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Proteasomes and Antigen Processing

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I. Introduction

The vertebrate immune system plays a primary role as a body defense–surveillance mechanism against numerous invaders (equivalent to non-self-antigens). One of the most important responses in this antigen-specific immune system is to distinguish correctly non-self-antigens from self-antigens for selective elimination because deviation from this recognition would lead to a variety of opportunistic infections or autoimmune diseases. In consideration of the fact that all antigenic proteins are composed of entirely the same amino acid residues, however, it is not so easy to discriminate exactly the difference between self- and non-self-antigens at the molecular level. No one contests that T lymphocytes play a central role in this strict recognition, but it is of note that T cells, unlike immunoglobulins, are incapable of recognizing antigens directly. By means of recognizing strictly non-self-antigens by T cells, the immune system has acquired an unusual mechanism through evolution. T lymphocytes with antigen-specific T cell receptors (TCRs), which acquire a great deal of diversity by gene rearrangement, can react specifically with the complex of foreign protein fragments (termed antigenic peptides) and their receptor molecules, the major histocompatibility complex (MHC), which are presented on the cell surface of antigen presentation and/or target cells. T lymphocytes activated through this process can initiate the immune response by secreting various cytokines (e.g., interferons and interleukins) to induce proliferation and differentiation of target T and/or B cells and cytotoxic molecules, such as perforin and granzymes, or by introducing the apoptotic cell death signal. Therefore, generation of antigenic peptides and their intracellular transport to surface membranes are thought to be initial events for the immune response. These actions would be necessary to distinguish only the lymphocyte that reacts with one specific antigen from numerous lymphocytes having diverse individual TCRs. However, it is noteworthy that the biological recognition between self- and non-self-antigens *in vivo* would be

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achieved by removal through programmed cell death of lymphocytes reactive with self-antigens during development of lymphocytes in the thymus, which results in the condition known as immune tolerance. Therefore, processing and presentation of self-antigens are also important for the acquisition of immune tolerance.

Non-self-antigens are commonly categorized into two classes depending on the source of the foreign proteins: one is intracellular (endogenous) and the other is extracellular (exogenous) antigens, which differ in their location in the body, being present respectively inside or outside cells after invasion. As a consequence of this difference, their peptide-binding receptor MHC molecules are also divided into two classes: MHC class I and class II proteins, respectively. Class I MHC molecules present peptides to T cells with the accessory molecule CD8, whereas class II molecules present peptides to CD4-positive T cells. MHC class II molecules are associated with peptides derived from exogenous proteins entrapped inside antigen-presenting cells, such as macrophages, dendritic cells, and B cells, by the endocytic pathway (Germain and Margulies, 1993; Germain, 1994). The endocytosed proteins are dealt with endosomes by proteases such as cathepsins B and D that reside there during their acidification, and the generated antigenic peptides become bound to class II molecules in a special peptide-loading organelle called MHC class II compartment or class II-containing vesicle. Such specialized endosomes contribute to facilitate the exchange between CLIP, a peptide that is derived from an invariant chain associated with a class II molecule and serves to protect binding against peptides derived from endogenous antigens in endoplasmic reticulum (ER), and antigenic peptides produced in endosomes. The resulting peptide–MHC complexes are transferred to the cell surface for presentation to $CD4^+$ helper T cells, which support proliferation and differentiation of specific immunoglobulin-producing B cells by secretion of interleukins (Germain, 1994). This system underlies the humoral immunity, but this antigen processing/presentation mechanism is outside the scope of this chapter.

The humoral immune mechanism is ineffective against viral and parasite proteins or tumor-specific proteins generated by mutation and malignant transformation. These antigens are presented with the MHC class I molecules on the surface of all somatic cells and are recognized by $CD8^+$ cytotoxic T lymphocytes (CTLs; also called killer T cells). The association of antigenic peptide ligands derived from intracellular proteins with their receptor class I molecules takes place in the ER. The degradation of such proteins is mediated by presumptive cytosolic proteases (see below) and the resulting antigenic peptides must therefore cross the ER membrane prior to their binding to class I molecules. Activated CTLs, which continu-

ously recirculate throughout the body, directly destroy target cells expressing endogenous non-self-antigens. This defense mechanism is termed cell-mediated immunity, which is constitutively active in most cells. However, the mechanism of processing of endogenous antigens has not been clarified until recently, although it has been suggested to differ from that of processing exogenous antigens for humoral immunity. Actually, lysosomal proteases are unlikely candidates for generation of the majority of class I-restricted peptides, even though lysosomes can take up cytosolic proteins by autophagy or molecular chaperone-mediated facilitated transportation, because various lysosomotropic inhibitors of lysosomal activity operating in living cells, including chloroquine or ammonium chloride and catheptic inhibitors of microbial origin, are ineffective against the processing pathway for endogenous antigens. Therefore, a novel proteolysis pathway responsible for processing of the endogenous antigens must be present in the cytosolic fraction of the cell.

