lymphocyte populations and can serve to regulate antibody production and affinity maturation, the generation of the B cell memory compartment, and peripheral self-tolerance. For the past 5 years, my colleagues and I have investigated the primary immune response of C57BL/6 and B10.A mice (both Igh^b) to immunogenic protein conjugates of the (4-hydroxy-3-nitrophenyl)acetyl (NP) hapten. In these strains, the response to NP is genetically restricted; the majority of primary anti-NP antibodies are heteroclitic (Imanishi-Kari and Makela, 1973), bear the λ1 L-chain (Makela and Karajalainen, 1977), and share a distinctive idiotype (Jack *et al.*, 1977; Makela and Karajalainen, 1977; Reth *et al.*, 1979). Early sequencing studies by Bothwell (1981) demonstrated that the distinctive phenotype of these antibodies was due to the predominant use of the V_H V186.2 and DFL16.1 gene segments to encode the H-chain. Thus, the restricted genotypic and phenotypic diversity of this response has made it ideal as a model to study the dynamics of the immune response *in situ*.

The methods we have used to study the immune response *in situ* are not novel and have been guided by the elegant histological studies of I. C. M. MacLennan and F. G. M. Kroese among others. However, we have gained considerable insight by combining more traditional histologic studies with *in situ* molecular genetic analyses dependent upon the poly-

merase chain reaction (PCR). Briefly, we have prepared histologic sections from blocks of frozen spleen and identified specific cell populations by immunohistology. It is possible to use micromanipulator-driven tools to dissect labeled cell populations and even single cells (Küppers *et al.*, 1994) from tissue sections and to amplify the recovered Ig or TCR rearrangements in specific PCRs. Indeed, we have been able to use multiplex PCRs to determine V_α/V_β and V_λ/V_H rearrangements in single T and B cells (B. Zheng, Q. Zhu, and G. Kelsoe, unpublished data).

Amplified V(D)J rearrangements are ligated into plasmid vectors for sequencing and may even be reexpressed as a transfectant protein (Miller and Kelsoe, 1995b), allowing the phenotype of the encoded antigen receptor to be studied. The ability to study the population genetics of immune responses presents considerable opportunity and should prove widely useful. This chapter will focus on the information made available by these studies in the hope of attracting the interest of others.

II. Histology of the Spleen

A. The Splenic Architecture

The spleen contains distinct regions of lymphoid and erythroid cells known as the white and red pulps, respectively. These zones are separated by a perforated marginal sinus that permits cellular trafficking between lymphoid and erythroid areas. It is in the lymphocyte-rich white pulp that the splenic immune response begins, although the red pulp may contain large numbers of B lymphoblasts and plasmacytes late in the primary immune response or after secondary challenge (Niewenhuis and Kuening, 1974; Jacob *et al.*, 1991; Liu *et al.*, 1991).

The splenic parenchyma can only be reached via the splenic artery, which branches several times before penetrating the splenic capsule at the hilus. After further ramifications into trabecular arteries, blood vessels enter the white pulp as central arterioles which extend for the length of these cylindrical lymphoid zones. Eventually, central arterioles branch to form thin pencilliary arterioles or arterial capillaries that open into complex venous sinuses. Thus, the circulatory system within the spleen is open.

In the spleen, the white pulp is organized coaxially about the central arteriole and consists of an inner zone of T lymphocytes known as the periarteriolar lymphoid sheath (PALS) and an outer zone rich in B lymphocytes. This outer region is known as the (primary) lymphoid follicle and it may contain populations of rapidly dividing B cells in germinal centers (GCs), or secondary follicles, surrounded by a follicular mantle of small resting B cells (MacLennan *et al.*, 1990) (Fig. 1). The B lymphocytes

within GCs avidly bind the lectin, peanut agglutinin (PNA), a property not shared by other peripheral B cells (Rose *et al.*, 1980; Reisher *et al.*, 1979).

The PALS and lymphoid follicle also contain distinct accessory cells that are highly specialized for antigen presentation to T and B lymphocytes, respectively. Within the PALS, interdigitating cells (IDCs) bearing abundant class II MHC (Frelinger *et al.*, 1979; Inaba *et al.*, 1984) CD40 (Clark and Ledbetter, 1986) and the leukocyte integrin CD11c (Metlay *et al.*, 1990) form an extensive network. IDCs are the most efficient population of nonlymphocyte antigen-presenting cells (APC) for T cells (Metlay *et al.*, 1990; Knight *et al.*, 1982); this property may be the result of their constitutive expression of the costimulatory molecule B7-2 (Inaba *et al.*, 1994; Caux *et al.*, 1994).

The lymphoid follicle contains follicular dentritic cells (FDCs) that bind complexes of antigen and can retain antigen in its native form for very long periods of time (Nossal *et al.*, 1964; Tew *et al.*, 1980). FDCs express a wide array of cell surface receptors (reviewed in Schriever and Nadler, 1992), including those for complement (CD11b, CD21) and immunoglobulin Fc (CD16, CD23) as well as the adhesion molecule, ICAM-1 (CD54) (Schriever and Nadler, 1992; Schriever *et al.*, 1989; Petrasch *et al.*, 1990). These molecules probably facilitate the capture of antigen–antibody complexes and the heterotypic cell interactions necessary for the GC reaction. Interestingly, CD19 and CD45 have also been reported as components of the FDC membrane (Schriever and Nadler, 1992; Schriever *et al.*, 1989; Petrasch *et al.*, 1990; Johnson *et al.*, 1986; Kosco *et al.*, 1986) and might function to activate FDC (Reth, 1992). FDCs express the CD40 molecule (Clark and Ledbetter, 1994; Clark *et al.*, 1992) and when activated in GCs, may interact with T cells expressing the CD40 ligand (CD40L) (Lederman *et al.*, 1993).

Macrophages are also present in the lymphoid follicles and are especially prominent in GCs where they become engorged with apoptotic B cells. GC macrophages constitute an unusual subpopulation in that they express Thy1 antigen and, when replete with the nuclei of dead lymphocytes, are known as tingible body macrophages (Smith *et al.*, 1988). While these macrophages express class II MHC, they are not believed to function as APC within the GC (Smith *et al.*, 1991).

B. Histology of Primary Humoral Responses

Primary humoral immune responses begin within the T cell-rich PALS; by 2 days postimmunization, antigen-binding B cells appear in the outer region of the PALS (Jacob and Kelsoe, 1992; MacLennan *et al.*, 1992). These cells are probably recent follicular immigrants (MacLennan *et al.*, 1992) and are interspersed among, and frequently in contact with, $CD4^+$

antigen-specific T cells (Kelsoe and Zheng, 1993; Zheng *et al.*, 1994). Interaction between antigen-specific T and B lymphocytes (Van den Ertwegh *et al.*, 1993) in the PALS leads to CD40–ligand-dependent B cell proliferation (Han *et al.*, 1995a); interruption of this phase of the response severely diminishes or abrogates subsequent production of antibody and the GC reaction (Noelle *et al.*, 1992; Hathcock *et al.*, 1993).

By day 4 of the primary responses, two differentiation pathways for antigen-driven B cell specialization are established. One path, continued local development into large foci of plasmacytes at the boundary of the PALS and follicle (Jacob *et al.*, 1991), is responsible for the great majority of antibody produced in the first 12 days of the response (Grobler *et al.*, 1974; Tsiagbe *et al.*, 1992). The other pathway, the GC reaction, occurs in the lymphoid follicle and is responsible for the generation of B cell memory that comprises selected populations of long-lived mutant B lymphocytes (Jacob *et al.*, 1991, 1993; Berek *et al.*, 1991; McHeyzer-Williams *et al.*, 1993).

1. The PALS-Associated Foci

Three or 4 days postimmunization, antigen-binding B cells in the PALS reach sufficient numbers to form foci of lymphoblasts and plasmacytes (Jacob *et al.*, 1991; Jacob and Kelsoe, 1992). Although initially present as loose clusters of cells among abundant $CD4^+$ T lymphocytes, these plasmacytic foci contain few T cells by day 6 of the response. Each focus is anatomically discrete and represents an oligoclonal population founded by some 4–10 B cells (Jacob *et al.*, 1991; Jacob and Kelsoe, 1992). *In situ* genetic studies of Ig heavy-chain genes from individual foci suggest that little or no B cell trafficking occurs between adjacent focus populations (Jacob *et al.*, 1991; Jacob and Kelsoe, 1992). The peak of the primary focus reaction occurs at about day 10 postimmunization when 100–200 foci may occupy as much as 1% of the total splenic volume (Jacob *et al.*, 1991). After this peak, the plasmacytic foci in the PALS rapidly disappear (Jacob *et al.*, 1991).

Formation of PALS-associated foci may be blocked or suppressed by the administration of anti-CD40L or anti-B7-2 antibody (Noelle *et al.*, 1992; Hathcock *et al.*, 1993), but by day 5 of the response these antibodies cannot inhibit foci nor reduce early antibody titers (Han *et al.*, 1995). Presumably, this late insensitivity reflects independence from contact-dependent proliferation and differentiation. This finding is consistent with the virtual exclusion of T cells from focus populations by day 6 postimmunization (Jacob *et al.*, 1991; Jacob and Kelsoe, 1992). Nonetheless, the antibody-producing B cells within these focus populations undergo Ig class switching; by day 8, the great majority of focus cells contain large

amounts of cytoplasmic IgG (Jacob *et al.*, 1991; Liu *et al.*, 1991). We have interpreted the absence of hypermutation and the rapid loss of these cells to mean that the focus population is transient and does not participate in the late primary or secondary antibody responses. Phenotypic change in focus populations (Jacob *et al.*, 1991) does suggest that antigen-driven selection takes place, however, probably as interclonal competition.

2. *Germinal Centers*

After their activation in the PALS, antigen-specific B cells begin to accumulate in the follicles (Jacob *et al.*, 1991; Liu *et al.*, 1991). By day 4 of a primary response, antigen-binding B cell blasts fill the FDC reticulum, forming nascent GCs (Fig. 1). In the rat, in which the histologic substructure of the GC is apparent, this early phase in the GC reaction is characterized by displacement of small, nondividing follicular B cells to form a follicular mantle surrounding the proliferating blasts (Liu *et al.*, 1991; MacLennan *et al.*, 1990). Shortly afterwards, the GC polarizes to form two distinct zones: a dark zone (DZ) proximal to the PALS that contains rapidly dividing centroblasts and a light zone (LZ) that contains nondividing centrocytes, the bulk of the FDC network, and most of the infrequent $CD4^+$ T cells present in GC (Liu *et al.*, 1991; Zheng *et al.*, 1994; Fuller *et al.*, 1993). Cell labeling studies with bromodeoxyuridine suggest division times of approximately 6 or 7 hr for centroblasts and indicate that the centrocyte population is continuously derived from cells in the DZ (Liu *et al.*, 1991). In recently formed GCs, centroblasts and centrocytes represent oligoclonal B cell populations (Jacob *et al.*, 1991a,b, 1993; Liu *et al.*, 1991; Jacob and Kelsoe, 1992); on average each mature GC is derived from one to three B cell clones. The GC reaction reaches its maximum by days 10–12 of a primary response and may occupy 1 or 2% of the total splenic volume (Jacob *et al.*, 1991a,b; Liu *et al.*, 1991; McHeyzer-Williams *et al.*, 1993). In the absence of further antigenic stimulation, the GC reaction begins to wane by 21 days postimmunization. The GCs lose volume and avidity for PNA and by 32 days after immunization, GC residua occupy $\leq 5\%$ of their peak volume and appear as infrequent collections of a few antigen-binding blast cells in association with FDCs (J. Przylepa and G. Kelsoe, unpublished data).

The relationship between the antibody-forming cells of the PALS-associated foci and GC B cells is disputed. My description has emphasized our own observations (Jacob *et al.*, 1991a,b, 1993; Jacob and Kelsoe, 1992) and those by the laboratories of MacLennan and Niewenhuis (Liu *et al.*, 1991; Kroese *et al.*, 1987). In contrast, N. Klinman and colleagues have reported a series of experiments (Linton *et al.*, 1989, 1992) that suggest that the B cells entering the extrafollicular pathway of differentiation to

plasmacytes constitute a separate cell lineage from those B cells that found GCs. However, if these B cells are distinct lineages the decision to enter one lineage or the other must occur within the periphery during the course of the immune response; more than 50% of adjacent foci and GCs share B lymphocytes with identical and complex V(D)J joints (Jacob and Kelsoe, 1992). A resolution of the origins of the focus and GC cell populations remains an important goal and will be necessary for a complete understanding of immunological memory.

III. The Germinal Center Reaction

A. Responses to Thymus-Dependent Antigens

The GC reaction is most prominent in primary responses to thymus-dependent (Td) antigens (Liu *et al.*, 1991) and represents the cellular process leading to the generation of high-affinity memory B cells (Grobler *et al.*, 1974; Han *et al.*, 1995a; Jacob *et al.*, 1991a,b, 1993; Berek *et al.*, 1991) (Fig. 2). This process is absolutely dependent on the presence of T helper cells (Jacobsen *et al.*, 1974; Gastkemper *et al.*, 1981; Vonderheide and Hunt, 1990; Stedra and Cerny, 1994); however, surprisingly low numbers of $CD4^+$ T cells can support the formation of histologically typical GCs (Stedra and Cerny, 1994; Miller *et al.*, 1995). B cell-reconstituted C.B17 *scid* mice do not respond to immunization with protein antigens but mount a virtually normal GC reaction when supplemented with 10^7 T cells 16 hr before immunization (Stedra and Cerny, 1994). Indeed, similar reconstitution experiments in congenitally athymic *nude* mice demonstrate that fewer T cells are necessary to reconstitute the GC reaction than to generate the antibody-forming cell foci in the PALS (Stedra and Cerny, 1994).

The nearly normal kinetics and magnitude of the GC reaction in the presence of limited numbers of T cells suggest that cognate T/B interactions may not be required to sustain follicular activation/proliferation. This GC-like reaction may even generate increased numbers of IgM^+, antigen-specific precursor cells, and prime for enhanced IgM responses on subsequent rechallenge (Stedra and Cerny, 1994; Diamantstein and Blitstein-Willinger, 1974; Roelants and Askonas, 1972). However, $\mu \rightarrow \gamma$ isotype switching is poorly supported by limiting T cell numbers and the extent of Ig hypermutation is directly proportional to the numbers of T helper cells available during the primary response (Miller *et al.*, 1995), probably reflecting the absence or reduction of critical helper signals during B cell proliferation and differentiation in the GC microenvironment.

The signals that give rise to GC formation are not well understood. Genetic defects (Freeman *et al.*, 1993; Allen *et al.*, 1993; Renshaw *et al.*,

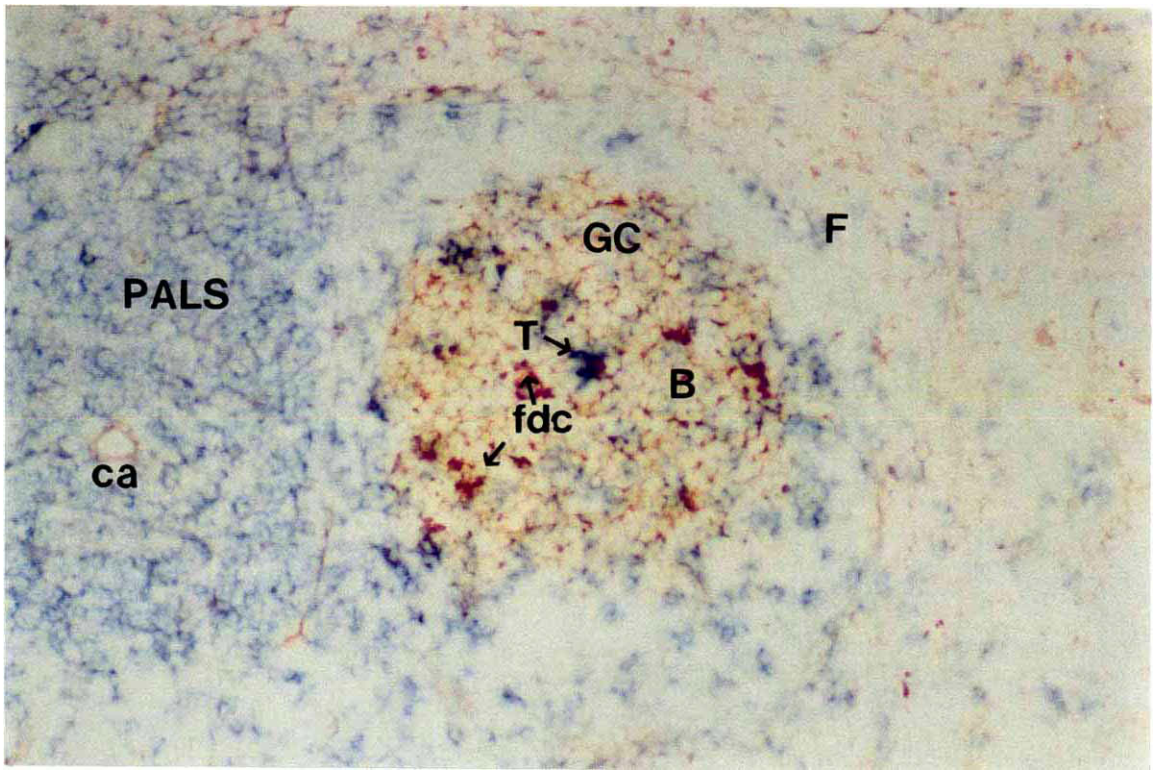

FIG. 1. Photomicrograph of a germinal center 12 days after primary immunization. The germinal center (GC) comprises a large population of antigen-specific B cells (B; stained tan) and smaller but requisite populations of follicular dendritic cells (fdc; stained red) and $CD4^+$ T cells (T; stained blue). Germinal centers form in the B cell region of the splenic white pulp, the lymphoid follicle (F), in close approximation to the T cell zone or periarteriolar lymphoid sheath (PALS). The periarteriolar lymphoid sheath contains many $CD4^+$ T cells (blue) that surround a central arteriole (ca); this blood supply is the only entry into the splenic compartment.

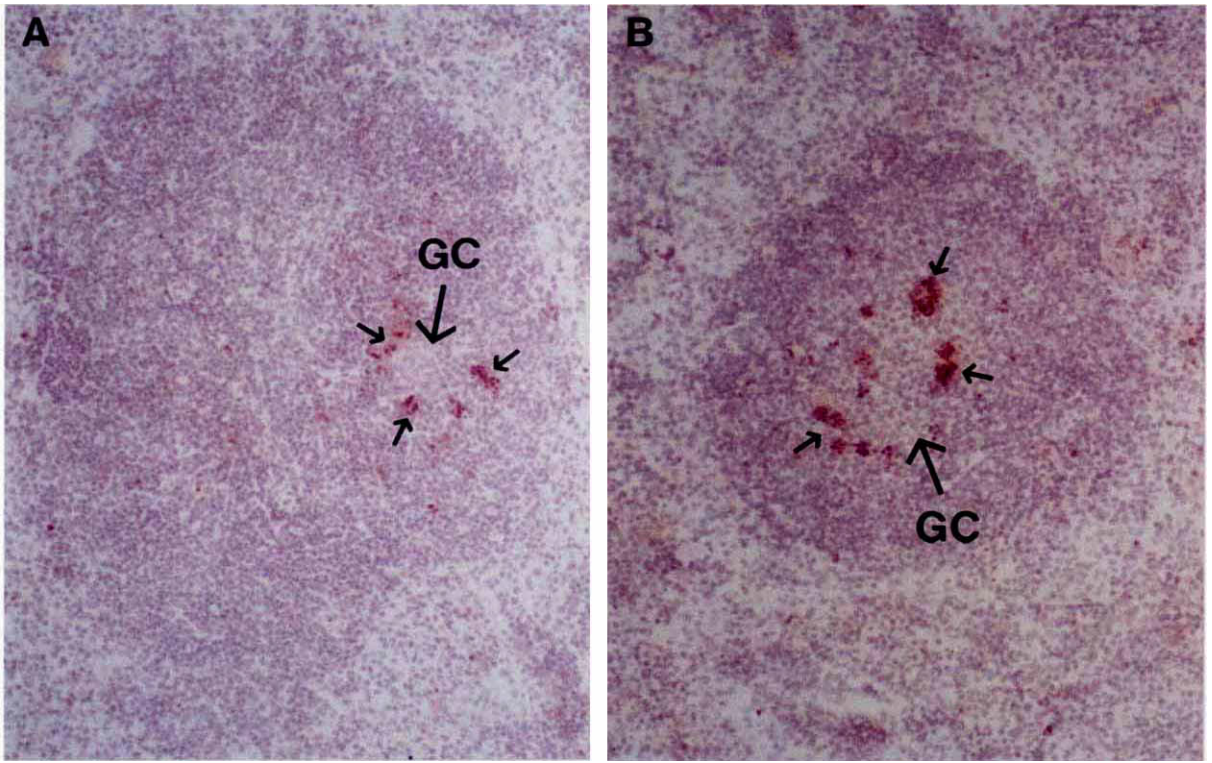

FIG. 5. Follicular regions within spleens of normal immune mice (A) nine days postimmunization or 6 hr after the injection of soluble antigen (B). Germinal centers (GC) are indicated and apoptotic cells (stained red) have been labeled by the TUNEL method (Gavrieli *et al.*, 1992). Normally, small numbers of germinal center cells become apoptotic and are rapidly engulfed by tingible body macrophages. Injection of soluble antigen induces rapid and massive B cell apoptosis that is antigen specific and avidity dependent. This process seems to be the result of cross-linking surface Ig on the light zone centrocytes either directly or by preventing necessary interactions with follicular dendritic cells (Han *et al.*, 1995b). This process may represent a mechanism for the maintenance of peripheral tolerance to abundant self antigens.

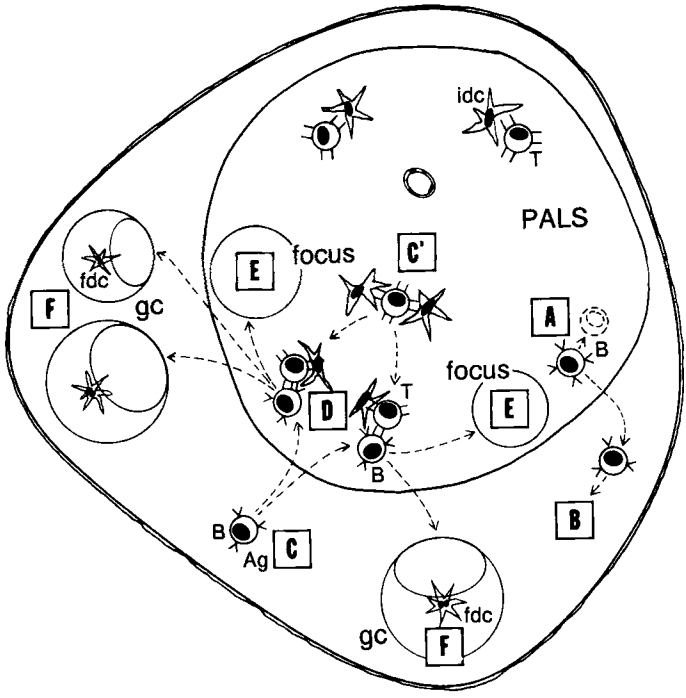

FIG. 2. Diagram of the splenic white pulp and the cellular reactions leading to a primary humoral response. The white pulp is enclosed within a perforated marginal sinus and is composed of a central T cell zone, the periateiolar lymphoid sheath (PALS) and a surrounding B cell area, the lymphoid, or primary, follicle. Newly produced, naive B lymphocytes migrate from the bone marrow to the PALS (A). Here they undergo a poorly understood "sorting" procedure that allows some cells to enter the follicle and causes apoptosis in others (Cyster *et al.*, 1994; MacLennan, 1995); favored cells enter the recirculating follicular pool (B). After immunization, follicular B cells are believed to acquire antigen and migrate into the PALS (C) while helper T lymphocytes first encounter antigen processed by interdigitating cells (idc) (C'). Antigen-activated B and T lymphocytes begin cognate interaction in the peripheral zone (D) of the PALS to initiate the humoral response. In the presence of specific T cell help, proliferating B cells either remain at the follicular boundary and differentiate into foci of lymphoblasts and plasmacytes (E) or migrate back into the follicle, forming germinal centers (gc) within a reticulum of follicular dendritic cells (fdc) (F).

1994) or the administration of antibodies (Hathcock *et al.*, 1993; Jabara *et al.*, 1990; Foy *et al.*, 1993, 1994) or fusion proteins (Lane *et al.*, 1994; Linsley *et al.*, 1992; Ronchese *et al.*, 1994; Lenshow *et al.*, 1992) that disrupt T/B collaboration mediated by CD40L or the B7 costimulators block or reduce antibody production and the GC reaction by disrupting the earliest interactions between T and B lymphocytes in the PALS (Lane

et al., 1994). Mice that cannot express MHC class II molecules exhibit little or no GC reaction (Cosgrove *et al.*, 1991) and the depletion of complement, either by the administration of cobra venom factor (Klaus *et al.*, 1977) or by genetic disruption (M. Carroll, Harvard University, personal communication), reduces the number of splenic GCs by interfering with the trapping of antigen–antibody complexes by FDC (Fig. 3).

Signals necessary for the maintenance of GC structure and continued follicular proliferation and differentiation have been studied by injecting antibody specific for CD40L or B7-2 6 days after the administration of the Td antigen, NP–CGG (Han *et al.*, 1995a). This late administration of passive antibody had no effect on serum antibody titers but significantly affected splenic GCs.

After treatment with anti-CD40L antibody, only the residua of GCs remained in follicles demonstrating that continuation of the GC reaction depends on the availability of CD40L. This disruption was virtually complete by 12 hr postinjection and, surprisingly, was not the result of B cell death (Liu *et al.*, 1989), but rather of the migration of IgD^- GC B cells from the follicles (Han *et al.*, 1995b). At least some of the migrant cells reentered the PALS (Han *et al.*, 1995b). Thus, while CD40L:CD40 interaction in GCs may be a necessary component in GC cell activation (Noelle *et al.*, 1992; Van den Ertwegh *et al.*, 1993), the *absence* of this interaction may itself provide the stimulus for selected memory B cells to migrate to other sites.

In contrast to the disruption of GCs by anti-CD40L antibody, administration of the anti-B7-2 antibody, GL-1 (Hathcock *et al.*, 1993), arrested GC development but did not destroy established GCs (Han *et al.*, 1995a). This

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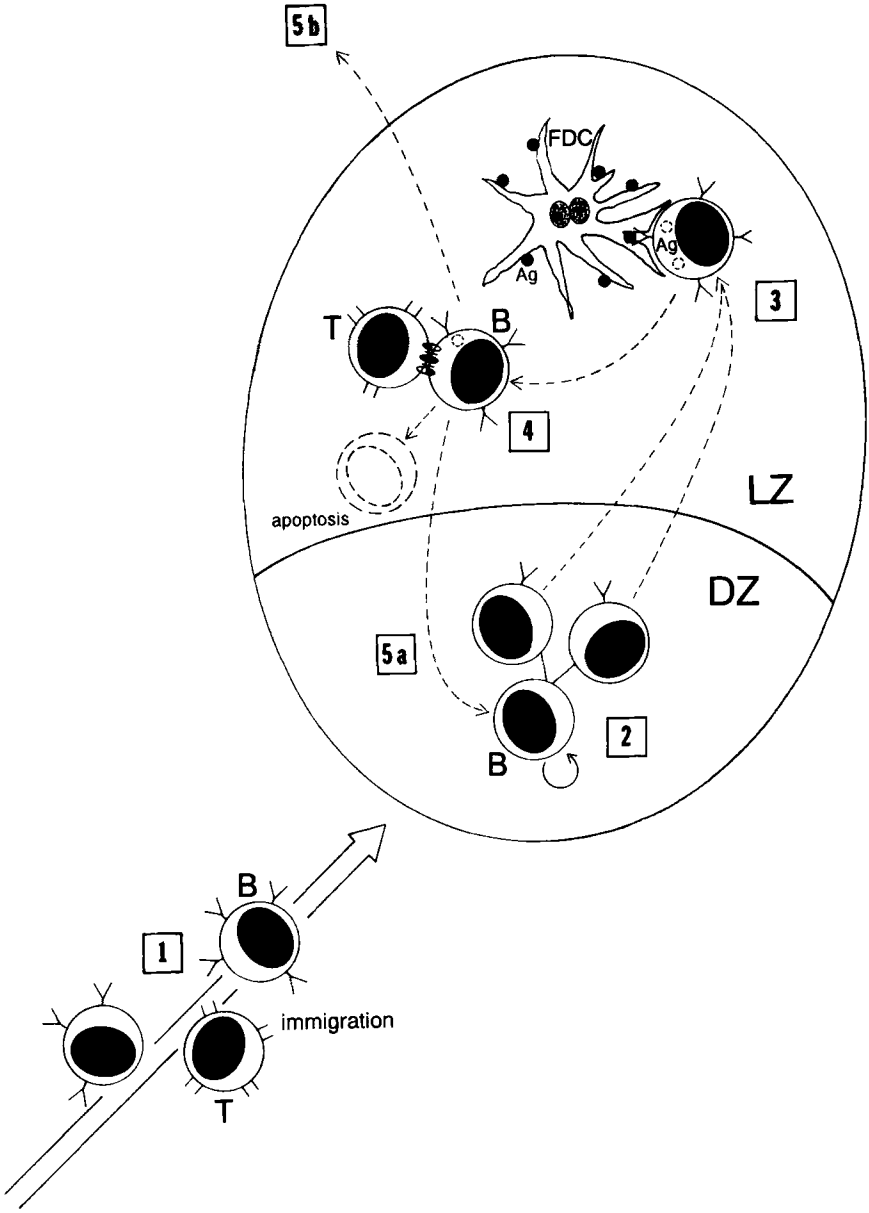
FIG. 3. A model for the dynamics of germinal center cell populations. Antigen-specific T and B cells migrate from the periarteriolar lymphoid sheath as a consequence of their activation (1). B cells initially proliferate as an undefined cell mass, but by Days 6 or 7 of primary responses, the germinal center becomes partitioned into a dark zone (DZ) of rapidly dividing centroblasts (2) and a light zone (LZ) containing nondividing B cell centrocytes, follicular dendritic cells (FDC), and $CD4^+$ T lymphocytes. It is thought that as the DZ B cells move into the LZ they acquire a higher density of surface Ig and become activated through encounters with antigen (Ag) held on FDC (3). Antigen is removed from the FDC surface, internalized and processed, and reexpressed in association with class II MHC. In this way, LZ centrocytes become antigen-presenting cells for the GC T lymphocytes and collaborate with them via interactions dependent on antigen, MHC, and costimulators such as CD40L:CD40 and B7-2:CD28/CTLA-4. In the absence of these events (4), centrocytes are expected to undergo apoptosis. Unknown signals return the selected centrocytes to the DZ for another round of proliferation/mutation (5a) or to leave the germinal center (5b) as a memory cell.


5b
FDC
Ag
Ag
3
T
B
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apoptosis
LZ
DZ
5a
2
B
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1
immigration
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developmental arrest was evident as retarded GC B cell proliferation, an approximately five-fold decrease in the number of Ig V-region mutations, and failure to generate humoral memory. These effects did not represent a generalized enfeeblement of the immune response since the GL-1 antibody had no effect on serum antibody levels or on T cell priming (Han *et al.*, 1995a). Signals mediated via the B7-2 costimulator appear to be responsible for driving GC B cells into the memory compartment.

B. Responses to Thymus-Independent Antigens

Although the GC reaction is strictly thymus dependent (Jacobsen *et al.*, 1974; Gastkemper *et al.*, 1981; Vonderheide and Hunt, 1990; Stedra and Cerny, 1994), several thymus-independent (Ti) antigens, including haptenated LPS (Liu *et al.*, 1991), some dextrans (Wang *et al.*, 1994), and a pneumococcal vaccine (Stedra and Cerny, 1994), have been demonstrated to elicit the formation of GCs or GC-like reactions within splenic follicles. Although the response to Ti antigens has several distinctive characteristics (Liu *et al.*, 1991; Pettersen *et al.*, 1967), many cellular events are shared between primary Td and Ti responses. For example, both antigen types induce an early accumulation of activated, proliferating B cells within the PALS (Stedra and Cerny, 1994; Liu *et al.*, 1991). This observation suggests that migration to T cell zones is the outcome of any activation event mediated through cell surface Ig. Similarly, reentry into the lymphoid follicle also appears to be a fundamental response of B cell proliferation/activation within the PALS (Liu *et al.*, 1991).

The kinetics of the GC reaction induced by Ti antigens is very similar to that seen in primary Td responses as judged histologically (Stedra and Cerny, 1994; Liu *et al.*, 1991) or by the appearance of PNA^+ splenocytes (Wang *et al.*, 1994). In the case of the GCs formed in response to LPS–hapten, the follicular reaction was not judged to be as intense as that observed in carrier-primed animals given a Td–hapten conjugate (Liu *et al.*, 1991). However, responses to pneumococcal vaccine are substantial, with some 60–100% of follicles containing antigen-specific GCs (Stedra and Cerny, 1994).

Humoral immune memory is not generally believed to be established by Ti antigens although increases in IgM precursors and increased levels of IgM serum antibody have been observed (Stedra and Cerny, 1994). Indeed, this nonclassical anamnesia can be established by Td antigens in the absence (or very low numbers) of T cells and with no detectable antibody response (Roelants and Askonas, 1972; Diamantstein and Blitstein-Willinger, 1974). Thus, it is interesting that immunization of rats with haptenated LPS results in the presence of hapten-specific B cells in splenic marginal zones for at least 3 months (Liu *et al.*, 1991); it is this

site that becomes colonized by the memory B lymphocytes generated during T cell-dependent antibody responses (Liu *et al.*, 1988).

The issue of somatic hypermutation within GCs induced by Ti antigens is not yet resolved. Mutation and selection have been observed in primary antidextran hybridomas (Wang *et al.*, 1990; Akolar *et al.*, 1987) but mutation to pneumococcal vaccine is absent in phosphorylcholine-specific GCs at times when Ig hypermutation to Td antigens has reached maximal levels (C. Miller and G. Kelsoe, unpublished data; Miller *et al.*, 1995). Perhaps type II Ti antigens, such as $\alpha(1 \rightarrow 6)$ dextrans or pneumococcal polysaccharides (Jacobsen *et al.*, 1974; Gastkemper *et al.*, 1981; Vonderheide and Hunt, 1990; Stedra and Cerny, 1994; Liu *et al.*, 1991; Wang *et al.*, 1994), induce mutation, but with a kinetics (and mechanism?) different from that observed in response to Td protein antigens.

IV. Somatic Hypermutation, Selection, and the Generation of Immunological Memory

A. Hypermution in Immunoglobulin H- and L-Chain Genes

In the spleen, it is only within the PNA^+ GCs that antigen-specific B cells acquire mutations in active, rearranged Ig V-region genes (Nossal *et al.*, 1964; Zheng *et al.*, 1994; Noelle *et al.*, 1992; Hathcock *et al.*, 1993). In conjunction with selection, this process of Ig somatic hypermutation (briefly reviewed in Berek and Ziegner, 1993) is necessary for the affinity maturation of serum antibody. Hypermutation is initiated on days 6–8 of the primary response, coincident with the polarization of the GC into a LZ and DZ (Liu *et al.*, 1991; MacLennan *et al.*, 1990). Mutations accumulate at a steady rate at least until day 16 by the iterative incorporation of approximately one to three nucleotide substitutions (Jacob *et al.*, 1991a,b, 1993; McHeyzer-Williams *et al.*, 1993; Clarke *et al.*, 1985). This process results in clonal genealogies that recapitulate the repeated rounds of mutation, selection, and proliferation that take place in the GC.

The mechanism of Ig hypermutation is unknown but it introduces a distinctive pattern of nucleotide misincorporations. Characteristically, hypermutation favors transition mutations and exhibits biased exchange at template A:T nucleotide pairs where the frequency of $A \rightarrow N$ exchange may be two- or threefold above the reciprocal $T \rightarrow N$ event (Jacob *et al.*, 1993; Golding *et al.*, 1987; Both *et al.*, 1990; Weber *et al.*, 1991; Pascual *et al.*, 1994; Betz *et al.*, 1993a,b). Independently, Betz *et al.* (1993) and Rogozin and Kolchanov (1992) identified sequence motifs (C/A AG C/T T) within $V_{H/L}$ exons that are intrinsic mutational hot spots; also, there is wide agreement that hypermutation is preferentially targeted to one strand

of the DNA molecule (Jacob *et al.*, 1993; Golding *et al.*, 1987; Both *et al.*, 1990; Weber *et al.*, 1991; Pascual *et al.*, 1994; Betz *et al.*, 1993a,b).

Soon after their introduction, the frequency and distribution of Ig V-region mutations are modified by phenotypic selection (Jacob *et al.*, 1993; Berek and Ziegner, 1993; Kocks and Rajewsky, 1988; Weiss and Rajewsky, 1990). Thus, early in the response, mutations are uniformly distributed within the V_H exon but with time they become focused within the complementarity-determining regions (CDRs). Similarly, ratios of replacement: silent (R:S) mutations initially approximate random values but with time become biased toward S mutations within the framework areas of V gene segments and toward R mutations in CDRs (Jacob *et al.*, 1993; Clarke *et al.*, 1985; Pascual *et al.*, 1994; Kocks and Rajewsky, 1988; Weiss and Rajewsky, 1990).

B. Locus-Specific Hypermutation in T Cell Antigen-Receptor Genes

Although the GC is widely accepted as the histologic site for Ig hypermutation, recent work (Zheng *et al.*, 1994) indicates that the V regions of TCR α-chains also mutate there.

B10.A(H-2^k) mice were immunized with pigeon cytochrome c (PCC) coupled with NP. In this strain, more than 70% of the $CD4^+$ T cells that react to PCC bear TCR encoded by the $V_\beta 3$ and $V_\alpha 11$ gene families (Winoto *et al.*, 1986). These cells may be identified by immunohistology (Kelsoe and Zheng, 1993) and recovered by the microdissection of five to seven T cells from the PALS or GCs. Rearranged $V_\alpha 11$, $V_\beta 3$, V_H (V186.2), and $V_\lambda 1$ genes in the recovered cells were amplified in a PCR using the *Pyrococcus furiosus* (*Pfu*) polymerase (Lundberg *et al.*, 1991). Cloned V(D)J inserts were then sequenced and compared to the germline $V_\alpha 11$, $V_\beta 3$, V186.2, and $V_\lambda 1$ genes to identify any sequence differences. The frequencies of misincorporations in $V_\alpha 11$ genes recovered from the PALS (2.1×10^{-4} bp^{-1}), in $V_\beta 3$ genes in GC cells ($<0.5 \times 10^{-4}$ bp^{-1}), and in $V_\lambda 1$ GC rearrangements ($<0.5 \times 10^{-4}$ bp^{-1}) did not significantly differ from that observed in a PCC-specific T cell line (0.7×10^{-4} bp^{-1}) maintained *in vitro* and probably reflected the intrinsic error rate of the *Pfu* polymerase. As expected, by day 16 postimmunization, V186.2 V_H genes recovered from NP-specific GCs contained a significantly higher frequency of mutations (53.8×10^{-4} bp^{-1}) demonstrating active V(D)J hypermutation in GCs. Surprisingly, $V_\alpha 11$ rearrangements amplified from GC T cells also contained a significant excess of mutations (14.2×10^{-4} bp^{-1}). These TCR α-chain mutations could not be accounted for by polymerase error, were confined to the V region of the TCR α-chain gene, and did not represent artifactual chimeric sequences generated *in vitro*. Mutated $V_\alpha 11$ exons

were not unrecognized members of the $V_\alpha 11$ gene family, as amplification of unrearranged genomic DNA from liver revealed no V_α exons that contained the mutant nucleotides. However, this search did reveal an undescribed member of the $V_\alpha 11$ family that resembled the known $V_\alpha 11.3$ gene segment (Zheng *et al.*, 1994; B. Zheng and G. Kelsoe, unpublished data). Importantly, related sequences containing shared and unique mutations could be isolated from single GCs, implying that mutations occurred as an active process in proliferating GC T cells.

Mutations in $V_\alpha 11$ genes were uniformly distributed within the V_α exon but were not random, exhibiting a marked bias for A → G transitions with A → N exchanges more than twice as common as the reciprocal T → N events. This pattern of biased nucleotide substitution and DNA strand polarity is characteristic of Ig hypermutation (Betz *et al.*, 1993a,b; Both *et al.*, 1990; Golding *et al.*, 1987; Jacob *et al.*, 1993; Pascual *et al.*, 1994; Weber *et al.*, 1991) and while the observed mutations differed significantly from spontaneous unselected meiotic point mutations, they were indistinguishable from mutations flanking the coding regions of hypermutated Ig genes (Zheng *et al.*, 1994).

Although the ratio of productive:nonproductive (P:nP) V_α J_α rearrangements in GCs was comparable to that observed for $V_\beta 3$ and the Ig H-chain (7.7:1, 5.8:1, and 17:1, respectively), the P:nP ratio within the set of *mutated* V_α rearrangements was much lower (0.9:1) (Zheng *et al.*, 1994 and unpublished data). We have intrepreted this finding to indicate that mutant TCRs have a higher probability of conferring a disadvantageous phenotype and, like GC B cells, are selected against. This speculation was based on the observation by MacLennan (1994) that GC T cells contain very low or undectable levels of the apoptosis inhibitor, Bcl-2.

To test this hypothesis, we used the recently described *T*dt-mediated d*U*TP-(digoxigenin) *n*ick *e*nd *l*abeling (TUNEL) method (Gavrieli *et al.*, 1992) to identify apoptotic GC cells doubly stained with anti-CD4, anti-CD3, or anti-$V_\alpha 11$ antibody. $CD4^+$ GC cells do undergo apoptosis. Apoptotic T cells in PCC-elicited GCs are five-fold more likely to express a $V_\alpha 11^-$, $CD4^+$ phenotype than to express the canonical $V_\alpha 11^+$ TCR; apoptosis is associated with a decrease in $V_\alpha 11^-$ GC T cells such that by day 16 postimmunization, most (>85%) $CD4^+$ GC cells bear canonical $V_\alpha 11^+$ TCR (Zheng *et al.*, 1995).

Virtually all GC T cells are $CD4^+$ and $CD3^+$ and in rats and humans, GC T cells express site-specific markers (Vonderheide and Hunt, 1990; Bowen *et al.*, 1991). In mice, monoclonal antibodies specific for V_β and V_α gene family products have been used to identify antigen-specific T cells *in situ* (Kelsoe and Zheng, 1993; Zheng *et al.*, 1994; Fuller *et al.*, 1993). This ability permits histologic monitoring of antigen-activated T lympho-

cytes and has revealed evidence for clonal competition and selection that may reflect differences in TCR avidity (Kelsoe and Zheng, 1993; Zheng *et al.*, 1994; Fuller *et al.*, 1993; Kearney *et al.*, 1994).

C. Phenotypic Selection in Germinal Centers

As the immune response progresses, evidence for the phenotypic selection of GC B cells becomes increasingly apparent. In the anti-NP response of C57BL/6 mice, early GCs (days 4 and 6 postimmunization) contain an average of six unique VDJ joint sequences, indicating that at least six B cell clones had colonized each GC site (Jacob *et al.*, 1993). By day 8 of the response, clonal diversity in GCs drops significantly, such that on average, only 1.5 distinct VDJ sequences can be recovered from each GC at days 8–16 postimmunization. This reduction in population diversity is coincident with the onset of Ig hypermutation and is correlated with the loss of B cells expressing noncanonical V_H gene segments or VDJ rearrangements that did not utilize the usual D element of the anti-NP response, FL16.1 (Bothwell *et al.*, 1981). A similar phenomenon of early selection PNA^+ B cells was noted by McHeyzer-Williams *et al.* (1993) as the reduction in V_H CDR3 lengths over the first week of the anti-NP response.

Selection in GCs can also be inferred from the analysis of mutations with GC B cell populations. The proportion of VDJ rearrangements containing crippling mutations, e.g., misincorporations leading to termination codons or replacement mutations at invariant amino acid residues, falls from a maximum of 33% on day 8 of the primary anti-NP response to less than 3% by day 14 (Jacob *et al.*, 1993). Berek and colleagues (1991) were the first to show that the frequency of high-affinity B cell mutants becomes enriched in the GC population, presumably reflecting their selection. This selection has also been observed in the anti-NP response (Weiss *et al.*, 1992; McHeyzer-Williams *et al.*, 1993) and the distribution of mutations late in the primary anti-NP response mirrors that observed in the secondary response (Weiss and Rajewsky, 1990).

D. Maintenance of Self-Tolerance

Antigen-driven somatic diversification of V(D)J genes within GCs will, at some unknown rate, produce antigen receptors that acquire new specificities; a fraction of these may be autoreactive and potentially detrimental (Diamond and Scharff, 1984; Shlomchik *et al.*, 1991). However, reduction of clonal diversity in GC T and B cell populations and the necessity of cognate interaction to maintain the GC response suggest a mechanism for the elimination of mutants that lose reactivity for the immunogen or gain some new specificity.

Consider an ideal GC containing single, selected T and B cell clones specific for the immunizing antigen (Fig. 4). B cells (centrocytes) emerge from the DZ and acquire antigen from FDC, causing MHC class II and B7-2 molecules to be upregulated. These cells can then present processed

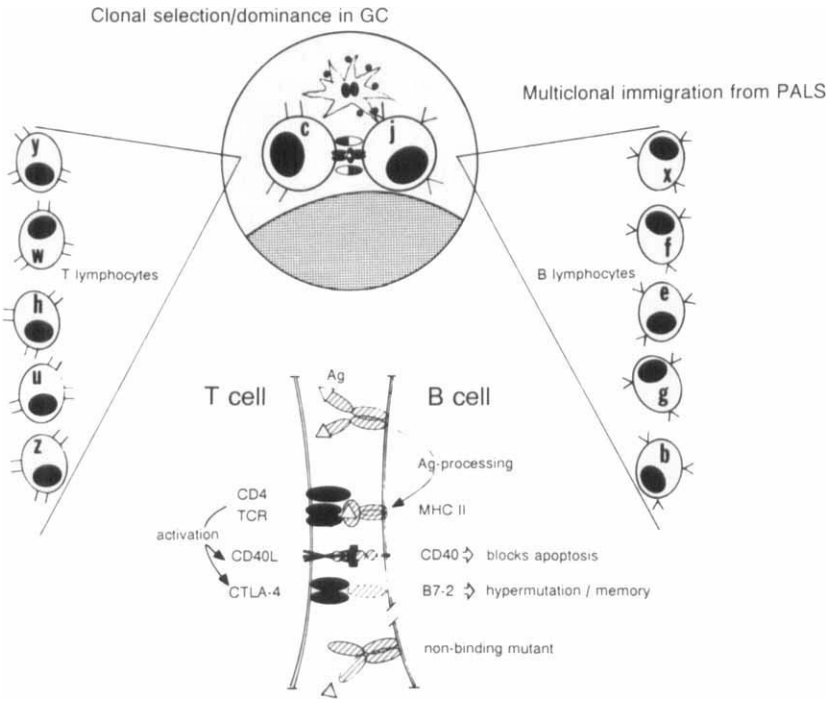

FIG. 4. Oligoclonality of T and B populations in germinal centers serves to prevent the expansion of mutant lymphocytes reactive to new determinants. B and T lymphocytes activated by antigen in T cell zones migrate into the germinal center. With time, single-dominant B and T cell clones arise, maintaining the germinal center through cognate recognition and signals provided by costimulators. Note that if a B cell loses the ability to bind the antigen for which both it and resident T cells are specific, it will be unable to receive the cognate T cell signals believed necessary to prevent apoptosis (Liu *et al.*, 1989). Likewise, should a B cell mutant acquire specificity for some other splenic antigen, *e.g.*, serum albumin, it will still fail to activate germinal center T cells and should undergo programmed cell death. Even if a cross-reactive mutant receptor arises, the reduced ability of the mutant cell to present the immunizing antigen would be impaired and such clones suffer a significant competitive disadvantage. These arguments apply equally well to mutant T cells. The specificity cross-checking inherent in the collaboration of single T and B cell clones would *not* prevent the expansion of mutant receptors that become cross-reactive for antigen(s) not present in the germinal center but retain high affinity for the immunogen or, in the unlikely case of simultaneous, complementing mutations in both T and B residents.

antigen to GC T helper cells to sustain the GC reaction. Note that B cells that lose the ability to recognize antigens on the FDC will be unable to participate in this collaboration and will die by apoptosis. More significantly, any B cells that acquire specificity for some new antigen *that is available within the GC* will also die as they too will be unable to collaborate with the single resident T cell clone. This simple process of specificity cross-checking should also serve to prevent the broadening of the GC response. B cell mutants that retain their ability to bind the immunizing antigen but gain cross-reactivity for self-determinants present in the GC (e.g., serum albumin) remain able to collaborate with their T cell partners, but with reduced efficiency. It seems likely that such cross-reactive populations would suffer a competitive disadvantage for critical T cell signals and would be overgrown by singly specific cells. These processes would operate equally well to ensure the specificity of GC T cells.