Recent studies on nonlysosomal, intracellular protein degradation have shown that the ATP-dependent proteolytic system consisting of ubiquitin and proteasomes is responsible for selective degradation of a variety of cytosolic and nuclear proteins (Hershko and Ciechanover, 1992; Coux *et al.*, 1996). Actually, the ubiquitin–proteasome pathway appears to be involved in the selective removal of regulatory proteins with a rapid turnover, such as cyclin, p53, c-Mos, c-Fos, and c-Jun, which are closely related to cell-cycle progression and/or metabolic regulation. Moreover, this degradation system catalyzes not only exhaustive proteolysis of these naturally occurring, short-lived proteins and abnormal/malfolded proteins generated in cells by environmental stresses or genetic mutation (Coux *et al.*, 1996; Ciechanover, 1994) but also processing of precursor NFκB to its mature form (Palombella *et al.*, 1994). Recently, the ubiquitin–proteasome system has been implicated in the MHC class I-restricted antigen processing responsible for generation of suitable peptides to associate with class I molecules (Goldberg and Rock, 1992; Monaco, 1992). Details of the MHC class I-restricted antigen processing and presentation pathway have been expertly reviewed (Yewdell and Bennink, 1992; Benham *et al.*, 1995; Heemels and Ploegh, 1995; York and Rock, 1996; Monaco and Nandi, 1995), but the field has continued to develop rapidly. In this review, therefore, we have focused our attention on the roles of proteasomes and their regulators in antigen processing with special reference to the influence of γ-interferon (γ-IFN) on both the structure and the functions of the proteasome. We cite mainly references concerning proteasomes and review articles for those readers in other fields as well as refer to other important papers recently published.

II. TAP System

At the end of 1990, several groups reported the existence of molecules responsible for peptide transport from the cytoplasm into the ER. These findings were based on studies of mutant cell lines that had a low expression of class I MHC because of a deficiency in the processing of endogenous antigens (Parham, 1990), suggesting that lack of suitable peptides in the ER results in impairment of class I assembly. The genes responsible for this defect were mapped within the class II MHC region. These molecules were initially named PSF1/2 (*p*eptide *s*upply *f*actor), Ham1/2 (*h*isto-compatibility *a*ntigen *m*odifier), RING4/11 (*r*eally *i*nteresting *n*ew gene), mtp1/2 (*M*HC-linked *t*rans*p*orter gene), but they have now been unified under the term TAP (*t*ransporter associated with *a*ntigen *p*rocessing). The TAP system consists of two highly homologous, but not identical, subunits, TAP1 and TAP2, that are members of the ATP-binding cassette family of transport proteins (Parham, 1990). The sequence of both TAP1 and TAP2 proteins shows that they have multiple membrane-spanning regions and consensus ATP binding sites in their cytoplasmic domain, near the C terminus. TAP1 and TAP2 appear to be associated physically, as suggested by their coimmunoprecipitation with antibodies against the individual proteins, implying that they form a peptide pump as a heterodimeric complex associated noncovalently, although their topology and molecular organization are still unknown (for reviews, see Townsend and Trowsdale, 1993; Momburg *et al.*, 1994; Howard, 1995; Hill and Ploegh, 1995; Androleweicz and Cresswell, 1996).

Several lines of evidence clearly demonstrate that the TAP system plays an indispensable role for transportation of antigenic peptides from the cytoplasm into the lumen of the ER, where these peptides then associate with MHC class I molecules (Townsend and Trowsdale, 1993; Momburg *et al.*, 1994; Howard, 1995; Hill and Ploegh, 1995). First, transfection of TAP-deficient cells with *TAP1* and *TAP2* cDNAs resulted in almost full restoration of proper presentation of endogenous antigens in the deficient cells. Second, the TAP proteins were found to be localized in the membranes of the ER and the *cis* Golgi. This subcellular location is consistent with the location in cells where MHC molecules bind peptides. Third, mice with disrupted *TAP1* showed deficiencies of surface expression of class I molecules and disappearance of $CD4^{-}8^{+}$ T cells in their peripheral blood (Van Kaer *et al.*, 1992). Moreover, a *TAP2* natural mutation was found in two severely immunodeficient patients (de la Salle *et al.*, 1994). Finally, *TAP* genes showed polymorphism as seen in various immunoregulatory genes. The polymorphism in TAP is responsible for transfer of distinct antigenic peptides in rats but does not seem to have a major effect on their specificities for peptides in animals other than rats. Such a pro-

found defect in antigen presentation in cells lacking *TAP1* or *TAP2* indicates that this transporter is responsible for supplying most peptides for class I presentation in order to initiate cell-mediated immunity.

Subsequently, *in vitro* assay systems using semi-intact, permeable cells or microsome vesicles have provided biochemical evidence for the functions of TAP (Townsend and Trowsdale, 1993; Momburg *et al.*, 1994; Howard, 1995; Hill and Ploegh, 1995; Androleweicz and Cresswell, 1996). Peptide translocation mediated by the TAP across the ER membrane is demonstrated to occur in an ATP-dependent fashion. Hydrolysis of ATP was required for the peptide translocation process but not for peptide binding to TAP proteins. The TAP translocator prefers to entrap peptides with hydrophobic amino acid residues at the C terminus. Correspondingly, C termini of class I-binding peptides are mostly hydrophobic amino acid residues (Engelhard, 1994; Rammensee *et al.*, 1995). Accordingly, the C-terminal amino acid is an important element not only for class I binding but also in the selectivity of the TAP peptide transportation. It should be noted, however, that the TAP system does not have a definite preference for peptide sequences, unlike the strict recognition by MHC molecules. In addition, the TAP transporter shows an appreciable preference for the length of peptides translocated. The efficiency of translocation drops markedly for peptides shorter than 8 and longer than approximately 14 amino acid residues in *in vitro* assays, although the transporter is able to transport considerably longer peptides with very low efficiency. Such preference of the TAP system with respect to length is apparently in agreement with the size of peptides associated with cell surface class I molecules, i.e., 8–10 amino acid residues (Engelhard, 1994; Rammensee *et al.*, 1995). Thus, TAP mediates ATP-dependent, sequence- and size-selective transportation of oligopeptides through membranes of the ER, and therefore the functions of TAP perhaps contribute somehow to peptide selection.

III. Ubiquitin System

The endogenous proteins must be limitedly digested prior to the binding of their fragments to class I molecules; the complex is then presented as the epitope. The indispensable role of the TAP peptide transporter system for the antigen presentation strongly suggests that endogenous antigens are processed by a cytosolic proteolytic system. Consequently, the cytosol has been shown to be the primary source of presentable peptides because normally synthesized cytosolic proteins or native proteins artificially introduced into the cytosol by means of osmotic loading, electroporation, or the expression vector system are properly processed for class I-restricted antigen presentation (Townsend and Trowsdale, 1993; Momburg *et al.*,

1994; Howard, 1995; Hill and Ploegh, 1995). However, the enzyme responsible for generation of antigenic peptides was not known until recent appreciable progress was made through studies of the mechanism for intracellular protein breakdown. Indeed, the proteolytic systems in the cytosol have not yet been well characterized. Although calpains, a family of cystein proteinases requiring Ca^{2+} for proteolysis, are distributed ubiquitously in the cytoplasmic compartment of various higher eukaryotic cells, they do not appear to be involved in the processing of intracellular antigens because their specific inhibitors are unable to suppress the class I peptide presentation. The available evidence indicates that the ubiquitin–proteasome proteolytic pathway, which operates universally in eukaryotic cell, may be responsible for MHC class I-restricted antigen processing (Goldberg and Rock, 1992). This energy-dependent degradation system is composed of two distinct, but sequential, processes. The first process is mediated by the ubiquitin system, which acts to tag the target protein for degradation. The subsequent degradative process is catalyzed by the proteasome, an ATP-dependent protease complex.

The ubiquitin-mediated pathway involves the covalent attachment of the ubiquitin moiety to target proteins through isopeptide linkage formation between the carboxyl terminus of ubiquitin and the ε amino group of the lysine residue on the protein substrate. Additional ubiquitin moieties can be ligated via the lysine residue of ubiquitin itself, resulting in the formation of a multiubiquitin chain that acts as a degradation signal for their selective breakdown, possibly being recognized by the 26S proteasome (see below). A simplified model of this pathway is shown in Fig. 1. Protein ubiquitination involves three classes of enzymes (Hershko and Ciechanover, 1992; Hochstrasser, 1997). The first step is the binding of ubiquitin to E1, the ubiquitin-activating enzyme, through thioester bond. ATP energy is required for this reaction. The second step is the catalyzation by the ubiquitin-conjugating enzymes, termed E2 (Jentsch, 1992). Alternatively, some E2s act as carrier proteins. The final step is the catalyzation by E3s, which are also classified into two species with different functions: one can recognize target proteins, and the other acts as a conjugating enzyme. The former E3 enzymes play a major role in defining the substrate specificity of the ubiquitin system, perhaps through direct binding of substrates. Ubiquitin attachment to a protein requires that a certain amino-terminal residue on the targeted protein be recognized by ubiquitin-recognition protein E3. This requirement is called the N-end rule (Varshavsky, 1992). The latter E3 enzymes were identified during studies on the system responsible for ubiquitination of tumor suppressor gene product p53; thereafter, various novel species of E3 involving the E1–E2–E3 cascade ubiquitination reaction were found to be present ubiquitously in various cells in yeast to human. In this pathway, ubiquitin is transferred sequentially in a thioester cascade from