The GC may also maintain tolerance to self directly by the elimination of autoreactive clones. Recently, Han (1995b) developed an experimental model to study the fate of GC B cells that acquire specificity for abundant soluble antigens present in the GC. C57BL/6 mice were immunized with NP–CGG; 9 days later animals received an intraperitoneal injection of 1–4 mg of NIP–BSA. The primary NP-reactive B cells in C57BL/6 mice are heteroclitic, binding NIP with 10-fold higher affinity (Imanishi-Kari and Makela, 1973). Administration of NIP–BSA induced massive and rapid apoptosis in GCs but not elsewhere in the spleen (Fig. 5). Cell death peaked by 5–8 hr postinjection and apoptosis was largely confined to the GC LZ, suggesting that the $Ig^{low/negative}$ centroblast population was unaffected. Apoptosis was antigen specific, dose dependent, and sensitive to hapten density, indicating that cell death was mediated directly by surface Ig engagement.

Nevertheless, as GC T and B cell populations are specific for the immunizing antigen (see above), the effects of NIP–BSA could have resulted from interference in cognate T/B collaboration. To test this possibility, we injected 4 mg of NIP coupled to the homologous carrier protein, CGG. NIP–CGG conjugates induced apoptosis in GCs with the same kinetics of NIP–BSA, ruling out any carrier effects (Han *et al.*, 1995b).

Additionally, two other experiments were carried out to determine the role of T/B interaction in soluble antigen-induced GC cell death. First, immune mice were injected with the MR1 anti-CD40L antibody instead of soluble antigen. Although doses of MR1 as high as 900 μg were given, GC apoptosis increased only slightly above background levels, immediately prior to the onset of MR1-induced cell migration (see above; Han *et al.*, 1995b). Second, identical experiments were performed in C57BL/6 mice congenic for the *lpr* locus. Mice homologous for *lpr* cannot express

the Fas death trigger and their lymphocytes are resistant to the pathway of programmed cell death initiated by Fas:FasL cross-linking (Watanabe-Futunaga *et al.*, 1992; Adachi *et al.*, 1993; Wu *et al.*, 1993; Chu *et al.*, 1993; Daniel and Krammer, 1994; Ju *et al.*, 1994; Rothestein *et al.*, 1995). C57BL/6 *lpr* mice were fully susceptible to antigen-induced GC apoptosis, further supporting our interpretation that free antigen causes death by directly cross-linking surgace Ig or by preventing centrocytes from interacting with the FCC.

Recent work by Pulendran *et al.* (1994) has also demonstrated a form of immunological tolerance mediated through the absence of T cell help within GCs. Interestingly, although this tolerance mechanism appears distinct from that described previously, this T-dependent process also affected only follicular responses, i.e., GCs but not PALS-associated foci. Thus, it is possible that GC B cells are uniquely sensitive to tolerance induction—as if recapitulating that susceptibility observed in primary lymphopoiesis.

E. Humoral Memory

Disruption of GCs within the first 6 days of the primary response blocks or impairs formation of B cell memory (Klaus *et al.*, 1980; Coico *et al.*, 1983; Lane *et al.*, 1994; Han *et al.*, 1995a). However, the memory state, the physiology of the selected B cells that leave the GC as the response wanes, is virtually unknown. Our own work has illustrated the tight linkage between Ig hypermutation and secondary response kinetics (Han *et al.*, 1995a) by blocking both with anti-B7-2 antibody. This treatment leaves GCs intact but appears to interfere with differentiation into the memory compartment.

Recent work by Pascual and colleagues (1994) describes the partitioning of human tonsilar GC B cells into five developmental compartments by multiparameter flow cytometry. This work offers the first real possibility for a molecular biologic study of humoral immune memory. Undoubtedly, the near future will bring much new information in this area.

V. Germinal Centers and Other Microenvironments for Diversification

In certain species, developmentally regulated, i.e., antigen-independent, diversification of Ig V-region genes continues after V(D)J recombination. In chicken (Reynaud *et al.*, 1985), rabbits (Becker and Knight, 1990; Weinstein *et al.*, 1994), and sheep (Reynaud *et al.*, 1991) B cells migrate from regions of primary lymphopoiesis and colonize epithelial crypts along the gut mucosa. Here the immigrant cells proliferate to form prominent lymphoid follicles and undergo postrearrangement V-region diversification by (i) nonreciprocal incorporation of sequence fragments from unre-

arranged V exons (chickens), (ii) the introduction of point mutations (sheep), or (iii) both (rabbits). Even though birds, rabbits, and sheep represent widely divergent taxonomic orders, the many conserved features of these processes imply a single underlying mechanism; it would be remarkable if this complex developmental pathway for enriching the primary antibody repertoire arose independently three times during evolution.

Reynaud and colleagues (1995) have also demonstrated that the spectrum of mutations introduced into the V regions of sheep ileal Peyer's patch B cells are similar to the unselected mutations observed in passenger κ transgenes in mice by Betz *et al.* (1993a,b). This finding links antigen-driven and antigen-independent V(D)J diversification and raises the possibility that the GC is homologous to the more primitive gut-associated microenvironments. In contrast to the phylogenetically ancient character of V(D)J hypermutation (Wilson *et al.*, 1992; Hinds-Frey *et al.*, 1993; Greenberg *et al.*, 1995), the GC reaction is not thought to be present in cold-blooded vertebrates (Kroese *et al.*, 1985). This leads to the intriguing possibility that the GC has evolved from the phylogenetically earlier gut-associated follicles by placing the response under antigen-dependent T cell control.

VI. Conclusions

GCs represent a remarkable lymphoid microenvironment that functions to expand and diversify antigen-reactive lymphocyte clones. Although the GC reaction is antigen dependent, GCs share many of the processes usually thought to occur during primary lymphopoiesis, including positive and negative selection, elimination of self-reactive cells, and enrichment of the repertoire of antigen receptors. The mechanisms that underlie these processes remain unknown but it is clear that these issues may be approached genetically; naturally arising mutants, such as *lpr* or *gld*, transgenesis, or targeted gene disruption have already proved invaluable in understanding mutation, selection, and memory. If GCs are homologous to the more primitive gut-associated lymphoid follicles of sheep, rabbits, or chickens, phylogenetic studies may also be helpful.

In the future, immunologists will face the reconciliation of two views of the immune response—immunity as the interaction of regulating factors within cells and immunity as interacting cell populations. Both views provide unique insights but neither will provide a full understanding in isolation.

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Cytotoxic T Lymphocytes: The Newly Identified Fas (CD95)-Mediated Killing Mechanism and a Novel Aspect of Their Biological Functions

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I. Introduction

Cytotoxic T lymphocytes (CTLs) are effector cells that eliminate cells infected with viruses (Maimone *et al.*, 1986). CTLs have also been implicated in clearing cells infected with intracellular bacterial pathogens (Beaty and Stephens, 1994; Kaufmann, 1988) and protozoan parasites such as *Toxoplasma gondii* (Chardès *et al.*, 1994; Hakim *et al.*, 1991; Kasper *et al.*, 1992; Subauste *et al.*, 1991). CTLs form tight conjugates with target cells (Geiger *et al.*, 1982) by producing projections which push into the target cell (Sanderson and Glavert, 1979) and induce zeiosis in the target cells (Matter, 1979), accompanying DNA degradation (Russell and Dobos, 1980), and loss of adhesion (Russell *et al.*, 1988).

$CD8^+$ CTLs specifically recognize class I MHC molecules complexed with peptide fragments derived from cellular proteins (Townsend *et al.*, 1984, 1985, 1986; McMichael *et al.*, 1986; Yewdell *et al.*, 1985) and then destroy them without affecting adjacent cells not expressing the relevant antigens (Maimone *et al.*, 1986; Takayama *et al.*, 1991) (Fig. 1). Such a mode of cytotoxicity is quite suitable for efficiently eliminating "bad" cells from surrounding normal cells *in vivo*. This mode of cytotoxicity is ensured by the antigen specificity defined by the antigen receptor (TCR) of a CTL and by focused exposure of cytotoxic effector molecule(s) to the target cells. Such a mode of cytotoxicity is observed not only in class I-restricted $CD8^+$ CTL-mediated killing but also in killing by soluble antigen-specific class II-restricted $CD4^+$ T lymphocytes (Maimone *et al.*, 1986; Takayama et al., 1991; Ozdemirli *et al.*, 1992; Ju *et al.*, 1988; Erb *et al.*, 1990). On this basis, therefore, both $CD8^+$ T cells and $CD4^+$ T cells, regardless of the CD4/CD8 phenotype or of the class I/II MHC restriction element, could be dealt with equally as antigen-specific CTLs. By these criteria, CTL-mediated target cell killing should be distinguished from the cytotoxicity of natural killer (NK) cells or lymphokine-activated killer (LAK) cells, since NK cells and LAK cells destroy certain malignant transformed cells in general without showing apparent antigen specificity.

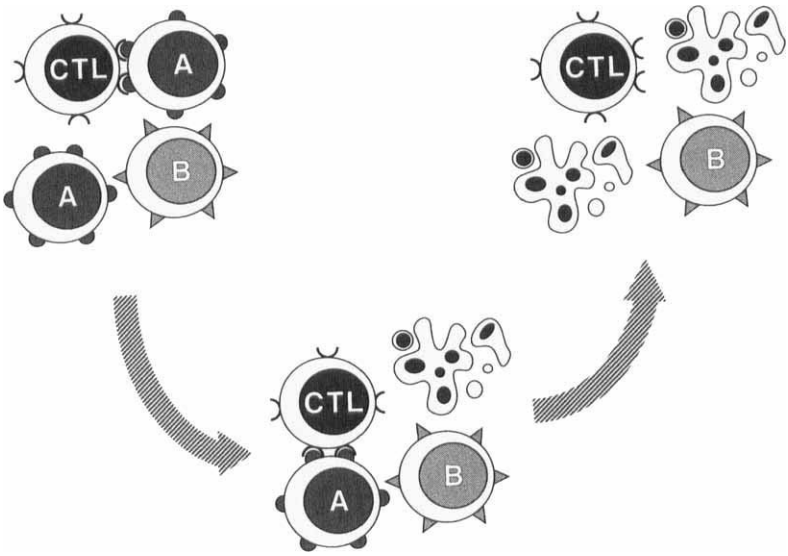

FIG. 1. Mode of target cell killing by cytotoxic T lymphocytes (CTL). CTL recognizes its target cells expressing specific antigen (target cells indicated as A), and destroys them one by one. This killing is highly directional and focused only on cells expressing the specific antigen; thus, the cells not expressing the antigen (indicated as B) are not damaged even if they are located near the target cell A.

The mechanisms of cytotoxicity operative in CTL-mediated killing have been extensively studied for years, and several candidates for the effector molecule have been reported. Any of these mechanisms should explain the specific and directional cytotoxicity mediated by CTLs. Soluble cytotoxic factors, such as tumor necrosis factor (TNF-α) and lymphotoxin (LT or TNF-β), produced by activated T cells would be toxic against all cells in the vicinity that carry receptors for these cytotoxins, regardless of the expression of specific antigen for recognition by the CTL. Therefore, it is not likely that these factors are involved in CTL-mediated antigen-specific directional killing (Tite, 1990). Perforin, a pore-forming protein first isolated from the lytic granules of a NK cell line and then from CTLs, has been indicated as the major effector molecule in cytotoxicity mediated not only by NK cells but also by CTLs (Masson and Tschopp, 1985; Tschopp *et al.*, 1986; Shinkai *et al.*, 1988a). Since perforin is stored along with the calcium-binding protein calreticulin (Dupuis *et al.*, 1993) in the cytosolic granules that are kept acidic by H^+-ATPase (Kataoka *et al.*, 1994), the "granule exocytosis model" had been claimed as the mechanism of lymphocyte-mediated cytotoxicity (Henkart, 1985; Tschopp and Nabholz, 1990;

Podack and Kupfer, 1991; Podack *et al.*, 1991; Yagita *et al.*, 1992). Indeed, the regulated secretion of a granule esterase, which is known to be colocalized with perforin (Peters *et al.*, 1989, 1991), has been demonstrated (Takayama and Sitkovsky, 1987; Takayama *et al.*, 1987; Pasternack *et al.*, 1986), and cinematographic analysis has supported the role of granule exocytosis in CTL function (Yannelli *et al.*, 1986). Nonphysiological elevation of intracellular calcium ion concentration in target cells after contact with CTLs has also indicated damage to plasma membrane integrity, possibly induced by perforation (Poenie *et al.*, 1987).

However, the existence of perforin-independent mechanisms in CTL-mediated cytotoxicity has also been supported by several lines of evidence (Strack *et al.*, 1990; Lancki *et al.*, 1991; Ostergaard and Clark, 1989; Berke *et al.*, 1993; Ucker *et al.*, 1994). One such line is the observation of CTL-mediated killing in the absence of extracellular calcium ion, which is mandatory for both exocytosis of cytolytic granules and polymerization of the released monomer perforin to polyperforin (Trenn *et al.*, 1987; Ostergaard *et al.*, 1987). In addition, anti-granule antibody preparation, which is capable of blocking NK cell-mediated killing, was found not to block CTL-mediated killing (Reynolds *et al.*, 1987). Thus, whether perforin is the major effector molecule in CTL-mediated cytotoxicity has been a major controversy (Berke *et al.*, 1991; Berke, 1994). Moreover, the fact that $CD4^+$ CTLs are capable of performing directional cytotoxicity in the absence of a detectable level of perforin expression also suggested the existence of perforin-independent, CTL–target contact-dependent killing mechanisms (Takayama *et al.*, 1991; Ju *et al.*, 1988; Erb *et al.*, 1990; Chang and Moorhead, 1986; Maimone *et al.*, 1986).

A perforin transfectant of rat basophilic leukemia (RBL) cells was shown to be hemolytic when the degranulation was triggered via FcεR (Shiver and Henkart, 1991). In addition, it was reported that after cotransfection with a granule serine esterase, granzyme A, RBL cells became cytotoxic against nucleated tumor cells as well as against red blood cells (Shiver *et al.*, 1992). Thus, this transfectant system appeared to serve as an elegant simulation of TCR-triggered CTL-mediated killing. Nevertheless, these experiments did not rule out the existence of perforin-independent mechanisms of CTL-mediated killing. On the other hand, it was reported that induction of cytolytic activity was only partially blocked by perforin antisense oligonucleotide when it was added to the CTL-inducing culture (Acha-Orbea *et al.*, 1990). Thus, it was possible that the residual activity was due to either the residual perforin activity or the perforin-independent cytotoxic mechanisms.

To overcome such ambiguity, it was obvious that knockout experiments with the perforin gene would be necessary. In 1994, four groups indepen-

dently reported the results of gene targeting experiments with perforin (Kojima *et al.*, 1994; Kägi *et al.*, 1994b; Lowin *et al.*, 1994a; Walsh *et al.*, 1994). They found that both perforin-dependent and perforin-independent mechanisms are operative in CTL-mediated cytotoxicity. The latter was found to be dependent on the Fas (CD95) molecule expressed on the target cells (Kojima *et al.*, 1994; Kägi *et al.*, 1994c; Lowin *et al.*, 1994b; Walsh *et al.*, 1994).

The basis for the redundancy of CTL killing mechanisms is not yet clear (Henkart, 1994; Ostergaard and Clark, 1989). However, it would be meaningful to postulate that, in addition to eliminating cells infected with intracellular pathogens, killing Fas-expressing B cells is a physiological function of CTLs that has not been fully considered previously (Vignaux and Goldstein, 1994). Since activated B cells are known to express the Fas molecule, and *lpr* and *gld* mice with mutations of Fas (Watanabe-Fukunaga *et al.*, 1992b) and Fas ligand (Takahashi *et al.*, 1994; Lynch *et al.*, 1994), respectively, are known to produce autoantibody, it appears possible that Fas-dependent $CD4^+$ CTLs play some role in eliminating undesirable B cells.

This chapter will summarize the recently revealed cytotoxic mechanisms operating in the antigen-specific target cell killing by CTLs. The possible immunoregulatory role of CTLs in antibody responses will also be discussed.

II. Cytolytic Mechanisms Operative in CTL-Mediated Antigen-Specific Killing

A. Inactivation of the Perforin Pathway of CTL-Mediated Cytotoxicity by Targeted Disruption of the Perforin Gene

Perforin cDNA was cloned in mouse (Lowrey *et al.*, 1989; Kwon *et al.*, 1989; Shinkai *et al.*, 1988b), rat (Ishikawa *et al.*, 1989), and human (Lichtenheld *et al.*, 1988; Shinkai *et al.*, 1989), and the functional domains in perforin were identified by peptide analysis (Ojcius *et al.*, 1991; Peitsch *et al.*, 1990). Monoclonal antibodies against murine perforin also became available, and thus the quantitative analysis of perforin expression and histochemical analysis became feasible (Kawasaki *et al.*, 1990). The perforin gene was mapped to chromosome 10 in mouse (Trapani *et al.*, 1990) and to 17q11–q21 in human (Shinkai *et al.*, 1989), and the genome structure was also revealed (Youn *et al.*, 1991; Lichtenheld and Podack, 1989, 1992; Trapani *et al.*, 1990), even though some discrepancy in the reported structures of the 5′ promoter region was evident (Podack *et al.*, 1991; Lichtenheld and Podack, 1992).

In these 5 years, generation of mice with an artificially introduced mutation in perforin gene became feasible. To obtain a clear answer to the

question of whether perforin is the mandatory effector molecule of CTL-mediated specific target cell killing, four groups used this gene targeting technology to obtain perforin-deficient mice (Kägi *et al.*, 1994b; Lowin *et al.*, 1994a; Walsh *et al.*, 1994) and CTLs (Kojima *et al.*, 1994). Three groups successfully generated perforin-deficient mice and examined CTL activity (Kägi *et al.*, 1994b; Lowin *et al.*, 1994a; Walsh *et al.*, 1994). Another group generated perforin-deficient CTL lines by *in vitro* allo-stimulation of lymphocytes isolated from chimeric mice in which only perforin-deficient lymphocytes could mature because of the lack of *RAG-2* gene product in the background somatic cells (Kojima *et al.*, 1994).

The first report by Kägi *et al.* demonstrated that, even though mature $CD8^+$ or $CD4^+$ T cells as well as $NK1.1^+$ NK cells developed normally in the absence of perforin, lymphocytic choriomeningitis virus (LCMV)-specific or vaccinia virus-specific CTL activities could not be induced, and NK cell-mediated killing measured by YAC-1 cell lysis was greatly impaired by the loss of perforin (Kägi *et al.*, 1994b). Cytolysis by alloreactive CTLs induced *in vitro* by primary MLC and cytolysis by *in vivo* primed CTLs in spleen and in peritoneal exudated lymphocytes were also undetectable. Thus, Kägi *et al.* concluded that perforin is indeed the major effector molecule required for clearance of virus infection and allospecific cytolysis. The possibility that the development or maturation of CTLs and not the killing activity was somehow impaired in perforin-deficient mice was ruled out by others. Spleen cells stimulated by MLC and established $CD8^+$ T cell lines from perforin-deficient mice have been found capable of producing as much interferon-γ as perforin-positive populations in response to the specific alloantigen (Kojima *et al.*, 1994; Lowin *et al.*, 1994a). In addition, granule serine esterase secretion by perforin-negative T cells induced by anti-CD3 antibody was also shown to be comparable to that by perforin-positive T cell preparations (Lowin *et al.*, 1994a). Therefore, perforin expression is not required for development and maturation of alloreactive $CD8^+$ T cells.

Notably, certain target cells, such as mastocytoma P815, lymphoma L1210, and RMA, were shown to be lysed in the absence of perforin (Kägi *et al.*, 1994b). Even though this cytolysis in the absence of perforin was not analyzed in detail in the first report, it was very likely that this killing indicated the existence of perforin-independent mechanisms, as correctly stated by Clark (1994). Indeed, cytolysis and DNA degradation by perforin-deficient T cells have also been described with P815, YAC-1, and 3T3 fibroblasts (Lowin *et al.*, 1994a) and with Con A blast target cells (Kojima *et al.*, 1994). These killing activities clearly indicated the existence of perforin-independent mechanisms in antigen-specific CTL-mediated cyto-

toxicity, even though these mechanisms were apparently dependent on the nature of target cells.

B. Fas/APO-1 (CD95) Pathway, a Perforin-Independent Mechanism of Antigen-Specific CTL-Mediated Target Cell Killing

Two groups independently first found that Fas and APO-1 antigen (CD95) were capable of transmitting signals that induce apoptotic cell death in the absence of complement (Yonehara *et al.*, 1989; Trauth *et al.*, 1989); these proteins were found to be identical by molecular cloning (Itoh *et al.*, 1991; Oehm *et al.*, 1992; Watanabe-Fukunaga *et al.*, 1992a). Accumulating evidence has indicated that Fas molecules on the target cells are involved in T-cell-mediated cytotoxicity, especially with T cell hybridoma and $CD4^+$ T cells (Hanabuchi *et al.*, 1994; Stalder *et al.*, 1994; Rouvier *et al.*, 1993; Ju *et al.*, 1994). Indeed, analyses of killing mechanisms mediated by perforin-deficient $CD8^+$ CTLs (Kojima *et al.*, 1994; Kägi *et al.*, 1994c; Lowin *et al.*, 1994b; Walsh *et al.*, 1994) have revealed that the Fas-dependent pathway is involved in perforin-independent $CD8^+$ CTL-mediated cytotoxicity.

Con A blasts prepared from mice carrying the *lpr* mutation, which have the insertion of a transposon in the Fas antigen gene (Adachi *et al.*, 1993) and thus express little or low levels of Fas (Watanabe-Fukunaga *et al.*, 1992b; Mariani *et al.*, 1994), were capable of stimulating CTLs for interferon-γ production, indicating that they expressed specific antigen for recognition by CTLs (Kojima *et al.*, 1994; Lowin *et al.*, 1994a). Nevertheless, target cells prepared from the *lpr* mutant mice were not lysed by perforin-deficient CTL lines, whereas the cells prepared from wild-type mice were lysed (Kojima *et al.*, 1994; Lowin *et al.*, 1994a; Kägi *et al.*, 1994c; Walsh *et al.*, 1994). Perforin-deficient CTLs also did not lyse target cells carrying the lpr^{cg} mutation (Kojima *et al.*, 1994), which has a substitution of an amino acid residue in the cytosolic domain of the Fas molecule (Matsuzawa *et al.*, 1990). These results indicated that cell death induced by perforin-deficient CTLs depends on expression of a functional Fas molecule on the target cells. Moreover, transfectants of Fas cDNA were lysed by perforin-deficient CTLs, whereas Fas-negative parent cells were not (Kojima *et al.*, 1994; Walsh *et al.*, 1994; Kägi *et al.*, 1994c). Therefore, lack of both perforin in CTLs and a functional Fas molecule on target cells totally abrogated CTL-mediated cytotoxicity, indicating that CTL-mediated antigen-specific killing can be explained by two distinct pathways: the perforin-dependent Fas-independent pathway and the perforin-independent Fas-dependent pathway (Fig. 2) (Henkart, 1994; Kojima *et al.*, 1994).

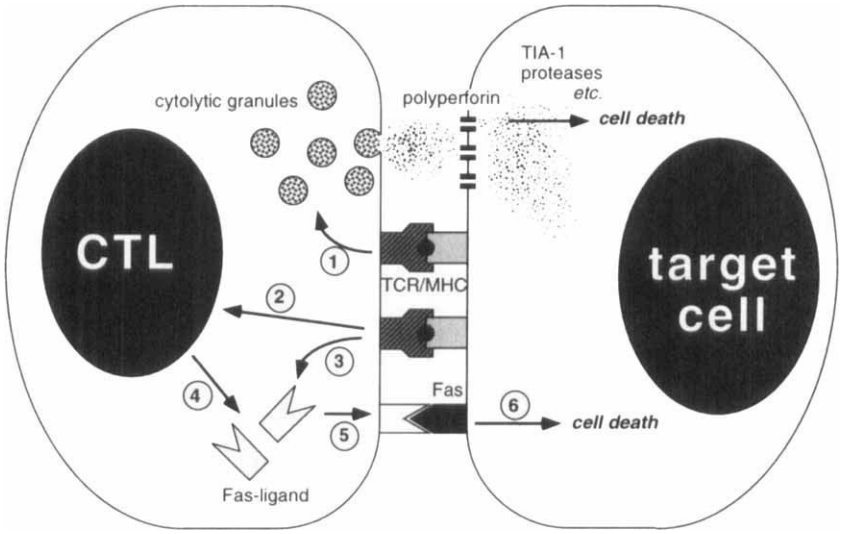

FIG. 2. Two pathways of target cell destruction mediated by CTLs. Upon recognition of specific antigen presented by MHC on the target cell, signals transmitted from the TCR/CD3 complex on the CTL, in cooperation with co-stimulatory signals, activate the cytotoxic machinery. One signal induces translocation and secretion of cytolytic granules (1), which contain perforin, proteases, and other cytotoxins such as TIA-1. These molecules cooperate to induce effective target cell death. The other signal links to activation of Fas ligand transcription (2) and/or to expression of functional Fas ligand molecules (3). It is not clear whether the *de novo* synthesis (4) and/or translocation of Fas ligand (5) is required for the Fas-mediated pathway (see Fig. 3). Upon interaction of the Fas ligand on CTLs and Fas on the target cells, Fas molecules transmit a "death signal" (6), biochemical details of which remain to be revealed.

Involvement of Fas in CTL-mediated cytotoxicity has been further confirmed by blocking experiments. An anti-Fas monoclonal antibody has been shown to block the lysis of Fas-transfected L1210 cells by perforin-deficient CTLs and by killer hybridoma cells, while cytolysis by perforin-positive CTLs was not blocked (Kägi *et al.*, 1994c). In contrast, it has also been reported that soluble Fas protein composed of the extracellular domain of the Fas molecule and an immunoglobulin Fc portion (Fas-Fc) failed to block perforin-deficient $CD8^+$ CTL-mediated killing, while $CD4^+$ T-cell-mediated killing was effectively blocked by the chimeric Fas protein (Kojima *et al.*, 1994).

A clear explanation for this discrepancy has not yet been provided. Different sensitivities between $CD8^+$ CTLs and $CD4^+$ T cells or T cell hybridomas to blocking by Fas-Fc chimera protein cannot be explained by the difference in the Fas ligands expressed on these T cells, since

soluble Fas should bind to any type of ligand for Fas and block the interaction between Fas and the ligand. If $CD8^+$ CTLs express Fas ligand at much higher levels than $CD4^+$ T cells or T cell hybidomas, it is possible that certain concentrations of soluble Fas block killing only by $CD4^+$ T cells and not by $CD8^+$ CTLs. However, staining of $CD8^+$ and $CD4^+$ T cells for Fas ligand expression indicated that the expression level on $CD8^+$ cells is very low even after stimulation (H. Yagita, personal communication).

Some other possibilities are also worth considering. It is suggested that functional Fas ligand is expressed only after CTLs are activated by the recognition of specific antigen, which can be bypassed by treatment with a combination of the phorbol ester PMA and the calcium ionophore ionomycin (Vignaux and Goldstein, 1994; Anel *et al.*, 1994; Suda *et al.*, 1993; Hanabuchi *et al.*, 1994). Therefore, if the antigen recognition and the expression of functional Fas ligand are confined to the tight contact area between the CTL and the target cell, exogenous soluble Fas-Fc would be excluded from the conjugation area and would not have access to the newly expressed Fas ligand (Fig. 3a). Since it is not clear so far whether the transcription of Fas ligand is obligatory for Fas-dependent CTL-mediated killing, it is also possible that triggering of the Fas ligand pathway involves translocation of ready-made Fas ligand from storage compartments to the cell surface (Fig. 3a) or transformation of Fas ligand from the inactive to the functional form. For instance, if the functional Fas ligand is blocked by an inhibitory "masking" protein in the resting state and is released upon activation, it would also be possible that the exogenous Fas-Fc is not capable of blocking Fas/Fas ligand interaction because of the lack of access to the masked epitope (Fig. 3b). Alternatively, it is also possible that $CD8^+$ CTLs utilize a ligand/receptor system other than the Fas/Fas ligand system, the receptor of which is physically or functionally associated with the Fas molecule and depends on the Fas signaling pathway in the target cells (Fig. 3c). If this is the case, soluble Fas molecules are not capable of blocking perforin-independent killing by $CD8^+$ CTLs.

C. Biochemical Events Involved in the Regulation of CTL Function

1. Role of Extracellular Calcium Ion

CTL-mediated target cell lysis in the absence of extracellular calcium ion was one of the used clues to identify the perforin-independent killing mechanism (Trenn *et al.*, 1987; Ostergaard *et al.*, 1987). It has been reported that Fas-mediated target cell killing by CTLs is an extracellular calcium-independent mechanism (Rouvier *et al.*, 1993). However, one report has shown that Con A blast targets are not lysed by a perforin-

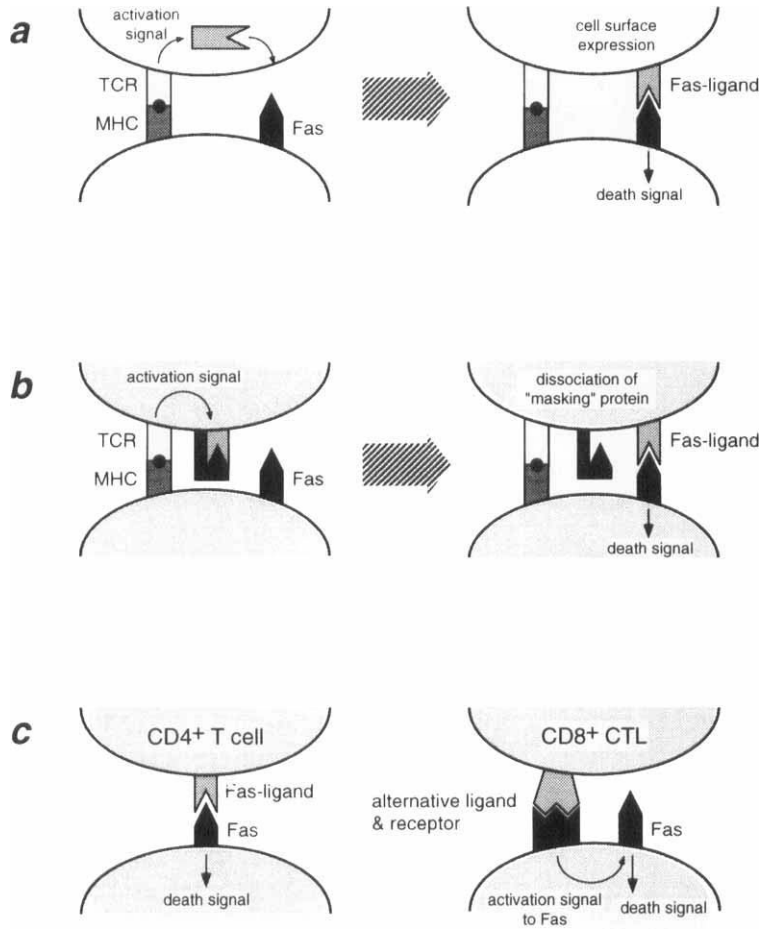

FIG. 3. Possible models of the interaction between Fas expressed on the target cells and Fas ligand expressed on CTLs. (a) Functional Fas ligand may be expressed only after CTL activation following recognition of the specific antigen. This induction may involve the *de novo* synthesis of Fas ligand or the translocation of "ready-to-go" ligand molecules to the cell surface. (b) Functional Fas ligand expression may be induced by transformation from the inactive to the active form. For example, dissociation of the "masking" inhibitory protein following recognition of the specific antigen could be hypothesized. This process does not require *de novo* protein synthesis. (c) $CD4^+$ T cells and $CD8^+$ CTLs may utilize different ligand/receptor systems. $CD4^+$ T cells and killer hybridoma express Fas ligand upon activation or constitutively, whereas $CD8^+$ CTLs may express ligand for the receptor that is functionally linked to the Fas-dependent signaling system to induce cell death.

deficient CTL line in the absence of extracellular calcium (Kojima *et al.*, 1994). One possible explanation for this discrepancy is that, while the CTLs reported earlier express Fas ligand constitutively, as does cytotoxic T cell hybridoma (Suda *et al.*, 1993; Rouvier *et al.*, 1993), the perforin-deficient $CD8^+$ CTL line described by Kojima *et al.* (1994) does not express Fas ligand constitutively and expresses it only after activation by antigen recognition. This activation of Fas ligand expression on CTL may require extracellular calcium, since cell death induced by anti-Fas antibody is known to be independent of extracellular calcium (Trauth *et al.*, 1989). Such activation of Fas ligand expression may involve signal transduction via TCR, transcription of Fas ligand and its translocation to the cell surface (Fig. 3a), or the transformation of Fas ligand from the inactive to the active form (Fig. 3b). Another, more likely, possibility is that the sensitivities of target cells for Fas-mediated cell death differ according to the nature of the cell and that extracellular calcium affects the sensitivity (Kojima *et al.*, manuscript in preparation). Further analysis will be required to resolve this issue.

2. *Protein Phosphorylation*

Protein phosphorylation by protein kinases is implicated in Fas ligand expression by CTLs, since treatment of CTLs with the combination of PMA and ionomycin induced prolonged expression of Fas ligand by CTLs (Vignaux and Goldstein, 1994; Anel *et al.*, 1994; Suda *et al.*, 1993; Hanabuchi *et al.*, 1994). Even though PMA/ionomycin treatment is also known to induce granule exocytosis (Takayama and Sitkovsky, 1987), it has been reported that such treatment preferentially induced Fas-dependent cytotoxicity rather than perforin-mediated cytolysis (Vignaux and Goldstein, 1994). This may be because of the difference in the biological stability of secreted perforin and that of cell-surface Fas ligand. Secreted perforin is immediately inactivated in the presence of calcium and a serum component (Podack *et al.*, 1991), while Fas ligand expressed on the cell surface does not lose its function immediately (Anel *et al.*, 1994; Vignaux and Goldstein, 1994; Suda *et al.*, 1993; Hanabuchi *et al.*, 1994). Nonspecific cytotoxicity by PMA-treated CTLs may also represent killing via the Fas pathway (Russell, 1986). Using inhibitors for protein tyrosine kinase (PTK), PTK has also been implicated in the regulation of Fas ligand expression induced by TCR signal (Anel *et al.*, 1994).

The activation of cyclic AMP-dependent protein kinases (PK-A) has been shown to downregulate CTL-mediated cytotoxicity as well as granule exocytosis (Gray *et al.*, 1988; Takayama *et al.*, 1988). The locus of PK-A action in this regulation of CTL function has been pinpointed to both early and late stages of signal flow (Gray *et al.*, 1988; Takayama *et al.*, 1988).

Protein phosphatases are also suggested to be involved in the regulation of CTL function. Okadaic acid, a specific inhibitor of type 1 and type 2A protein phosphatases, showed a biphasic effect on CTL-mediated killing and granule exocytosis, suggesting that at least two protein phosphatases participate in the CTL function, one in the downregulation of activities dependent on CTL–target contact (Taffs *et al.*, 1991). A Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, is also implicated (Trenn *et al.*, 1989; Lancki *et al.*, 1989), since cyclosporin A inhibited red blood cell lysis induced by redirected TCR signaling and granule exocytosis without affecting phosphatidylinositol turnover, but only partially or marginally inhibited antigen-specific target lysis by CTLs. Therefore, calcineurin may play some role in the late stages of granule exocytosis-dependent, i.e., perforin-dependent, cytotoxicity, but not in the Fas pathway (Trenn *et al.*, 1989; Lancki *et al.*, 1989).

D. Biochemical Events Involved in the Target Cell Death

1. Death Signal Transmitted from Fas

Until recently, biochemical signals transmitted from Fas antigen have been totally obscure (Schulze-Osthoff *et al.*, 1994). Many metabolic inhibitors, including PTK inhibitors, have failed to block Fas-induced death of murine fibrosarcoma L929 cells and human histiocytic cell line U937 (Schulze-Osthoff *et al.*, 1994). However, PTK activity has recently been implicated in the death signal transmitted from Fas antigen using PTK inhibitors on human lymphoma Jurkat cells and even U937 cells (Eischen *et al.*, 1994). The basis of the discrepancy between these two reports is not clear, since similar concentrations of reagents were used in both. The major difference is the detection of cell death; one study used flow cytometry to detect DNA degradation of dead cells (Eischen *et al.*, 1994), and the other used colorimetric assay with crystal violet to detect dead cells (Schulze-Osthoff *et al.*, 1994). This difference may affect the judgment of the effects of the inhibitors. Multiple proteins have been shown to be tyrosine phosphorylated within minutes after cross-linking of Fas antigen, and then dephosphorylated gradually (Eischen *et al.*, 1994). So far, the eventual consequence of these protein phosphorylations is not clear. Nevertheless, it could be postulated that the inhibitory function of a factor that blocks the death signal from Fas antigen is regulated by its phosphorylation/dephosphorylation (Rudert *et al.*, 1994).

2. Sphingomyelin (SM) Turnover

It has recently been reported that sphingomyelinase (SMase) is activated by cross-linking of Fas antigen (Cifone *et al.*, 1993). Since SM turnover has been implicated in the TNF receptor-mediated cell death signal, and

ceramide, a product of hydrolysis of SM by SMase, has effects on cells similar to those of TNF itself (Jarvis *et al.*, 1994), it was suggested that the death signal from the TNF receptor and Fas may share a common pathway (Clement and Stamenkovic, 1994). However, this observation has been challenged by contradictory reports (Schulze-Osthoff *et al.*, 1994; Grell *et al.*, 1994). The lack of involvement of NF-κB activation in the Fas signal is the major difference from the TNF signal (Schulze-Osthoff *et al.*, 1994). Since activation of NF-κB is known to be involved in both TNF-induced and ceramide-induced cell death (Reddy *et al.*, 1994), one would expect NF-κB to be activated by a Fas signal if Fas does promote SM turnover and release ceramide.

3. *Other Factors*

It has already been reported that *bcl-2* expression does not prevent cell death induced by CTLs (Vaux *et al.*, 1992; Strasser *et al.*, 1991) and that DNase I-like endonuclease responsible for apoptosis (Peitsch *et al.*, 1993) may participate in CTL-mediated DNA fragmentation (Ucker *et al.*, 1992). The involvement of these factors, however, was studied without considering the two pathways of cytotoxicity by CTLs; thus, it is possible that only one of the two involves these factors.

Involvement of DNA topoisomerases I and II has also been implicated in the target cell DNA fragmentation induced by lectin-mediated killing by CTLs (LDCC) (Nishioka and Welsh, 1992), although the observation was not confirmed with antigen-specific killing by a CTL clone (Takayama *et al.*, unpublished observation). This may also suggest that DNA toposiomerases are required for DNA fragmentation only in the Fas pathway or in the perforin pathway and that LDCC and antigen-specific killing by CTLs induce DNA fragmentation preferentially by only one of these pathways.

E. Other Effector Molecules Involved in CTL-Mediated Cytotoxicity

Several molecules other than perforin and Fas have been reported as candidates for the effector molecule responsible for CTL-mediated cytotoxicity. Among these, granzymes, proteases stored in the cytolytic granules of CTLs (Masson and Tschopp, 1987), have been shown to have important roles in effective killing and in inducing DNA degradation of target cells by CTLs (Nakajima and Henkart, 1994). Granzyme expression, as well as perforin (Nagler-Anderson *et al.*, 1989; Seko *et al.*, 1991; Young *et al.*, 1989a,b), has been detected *in vivo*, where CTLs are supposed to be activated and functioning (Griffiths *et al.*, 1991; Müller *et al.*, 1989). Granzyme has been shown to be secreted from granules into medium upon activation of CTLs (Takayama *et al.*, 1987; Takayama and Sitkovsky, 1987;

Pasternack *et al.*, 1986), providing evidence for the granule exocytosis model of CTL-mediated killing. Indeed, transfected granzyme A augmented perforin-mediated cytolytic activity, especially against nucleated target cells in the mast cell degranulation model system (Shiver *et al.*, 1992), while perforin alone was sufficient for lysis of red blood cells but not for lysis of nucleated target cells (Shiver and Henkart, 1991). Granzyme activity has been shown to be linked to induction of DNA degradation in permeabilized cells (Hayes *et al.*, 1989). This function was verified not only with granzyme A, but also with granzyme B by a knockout experiment using gene targeting technology (Heusel *et al.*, 1994). Even though the physiologic substrates for granzymes have not been identified, it has been shown that granzyme A binds to a nuclear protein, nucleolin, and cleaves it (Pasternack *et al.*, 1991). Such degradation of nuclear proteins by granzymes may contribute to the induction of DNA fragmentation induced by CTLs. Another protease, fragmentin, is also implicated in the induction of DNA degradation in cooperation with perforin (Shi *et al.*, 1992). Fragmentin-induced DNA degradation has been reported to involve the activation of $p34^{cdc2}$ kinase, suggesting that aberration from highly regulated cell cycles causes apoptotic cell death (Shi *et al.*, 1994).

Another group of granule proteins TIA-1 (Tian *et al.*, 1991) and related proteins (Kawakami *et al.*, 1992), is also reported to be capable of inducing DNA fragmentation when the target cells are permeabilized. The high-molecular-weight form of TIA-1 has a polyadenylate binding motif and thus may have an affinity to polyadenylated nuclear proteins (Tian *et al.*, 1991). However, since the small form of TIA-1 does not have the motif (Tian *et al.*, 1991), the implication of a polyadenylate binding motif of TIA-1 in CTL-mediated killing is not clear.

These intragranule proteins described above are likely to be delivered to target cell cytosol through polyperforin pores, since these are reported to induce DNA degradation only in permeabilized cells (Hayes *et al.*, 1989; Shi *et al.*, 1992; Tian *et al.*, 1991). Thus, these proteins may play some role in the perforin pathway of CTL-mediated killing. Fas-mediated cytotoxicity also causes cytolysis measurable by ^{51}Cr-release assay, even though apoptosis is generally considered cell death without changes in the permeability of the plasma membrane. If the membrane damage is the result of intracellular disintegration caused by the Fas signal, DNA degradation in the Fas pathway must involve mechanisms other than those of intragranule proteins.

Extracellular adenosine triphosphate (ATP) is another candidate because of its cytolytic activity (Zheng *et al.*, 1991; Zanovello *et al.*, 1990; Di Virgilio *et al.*, 1993). It has been shown that CTLs are capable of producing ATP in the medium upon activation (Filippini *et al.*, 1990b) and that CTLs

protect themselves from ATP-mediated damage by degrading it with ecto-ATPase (Filippini *et al.*, 1990a; Di Virgilio *et al.*, 1989; Redegeld *et al.*, 1993). To date, it is not clear whether ATP plays a major role in CTL-mediated killing as a cytotoxic factor, since some differences between ATP-induced cell death and CTL-induced cell death have been noted (Redegeld *et al.*, 1991; Avery *et al.*, 1992). Nevertheless, ATP may serve as a cofactor that promotes cell death effectively, especially in the Fas-mediated pathway, since the molecular mechanism of the destruction of membrane integrity in the Fas pathway is totally unknown. It is also possible that ATP is provided as a substrate for protein kinases that regulate CTL activity and target cell death through protein phosphorylation (Redegeld *et al.*, 1993; Sugiyama *et al.*, 1993).

A TNF-like factor bound on the CTL surface has been reported (Ferluga and Allison, 1975; Liu *et al.*, 1987, 1989). Even though the molecular biological entity of the protein remains unknown to date and the molecular weight described in the report does not fit, it is possible that the protein is either the cell surface lymphotoxin complex composed of TNF and lymphotoxin-β (Browning *et al.*, 1993; Kinkhabwala *et al.*, 1990), the receptor of which has been recently identified (Crowe *et al.*, 1994), or the constitutively expressed Fas ligand.

F. General Considerations of Specific vs Nonspecific Killing

As described in the introduction, the physiological function of CTLs is generally considered the antigen-specific elimination of cells infected with intracellular pathogens without affecting surrounding normal cells (Fig. 1). Thus, directional and focused target cell killing is a mandatory requirement for CTL-mediated antigen-specific cytotoxicity. Neither perforin nor Fas ligand can dictate target specificity. Preactivation of CTLs with nonpolar stimulation, such as PMA + ionomycin, converts CTLs into nonspecific killers. Despite the nonspecific nature of these effector molecules, however, specific killing by $CD4^+$ and $CD8^+$ CTLs is directed solely to the specific target and does not involve adjacent bystanders. This fact indicates that delivery or expression of these attacking molecules by CTLs is under strict regulation to avoid unwanted attack on nonspecific cells even after activation through TCR engagement. Elucidation of such regulatory mechanisms is essential to understanding the specific killing performed by CTLs.

Nevertheless, CTLs occasionally express antigen-nonspecific killing activity, so-called "LAK activity," as do NK cells and LAK cells. Nonspecific killing may be caused by any type of cytotoxic effector, i.e., soluble factors such as TNF and LT produced by activated T cells, especially with the CD4 phenotype (Schmid *et al.*, 1986; Ju *et al.*, 1990; Tite, 1990). These factors may also contribute to the killing of bystander cells on some occasion

that do not express the specific antigen for CTLs (Tite and Janeway, 1984; Chang and Moorhead, 1986). Fas ligand constitutively expressed on CTLs by PMA/ionomycin treatment or on killer hybridomas also causes nonspecific cytotoxicity. However, such constitutive expression of the Fas ligand *in vivo* would be very dangerous, since any Fas-positive tissue would be destroyed by circulating Fas-ligand-expressing lymphocytes. Indeed, injection of an anti-Fas antibody has been reported to be lethal for mice because of severe damage, especially in liver (Ogasawara *et al.*, 1993). Therefore, constitutive expression of Fas ligand is likely to represent an aberration from physiological control and an artifact of *in vitro* culture or of cell fusion.

III. CD4$^+$ CTLs: Fas-Dependent Class II MHC-Restricted CTLs and Their Possible Involvement in the Negative Regulation of Immune Responses

A. General Considerations

The primary role of the CTL *in vivo* is the elimination of pathologic somatic cells infected by intracellular parasitic organisms through recognition of class I MHC molecules complexed with peptide fragments derived from intracellular proteins. The biological implication of the redundancy of killing mechanisms in CD8$^+$ CTLs is not clear at present. Perforin, not requiring expression of a special receptor, is a universal weapon for attacking the cell membrane, a basic component of cells. On the other hand, the attack by Fas ligand requires expression of Fas on the surface of target cells and therefore is not a universal weapon. Primary *in vivo* CTLs have been reported to be free of perforin and dependent on the Fas pathway (Berke *et al.*, 1991, Rouvier *et al.*, 1993). Nevertheless, it is obvious that such CTLs cannot attack Fas-negative target cells. True understanding of the redundancy awaits further studies on the differences between perforin-mediated and Fas-mediated cell lysis, the regulation of expression of perforin and Fas ligand on the CTL, and regulation of expression of Fas on target cells in pathological situations. On the other hand, there are perforin-free T cells with lytic activity that recognize class II MHC molecules. Numerous CD4$^+$ T cells have been reported to be cytolytic (Tite and Janeway, 1984; Tite *et al.*, 1985; Nakamura *et al.*, 1986; Chang and Moorhead, 1986; Ozaki *et al.*, 1987; Watanabe *et al.*, 1987; Bourgault *et al.*, 1989; Shinohara *et al.*, 1991; Takayama *et al.*, 1991). The killing activity of such cells is antigen specific and strictly directional (Takayama *et al.*, 1991). Most of these cells are perforin free and their killing activity is entirely dependent on the Fas-dependent pathway (Takayama *et al.*, 1991; Hanabuchi *et al.*, 1994; Kojima *et al.*, 1994). Consequently, the targets for their killing are confined to Fas-expressing cells. Considering that class II

MHC molecules are involved mainly in presentation of extracellular antigens, it is rather difficult to imagine that such class II-restricted CTLs are capable of specific elimination of infected cells. Instead, accumulating evidence has raised the novel possibility of involvement of such CTLs in the specific negative regulation of immune responses (Shinohara *et al.*, 1988b, 1991).

B. Cognate Target Lysis by $CD4^+$ CTLs

There is ample evidence for induction of $CD4^+$ class II-restricted T cells with cytolytic activity in response to antigenic stimulation by soluble proteins (Tite and Janeway, 1984; Tite *et al.*, 1985; Nakamura *et al.*, 1986; Bourgault *et al.*, 1989). Furthermore, many cloned $CD4^+$ helper T cells were shown to be capable of lysing class II-positive B cells in the presence of the specific antigens (Chang and Moorhead, 1986; Ozaki *et al.*, 1987; Watanabe *et al.*, 1987; Shinohara *et al.*, 1991; Takayama *et al.*, 1991). Although early studies indicated that cytolysis by CD4 T cells was the secondary result of antigen-triggered secretion and accumulation of cytotoxic lymphokines such as TNF (Tite and Janeway, 1984; Tite, 1990), later studies revealed that the cytotoxic effect is mediated through cognate interaction and the killing effect is highly specific and directional (Maimone *et al.*, 1986; Ju *et al.*, 1988; Erb *et al.*, 1990; Takayama *et al.*, 1991; Ozdemirli *et al.*, 1992). It is now clear that in short-term (6–8 hr) assays, target cell lysis does not involve nonspecific lymphokines, such as TNF, and does not exert the effect on bystander nonspecific cells. When CML assays were prolonged to overnight (16 hr), TNF-mediated bystander killing activity became detectable (Takayama *et al.*, 1991; Ozaki *et al.*, 1987). Nevertheless, it appears unlikely that such a time-consuming pathway involving extracellular accumulation of lymphokines plays any significant role in the body, where there is constant flow of intercellular fluid. Indeed even in long-term cultures of antibody responses in which cells were not packed, the effect on B cells was extremely specific, and a nonspecific bystander effect was not observed (see below). Target cell lysis by $CD4^+$ CTLs results in DNA fragmentation and increased membrane permeability, as with $CD8^+$ CTLs (Takayama *et al.*, 1991).