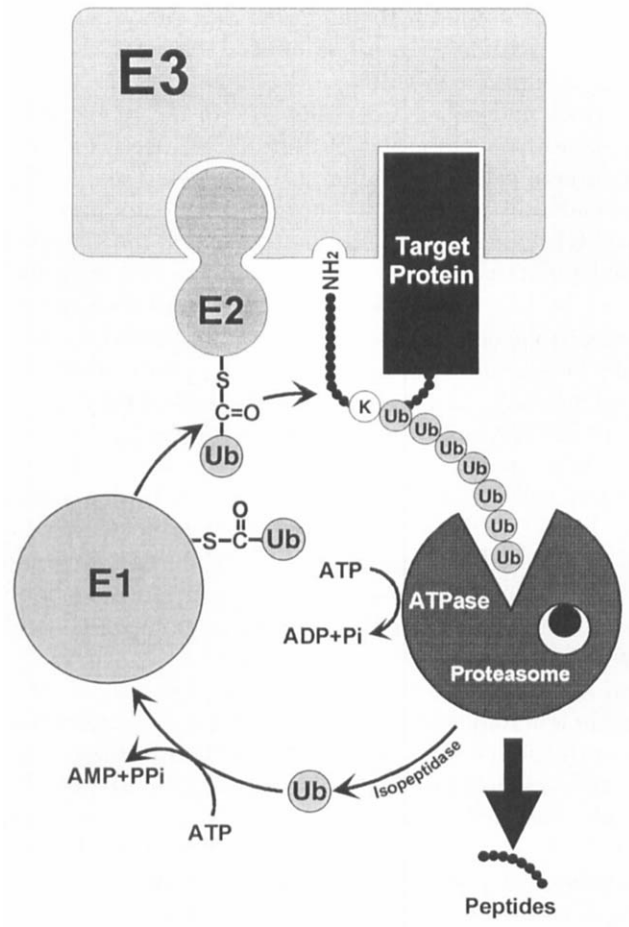

FIG. 1. Model of intracellular protein degradation mediated by the ubiquitin–proteasome system. Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme (also termed carrier protein); E3, ubiquitin–protein ligase (recognition protein).

E1 to a specific E2, and finally to E3, which then transfers the ubiquitin directly to substrates in the form of a stable isopeptide bond (Scheffner *et al.*, 1995). In addition, a novel E3 ligase for ubiquitination of cyclin B that allows cells to exit from the M-phase of the cell cycle was found that consists of a large complex termed the 20S APC complex (*a*naphase-promoting complex) and that is equivalent to cyclosome (Sudakin *et al.*, 1995; King *et al.*, 1995). Thus, the ubiquitin system is still expanding into a variety of cell biology fields.

Initial experiments indicating the involvement of the ubiquitin pathway in antigen processing showed that the enhanced degradation of viral pro-

tein fused with the ubiquitin moiety increased presentation of the proteins recognized by CTLs (Townsend *et al.*, 1988). More direct evidence addressing the importance of ubiquitination in antigen presentation was reported by Rock and colleagues (Michalek *et al.*, 1993), who studied a temperature-sensitive (ts) mutant of the ubiquitin-activating enzyme E1. The presentation of native ovalbumin (OVA) injected into an E1 ts mutant cell line was markedly inhibited at the restrictive temperature. The defect induced by ts E1 appeared to be in the processing reaction itself without affecting the later steps such as TAP transportation of antigenic peptides, assemblage of the latter with class I molecules, and vesicular transport of the complex onto the cell surface; if the need for proteolysis was bypassed by expressing the relevant OVA peptide directly in the cytosol, the presentation occurred normally in the ts E1 mutant cells. However, the role of ubiquitination in OVA presentation was questioned by Cox *et al.* (1995), who failed to detect an E1-related decrease in the presentation of biosynthesized or osmotically loaded OVA to a T cell hybridoma after thermal inactivation of ts E1. Although the reason for the discrepancy is unknown, the level of suppressed E1 activity might be different, depending on the conditions used. Indeed, it is known that ubiquitin conjugation cannot be completely inhibited in such cells carrying ts mutated E1 and thus the phenotypes of individual ts E1 cells may vary.

Moreover, Grant *et al.* (1995) found that the rate of MHC class I presentation of microinjected antigenic epitope β-galactosidase was enhanced when this antigenic protein was modified to have a destabilizing terminal amino acid residue according to the N-end rule (Varshavsky, 1992). Actually, the rates of presentation of β-galacosidases that can induce CTL specific for the epitope are roughly correlated with their degradation rates, suggesting that proteolysis mediated according to the N-end rule plays a critical role in antigen processing and that ubiquitin conjugation is a key rate-limiting step in antigen presentation. However, it is unknown whether degradation of most cellular proteins mediated by the ubiquitin pathway leads to the generation of antigenic peptides. It should be examined whether many species of proteins including viral proteins are degraded by the ubiquitin pathway and their products utilized as antigenic peptides.