C. Fas Dependence of Cell Lysis by $CD4^+$ CTLs

Despite their specific cytolytic activities, many $CD4^+$ CTL clones turned out to be free of perforin and perforin mRNA (Takayama *et al.*, 1991). Therefore, it was proposed that T cells are furnished with a mechanism of specific target cell lysis independent of perforin. This notion awaited the revelation of the Fas-mediated pathway and destruction of the perforin gene (Hanabuchi *et al.*, 1994; Kojima *et al.*, 1994). Hanabuchi *et al.* showed

that lectin or anti-TCR antibody-mediated killing of cells by $CD4^+$ CTLs could be blocked by soluble Fas molecules and that killing activity was dependent on the expression of Fas on the surface of the target cells. Furthermore, antigen-specific killing of a B cell tumor by a $CD4^+$ CTL clone was sensitive to the blocking effect of soluble Fas, and an antigen-specific $CD4^+$ T cell line established from a Fas ligand mutant mouse, C3H-*gld/gld*, could not lyse target B cells pulsed with the antigen (N. Shinohara, personal observation). These observations indicate that target cell lysis by $CD4^+$ CTLs is perforin independent and mediated through Fas/Fas ligand interactions. B cells express Fas molecules on the surface only when they are activated (Trauth *et al.*, 1989), and therefore resting B cells are not sensitive to such killing. Expression of Fas antigen on macrophages and Langerhans/dendritic cells is not clear. Therefore, at this moment it is not clear whether these versatile antigen presenters are sensitive to killing by $CD4^+$ CTLs.

D. Specific Killing of Antigen-Reactive B Cells by Class-II-Restricted T Cells

B cells can present exogenous antigens. They are, however, rather poor antigen presenters and require extremely high concentrations of soluble antigens. On the other hand, once they encounter antigens reactive with their own surface immunoglobulin (Ig) receptors, they become extremely efficient antigen presenters, being far more efficient than versatile macrophages (Rock *et al.*, 1984; Abbas *et al.*, 1985; Watanabe *et al.*, 1986). It appears that the major pathway of antigen presentation in the B cell is designed for antigens bound to the Ig receptor. Therefore, B cell clones reactive with a given antigen should be preferential targets for specific lysis by T cells recognizing the peptide fragments derived from the same antigen molecule. This notion, indeed, has been experimentally supported by utilizing A20HL, a class II MHC-positive B cell lymphoma line transfected with the genes of anti-TNP IgM (Fig. 4). To sensitize A20HL for $I\text{-}E^d$-restricted specific lysis by a KLH-specific Th1-type $CD4^+$ T cell clone, BK1, only 0.01 μg/ml of TNP–KLH was sufficient, whereas 100 μg/ml of KLH was required. The sensitization of the target B cells was inhibited by a variety of processing inhibitors, and recognition of the target by the CTL was shown to be directed to the class II molecule of the target (N. Shinohara, unpublished observations). The tremendous difference between the two antigen preparations in the minimal effective concentration required to sensitize the TNP-reactive B lymphoma cells suggests that the primary *in vivo* target for this type of specific killing is the antigen-reactive B cell (Fig. 5). Thus, the specific lysis of B cells by class II MHC-restricted CTLs involves dual specificity, i.e., the specificity defined by TCR and that

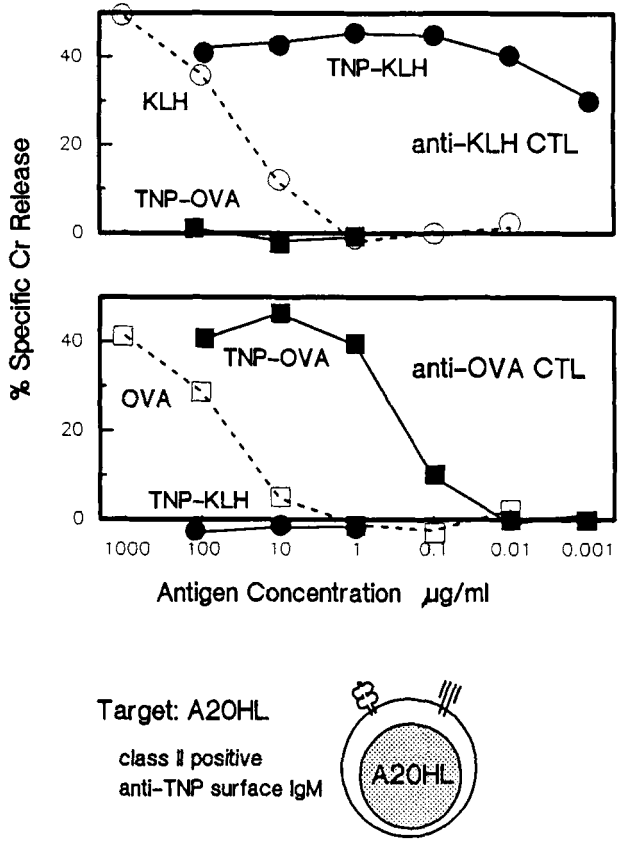

FIG. 4. Specific lysis of antigen-presenting B cells by class II MHC-restricted CTL. An anti-KLH (I-E^d + KLH) $CD4^+$ CTL clone and an anti-OVA (I-A^d + OVA) $CD4^+$ CTL clone specifically lysed A20HL in the presence of high concentrations of the relevant antigens. However, TNP-conjugated antigens sensitized the target far more efficiently owing to anti-TNP surface IgM receptor expressed on A20HL. Note the tremendous differences in the minimal sensitizing doses between the native and the TNP-conjugated antigens. (Reproduced from Shinohara *et al.*, 1991.)

defined by the Ig receptor of the B cell (Fig. 5). Therefore, it was speculated that such T cells might be capable of antigen-specific suppression of antibody production.

E. *In Vitro* Suppression of Antibody Responses by Class II-Restricted Soluble Antigen-Specific CTLs

When cloned class II-restricted soluble antigen-specific CTLs were incorporated into *in vitro* secondary antibody responses, carrier-specific sup-

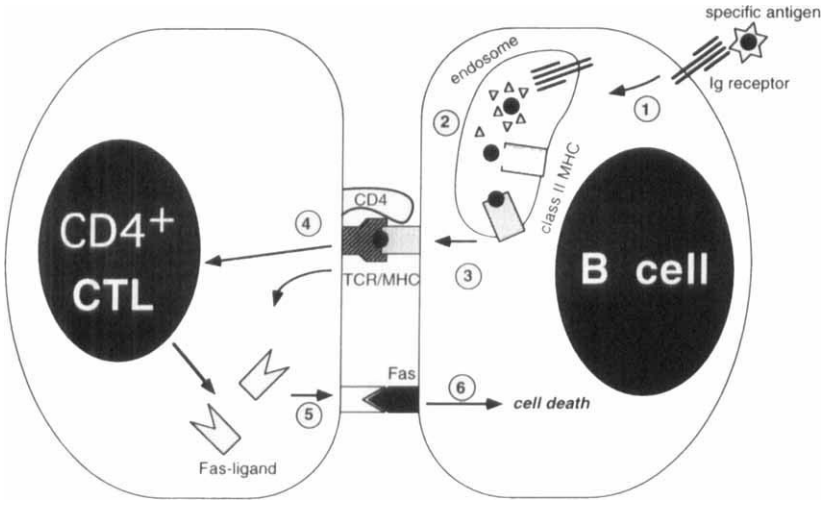

FIG. 5. Schematic diagram of cytolysis of an activated B cell by a $CD4^+$ CTL. Unless the antigen dose is extremely high, B cells are specific antigen presenters. Thus, B cells internalize extracellular antigens specifically trapped on the Ig receptor (1). The internalized protein antigens are processed in the endosome, and approriate peptide fragments form complexes with class II MHC molecules (2). The peptide/class II complexes are expressed on the surface (3). The specific recognition of the complex by the $CD4^+$ CTL triggers a signal to promote expression of the Fas ligand on the surface (4, 5). Fas/Fas ligand interaction is crucial for the cytolytic activity of $CD4^+$ CTLs because of the lack of the perforin pathway in this type of CTL (6).

pression of anti-hapten antibody production was reproduced (Shinohara *et al.*, 1991). As shown in Fig. 6, a KLH-specific I-E^d-restricted $CD4^+$ CTL clone exerted a profound suppressive effect on anti-TNP antibody production in the *in vitro* secondary response to TNP–KLH, whereas it did not suppress the anti-TNP–OVA response. An OVA-specific I-A^d-restricted CTL clone caused suppression of the reciprocal specificity. Since this suppression is dependent on the specific trapping of the antigen by hapten-specific B cells, incorporation of TNP–OVA into anti-TNP–KLH culture enabled the anti-OVA CTL to suppress anti-TNP antibody production, whereas anti-KLH antibody production was not suppressed in the same culture. Thus, available evidence indicated that these CTL clones specifically suppressed antibody production by killing hapten-specific B cells that had specifically trapped and presented the haptenated proteins (Shinohara *et al.*, 1991). As might be immediately noticed, the observed suppression carried out by these CTLs simulated classical observations on T-cell-mediated suppression of antibody responses. Both are carrier protein-specific suppressions of anti-hapten antibody production requiring

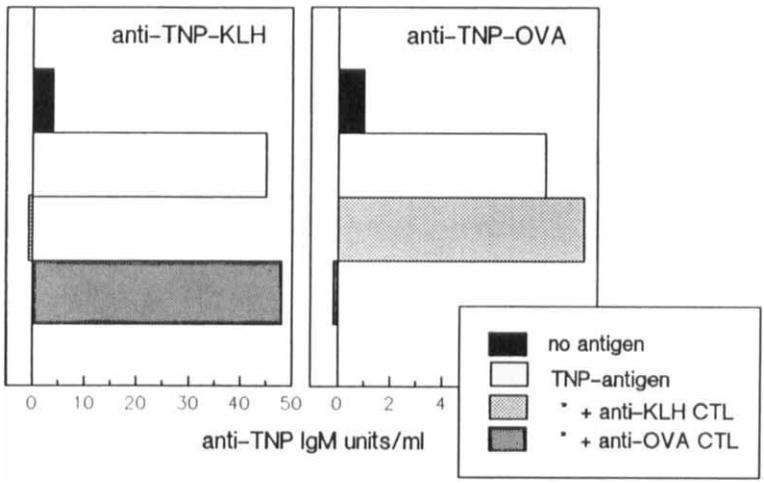

FIG. 6. Carrier-specific suppression of anti-hapten antibody responses by class II MHC-restricted soluble antigen-specific $CD4^+$ CTLs. Anti-KLH and anti-OVA CTL clones were added to *in vitro* secondary antibody responses of BALB/c spleen cells to TNP conjugate antigens. Amounts of anti-TNP antibody in the Day 9 supernatant were determined. Carrier-specific suppression of anti-hapten antibody production was consistently observed. (Reproduced from Shinohara *et al.*, 1991.)

coexistence of haptenic determinants and carrier determinants (peptides) on the same molecule and require MHC compatibility between T and B cells. Thus, a part of T-cell-mediated suppression of antibody responses might be attributable to induction of class II MHC-restricted CTLs.

F. Speculations on the Etiology of Autoimmunity in *lpr* Mice

Selective lysis of antigen-reactive B cells by $CD4^+$ CTLs through Fas/Fas ligand interaction is summarized schematically in Fig. 5. $CD4^+$ T cells are known as helper T cells and consequently are believed to promote immune responses rather than to exert negative effects. Sporadic reports on the suppressive effect of $CD4^+$ T cells on immune responses (Bottomly *et al.*, 1983; Asano and Hodes, 1983) have not been sufficient to turn general attention to the potential role of CD^+ T cells in negative regulation. Nevertheless, Fas ligand-mediated killing activity of the Th1-type $CD4^+$ T cells indicates that they will probably kill Fas-expressing antigen-presenting cells upon encounter in the presence of the relevant antigens. Among $CD4^+$ T cells, Th1-type cells produce IL-2 and IFN-γ, which primarily stimulate cellular responses, but not IL-4 or IL-5, which primarily stimulate B cells (Mosmann *et al.*, 1991). Together with their lymphokine profile, the killing ability of Th1-type $CD4^+$ T cells suggests that their effect on

humoral responses can be negative. As it now stands, it is extremely difficult to delineate precisely the effect of Th1 cells on B cells *in vivo,* but it is worth considering the possible involvement of $CD4^+$ CTLs in the negative regulatory circuit of humoral immune responses. Since target destruction by $CD4^+$ CTLs has been shown to be entirely dependent on the Fas/Fas ligand interaction due to the lack of perforin (Takayama *et al.,* 1991; Hanabuchi *et al.,* 1994; Kojima *et al.,* 1994), this putative regulatory pathway should fail to function in animals carrying a genetic defect in Fas/Fas ligand interactions, such as *lpr/lpr* or *gld/gld* mice (Watanabe-Fukunaga *et al.,* 1992b; Allen *et al.,* 1990; Takahashi *et al.,* 1994). From this point of view, it appears quite possible that autoantibody production in these mice is actually the consequence of the defect in regulatory surveillance of B cells by $CD4^+$ CTLs in addition to, or rather than, a defect in the negative selection of autoreactive T cells. Several studies reported the importance of B cells rather than T cells in development of autoimmunity in *lpr/lpr* mice (Perkins *et al.,* 1990; Sobel *et al.,* 1991), and available evidence indicates that negative selection of T cells in these mice is operative (Singer *et al.,* 1989; Mountz *et al.,* 1990; Zhou *et al.,* 1991). Although transgenic expression of the Fas antigen exclusively on T lymphocytes in *lpr/lpr* mice abrogated abnormal proliferation of double-negative T cells, it did not stop autoantibody production (Wu *et al.,* 1994). Thus, it appears quite possible that failure of $CD4^+$ CTLs to eliminate unwanted B cells underlies the manifestation of autoantibody production in *lpr/lpr* and *gld/gld* mice. Taken collectively, the existence of soluble antigen-specific class II MHC-restricted $CD4^+$ CTLs suggests involvement of T-cell-mediated killing in the regulatory pathway of immune responses in addition to elimination of pathologic somatic cells.

G. $CD8^+$ Class II-Restricted CTLs

In allogeneic immunizations with class II MHC disparity, a large number of $CD8^+$ as well as $CD4^+$ CTLs can be induced (Vidovic *et al.,* 1981; Miller and Stutman, 1982; Shinohara and Kojima, 1984; Shinohara, 1987; Pierres *et al.,* 1984; Golding and Singer, 1985). The genuine class II specificity of such CTLs has been supported by several lines of evidence (Shinohara *et al.,* 1988b). However, transgenic mice bearing TCR of one $CD8^+$ class II MHC-specific allogeneic CTL clone indicated that such T cells belong to the conventional $CD8^+$ subpopulation of mature T cells that is dependent on positive selection by self class I MHC molecules (Suzuki *et al.,* 1994). Therefore, the reactivity of these cells to allogeneic class II MHC molecules is considered an accidental cross-reaction in an artificial situation and probably has no physiological relevance. On the other hand, there are a few reports describing $CD8^+$ CTLs that recognize

exogenous antigens presented on self class II MHC molecules (Morrison *et al.*, 1985; Shinohara *et al.*, 1988b; Hioe and Hinshaw, 1989). Since such cells are capable of specifically lysing antigen-specific B cell clones (as explained previously), it was proposed that they might explain classical observations concerning $CD8^+$ T-cell-mediated antigen-specific suppression of antibody responses (Shinohara *et al.*, 1988b). Nevertheless, later investigations indicated that the frequency of T cells with such specificities is quite low and a very limited number of antigens can induce such $CD8^+$ cells (Shinohara, unpublished observations). Therefore, it is rather difficult to attribute a general role to this type of CTL. Since KLH is one of the few antigens that can induce $CD8^+$ CTLs, this type of CTL may play some role in the regulation of the antibody response to this antigen.

IV. Perspectives

The revelation of the two distinct pathways of killing necessitates a thorough reevaluation of a significant portion of our knowledge on specific target lysis by CTLs. Interpretation of an observation of CTL-mediated killing in conventional experimental situations in which the two killing systems could have been operating is not simple. Now the two pathways of CTL-mediated cell destruction can be analyzed separately in isolated situations. CTLs derived from *gld* mice should exert cytotoxicic effects only via the perforin pathway because of the lack of functional Fas ligand (Allen *et al.*, 1990; Lynch *et al.*, 1994; Takahashi *et al.*, 1994; Ramsdell *et al.*, 1994), whereas CTLs derived from perforin-deficient mice have an intact Fas pathway.

Biochemical events leading to cell death are not fully understood. In particular, the information on Fas-mediated cell death is extremely limited. Signal transduction in the Fas pathway will be substantiated by the search for interacting proteins, as recently reported for the TNF receptor system (Rothe *et al.*, 1994). Cytotoxic factors such as ATP may have direct effects on target cell killing in a perforin- and Fas-independent manner or serve as cofactors that work in concert with perforin and/or Fas.

The highly specific and selective nature of CTL-mediated killing despite the nonselectivity of the effect of these molecules indicates the existence of a strict control mechanism that focuses the effector molecules solely on the specific target cells. Costimulatory signal from the CD28/B7 system (Azuma *et al.*, 1992) and/or signals from so-called accessary molecules, such as CD4/8, LFA-1 (CD11a/CD18), CD2, and CD45, are possibly involved in the determination of the killing vector, yet this remains to be analyzed. Elucidation of such mechanisms is essential to understanding the physiological role of CTLs.

A true understanding of the biological significance of the redundancy of killing mechanisms requires extensive studies on animals with genetic defects in one of the two systems. It is of interest to know whether the Fas pathway alone is sufficient to eliminate infection caused by intracellular pathogens, since $CD4^+$ T cells can complement the lack of $CD8^+$ CTLs in β_2-microglobulin-deficient mice to clear the LCMV infection (Muller *et al.*, 1992) and the Fas pathway may play a role in eliminating infection by *Listeria monocytogenes* in the absence of perforin (Kägi *et al.*, 1994a). Exploring the possibility of the involvement of class II MHC-restricted CTLs in the negative regulation of immune responses may provide new insight into the physiological role of T-cell-mediated killing.

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The Role of Nitric Oxide in Inflammation

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I. Introduction

Nitric oxide (NO) is a highly reactive radical produced from the amino acid arginine by the enzyme nitric oxide synthase (NOS) (for review see Knowles and Moncada, 1994; Marletta, 1993a). Its role is currently of interest to many groups because of its postulated physiological and pathological roles in immune effector mechanisms (Nathan and Hibbs, 1991), intra- and intercellular communication (Lee *et al.*, 1994; Lowenstein and Snyder, 1992), leukocyte adhesion, vascular integrity, blood pressure homeostasis (Moncada and Palmer, 1991), and neurotransmission (Bredt *et al.*, 1990; Garthwaite *et al.*, 1989; Izumi *et al.*, 1992; Knowles *et al.*, 1989). This chapter will examine the multiple roles that NO may play during the inflammatory response.

The reaction which leads to NO production occurs in multiple cell types, results in oxidation of the guanidino nitrogen of L-arginine to NO and production of L-citrulline (Hibbs *et al.*, 1987a), and is catalyzed by the enzyme NOS. NO production is measured in a variety of ways, but the two most common methods involve either monitoring the conversion of ^{3}H- or ^{14}C-labeled arginine to citrulline (Bredt and Snyder, 1989) or the spectrophotometric detection of nitrite, a spontaneous oxidation product of NO, by the Griess reaction (Green *et al.*, 1982). The isotopic method is more sensitive (detecting <100 n*M* of product) and is unaffected by the presence of molecules capable of binding NO (i.e., hemoglobin). The lower limit of sensitivity of the Griess reaction is ~ 500 n*M*. To detect NO production *in vivo* by the Griess reaction, nitrates in the body fluid sample must first be reduced to nitrites by a nitrate reductase because the predominant NO oxidation product in mammals is nitrate.

Three NOS isoforms have been identified and their genes have been cloned (Bredt *et al.*, 1991; Janssens *et al.*, 1992; Lyons *et al.*, 1992; Michel and Lamas, 1992; Sessa *et al.*, 1992; Xie *et al.*, 1992). Two distinct NOS isoforms (cNOS) are constitutively expressed at basal levels primarily in neuronal cells (Bredt and Snyder, 1989) and in endothelium (Palmer *et al.*, 1987). A third isoform, inducible NOS (iNOS) is transcribed in response to cytokines (Stuehr and Marletta, 1985). An identical iNOS is expressed in multiple cell types including macrophages (Mø), vascular smooth muscle

cells (VSMC), and hepatocytes (Wood *et al.*, 1993). Initially, the two cNOS isoforms were distinguished from iNOS based on the dependence of cNOS activity on calmodulin (Bredt and Snyder, 1990); recent evidence indicates that iNOS also requires calmodulin for function (Cho *et al.*, 1992). Calmodulin binding by cNOS leads to a quenching of the intrinsic protein fluorescence suggesting that the interaction between calmodulin and cNOS leads to a change in the enzyme conformation which may provide for more efficient transfer during oxidative reactions (Abu-Soud and Stuehr, 1993; Sheta *et al.*, 1994). Other cofactors required for activity of all three isoforms are flavin mononucleotide, flavin adenine, dinucleotide, heme (iron–protoporphyron IX), and tetrahydrobiopterin (BH_4) (Bredt and Snyder, 1990; Forstermann *et al.*, 1994; Stuehr *et al.*, 1991; White and Marletta, 1992). The cNOS isoforms require Ca^{2+} for activity while iNOS does not (Bredt and Snyder, 1990; Schmidt *et al.*, 1991).

It is likely that all three NOS isoforms play some role during inflammation. Because tissue expression and regulation of NOS isoforms occur through distinct pathways, it is important to define whether cNOS or iNOS generate NO in an experiment. Some critical differences which allow differentiation between cNOS versus iNOS activity are the kinetics and quantity of NO production (Marletta, 1993b; Moncada *et al.*, 1991). cNOS activity is regulated post-translationally (Bredt and Snyder, 1990) and requires a Ca^{2+} influx (Forstermann *et al.*, 1990; Groschner *et al.*, 1992; Singer and Peach, 1982); NO production by cNOS is observed within seconds of administering a cell activation signal (Bredt and Snyder, 1989; Forstermann *et al.*, 1991). It is hypothesized that Ca^{2+} influx induced by external signals results in kinase activation with subsequent cNOS phosphorylation (Brune and Lapetina, 1991) and activation (Michel *et al.*, 1993; Tsukahara *et al.*, 1993). NO production by cNOS is usually in picomolar concentrations (Kelm *et al.*, 1988; Palmer *et al.*, 1988a). In general, cNOS activity by endothelial cells and neurons is responsible for maintenance of physiological homeostasis such as fine tuning blood pressure and blood flow, controlling leukocyte–endothelial interactions, and signaling among neurons (Moncada *et al.*, 1991). In contrast, iNOS activity is transcriptionally regulated (Lorsbach *et al.*, 1993) and stimulated cells require minutes to hours to synthesize iNOS (Stuehr and Marletta, 1987). Cellular iNOS activity can remain elevated for hours and produce micromolar concentrations of NO. Due to the production of extremely high levels of NO, the biological consequences of iNOS activity may be completely different from the biological functions of cNOS activity. For example, it has been suggested that at high levels, NO may interact with superoxides to produce tissue-damaging peroxynitrites and hydroxyl radicals (Hogg *et al.*, 1992; Ischiropoulos *et al.*, 1992; Radi *et al.*, 1991a,b). Thus, while cNOS activity

maintains normal physiology, the iNOS high-output system may be responsible for the pathological consequences of inflammation. Besides differences in the kinetics and quantity of NO production by cNOS and iNOS, glucocorticoid sensitivity is another method for separating the role of cNOS from iNOS. iNOS expression is inhibited by glucocorticoids, while cNOS activity is resistant (Di Rosa *et al.*, 1990; Geller *et al.*, 1993b; McCall *et al.*, 1991; Moncada and Palmer, 1991; O'Connor and Moncada, 1991; Palmer *et al.*, 1992, 1993; Radomski *et al.*, 1990a).