IV. Proteasome System

A. Structural and Catalytic Features

The proteasome is a major cytosolic proteinase catalyzing energy-dependent, extralysosomal proteolysis. It appears to be present in the cytosol and nucleus of all eukaryotic cells. Eukaryotic proteasomes are unusually large complexes with a heterogeneous subunit composition and

have been classified into two isoforms with apparently distinct sedimentation coefficients of 20S and 26S (Goldberg and Rock, 1992; Coux *et al.*, 1996). The molecular masses of the 20S and 26S proteasomes were determined to be approximately 750 and 2000 kDa, respectively, by various physical techniques (Tanaka *et al.*, 1988; Yoshimura *et al.*, 1993).

The 20S proteasome is composed of a set of small subunits with molecular masses of 21–32 kDa and is also termed a multicatalytic/multifunctional proteinase complex because it has multiple active sites responsible for endoproteolytic cleavage of peptide bonds on the carboxyl side of acidic, basic, and hydrophobic amino acid residues of proteins (Orlowski, 1990; Rivett, 1993). The manifestation of multiple catalytic activities at independent active sites within a single enzyme complex appears advantageous for the rapid and complete breakdown of various types of cellular proteins. Near the end of 1994, the primary structures of all subunits for yeast and human proteasomes were determined by cloning of their cDNAs and/or genes (Tanaka, 1995; Hilt and Wolf, 1995). All proteasomal genes examined so far encode previously unidentified proteins, which show considerable intersubunit homology in the same species and high evolutionary conservation in various eukaryotes (Tanaka *et al.*, 1992). Electron microscopy showed that the 20S proteasome appears to be a cylindrical particle with a dimeric structure of two distinct rings: their gross structure has been conserved during evolution, which is similar in organisms ranging from eubacteria and archaebacteria to eukaryotes (Lupas *et al.*, 1993; Tamura *et al.*, 1995). The archaebacterial 20S proteasome is a cylindrical particle consisting of four rings, each of which comprises seven identical α or β subunits: The rings are assembled in the order $\alpha\beta\beta\alpha$. (Löwe *et al.*, 1995) Analogously, the subunit organization or eukaryotic proteasomes is presumed to be $\alpha n(1\text{–}7)\beta n(1\text{–}7)\beta n(1\text{–}7)\alpha n(1\text{–}7)$, where n indicates the number of seven heterogeneous subunits (Tanaka, 1995).

The 26S proteasome is a multimolecular assembly consisting of a central 20S proteasome with catalytic functions and two terminal subsets with possible regulatory roles (Rechsteiner *et al.*, 1993; Coux *et al.*, 1996). By electron microscopy in conjunction with digital image analysis, the 26S proteasome appears to be a dumbbell-shaped particle with two large V-like or U-like terminal structures in opposite orientations attached to a smaller, four-layered central 20S proteasome. The 26S proteasomal configuration has been highly conserved in various eukaryotes, including yeast (Rubin *et al.*, 1996), plants (Fujinami *et al.*, 1994), amphibia (Peters *et al.*, 1993), and mammals (Yoshimura *et al.*, 1993), but it has not yet been found in other lower organisms. The 26S proteasome complex can be formed *in vitro* by the ATP-dependent association of the 20S proteasome with another large complex composed of approximately 20 proteins that

range in molecular masses from 25 to 112 kDa; this complex has been isolated by several laboratories and given diverse names, such as the ball, 19S cap complex, ATPase complex, μ particle, PA700, and 22S complex (for details, see reviews by Peters, 1994; Dubiel *et al.*, 1995; Coux *et al.*, 1996).

The proteolytic process mediated by the 26S proteasome is schematically presented in Fig. 2. The terminal regulatory unit would be expected to have multiple functions considering the unusual heterogenous subunit organization. The large, roughly V-shaped structure may be the site of entry of the substrate protein; that is, it seems to function as the "mouth" for the 20S's digestive machinery (Lupas *et al.*, 1993; Rubin and Finley, 1995; Jentsch and Schienker, 1995; Coux *et al.*, 1996). Indeed, the regulatory unit provides several proteins necessary for selectively degrading ubiquitinated proteins, including a ubiquitin receptor that acts as a binding site for multiubiquitin chains (Deveraux *et al.*, 1994) and an isopeptidase for reutilization of the ubiquitin moieties (Hochstrasser, 1995). Moreover, at least six distinct, but related, ATPase family proteins are associated with the regulatory unit (Rechsteiner *et al.*, 1993; Tanaka, 1995; DeMartino *et al.*, 1996). It is currently unclear why so many ATPases are present in the 26S proteasome. One probable explanation is that each of the multiple

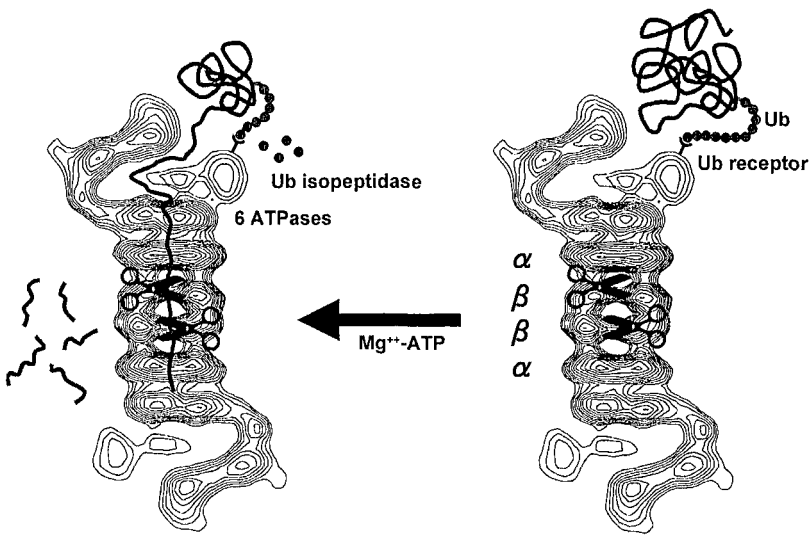

FIG. 2. Schematic view of degradation of ubiquitinated proteins by the 26S proteasome. α and β, α- and β-subunit rings, respectively, of the 20S proteasome; Ub, ubiquitin; Ub receptor, multi-Ub binding protein. For details, see text.

ATPase subunits interacts with individual subunits of the outer α-subunit ring of the central 20S proteasome to form the 26S proteasome, but it remains to be established that all these ATPases are found in the same 26S complex. Alternatively, it is still possible that the isolated 26S proteasomes are in fact a heterogeneous population of very similar complexes, except for their ATPase content.

The 26S proteasomal ATPases appear to be essential for proteolytic function, but the molecular basis of the mechanisms with this energy requirement is still unknown. One role of ATP hydrolysis is to supply energy for the association of the 20S catalytic proteasome and the terminal regulator unit to form the 26S complex. It is unclear whether this assembly reaction occurs only during the biosynthesis of the 26S complex or whether dissociation and reassembly of the particle occur frequently according to the proteolytic reaction cycle. The energy requirement for the proteolytic functioning of the 26S proteasome is unexpected on thermodynamic grounds because peptide bond hydrolysis is an exergonic reaction catalyzed by numerous proteases that show no ATP requirement. One possible function of the ATPases could be to inject the substrate protein into the 20S particle and thus to promote entry of a polypeptide into the central 20S proteasomal degradative chamber. In addition, the progressive degradation of ubiquitinated substrates, which may be globular or partially unfolded, may require additional unfolding reactions because active sites were shown to be localized inside the β-subunit ring, which has very limited space and is thus unable to entrap or allow penetration of a target protein with tertiary structure (Löwer *et al.*, 1995). Thus, a likely role for ATP hydrolysis in the 26S complex could be to promote the full unfolding of substrates in a chaperone-like manner (Lupas *et al.*, 1993; Rubin and Finley, 1995).

B. Effect of Selective Proteasome Inhibitors

Involvement of the proteasome in the processing reaction for endogenous antigen has been directly indicated by the suppressive effect of various proteasome inhibitors. A peptide aldehyde inhibitor was the first used to show that the proteasome catalyzed the generation of antigenic peptides without affecting subsequent processes—that is, intracellular transportation from the cytoplasm to ER and ER to surface membranes (Rock *et al.*, 1994). Dipeptide aldehyde also was reported to similarly inhibit not only the proteasomal activity but also the antigen processing mechanism (Harding *et al.*, 1995). However, these inhibitors affected the activities of various cystein proteases, such as calpains and lysosomal cathepsins, in addition to the proteasomal activities, although it was ascertained that other inhibitors specific for cysteine proteases had no effect on the processing

mechanism. Therefore, the possibility was completely ruled out that uncharacterized proteases sensitive to these inhibitors were involved. The novel proteasome-specific microbial inhibitor lactacystin (Ōmura *et al.*, 1991; Fenteany *et al.*, 1995) was also found to inhibit the antigen processing activity (Coux *et al.*, 1996; York and Rock, 1996). These results suggest that the proteasome plays a major role in supplying peptides to class I molecules.