The majority of data implicating a role for NO in inflammation is based on experiments using NOS inhibitors. In general, these inhibitors are arginine analogs which competitively inhibit all three NOS isoforms. Several arginine analogs have been developed including N^G-monomethyl-L-arginine (L-NMMA) (Hibbs *et al.*, 1987a,b; Rees *et al.*, 1990), N^G-nitro-L-arginine (L-NOARG) (Moore *et al.*, 1989), N^G-nitro-L-arginine methyl ester (L-NAME) (Rees *et al.*, 1990), and N^G-iminoethyl-L-ornithine. Some progress has been made in developing selective inhibitors of the different NOS isoforms. For example, L-N^G-nitroarginine-*p*-nitroanilide appears to have a selective effect for neuronal cNOS while aminoguanidine has a 10- to 100-fold selectivity for iNOS (Griffiths *et al.*, 1993; Joly *et al.*, 1994). The identification of selective NOS isoform inhibitors is important for both basic research and for therapeutic potential (Gross *et al.*, 1990). For example, inhibiting iNOS activity during sepsis may increase mean arterial pressure, but the coincident inhibition of endothelial cNOS may result in enhanced platelet aggregation and leukocyte adherence to endothelium resulting in thrombosis and damage to normal tissue (Shultz and Raij, 1992). It is also important to remember that arginine analogs can also inhibit arginine transport into cells (Schmidt *et al.*, 1993). Identification of NOS inhibitors that do not interfere with arginine transport will be important because even if selective NOS isoform inhibitors are identified, depletion of intracellular arginine may continue to effect NO production by the other NOS isoforms.

Before summarizing the literature on the role of NO in the pathophysiology of inflammation it is useful to define inflammation and construct the sequence of events that occurs during this process. Inflammation was described by Celsus in the first century AD as demonstrating four cardinal clinical signs: rubor (redness), tumor (swelling), calor (heat), and dolor (pain). The cellular events which cause these clinical stages of inflammation are being extensively studied. Thus, inflammation results from increases in vascular flow, alterations in endothelium that can lead to plasma protein and leukocyte extravasation, and finally, the emigration of leukocytes from the circulation to the site of injury with resultant activation of effector cell mechanisms. There is evidence that NO can play a role during each stage of inflammation and this chapter will discuss NO in the context of each

of these stages. A recurring theme is that conflicting data exist in the literature regarding the role of NO during inflammation. Some conflicts arise because of differences in the experimental systems studied, such as in the animal species used or in the type of inflammatory stimulus. However, other differences may represent distinct physiologic and pathologic effects of NO during different phases of inflammation.

II. Vasodilatation

One of the earliest events in inflammation is vasodilatation. Endothelial-derived relaxation factor (EDRF) is the primary mediator of VSMC vasorelaxation (Furchgott, 1984; Furchgott and Zawadzki, 1980). The demonstration that NO and EDRF were identical was a major breakthrough in vascular biology. Initial studies had demonstrated that relaxation of isolated arteries induced by acetylcholine was endothelium dependent (Furchgott and Zawadzki, 1980). Thus, acetylcholine-induced relaxation of constricted aortic rings was lost if the endothelium was mechanically removed. These experiments suggested that the stimulation of muscarinic receptors on endothelial cells by acetylcholine resulted in the local release of a diffusible factor that acted to relax smooth muscle. Further studies demonstrated that EDRF also inhibited platelet aggregation and platelet adhesion and that these effects were mediated by an increase in intracellular cyclic guanosine 3′,5′-monophosphate (cGMP) (Azuma *et al.*, 1986; Furlong *et al.*, 1987; Radomski *et al.*, 1987). It was also noted that methylene blue blocked the effect of EDRF (Martin *et al.*, 1985) by inhibiting the activation of guanyl cyclase, whereas superoxide dismutase (Gryglewski *et al.*, 1986; Rubanyi and Vanhoutte, 1986) enhanced the action of EDRF. Based on a compilation of the characteristics and similarities in biological activity between EDRF and NO, two groups simultaneously suggested that EDRF and NO were identical (Furchgott, 1986; Ignarro *et al.*, 1987). Since then, several studies have confirmed that in most circumstances, the observed effects of EDRF are through NO. Under noninflammatory conditions, endothelial NO is made from cNOS. In its basal state, cNOS protein is stored in endothelial cells as a membrane-associated enzyme (Forstermann *et al.*, 1991). A variety of stimuli can induce cNOS activity leading to a measurable NO release by endothelial cells (Table I).

Studies suggesting that these stimuli cause a Ca^{2+} flux into cells resulting in kinase activation and serine phosphorylation of cNOS protein which is then translocated from its membrane location into the cytosol (Michel and Busconi, 1993). This translocation may be important in regulating the biological effects of endothelial cNOS. NO has a half-life of approximately 6 sec (Cocks *et al.*, 1985; Griffith *et al.*, 1984). The mechanism of NO-

TABLE I
MEDIATORS REPORTED TO RELEASE NO FROM ENDOTHELIAL CELLS

Mediator	Reference
Bradykinin	Bogl *et al.* (1991), Gryglewski *et al.* (1988), Kelm *et al.* (1988), Palmer *et al.* (1988a), Tsukahara *et al.* (1993)
Interleukin-1	Suschek *et al.* (1993)
Endothelin-3	Sudjarwo *et al.* (1992)
Acetylcholine	Furchgott and Zawadzki (1980)
Histamine	Sakuma *et al.* (1988)
Thrombin	Boulanger and Luscher (1990), Gryglewski *et al.* (1988), Tsukahara *et al.* (1993)
5-hydroxytryptamine	Gryglewski *et al.* (1988)
Angiotensin I metabolites	Porsti *et al.* (1994)
Increased blood flow	Cooke *et al.* (1991)

induced VSMC relaxation occurs when NO interacts with the heme moiety of soluble guanylyl cyclase in VSMC (Ohlstein *et al.*, 1992). The NO–heme interaction results in a conformational change which activates the catalytic site of guanylyl cyclase (Garbers, 1990, 1992; Wolin *et al.*, 1982) resulting in an increase in intracellular cGMP. The increase in cGMP stimulates cGMP-dependent protein kinase resulting in VSMC relaxation.

Many of the mediators listed in Table I are released during the early stages of inflammation. For example, the interaction of many organisms with tissue macrophages results in an immediate release of IL-1, and the interaction of antigen with IgE on a mast cell induces the release of histamine. Thus, some of these cytokines and molecules likely act on local vasculature to induce cNOS activity and NO production in the early stages of inflammation to cause vasodilatation. Once increased flow is initiated by these mediators, vasodilatation can be maintained even in the absence of inflammatory mediators by an autocrine effect of increased blood flow (Cooke *et al.*, 1991).

III. Alterations in Endothelial Integrity

There has been increasing speculation regarding a role for NO as an agent involved in the induction of vascular leak syndromes. These syndromes are particularly evident in clinical treatment protocols that use or result in a high circulating level of cytokines, particularly IL-2. The vascular leak syndromes can lead to accumulation of large amounts of fluid in the interstitial tissues and lung (Rosenberg *et al.*, 1988). Hibbs *et al.* demonstrated that, in human subjects undergoing IL-2 therapy for malignancies, serum and urine nitrate levels were significantly increased and correlated

with an increase in the serum level of citrulline (Hibbs *et al.*, 1992). The diet in the study was stringently controlled to avoid spurious alterations of serum and urine nitrate measurements. Further, renal function was monitored to control for altered renal clearance that could increase the circulating nitrate level. Thus, it is reasonable to suggest that some of the physiological changes, particularly hypotension (Kilbourn *et al.*, 1992), observed in patients undergoing cytokine therapy may be caused by or exacerbated by increased NO (Ochoa *et al.*, 1992). However, experimental evidence for a direct role for NO causing a vascular leak is sparse.

A. Paw Edema Model

Some inert substances injected subcutaneously can induce an inflammatory response mediated by the local generation of vasoactive mediators including histamine and prostaglandins. Two agents capable of eliciting such a response are dextran and carageenin (Winter *et al.*, 1962). These agents, when injected into the footpad of a rat, produce a local plasma extravasation with associated edema. The response to dextran is rapid and occurs steadily over 3 or 4 hr. Carageenin-induced edema has an early and a late phase. The early phase involves rapid edema formation over 1 or 2 hr and is dependent on histamine, 5-hydroxytryptamine, and bradykinin. The late phase of edema formation occurs between 3 and 4 hr and is dependent primarily on arachidonic acid metabolites (DiRosa *et al.*, 1971).

A role for NO in both carageenin-induced and dextran-induced edema was suggested in experiments with NOS inhibitors (L-NMMA or L-NAME) (Ialenti *et al.*, 1992). The effect of NOS inhibitors on carageenin-induced vascular permeability was monitored using fluoresceinated bovine serum albumin (F-BSA) as a marker. NOS inhibitors had no effect on vascular permeability at control saline-injected sites while they inhibited F-BSA extravasation at carageenin-injected sites in a dose-dependent manner. The inhibition of carageenin-induced F-BSA extravasation by L-NMMA or L-NAME was reversible by L-arginine, but not by D-arginine. Because cNOS is resistant to steroids, whereas iNOS is not; pretreatment of the rats with dexamethasone prior to injecting the dextran or carageenin allowed separation of the NOS isoform activities during different stages of edema formation. The authors hypothesized that if cNOS were required during edema formation then exogenous arginine should enhance edema formation even in the presence of steroids, while if iNOS were required, then steroids should prevent iNOS expression and no arginine enhancement of edema should be observed. L-Arginine enhanced dextran-induced edema formation throughout its entire course as well as the early phase (<1 hr) of the carageenin-induced edema in dexamethasone-treated rats. In contrast, L-arginine did not affect the late phase (>3 hr) of the

carageenin-induced edema in dexamethasone-treated rats. Arginine enhancement in the presence of steroids indicated cNOS involvement in dextran-induced edema as well as the early phase of carageenin-induced edema. An inability of L-arginine to enhance the steroid-sensitive late phase of carageenin-induced edema indicated that iNOS likely plays a dominant role during the late phase of the carageenin-induced edema formation. The cells responsible for the release of NO in this model were not addressed, but it was speculated that cNOS activity in vascular endothelial cells was likely responsible for the NO release in the early phase of carageenin as well as dextran-induced edema, whereas iNOS activity during the late phase of carageenin-induced edema could be from either local tissue or from infiltrating polymorphonuclear neutrophils (PMN). A role for PMN as a potential source of iNOS was suggested by earlier studies which demonstrated a generous influx of PMN after carageenin injection, whereas dextran resulted in a minimal influx of PMN (DiRosa *et al.*, 1971).

B. Mesenteric Microvascular Models

Using an *in vivo* vascular leak model, Kubes and Granger (1992) investigated the role of NO in microvascular permeability of the feline mesentery. He monitored lymph flow, lymph and plasma protein concentrations, and vascular protein clearance in the mesentery to determine a lymph flux as a measure of microvasculature leakage. In contrast to the carageenin and dextran edema models, he observed a significant *increase* in lymph flux when NO inhibitors were infused into the mesenteric artery. Thus, infusion of L-NAME resulted in a fivefold increase in vascular fluid and protein fluxes while capillary pressure remained unchanged. The increase in lymph flux was reversed by infusion of NO donors, including nitroprusside. Previous studies indicated that a decrease of NO in mesenteric vessels resulted in enhanced leukocyte adherence to the endothelium via a CD18 mechanism (see below). In order to evaluate this adherence mechanism in the microvasculature leak model, Kubes *et al.* pretreated animals with a monoclonal antibody to CD18 and monitored lymph flux. Anti-CD18 treatment had no significant effect on vascular leakage during the initial 15 min of L-NAME infusion compared to animals that received only L-NAME. However, anti-CD18 did significantly decrease the vascular leakage during the latter phase (30–60 min) of L-NAME infusion and a time period which correlated with peak leukocyte adherence during L-NAME infusion. That anti-CD18 treatment only *partially* reversed vascular leakage suggested that inhibition of NO synthase led to a reversible increase in microvasculature leakage that involved both leukocyte-dependent and -independent

mechanisms. The role of NO in this model, therefore, is to preserve the integrity of the endothelium.

On the other hand, in an endotoxin-induced gut vascular leakage model, NO increased vascular leakage rather than protecting endothelial integrity (Boughton-Smith, 1993). Using radiolabeled albumin as a marker for serum proteins, increased leakage of albumin was observed from colon and jejunum 3 hr after intraperitoneal lipopolysaccharide (LPS) (3 mg/kg) injection of rats. Albumin leakage correlated with iNOS induction in organ homogenates from the LPS-treated rats and was inhibited by pretreatment with dexamethasone (1 mg/kg) or L-NMMA (12.5–50 mg/kg, sc), which inhibit iNOS expression and activity, respectively. The decrease in vascular leakage by L-NMMA was reversed by excess arginine. The authors suggested that iNOS induction was associated with the vascular injury observed after endotoxin injection.

An important distinction between the two gut vascular leakage models was that Kubes feline mesenteric model addressed the role of cNOS in the normal homeostasis, while the endotoxin model induced high levels of NO via iNOS induction. Thus, the differences in results between the two models were undoubtedly related to the levels of NO produced.

IV. Vascular Adhesion and Emigration of Inflammatory Cells

The emigration of leukocytes and lymphocytes from vessels into sites of inflammation has itself become a science. This process involves the regulation of multiple adhesion molecules on both migrating cells and the endothelium. The role of NO in this process has only recently been appreciated, and it is likely that further studies will demonstrate an increased role of NO in cell adhesion and migration.

One of the first studies demonstrating a potential role for NO in regulating cell–cell interactions was shown in platelet aggregation assays. Radomski demonstrated that platelet aggregation was enhanced by L-NMMA treatment and the addition of excess arginine-reversed aggregation (Azuma *et al.*, 1986; Furlong *et al.*, 1987; Radomski *et al.*, 1990b). It was postulated that the role of NO in the antiaggregation effect was mediated by an increased level of cyclic GMP in the platelets (Mellion *et al.*, 1981; Radomski *et al.*, 1987).

Perhaps the most well-defined system used to investigate the role of NO in leukocyte adhesion and migration was an ischemia–reperfusion model in mesenteric vessels (Kubes *et al.*, 1991; Kurose *et al.*, 1994). Due to the rapid responses observed in these studies, it is likely that these models monitored cNOS activity in endothelium and the effect of NO on leukocyte adherence and migration. Using intravital video microscopy to

visualize single vessels, the ability of leukocytes to adhere to the vessel wall was determined in *in vivo* experiments. The basic protocol involved blocking blood flow to the mesenteric artery for a defined period of time to induce ischemia. Blood flow was then allowed to return to the area. During reperfusion, an enhanced adherence of PMN cell to vascular endothelium was identified by an increase in numbers of cells "rolling" on the endothelium. This system was manipulated by infusing inhibitors of inflammatory mediators and monoclonal antibodies to adhesion molecules to observe changes in PMN–endothelium interactions. Initial experiments indicated that NOS inhibitors decreased venular wall shear rate (Kubes *et al.*, 1991). (Venular wall shear rate is a measure of the forces that tend to remove PMNs from the vascular wall determined from red blood cell velocity and vessel diameter). NOS inhibitors also increased PMN adherence by greater than 15-fold over baseline value with a concomitant increase in PMN emigration into the interstitium. Increased adherence was not due solely to a decrease in venular wall shear rate because mechanical reductions in wall shear rate produced by partial occlusion of the arterial circuit had no effect on PMN adherence. However, coadministration of monoclonal antibody to CD18 with NOS inhibitors eliminated the increased leukocyte adherence which suggested a link between expression of adhesion molecules and NO production. Kubes *et al.* (1991) have continued to define the mechanisms by which NO decreases leukocyte adherence, and their data suggest a complex interaction between cNOS activity, superoxides, and mast cells in the vascular wall that is responsible for regulating leukocyte adhesion in an ischemic inflammation model and will be discussed in further detail (Gaboury *et al.*, 1993; Hogaboam *et al.*, 1993; Kubes *et al.*, 1993; Kurose *et al.*, 1994; Salvemini *et al.*, 1991).

Studies have shown that neutrophil-produced oxidants promote leukocyte adhesion to endothelium. *In vitro* studies indicated that reactive oxygen metabolites as well as H_2O_2 promote neutrophil adherence to endothelial monolayers and that superoxide dismutase, a scavenger of oxygen radicals, can inhibit these adhesive events (Salvemini *et al.*, 1991). The increase in oxidative-induced adherence may be secondary to increased and prolonged expression of P-selectin on endothelial cells (Geng *et al.*, 1990; Patel *et al.*, 1991). The mechanisms by which the endothelium increases adhesion molecules during the early stages of inflammation involve interactions between mast cells and endothelial cells. Mast cells surround vessels of the mesentery. Mast cells secrete a variety of factors including platelet-activating factor and histamine which can upregulate adhesion molecules on endothelium (Hogaboam *et al.*, 1993; Salvemini *et al.*, 1991). Mast cells are triggered by oxidative events and NO can inactivate superoxides (Kanner *et al.*, 1992; Rubyani *et al.*, 1991). One hypothesis

suggests that under noninflammatory conditions, endothelial-derived NO neutralizes oxygen radicals produced as by-products of normal cellular biochemical reactions and prevents mast cells from degranulating (Kubes *et al.*, 1993). Thus, this neutralizing action of NO prevents superoxide-induced mast cell release of mediators that can upregulate adhesion molecules on endothelial cells. This hypothesis was tested in the ischemia–reperfusion model in rat mesentery. The data demonstrated that NOS inhibitors, excess superoxides produced from infusion of hypoxanthine/xanthine oxidase, or mast cell activators could each enhance PMN adhesion to endothelium during the reperfusion period. Ketotifen, a mast cell stabilizer, or NO donors inhibited increased leukocyte adherence induced by either superoxides or NOS inhibitors (Kubes *et al.*, 1993; Kurose *et al.*, 1994). Taken together the data suggested that during inflammatory states decreased NO production, increased superoxide generation, or both resulted in increased vascular leakage, leukocyte adhesion, and migration via superoxide-triggered mast cell release of products that induce adhesion molecules on endothelial cells and promote leukocyte adhesion (Kurose *et al.*, 1994).

The important interrelationship of NO in regulating mast cell degranulation may have implications for chronic inflammatory diseases involving organ systems that are known to contain increased numbers of mast cells including inflammatory bowel disease (Dvorak *et al.*, 1980) and asthma (Barnes and Belvisi, 1993).

V. NO as an Effector Molecule during Inflammation

This discussion will focus on the effector phase of a cell-mediated immune response (CMI) and the regulatory events which play a role in iNOS expression and activity by effector cells, particularly Mø. The primary host response against intracellular pathogens and many extracellular organisms occurs via CMI (Mackaness, 1964). CMI is regulated by an interplay of T cells and cytokines which leads to secondary effects including recruitment of effector cells, particularly macrophages, and release of cytokines which upregulate and/or suppress the immune response. The host's ability to regulate immune responses appropriately can determine the difference between resolution of infections, a chronic inflammatory state with or without chronic infection, or death due to overwhelming infection or effects of a dysregulated immune response. The appropriate regulation of NO during CMI may be an important determinant for deciding which of these outcomes occur.

The discovery of a role for NO as an effector molecule is an interesting story. Early studies demonstrated a link between nitrosamines and cancer

which led to the investigation of nitrogen metabolism in man (Green *et al.*, 1981a). Experiments performed in humans fed a low nitrate diet indicated that they produced nitrate. The role of gut flora as a source of nitrate production was ruled out by demonstrating that germ-free mice on nitrogen-free diets produced nitrate (Green *et al.*, 1981b). This finding led to a search for cells capable of producing nitrate. A potential link between immune effector systems and NO was forged when studies demonstrated that intraperitoneal (ip) administration of LPS to mice resulted in dramatic increases in urine nitrate production (Stuehr and Marletta, 1985). These studies were extended when ip injection of LPS in LPS-sensitive mice, but not LPS-resistant mice, resulted in an increase in urine nitrate levels (Stuehr and Marletta, 1985). These authors also showed that LPS-activated peritoneal Mø from mice produced nitrite (NO_2) and nitrate (NO_3) (Stuehr and Marletta, 1987). The finding that the Mø cell line RAW 264.7 made NO_2 and NO_3 when stimulated with IFN-γ and LPS was a major advance in nitrate biology because it provided a reproducible cell source for the biochemical study of nitrate metabolism (Ding *et al.*, 1988). Using isotopically labeled arginine, it was shown that the NO_2 and NO_3 produced from macrophages originated from a common intermediate (Iyengar *et al.*, 1987). Two groups, one working on cell-mediated tumor cytotoxicity and the other on endothelial cell-induced vasorelaxation, made nearly simultaneous discoveries. Hibbs *et al.* (1987b) showed that the mechanism for tumor cytotoxicity by activated Mø was arginine dependent and could be inhibited by L-NMMA. At about the same time, Palmer *et al.* (1988a,b) demonstrated that endothelial cells produced NO from arginine. When the similarities in the data observed in the study of nitrate metabolism among these researchers were compared, it was apparent that NO was the likely intermediate in the synthesis of NO_2 and NO_3 by macrophages and subsequent experiments by several groups confirmed this hypothesis (Stuehr *et al.*, 1989).

A. Regulation of iNOS

1. Cytokine and LPS Enhancement of iNOS Activity

Several reviews have discussed the importance of cytokines during all phases of the immune response. Infection models have clarified what appears to be a common pathway for initiation of CMI during an inflammatory response to a pathogen. Macrophages phagocytose, kill and degrade pathogens. Depending on the structural components of the organism, Mø may be directly activated by this interaction. Mø process proteins of the organism and release them locally for presentation to T cells by dendritic cells and B cells. The type of T cells that respond to the pathogen appear

to be governed by cytokines produced during inflammation (Mosmann and Coffman, 1989b). For an effective CMI to occur, IL-12, IL-2, and IFN-γ are critical (Mosmann and Coffman, 1989a). The source of IL-12 appears to be primarily Mø and natural killer (NK) cells (Chan, 1992; Hsieh *et al.*, 1993). IFN-γ may come from a variety of cells including natural killer cells and T$\gamma\delta$ cells. These cytokines enhance the development of a Th1 response. A Th1 response is important because high levels of IFN-γ are produced by Th1 cells which, as discussed below, are vital for appropriate activation of Mø and NO production and this has significant implications in Mø-mediated defenses against a variety of organisms.

IFN-γ-activated peritoneal Mø make NO (Ding *et al.*, 1988). When RAW 264.7 cells and peritoneal exudate Mø were stimulated with a battery of cytokines, only the interferons resulted in production of NO by the cells. Further experiments demonstrated synergism in NO production with the addition of LPS to IFN-γ (Ding *et al.*, 1988). Since these initial observations, a wealth of information has emerged regarding the role and interactions of cytokines in regulating NO production. Depending on the cytokine and cell types, enhancement or inhibition of iNOS activity may result through mechanisms affecting levels of protein expression, transcription, mRNA stability and translation, and protein stability (Nathan and Xie, 1994).

Mø are the most well-studied cells that respond to cytokines by NO production. However, many studies have utilized virally transformed cell lines and some data may not pertain to normal macrophages. Furthermore, studies often remove Mø from their normal milieu, and Mø activity in culture systems may be quite different from their *in vivo* activity due to the change in their local environment. Finally, Mø can secrete many cytokines in response to agents that also induce iNOS activity. Mø-released cytokines can influence iNOS activity in an autocrine fashion. Autocrine effects are readily missed unless considered and properly controlled for (Fujihara *et al.*, 1994b; Oswald *et al.*, 1992). For example, Mø release interferon-β (IFN-β) after stimulation with LPS, and IFN-β may enhance NO production in the presence of exogenous cytokines. Indeed, some studies suggest that the release of IFN-β by macrophages is both necessary and sufficient to induce NO production (Zhang *et al.*, 1994). Autocrine effects from cytokines may account for some of the differences observed in the NO stimulatory cytokine profiles among different cell types.