C. LMP2/LMP7 AND γ-IFN REGULATION

Monaco and McDevitt (1982) initially described large complexes of low-molecular-weight proteins (LMPs), which were encoded in the MHC class II region and induced by γ-IFN (for reviews, see Monaco, 1992; Monaco and Nandi, 1995). The first evidence implicating these particles in antigen presentation came from the cloning of the *LMP2* and *LMP7* genes, whose sequences were found to closely resemble those of the β subunits of the 20S proteasome. In addition, two of the multiple LMP proteins, corresponding to LMP2 and LMP7, were shown to have a polymorphic nature (Monaco, 1992). Later chromosomal walking and jumping in the MHC *TAP* genes led to the identification of two novel proteasomal subunits that were identical to LMP2 and LMP7 (Robertson, 1991).

Studies on the effect of γ-IFN are of particular interest for revealing the exact role of the proteasome in the immune system because γ-IFN is a well-known major immunomodulatory cytokine (Billiau, 1996). Actually, γ-IFN alters the proteolytic specificity of proteasomes, increasing their activities for cleavage of peptide bonds on the carboxyl side of basic and hydrophobic amino acid residues of fluorogenic substrates but decreasing their activities for peptides containing acidic amino acid residues, without affecting the activity for ATP-, ubiquitin-dependent proteolysis (Gaczynska *et al.*, 1993; Driscoll *et al.*, 1993; Aki *et al.*, 1993). These modifications of peptidase activity suggest that, during protein breakdown, the proteasomes from γ-IFN-treated cells should generate more peptides that have hydrophobic or basic carboxyl termini and fewer peptides with acidic carboxyl termini. This change in cleavage specificity would favor peptides with carboxyl termini most commonly found bound to class I molecules because a hydrophobic or basic carboxy-terminal residue is required for strong binding of a peptide to MHC class I molecules (Engelhard, 1994; Rammensee *et al.*, 1995). In addition, such peptides are selectively transported into the ER by the TAP transporter (Townsend and Trowsdale, 1993; Momburg *et al.*, 1994; Howard, 1995; Hill and Ploegh, 1995). Although the magnitude of these effects varies, these findings have been confirmed by various laboratories using multiple cell types. However, conflicting results showing a reduction in chymotryptic activity by γ-IFN were re-

ported by other investigators (Boes *et al.*, 1994; Ustrell *et al.*, 1995). This discrepancy may be due to technical differences in the procedures used for isolation of the proteasomes. Actually, it seems to be quite difficult to determine the characteristics of the unmodified proteasome because enzymatic properties of proteasomes change considerably, depending on the means of purification. It may be better to use the ultracentrifuged precipitate of the cells including proteasomes as the enzyme source. However, it should be noted that the activity in this fraction may not reflect the accurate values for the proteasomes themselves because this enzyme source is thought to contain not only both 20S and 26S proteasomes but also the activator proteins that are also modified by γ-IFN (see below). An alternative explanation for these controversial results is that there were also differences in both the types of cells used and the conditions employed for treatment with γ-IFN, but the exact reason is currently unclear.

Presumably, transfection of MHC-encoded *LMP* cDNAs would provide a more direct means to ascertain their contribution to the proteasomal functions. When the *LMP7* gene was transfected into lymphoblasts of HeLa cells (Gaczynska *et al.*, 1994), the LMP7 content increased; in parallel, the chymotryptic and tryptic activities of the multifunctional proteasome were enhanced. Moreover, transfection with the *LMP2* gene increased the LMP2 content and caused a proportional suppression of the peptidylglutamyl peptide-hydrolyzing activity. On the other hand, Kloetzel and colleagues (Kuckelkorn *et al.*, 1995) reported opposite effects on chymotryptic activity by similar transfections with *LMP2/7* cDNAs in T2 cells lacking MHC-encoded *LMP* and *TAP* genes. However, they also found that the presence of these LMP subunits induced a drastic increase in positive cooperativity between the proteasomal subunits for cleavage-site preference of a synthetic 25-mer polypeptide, implying that γ-IFN considerably induces a shift of cleavage specificity.