Mø are the primary effector cells involved in a CMI response against infections and thus, it is not surprising that multiple combinations of cytokines can influence Mø activity, including NO production (Deng, 1993; Ding *et al.*, 1988). As mentioned previously, IFN-γ is a key element for production of NO by Mø during the immune response and *in vitro* studies

have shown that IFN-γ is required for efficient NO production. The importance of IFN-γ in regulation of NO production was studied in IFN-γ–receptor knockout mice [IFN-γ–R(0/0)] (Kamijo *et al.*, 1993). Peritoneal Mø from IFN-γ–R(0/0) mice did not make NO in response to IFN-γ or TNF-α. IFN-α/β alone elicited poor and equal responses in isolated Mø from both receptor-deficient and wild-type mice. The addition of LPS to IFN-α/β resulted in an enhancement of NO production by Mø from both strains of mice, but remained significantly less than that of wild-type Mø stimulated with IFN-γ and LPS. Further studies challenged IFN-γ–R(0/0) mice with *Mycobacterium bovis* (BCG) systemically and monitored the level of Ia expression on peritoneal Mø as a measure of Mø activation during an infection. Peritoneal Mø from BCG-infected IFN-γ–R(0/0) mice exhibited significantly less Ia than peritoneal Mø from infected wild-type mice. The authors interpreted this latter experiment as an inability of IFN-α/β to efficiently compensate for the lack of IFN-γ during an active immune response. In a related study, IFN-γ knockout mice demonstrated an inability to defend against *Mycobacterium tuberculosis* and this correlated to an inability of Mø from infected mice to produce NO (Flynn *et al.*, 1993). The recent cloning of the iNOS promoter together with its functional analysis have provided insight into the molecular basis for cytokine control of Mø iNOS expression (Lowenstein *et al.*, 1993; Xie *et al.*, 1993). Initial sequence data of the iNOS promoter indicated several potential regions that could play a role in the regulation of iNOS gene expression by IFN-γ. An interferon-specific response element (ISRE) capable of interacting with interferon response factor-1 (IRF-1) was identified approximately 1000 bp upstream from the transcriptional start site. Vilcek *et al.* demonstrated that IRF-1 knockout mice were unable to produce NO in response to IFN-γ (Kamijo *et al.*, 1994), indicating an important role for this ISRE site in iNOS regulation. Subsequent promoter analysis studies confirmed the importance of the IRF-1 binding motif in iNOS transcriptional regulation in response to IFN-γ (Martin *et al.*, 1994). Thus, IFN-γ regulates the iNOS expression in Mø through IRF-1 binding to the ISRE in the iNOS promoter. The mechanisms by which LPS induces transcriptional signals for iNOS induction are currently under investigation. Recent studies suggest a role for protein kinase C as part of the signal transduction (Fujihara *et al.*, 1994a). Previous studies indicate that LPS is a very efficient activator of NF-κB (Muller *et al.*, 1993). Sequence analysis of the iNOS gene promoter demonstrated two NF-κB motifs (Lowenstein *et al.*, 1993; Xie *et al.*, 1993) and there is evidence that at least one of these NF-κB sites is required for iNOS induction by LPS (Xie *et al.*, 1994). LPS has significant effects on post-translational mechanisms as well; LPS and IFN-γ activation of Mø resulted in a prolonged half-life of iNOS mRNA compared to IFN-

γ stimulation alone (Weisz *et al.*, 1994). This latter mechanism could at least partially account for the synergism in NO expression (Lorsbach *et al.*, 1993) and activity (Ding *et al.*, 1988) by Mø stimulated with both agents *in vitro*.

IFN-γ by itself is a relatively poor stimulator of NO production by Mø and the addition of a second signal can significantly enhance NO production (Ding *et al.*, 1988). In the case of gram-negative organisms, LPS provides an obvious second signal. The role of LPS in NO production has been the subject of intense study by several groups. Initial studies indicated that addition of LPS significantly enhanced NO production by IFN-γ-treated Mø (Ding *et al.*, 1988). Studies suggest that the timing (Bogdan *et al.*, 1993; Lorsbach and Russell, 1992) and quantity of LPS (Bogdan *et al.*, 1993; Rojas *et al.*, 1993b; Zhang and Morrison, 1993) stimulation are important for effective NO production. If LPS stimulation was given during or after IFN-γ treatment, there was effective induction of NO. However, if LPS was given prior to IFN-γ, little or no NO production occurred. Furthermore, prior treatment of Mø with LPS prevented stimulation by IFN-γ at a later time point. This mechanism may allow for downregulation of iNOS after the pathogen has been cleared but IFN-γ levels continue to remain elevated during the immediate postinflammatory state.

The *in vivo* administration of LPS in several animal models is used to simulate a hypotensive shock because a dramatic fall in mean arterial blood pressure occurs after LPS injection. Intraperitoneal injection rats with LPS rats resulted in widespread iNOS expression in multiple tissues, including lung, liver, skeletal muscle, and kidney, which could account for the significant vasodilatation and hypotension observed after LPS injection (Liu *et al.*, 1993). Antibody to TNF-α has been shown to block LPS-induced decreases in blood pressure in some studies (Billiau and Vandekerchove, 1991). The role of TNF-α in regulating iNOS in a rat endotoxic shock model was evaluated (Thiemermann *et al.*, 1993). Intraperitoneal administration of LPS to rats resulted in an immediate decrease in mean arterial blood pressure (MAP) over a 60-min period followed by a delayed, prolonged decrease in MAP. Isolated aortic rings froms LPS-injected rats, which had their endothelium removed, exhibited a decreased contractile response to noradrenaline. TNF-α antibody treatment ameliorated the delayed (60–180 min) hypotensive response and *partially* restored aortic ring reactivity to noradrenaline. Aortic ring reactivity to noradrenaline from LPS-treated rats was significantly increased if L-NAME was present, while the NOS inhibitor treatment had no effect on noradrenaline reactivity of aortic rings from rats treated with both LPS and TNF-α antibody. iNOS expression was significantly increased in lung homogenates from endotoxemic animals compared to sham-treated animals and TNF-α antibody pre-

treatment reduced lung iNOS expression by 40% (Thiemermann *et al.*, 1993). The authors suggested that this study confirmed the importance of NO in septic shock but concluded that, in their model, iNOS expression was only partially responsible for decreasing blood pressure in response to LPS. They also suggested, however, that because TNF-α antibody did not completely restore noradrenaline reactivity and the NOS inhibitor did not improve the TNF-α antibody response, other mechanisms for endotoxic shock besides the iNOS pathway must have played a role. Therefore, future development of therapeutic modalities for sepsis must also consider other contributors to vascular vasodilatation including prostanoids and/or platelet-activating factor.

In a TNF-α-induced hypotensive dog model, Kilbourn *et al.* (1990) demonstrated that TNF-α-induced hypotension was completely abrogated by the NOS inhibitor, L-NMMA. They extended this study to include LPS-induced (40 μg/kg) (Kilbourn *et al.*, 1990) and IL-1-induced (Kilbourn *et al.*, 1992) hypotension and again, the decrease in blood pressure after LPS injection and IL-1 infusion in dogs was prevented by administration of L-NMMA. These studies may have significance for the treatment of endotoxic shock or hypotension observed in individuals undergoing cytokine immunotherapy for malignancies. Indeed, anecdotal data exist regarding a role for L-NMMA in treatment of hypotension during endotoxic shock (Petros *et al.*, 1991).

The mechanism(s) by which LPS-lacking organisms might induce high levels of NO during infection is less clear. Several *in vitro* studies indicate that these organisms may induce the production of TNF-α by Mø which can then enhance NO production by Mø in an autocrine fashion (Suschek, 1993), but the most persuasive data for a role for TNF-α in NO production come from *in vivo* infection models (see Section V,D). During immune inflammation, TNF-α is produced by a variety of cells including Mø and T cells.

The cytokines required for iNOS induction by different tissues vary. Smooth muscle cells produce NO in response to IL-1 and the response is enhanced by TNF-α and/or IFN-γ (Beasley and Bridge, 1994; Beasley *et al.*, 1991; Kilbourn *et al.*, 1992). Endothelial iNOS is produced in response to LPS and IFN-γ similar to the Mø (Marumo *et al.*, 1993). Rat hepatocytes and Kupffer cells respond to LPS (Wood *et al.*, 1993). In contrast, in order to produce significant amounts of NO, human hepatocytes (Geller *et al.*, 1993b; Nussler *et al.*, 1992) and human VSMC (Geller *et al.*, 1993a) require a cytokine cocktail consisting of LPS, IFN-γ, TNF-α, and IL-2. Whether these tissue responses represent differences in receptor expression, transcriptional activators, or both has not been determined.

2. *Cytokine Inhibition of iNOS*

During inflammation, the ability to downregulate an effector molecule may be as important as its upregulation in order to avoid excess damage to normal tissue. This could be particularly important in organs such as the lung in which normal function is dependent on maintaining a delicate tissue structure. Information gathered over the past decade suggests that there are a number of cytokines that downregulate the immune response after removal of the pathogens. Most studies that have investigated cytokine inhibition of iNOS activity have evaluated Mø NO production since this is the primary effector cell involved in a CMI response and is likely to be controlled by both T cell cytokines and autocrine effects from cytokines released from activated Mø.

Transforming growth factor-β (TGF-β) was the initial cytokine described that inhibited NO production by Mø (Ding *et al.*, 1988). Incubating TGF-β with Mø in the presence of LPS decreased NO production by decreasing stability and translation of mRNA as well as increasing iNOS protein degradation (Vodovotz *et al.*, 1993). However, the mechanism of TFG-β-induced inhibition of NO production is tissue specific. Thus, in VSMC, TGF-β inhibited IL-1β/TNF-α-induced iNOS transcription (Perrella *et al.*, 1994). TGF-β2 inhibited NO production by IL-1/TNF-α-stimulated renal mesangial cells (Pfeilschifter and Vosbeck, 1991). Surprisingly, TGF-β was reported to *enhance* NO production by Swiss 3T3 fibroblasts (Gilbert and Herschman, 1993). Thus, the role of TGF-β in regulating NO production may be determined by the specific functions of a cell type.

Interleukin-10 is primarily produced by activated Mø and plays a significant role in downregulating multiple aspects of immune responses (Mosmann, 1991). IFN-γ-treated Mø can kill *Schistosoma mansoni in vitro.* Addition of IL-10-inhibited IFN-γ-induced killing of *S. mansoni.* IL-10 treatment caused a decrease in TNF-α–RNA expression by IFN-γ-treated Mø, and addition to exogenous TNF-α restored the ability of IFN-γ-treated Mø to kill parasites in the presence of IL-10. These studies suggested that IL-10-inhibited iNOS transcription by inhibiting a TNF-α-regulated pathway (Oswald *et al.*, 1992; Gazzinelli *et al.*, 1992). Recent *in vivo* studies demonstrated that administration of IL-10 antibody to mice protected susceptible mice from a lethal infection with *Candida albicans* (Romani *et al.*, 1994). The protection correlated with an enhanced ability of the mice to produce NO.

Interleukin-4 has several important immunomodulatory activities during the immune response. It facilitates the development of a Th2 response (Mosmann and Coffman, 1989b), leads to enhancement of humoral immunity, and may be important in the development of IgE responses in asth-

matics. IL-4 also suppresses iNOS activity in IFN-γ and LPS-activated Mø (Bogdan *et al.*, 1994). The suppressive mechanism occurs at two levels; initially, IL-4 decreases iNOS protein concentration without affecting iNOS mRNA expression. At later times, however, IL-4 decreases iNOS mRNA production. Other *in vivo* studies characterized the inability of mice vaccinated with an attenuated *Salmonella typhimurium* to mount an antibody response to heterologous antigens. These studies demonstrated that a Mø-released product caused the inhibition and NOS inhibitors or IL-4 reversed the inhibition (al-Ramadi *et al.*, 1992). Thus, IL-4 and IFN-γ have reciprocal actions on NOS activity by Mø. Finally, IL-13 has also been demonstrated to inhibit NO production by activated Mø by an undetermined mechanism (Doherty *et al.*, 1993). A balance among these cytokines during infections may determine what role, if any, NO will have during the immune response.

New classes of immunoregulatory molecules, the chemokines, have recently been described (Miller and Krangel, 1992). These molecules are important for the initial recruitment and activation of neutrophils and monocytes early in the course of an inflammatory response. Monocyte chemotactic protein-1 treatment of the murine J774 cell line stimulated with IFN-γ and LPS inhibited the production of NO (Rojas *et al.*, 1993a). Similarly, interleukin-8 (McCall *et al.*, 1992) inhibited NO production by rat neutrophils. The mechanisms for the inhibitory action of these agents have not been defined.

3. *Cofactor and Substrate Regulation of iNOS*

Although the primary mode of iNOS regulation during an immune response is mediated by cytokines and microbial constituents, other mechanisms may affect iNOS activity. As discussed previously, iNOS activity requires arginine and several cofactors including NADPH and BH_4. Alterations in cofactor levels during inflammation may regulate the activity of NOS. Thus, Mø can secrete arginase (Benninghoff *et al.*, 1991) and arginase production was enhanced by LPS (Benninghoff *et al.*, 1991). Increased arginase levels in the inflammatory milieu may limit the substrate required for NOS activity (Albina *et al.*, 1988). A possible mechanism for regeneration of arginine within activated cells was reported. Cytokine activation of VSMC induced synthesis of argininosuccinate synthetase (Hattori *et al.*, 1994; Nussler *et al.*, 1994). This enzyme, together with argininosuccinate lyase, can regenerate arginine from citrulline and provide an endogenous source of arginine for NO production. Other studies demonstrated that L-arginine transport is elevated in LPS-activated Mø providing another mechanism for sustained substrate levels during inflammation (Bogle *et al.*, 1992). BH_4 availability is limiting in some tissues (Gross and Levi,

1992; Gross *et al.*, 1993; Sakai and Milstien, 1993; Schmidt *et al.*, 1992; Schoedon *et al.*, 1993; Werner-Felmayer *et al.*, 1993). Others have shown that iron availability also significantly alters iNOS transcription (Kubrina *et al.*, 1993; Weiss *et al.*, 1994). Thus, the regulation of proteins that affect levels of substrate and NOS cofactors may provide yet another level of NO regulation during an inflammatory response.

4. Effector Mechanisms of NO

NO microbistatic and microbicidal mechanisms have not been well defined. However, the effects of NO on eucaryotic cells suggest clues to potential mechanisms. Activated Mø inhibited DNA synthesis by tumor cells and one target in the tumor cell was mitochondrial respiration (Dijkmans and Billiau, 1991; Geng *et al.*, 1994; Granger *et al.*, 1980). Thus, Mø-released substances inhibited complex I and complex II as well as aconitase (Heiss *et al.*, 1994). An association of NO as the effector molecule in this system was demonstrated when Hibbs *et al.* (1987a) showed an inhibition of mitochondrial respiration in hepatoma cells treated with NO. These studies were subsequently confirmed (Stuehr *et al.*, 1989) and extended to include NO inhibition of ribonucleotide reductase, an enzyme vital in DNA synthesis (Lepoivre *et al.*, 1990). A common link among all the enzymes inhibited by NO is that they contain an associated iron moiety. Experiments have shown that upon exposure of Fe–S groups to NO an iron–nitrosyl compound forms. *In vivo* studies have confirmed that iron–nitrosyl compound formation is arginine dependent in activated, but not in resting hepatocytes (Nussler and Billiar, 1993). NO also inhibited zinc-finger transcription factors by interacting with and destroying the required zinc–sulfur clusters in these proteins (Kroncke *et al.*, 1994). These data suggest that one important mechanism by which NO inhibits growth or kills pathogens is through inhibition of metal-requiring enzymes in the invading organisms.

At least two studies suggested that NO radicals are not the direct effector molecules. The first study utilized 2-phenyl-4,4,5,5,-tetramethyllimidazoline-3-oxide-1-oxyl (PTIO), an oxygen donor, to scavenge free NO radicals during the killing of *Cryptococci neoformans* by activated Mø (Yoshida *et al.*, 1993). The study indicated that PTIO enhanced fungicidal activity and suggested that PTIO interacted with NO to produce other nitrogen metabolites such as NO_2 radicals. The authors hypothesized that instead of NO providing fungicidal activity, other nitrogen metabolites were responsible for microbicidal activity observed in NO-generating systems. Supporting the hypothesis was a second study that examined the ability of NO and its metabolites to kill *Plasmodium falciparum* (Rockett *et al.*, 1991). This study demonstrated that while a saturated solution of NO was

unable to inhibit parasite growth, both nitrite and nitrate were toxic to the parasite. Rockett *et al.* (1991) extended this study to show that nitrosothiol derivatives of cysteine and glutathione were also extremely potent inhibitors of parasite growth.

B. Infection Models

The role of NO as an antimicrobial molecule during infection is under intensive study. As discussed previously, it is likely that responding T cells and their secreted cytokines result in Mø activation (Mosmann and Coffman, 1989a). Mø activation appears to be particularly important for defending against infection by intracellular organisms including bacteria, *M. tuberculosis* (J. Chan, 1992; Flesch and Kaufmann, 1991), *Francisella tularensis* (Fortier *et al.*, 1992), and parasites such as *Leishmania major* (Liew, 1990) and *Toxoplasma gondii* (Adams *et al.*, 1990). Most studies that demonstrate a role for NO as an effector molecule involve these organisms. A more complete list of the organisms for which NO demonstrated a role as an effector molecule is shown in Table II.

Initial studies demonstrated that NO from activated Mø inhibited or killed multiple organisms. LPS- and IFN-γ-activated Mø inhibited the growth of the fungus, *C. neoformans* (Granger *et al.*, 1986) and growth inhibition was arginine dependent, inhibited by L-NMMA, and correlated with NO_2 and NO_3 production (Granger *et al.*, 1990). Direct exposure of Cne to NO also resulted in growth inhibition (Alspaugh and Granger, 1991). Activated Mø also inhibited *T. gondii* (Adams *et al.*, 1990) and *L. major* (Liew *et al.*, 1990, 1991) in an arginine-dependent manner. *In vivo* studies using *Corynebacterium parvum*-injected mice, which demonstrated enhanced resistance to *T. gondii* infection, indicated that *C. parvum*-induced resistance was eliminated if L-NMMA was administered to mice prior to inoculation with *T. gondii* (Adams *et al.*, 1990). Thus, enhanced resistance in this model was due to NO production by the *C. parvum*-primed Mø.

Several animal models examined the role of NO during *in vivo* infections. C.B.17–SCID mice, which lack functional T and B cells, and normal C.B.17 mice are both resistant to Listeria. T cell-independent resistance to *L. monocytogenes* by SCID mice relies on IFN-γ production by IL-1 and TNF-α-stimulated NK cells to activate Mø (reviewed in Bancroft *et al.*, 1991). A role for Mø-produced NO in T cell-independent resistance to *L. monocytogenes* infection was examined (Beckerman *et al.*, 1993). Spleen cells from noninfected SCID mice incubated with heat-killed *L. monocytogenes* for 48 hr produced NO and NO production was inhibited by aminoguanidine, anti-IFN-γ antibody, or anti-TNF-α antibody. In this system, anti-TNF-α antibody inhibited NO production by eliminating

TABLE II

Organisms	References
	Bacteria
Chlamydia trachomatis	Mayer *et al.* (1993)
Legionella pneumophilia	Summersgill *et al.* (1992)
Listeria monocytogenes	Beckerman *et al.* (1993)
Mycobacterium tuberculosis	Chan *et al.* (1992), Denis (1991a)
Mycobacterium leprae	Adams *et al.* (1991)
Mycobacterium bovis	Flesch and Kaufman (1991)
Ehrlichia risticii	Park and Rikitisa (1992)
Francisella tularensis	Green *et al.* (1993b)
	Protozoa
Plasmodium chabaudi chabaudi	Taylor-Robinson *et al.* (1993)
Leishmania major	Liew *et al.* (1990) (#437), Green *et al.* (1990), Evans *et al.* (1993), Green *et al.* (1993), Birkland *et al.* (1992), Stenger *et al.* (1994)
Leishmania donovani	Roach *et al.* (1991)
Toxoplasma gondii	Adams *et al.* (1990)
Trypanosoma cruzi	Munoz-Fernandez *et al.* (1992b)
Trypanosoma musculi	Vincendeau and Daulonede (1991)
Naegleri fowleri	Fischer-Stenger and Marciano-Cabral (1992)
	Fungi
Cryptococcus neoformans	Granger *et al.* (1986), Alspaugh and Granger (1991)
Histoplasma capsulatum	Nakamura *et al.* (1994)
Candida albicans	Romani *et al.* (1994)
	Helminths
Schistosoma mansoni	James and Glaven, (1989)

NK cell production of IFN-γ. The addition of live *L. monocytogenes* and IFN-γ to peritoneal macrophages from SCID mice resulted in reduced growth of *L. monocytogenes* and growth inhibition was inhibited by aminoguanidine. Administration of aminoguanidine (50 mg/kg) twice daily to *L. monocytogenes*-infected immunocompetent C.B.17 and C.B.17–SCID mice resulted in a significant increase in mortality compared to sham-treated animals. Aminoguanidine treatment also resulted in higher numbers of *L. monocytogenes* colony-forming units recovered from the spleens of infected animals. These experiments indicated that Mø NO production played a critical role in T cell-independent resistance to *L. monocytogenes.*

Leishmania major is an intracellular parasite that requires T cell immunity and cytokine-induced Mø activation for effective host resistance. Infected mice maintained on a nitrite/nitrate-free diet were monitored for increased urine nitrate production as an *in vivo* measure of NO production (Evans *et al.*, 1993). Resistant C3H/HeN mice had significantly higher

levels of urine nitrate production compared to susceptible BALB/c mice. The urine nitrate level decreased and the parasitic load increased in C3H/HeN mice given L-NMMA in their drinking water. This data confirmed *in vitro* data suggesting the importance of NO in Mø resistance to *L. major*. A recent study used immunohistochemistry to monitor the tissue expression of NO during a footpad *L. major* infection. These studies showed that resistance to *L. major* correlated with tissue expression of NO at the inflammatory site, and at least in the strains of mice used in this study, NO expression was inversely proportional to TGF-β expression (Stenger *et al.*, 1994). These experiments were interpreted as suggesting that one element of a susceptible phenotype may relate to downregulation of iNOS by TGF-β.

The importance of cytokine regulation of NO during an infection was demonstrated in a mouse model of *F. tularensis* (Green *et al.*, 1993b). Intraperitoneal inoculation of normal mice with *F. tularensis* results in a lethal infection. Resistance to infection was induced by ip inoculation of BCG prior to *F. tularensis* inoculation and correlated with an elevated urinary nitrate production. Administration of anti-TNF-α or anti-IFN-γ antibodies at the time of BCG inoculation eliminated the increase in urinary nitrate as well as prevented protection against *F. tularensis*.

The role of NO in a *C. neoformans* pulmonary infection model was examined. In this model, yeast were intratracheally inoculated in small amounts and CFU were determined from lung homogenates. In C.B.17 mice, inoculated yeast grow rapidly in lungs during the initial 7 days postinoculation. After 7 days, C.B.17 mice exhibited a rapid T cell-dependent clearance of yeast from lung (Huffnagle *et al.*, 1991). An analysis of the cytokine response during infection demonstrated that yeast clearance correlated with IFN-γ production by lung-associated lymph node cells (Hoag *et al.*, 1994). Other experiments examining the role of NO in pulmonary infection by *C. neoformans* indicated that clearance correlated with iNOS expression by lung cells, increased levels of urine nitrate, and the clearance was inhibited by either anti-IFN-γ antibody of by feeding L-NMMA (Lovchik *et al.*, 1994). Taken together, these *in vivo* studies indicated an important role for these cytokines in the physiological induction of reactive nitrogen oxides during infection.

C. Inflammatory Disease Models

1. Arthritis

Rheumatoid arthritis is a joint disease characterized by chronic destructive inflammation in joint spaces. Morphological and cellular studies suggest that the disease is initiated by activation of T cells in joints (Simon *et al.*,

1994) which release cytokines that recruit and activate Mø to perpetuate inflammation. One result of Mø activation is NO production. Further, both articular chondrocytes (Stadler *et al.*, 1991b) and synovial fibroblasts can produce substantial amounts of NO in response to cytokines which can lead to high levels of intraarticular NO. Indeed, two separate groups of researchers have verified that intraarticular NO is increased in arthritic joints in humans (Farrell *et al.*, 1992; Jacob *et al.*, 1992).

A potential role for NO-induced joint destruction was evaluated in a rat arthritis model (McCartney-Francis *et al.*, 1993). Susceptible Lewis rats were given streptococcal cell wall fragments to induce chronic, erosive arthritis. The acute phase (Days 0–12) of the disease is characterized by infiltration of neutrophils followed by a chronic phase (Days 12–26) characterized by an influx of T cells and Mø. If L-NMMA was given to rats during either the acute or the chronic phase, joint swelling and cell infiltration was significantly decreased. During the acute phase, recruitment of leukocytes was unaffected by L-NMMA, while joint swelling was significantly decreased. During the chronic phase, however, T cell and Mø recruitment were significantly reduced in joints of rats treated with L-NMMA and joint swelling was substantially reduced as well. Thus, clinical improvement observed during the chronic phase might have been caused by both decreased production of NO locally as well as decreased recruitment of inflammatory cells. Since Northern analysis of iNOS RNA from joints of control and L-NMMA-treated rats were similar yet cell recruitment was decreased in L-NMMA-treated rats, it suggested that local resident joint cells, such as articular chondrocytes and fibroblasts, rather than infiltrating inflammatory cells, were the predominant cells expressing iNOS.

A similar decrease in clinical arthritis was observed in an adjuvant arthritis model of Lewis rats (Ialenti *et al.*, 1993). Lewis rats develop a chronic polyarthritis after intradermal injection of an oil emulsion of dead *M. tuberculosis*. The arthritis results from a T cell-mediated delayed-type hypersensitivity reaction (Cohen, 1991). Rats that were fed L-NAME in their drinking water prior to and after adjuvant injection had significantly less arthritis and decreased systemic symptoms (i.e., loss of weight compared to control arthritic rats and arthritic rats fed excess arginine). The clinical improvement correlated with a decrease in spontaneous NO production by peritoneal Mø from L-NAME-treated rats compared to control and arginine-fed groups (Ialenti *et al.*, 1993).

2. *Diabetes Mellitus*

An appreciation for a possible role for NO in diabetes is evolving. The hypothesis that type I diabetes is an autoimmune disease that results in

islet cell destruction has led to investigations identifying potential mediators of islet cell damage. Recent studies suggested that cytokines can induce islet cells to secrete NO (Corbett *et al.*, 1991), which acts in an autocrine fashion to inhibit insulin release (Corbett *et al.*, 1992, 1993). Streptozocin is a diabetogenic compound known to decrease islet cell function although the exact mechanism remains unknown. In the streptozocin-induced diabetes model, NO may be one mediator that causes islet cell dysfunction. Administration of L-NMMA to mice given repeated doses of streptozocin protected mice from developing diabetes. Histological examination of pancreata from L-NMMA-treated mice demonstrated little or no cellular infiltration compared to pancreata from control mice (Lukic *et al.*, 1991). Other studies have demonstrated that NO donors produced fragmentation of islet cell DNA, even in the presence of endonuclease inhibitors, suggesting that DNA was a direct target of NO (Fehsel *et al.*, 1993). Thus, the authors suggested that part of the islet cell damage incurred during local inflammation may be secondary to NO interaction with islet cell DNA.

3. *Immune–Complex Disease*

A rat model of pulmonary immune–complex disease was developed (Mulligan *et al.*, 1991). Intratracheal infusion of preformed complexes of antigen and antigen-specific antibodies resulted in immune complex deposition with associated inflammation. Administration of complexes containing either IgG (Mulligan *et al.*, 1991) or IgA (Mulligan *et al.*, 1992) induced acute lung inflammation that resulted in red blood cell leakage and protein extravasation into lung parenchyma. IgG immune–complex-induced inflammation required an intact complement system, TNF-α induction, expression of ELAM-1, and neutrophils. IgA immune–complex-induced inflammation was also complement dependent, but did not require TNF-α induction, ELAM-1 expression, or neutrophils. A role for NO or its metabolites in inducing inflammation by both IgG and IgA complexes was assessed by treating animals with L-NMMA and measuring the effect on pulmonary inflammation. Mice treated with L-NMMA prior to either IgG or IgA complex administration had a significant reduction in plasma extravasation which correlated with a decrease in NO_2 recovered in bronchoalveolar lavage fluids. It was unclear whether NO was responsible for pulmonary damage or, as suggested by the authors, damage was caused by a combination of superoxides and NO with resultant generation of peroxynitrite and hydroxyl radicals (Radi *et al.*, 1991b).

D. Role of NO in Inflammation-Induced Carcinogenesis

Chronic inflammation is an important risk factor in cancer development. Two examples are the association of chronic hepatitis infection with hepato-

cellular carcinoma and the linkage between bladder infection with *S. haematobium* and bladder cancer. One hypothesis is that the chronic activation of effector cells results in the release of products, particularly reactive radicals, which contributes to carcinogenesis. The demonstration that activated Mø and neutrophils can produce NO led to studies examining the potential role of NO in carcinogenesis (Bartsch *et al.*, 1992). An epidemiological association between liver flukes, *Opisthorchis viverrini* or *Clonorchis sinensis*, and cholangiocarcinoma exists (Parkin *et al.*, 1991). Urine nitrate levels from liver fluke-infected patients were found to be elevated (Srianujata *et al.*, 1987). Similarly, patients with liver cirrhosis, a population with an increased risk for development of hepatocellular carcinoma, were shown to have an elevated urine nitrate level compared to normal control subjects (Ohshima and Bartsch, 1994). In a Woodchuck model of chronic hepatitis infection, hepatocytes isolated from infected animals produced significantly more N–nitroso compounds in response to LPS than did hepatocytes from noninfected animals suggesting that the chronic inflammation associated with this infection was continually "priming" hepatocytes (Liu *et al.*, 1992). The effect of this priming could lead to excessive amounts of NO production in response to inflammatory stimuli compared to NO production by nonchronically infected livers with a resultant increase in NO-induced carcinogenic events.

Mechanisms by which NO could contribute to carcinogenesis during chronic infections have not been precisely identified. One possible mechanism would be to increase local concentrations of nitrosamines, which are well-known animal carcinogens. Nitrosamines can be formed directly through the reaction of amines with NO (Grisham *et al.*, 1992; Iyengar *et al.*, 1987; Miwa *et al.*, 1987). NO also directly damaged DNA and induced mutations in DNA strands (Nguyen *et al.*, 1992). Treatment of *Salmonella typhimurium* with NO donors or NO gas resulted in DNA mutations (Maragos *et al.*, 1993; Wink *et al.*, 1991) and direct exposure of DNA to NO resulted in a time-dependent deamination of nuclear bases (Wink *et al.*, 1991). Further study on this potentially important mechanism is needed to define the role of NO in carcinogenesis.

VI. NO as a Protective Molecule during the Inflammatory Response

Most studies have suggested that NO acts as a tissue-damaging molecule during inflammation, although other data suggest that NO plays a protective role during inflammation (Nussler *et al.*, 1993). A role for NO in providing a protective response was shown in a mouse sepsis model and a rat gastric ischemia–reperfusion model. In the sepsis model, mice were given an ip injection of LPS and hepatic damage was assessed by monitoring blood

levels of AST and LDH as well as ornithine carbamoyltransferase, a more specific marker of hepatic parenchyma injury. Dose-dependent hepatic damage occurred in response to LPS (Harbrecht *et al.*, 1992). Blood nitrate levels were elevated indicating the expected LPS-induced increase in NO production by several cell types (Liu *et al.*, 1993). However, when NOS activity was inhibited by L-NMMA injection 6 hr after LPS inoculation, when iNOS mRNA levels are maximally expressed, liver damage increased and was proportional to the amount of LPS given. An important control was the injection of L-NMMA without LPS which did not change the normal level of hepatic enzyme blood levels, indicating that L-NMMA itself was not hepatotoxic. These authors had previously reported that NO could decrease hepatic protein synthesis (Billiar *et al.*, 1989) and mitochondrial respiration (Stadler *et al.*, 1991a) and suggested that it was through these mechanisms that NO provided a protective response during endotoxemia. Other potential protective mechanisms could be to either inhibit neutrophil aggregation and mobility to suppress the leukocyte component of the inflammatory response or to enhance local vasodilatation and maintain hepatic blood supply.

A protective role for NO in maintaining intestinal vascular integrity during an acute high-dose LPS–sepsis (50 mg/kg) model was found by others (Hutcheson *et al.*, 1990). In these studies, rats were injected with LPS, and 15 min later macroscopic and histological intestinal damage and changes in vascular permeability determined by leakage of ^{125}I-labeled albumin were assessed. Pretreatment with L-NMMA prior to LPS challenge resulted in a significant increase in mucosal damage and an increase in vascular leak compared to rats injected with LPS alone. The protective effect of NO in this model is in contrast to that of the rat sepsis model (Boughton-Smith *et al.*, 1993) discussed under Section III which suggested a mucosal damaging role for NO. The authors suggest that the differences might be due to the different amounts of LPS given (50 vs 3 mg/kg) in the two different experiments.

A similar protective effect for NO was observed in a rat gastric–ischemia model (Andrews *et al.*, 1994). In this model, the stomach blood supply was interrupted while the stomach lumen was maintained in an acidic state. After 25 min of ischemia the acid was removed from the stomach, the blood supply was allowed to reperfuse tissues for 15 min, and the rats were sacrificed. The extent of gastric mucosal damage incurred as a result of ischemia was assessed using histological techniques. The ischemia-induced mucosal damage was decreased by infusion of NO donors, including nitroprusside, during the ischemic time period. Acetylcholine also provided protection and the protection was eliminated if L-NMMA was given prior to acetylcholine, suggesting that local acetylcholine-induced NO release

was responsible for the protection. NO-induced vasodilatation apparently was not responsible for the protection because administration of papavarine, a NO-independent vasodilator, did not provide protection from ischemia. Interestingly, administration of L-NMMA prior to and during the ischemic time period did not worsen mucosal damage, and the authors suggested that this might be due to an already significant reduction of NO due to endothelial injury and/or effects of locally elevated superoxide. Histological examination of mice given NO donors or acetylcholine indicated that there was a significant reduction in the number of infiltrating mucosal neutrophils. Thus, at least one mechanism for protection by elevation of NO in this model might be an inhibition of neutrophil extravasation into the mucosa. Whether this was a result of NO effects on the neutrophil or on the endothelium was unclear.

VII. NO in Immunoregulation

The role of NO as an immunoregulatory molecule continues to be an active area of research. Initial studies indicated that NO inhibited T cell proliferation to mitogens (Fu and Blankenhorn, 1992; Mills, 1991; Tomioka and Saito, 1992) and antigens (Albina *et al.*, 1991; Denham and Rowland, 1992). These studies have now been extended to examine the role of NO in regulating distinct T cells capable of enhancing a CMI response (Th1) versus a predominant humoral response (Th2) during an immune response. Clearly, the predominant regulation of T cell subset regulation is mediated by specific cytokines that drive responses, but recent studies suggest that NO may also play a role. When cloned Th1 and Th2 cells were exposed to NO, the secretion of IFN-γ and IL-2 by Th1 clones was inhibited, while IL-4 production by Th2 clones remained intact (Taylor-Robinson *et al.*, 1994). Thus, NO production during an inflammatory response may downregulate Th1 cytokine responses and decrease local inflammatory mediators.

In support of an immunomodulatory role for NO are recent studies that analyzed a protective role for NO in a staphylococcal entertoxin B-induced (SEB) shock model (Florquin *et al.*, 1994). Mice administered 100 μg of SEB survived. Following injection of SEB, blood nitrate levels increased and the increase was inhibited by antibodies to TNF-α or IFN-γ. This finding was compatible with the known "superantigen" effect of SEB to stimulate a high percentage of T cells resulting in the release of T cell cytokines (Buelow *et al.*, 1992). High levels of IFN-γ and TNF-α should induce iNOS in Mø. Surprisingly, survival of SEB-injected mice was *decreased* in mice given L-NAME; 80% of L-NAME-treated mice were dead within 96 hr after SEB inoculation. TNF-α and IFN-γ were significantly

elevated in L-NAME/SEB-treated mice compared to SEB-treated mice. Treatment of L-NAME/SEB-inoculated mice with antibodies to IFN-γ and TNF-α reduced the mortality observed in L-NAME/SEB-treated mice. The investigators interpreted these data as demonstrating an important role for NO in downregulating excess IFN-γ and TNF-α levels which decreased the pathogenic effects of SEB (Florquin *et al.*, 1994).

Alternatively, an important mechanism by which NO might modulate an immune response to downregulate NO production is by acting on macrophages in an autocrine fashion. At least two different mechanisms may be involved in downregulating Mø NO production. Studies demonstrated that NO treatment of activated Mø-induced cells to undergo apoptosis, thus removing Mø from sites of inflammation (Albina *et al.*, 1993). Still another study indicated that NO donors irreversibly inhibited NOS activity in a dose-dependent manner (Assreuy *et al.*, 1993). One might speculate that, because iNOS requires heme for activity, the mechanism of this inhibition is through interaction with the heme moiety in the same manner as NO inhibits other Fe-requiring enzymes as discussed previously.

VIII. NO as a Pain Mediator

Pain is a classic hallmark of inflammation. One hypothesis for pain during inflammation is that nociceptors that are usually dormant are influenced by mediators, including cyclooxgenase metabolites and sympathomimetic amines within the local milieu, to produce a hyperalgesia state. For example, IL-1β and IL-8 induce nociceptors into a hyperalgesia state (Cunha *et al.*, 1991; Ferreira *et al.*, 1988). Hyperalgesic nociceptors may then be activated by a variety of stimuli, which under noninflammatory conditions would not generate a painful response.

Hyperalgesia states result from increased intracellular cAMP within nociceptors (Ferreira and Nakamura, 1979). The most well-studied inducers of hyperalgesia are cyclooxygenase metabolites such as prostaglandins. It is widely accepted that the mechanism by which aspirin-like drugs prevent the sensitization of nociceptors is by preventing prostaglandin synthesis. A possible indirect role for NO in this process was recently described. Prostaglandin production relies on the activity of cyclooxygenase (COX) enzymes. Some COX enzymes are constituitive (COX-1) while others are induced (COX-2) by inflammatory mediators which may exacerbate the hyperalgesia state in inflammation. A recent paper examined the role of NO on the activity of both COX-1 and COX-2 forms in the Mø cell line, RAW 264.7 (Salvemini *et al.*, 1993). The production of nitrite and PGE2 was monitored as a measure of iNOS and COX activity, respectively. RAW 264.7 produced both nitrite and PGE2 in response to LPS

(1 μ/ml). Both nitrite and PGE2 production by LPS-stimulated RAW 264.7 cells were inhibited by L-NMMA or aminoguanidine. In separate studies, NO donors were shown to enhance the activity of recombinant COX-1 and -2. Thus, in the early stages of inflammation, local generation of NO could enhance the development of nociceptor hyperalgesia by increasing COX activity resulting in higher levels of proinflammatory prostaglandins (Salvemini *et al.*, 1993).

Recent experiments demonstrated that administration of cGMP blocked hyperalgesia suggesting that the state of nociceptor activation was determined by intracellular cAMP/cGMP ratio. Analgesics that can directly block the ongoing hyperalgesia include peripheral opiates, dipyrone and diclofenac (Ferreira, 1993). It has recently been shown that these analgesic agents directly downregulate the hyperalgesia state by increasing intracellular cGMP and studies indicated that the elevation of cGMP was via activation of an arginine/nitric oxide/cGMP pathway within the sensory neurons (Duarte *et al.*, 1990, 1992; Tonussi and Ferreira, 1994). Thus, NO appears to have two distinct roles in inflammatory pain depending on the site of production; first, NO produced by effector cells in response to cytokines can enhance the induction of hyperalgesia by increasing COX enzymes, and second, analgesic stimulation of sensory neurons activates a NO-producing pathway that can decrease the hyperalgesic state.

IX. NO in Wound Healing

Arginine was shown to be important for efficient wound healing in a rat wound healing model (Albina *et al.*, 1988). Arginine can be metabolized via two different routes: the NO pathway results in citrulline and reactive nitrogen metabolites and the arginase pathway results in production of urea and ornithine. Using these end-products as a measure of the predominant arginine metabolic pathway, Albina *et al.* (1990) examined the different pathways of arginine metabolism during an experimental wound healing model in the rat. Early (<3 days) in the wound healing process, citrulline and nitrate predominated in the secretions suggesting an active role for the NOS pathway. At later time points (>3 days) the predominant arginine metabolite was urea and ornithine. This sequence of events seems logical, because during the period immediately after wounding, NO may provide vasodilatation and microbiostasis, both important events for efficient wound healing. However, during later stages of wound healing, arginine might be needed in tissue repair and protein synthesis.

A recent paper connected NO production, epidermal growth factor (EGF) and regulation of keratinocyte growth during the wound healing process (Heck *et al.*, 1992). Keratinocytes stopped proliferating in re-

sponse to LPS and the inflammatory cytokines, IFN-γ and TNF-α, and growth inhibition correlated with the expression of a Ca^{2+} and calmodulin-dependent NOS. When EGF was added to the keratinocyte culture with the inflammatory cytokines, NOS activity was inhibited and the growth inhibition was reversed. Thus, EGF may be an important downregulator of NO activity during the wound healing process to allow for fibroblast growth.

X. Human Studies

The importance of cNOS activity in neural and vascular biology is clear and beyond the scope of this review (Knowles and Moncada, 1994). This section will concentrate on the expression iNOS in human effector cells. The first experiments to suggest that humans could generate NO came from studies examining nitrate biosynthesis in young men. In 1916, Mitchell *et al.* noted excess nitrates in human urine. In 1981 Green *et al.* placed men on a low nitrate diet and monitored urinary nitrate (Green *et al.*, 1981a). These results indicated that regardless of the amount of nitrogen consumed endogenous nitrate biosynthesis occurred. With the discovery of several different NOS isoforms, a search for tissue expression of different NOS isoforms in humans readily demonstrated constituitive forms (Buttery *et al.*, 1994; Janssens *et al.*, 1992; Knowles and Moncada, 1994; Marsden *et al.*, 1992; Miyahara *et al.*, 1994; Sessa *et al.*, 1992), but the iNOS was more elusive. In a study designed to evaluate nitrogen oxide production in sepsis and trauma, septic patients generated significantly more urine nitrates than patients with nonseptic trauma (Ochoa *et al.*, 1991). Perhaps the best evidence for the expression of human iNOS came from a previously mentioned study that monitored the production of nitrate in the serum and urine of patients receiving interleukin-2 therapy for malignancies (Hibbs *et al.*, 1992). Patients were kept on a defined diet and renal function was carefully monitored to rule out changes in renal function as a cause for the changes in urine nitrate. In metabolic tracer studies, patients were given L-[guandino-$^{15}N_2$]arginine which demonstrated that the nitrate production was derived from the guanidino nitrogen of L-arginine, consistent with production of the nitrates through the iNOS pathway.

In vitro studies demonstrated iNOS production by human hepatocytes (Nussler *et al.*, 1992, 1993) and VSMC (Bernhardt *et al.*, 1991; Qi *et al.*, 1993). Cytokines required for optimal stimulation included TNF-α, IL-1, IFN-γ, and LPS. This cytokine requirement was much more complex than the requirements to induce iNOS expression in rodent Mø. Human iNOS was finally cloned from a cDNA library generated from cytokine-stimulated hepatocytes and a labeled human iNOS probe detected iNOS mRNA from cytokine-stimulated human hepatocytes (Geller *et al.*, 1993a) and human

VSMC (Geller *et al.*, 1993a). At about the same time, another group, using a cDNA library from IL-1-stimulated human articular chondrocytes, also cloned human iNOS (Charles *et al.*, 1993; Maier *et al.*, 1994). Data comparing human iNOS with murine Mø iNOS suggested that iNOS was strongly conserved across different cell types and species. No data were shown for expression of iNOS RNA in stimulated human Mø in either of the above reports.

Several studies indicated that human neutrophils generate NO (Schmidt *et al.*, 1989; Wright *et al.*, 1989). Dichlorofluorescein (DCFH) oxidation was used to monitor NO production by neutrophils (Rao *et al.*, 1992). In these studies, when neutrophils were incubated with the calmodulin antagonist W-13, an inhibitor of superoxide anion generation, the oxidation of DCFH was enhanced. That this unexpected finding was due to neutrophil production of NO was confirmed by demonstrating that incubating neutrophils with W-13 and L-NMMA, which inhibit both superoxides and NO, respectively, completely abrogated DCFH oxidation. A different study demonstrated NOS activity in neutrophils by analyzing the ability of anucleate, granule-poor fragments [cytokinoplasts (CPK)] isolated from neutrophils to kill CPK-associated staphylococci (Malawista *et al.*, 1992). L-NMMA treatment of CPK resulted in a significant decrease in the CPK's ability to kill CPK-associated staphylococci. Decreased killing was reversed when excess arginine was added. Interestingly, staphylococci killing by intact neutrophils was not inhibited by L-NMMA. Therefore, these authors suggest that NO generation by neutrophils may represent a secondary antimicrobial mechanism. Another possibility is that oxygen radicals produced by intact neutrophils inactivated NO and masked NO production.

Whereas the production and relevance of NO synthesis by rodent Mø is firmly established (Nathan, 1992), the role of NO synthesis in human Mø remains extremely controversial. Early studies failed to show a role for NO production by human alveolar Mø and monocytes as a defense against fungus (Cameron *et al.*, 1990), bacteria (Murray and Teitalbaum, 1992), or parasites (James *et al.*, 1990). This was followed by a careful analysis of NO production by human monocytes/macrophages by Schneemann *et al.* (1993). In this study, the ability of human mononuclear phagocytes from blood and peritoneum to produce NO in response to LPS and multiple cytokines, including IFN-γ, GM-CSF, and TNF-α, was monitored (Schneemann *et al.*, 1993). BH_4 was added to culture because of speculation that human Mø might not make BH_4 efficiently (Schoedon *et al.*, 1987). However, later data indicated that human Mø do make BH_4 (Sakai and Milstien, 1993). NO production by human mononuclear phagocytes was not detected by either the Griess reaction or citrulline production. Moreover, a similar study by a different group also failed to show NO synthesis by

IFN-γ-activated human monocytes in the presence of added BH_4 (Werner *et al.*, 1991).

Alternatively, a number of studies suggest that monocytes can synthesize NO. TNF-α-activated monocytes growth inhibit or kill virulent and avirulent *M. avium* and this effect was increased by GM-CSF (Denis, 1991b). The combination of cytokines and *M. avium* resulted in nitrite generation by monocytes. Nitrite production and growth inhibition was decreased by L-NMMA suggesting that NO production was from arginine (Denis, 1991b). In a related study, another group demonstrated that infection of monocytes with live, but not gamma-irradiated, virulent *M. avium* resulted in production of nitrite (Dumarey *et al.*, 1994). Nitrite production was inhibited with L-NMMA and, interestingly, was not enhanced by the addition of cytokines. However, monocyte growth inhibition of *M. avium* was not affected by L-NMMA suggesting that NO synthesis in this model was not required for antibacterial activity. Nitrite production was also shown in studies examining *Pneumocystis carinii* infection of human alveolar Mø (Sherman *et al.*, 1991) and *T. cruzi* infection of human monocytes (Munoz-Fernandez *et al.*, 1992a). An immunohistochemistry analysis of human lung also identified iNOS protein in human alveolar Mø (Kobzik *et al.*, 1993). A recent study using reverse-transcriptase polymerase chain reaction techniques demonstrated that human iNOS was induced in purified monocytes and multiple monocytoid cell lines following LPS stimulation (Reiling *et al.*, 1994) although no enzymatic functional activity was demonstrated.

Three different studies have suggested that monocytes released NO in their basal state. Monocytes from individuals with alcoholic liver disease secreted a basal level of nitrite which was enhanced by treatment with LPS (Hunt and Goldin, 1992). Both the basal level and the LPS-induced nitrite production were abrogated with addition of L-NMMA. In another study, monocytes which were allowed to undergo differentiation in culture also secreted basal nitrite. Spontaneous nitrite production from monocytes of multiple donors was compared and donors fell into two groups, low-nitrite producers (<10 nmol/2 $\times$ 10^6 cells) and high-nitrite producers (>10 nmol/2 $\times$ 10^6 cells), as judged by monocyte nitrite production during 12 *days* in culture (Mautino *et al.*, 1994). The basal production of nitrite by both low- and high-nitrite producers was inhibited by L-NMMA. The authors extended these studies to examine the effect of IL-4 on nitrite production by monocytes from the low and high producers. IL-4 inhibited nitrite production by high-producer monocytes, while IL-4 enhanced nitrite production by low-producer monocytes. In a recent study, the gp120 HIV envelope glycoprotein induced NO production by cultured monocytes as measured by electron paramagnetic resonance spin trapping (Pietraforte *et al.*, 1994). The adduct measured in these studies was a result of hydroxyla-

tion of the substrate 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). The hydroxylation of DMPO by gp120-treated monocytes was inhibited with L-NMMA. An important aspect of stimulated monocyte NO production, when it is observed, is that the level of NO is significantly less (0.1–20 nmol/24 hr) compared to rodent macrophages (2–60 μmol/24 hr). Thus, it is possible that NO production by human monocytes might play a signaling role via cGMP instead of as a direct microbicidal mechanism.

In conclusion, the role of iNOS in human Mø remains unsettled. The fact that the levels of nitrite produced by the monocyte are significantly lower than those of rodent Mø may indicate a minimal role for direct microbicidal activity although no one has yet determined how much NO is actually required to provide a protective response. Indeed, NO may be only one of several microbicidal mechanisms that the monocyte uses to defend the host. Hopefully, further study of NO regulation in the human as well as continued investigation into the mechanisms of human monocyte microbial defenses will shed much needed light on this controversial topic.

XI. Conclusions

NO certainly appears to have earned its 1992 (Culotta and Koshland, 1992) molecule of the year status. NO plays a multifaceted role in all phases of the inflammatory immune response depending on the source and quantity of NO produced. It is important in the development of therapeutic modalities based on NO biochemistry and biology to take all roles of NO into consideration when designing potential treatments.

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