Mutant cell lines with a large deletion in the MHC, encompassing both *LMP* genes and *TAP* genes, display grossly impaired class I assembly and are incapable of presenting endogenously synthesized antigens. Class I expression was restored fully upon transfection of these mutant cells with the cDNAs encoding *TAP1* and *TAP2* (Arnold *et al.*, 1992; Momburg *et al.*, 1992; Yewdell *et al.*, 1994; Zhou *et al.*, 1994). These results were initially interpreted to indicate that these subunits were not important for antigen presentation. In an attempt to clarify the role of LMPs in antigen presentation, mice with disrupted *LMP2* or *LMP7* genes were generated by means of homologous recombination. Mice lacking *LMP7* showed reduced surface levels of MHC class I molecules and impaired presentation of viral antigens, thereby firmly establishing the role of LMP7 for supplying peptides necessary for class I MHC assembly (Fehling *et al.*, 1994). Indeed,

the reduced class I levels were restored by incubating *LMP7*-deficient cells with the appropriate peptides, indicating that peptide supply is the limiting factor in class I assembly. These findings seen in the *LMP7* knockout animals are not consistent with the conclusion reached in earlier studies using mutant cell lines, i.e., that LMP7 is not necessary for antigen presentation (Arnold *et al.*, 1992; Momburg *et al.*, 1992; Yewdell *et al.*, 1994; Zhou *et al.*, 1994). Presumably, the deletion mutants lacking *LMP*s and *TAP*s were transfected with the latter, and transfectants might have been selected for high class I expression, thereby favoring cells expressing multiple copies of the peptide transporters (Heemels and Ploegh, 1995). The deletion of the *LMP2* gene did not result in a profound phenotypic change, including changes in cell surface class I expression, unlike the disruption of the *LMP7* gene (Van Kaer *et al.*, 1994). However, the *LMP2*-deficient mice revealed features subtly distinct from those of the wild-type ones. Proteasomes from lymphoblastoid lines lacking LMP2 exhibited enhanced post-glutamyl activity and reduced chymotryptic and tryptic-like activities. Also, these latter activities were unaffected by γ-IFN treatment. The macrophages and spleen cells exhibited a clear defect in presentation of a viral antigen, including an epitope from influenza nucleoprotein that is able to induce a specific CTL, in mice carrying a deletion in *LMP2*. Moreover, in a T-cell lymphoma that displayed a selective defect in MHC class I-restricted presentation of influenza virus antigens and underexpressed only LMP2, γ-IFN simultaneously restored LMP2 expression and antigen presentation, implying that LMP2 can directly influence both MHC class I-restricted antigen presentation and class I surface expression (Sibille *et al.*, 1995). The role of LMP7 in the generation of a certain viral epitope was also confirmed (Cerundolo *et al.*, 1995). These observations indicate clearly that the γ-IFN-induced modification of proteasome composition can increase the efficiency of immune surveillance.

D. Subunits X/Y and Immunoproteasomes

During genetic analysis of proteasome genes, the involvement of proteasomes in immunoregulation was fortuitously discovered. As mentioned previously, the subunit organization of eukaryotic proteasomes is presumed to be $\alpha n(1\text{–}7)\beta n(1\text{–}7)\beta n(1\text{–}7)\alpha n(1\text{–}7)$, where n indicates the number of 7 heterogeneous subunits. Indeed, 7 human cDNAs encoding α-type subunits have been cloned, each of which has been conserved from that of its presumed yeast counterpart. Moreover, in yeast 7 genes encoding β subunits, have been isolated, in accord with the molecular organization of the β ring, which is organized from seven homologous subunits. On the other hand, 10 human cDNAs of β subunits have been found so far. The existence of 10 β subunits is curious considering that one β-type ring is

composed of 7 subunits. Why are more than 7 β-type subunits present in multicellular organisms but not in yeast? Clearly, there are three extra species of proteasomal β genes in the vertebrate animal and they are suspected to be involved in the immune system because two polymorphic MHC-encoded proteasomal genes, *LMP2* and *LMP7*, were shown to belong to the β-type subfamily.

Subsequently, γ-IFN was shown to affect the subunit composition of the proteasome (Yang *et al.*, 1992; Aki *et al.*, 1993; Boes *et al.*, 1994; Brown *et al.*, 1993). Indeed, the expressions of LMP2 and LMP7 were found to be greatly induced in response to γ-IFN, as seen for *TAP* and MHC genes, although the degree of inducibility and/or basal expression levels differed depending on the type of cells and tissues examined. However, the expressions of LMP2 and LMP7 by γ-IFN are curious, considering that the proteasomal β ring is organized by seven β subunits. Intriguingly, in contrast to the expressions of LMP2 and LMP7, two subunits of proteasomes immunoprecipitated by antiproteasomal antibodies were found to be regulated negatively in response to γ-IFN; nevertheless, the patterns of almost all other subunits remained unchanged. These two subunits were designated X and Y (Akiyama *et al.*, 1994a). Similar results were also obtained with various other types of cells (Boes *et al.*, 1994). Molecular cloning of cDNAs encoding X and Y showed that X (also named MB1 or ε) and Y (equivalent to δ) are novel proteasomal subunits with high homology to LMP7 and LMP2, respectively (Akiyama *et al.*, 1994b; Belich *et al.*, 1994). This adaptation is proposed to be due to γ-IFN-dependent subunit replacement of X and Y by the structurally similar subunits LMP7 and LMP2, respectively, because these pairs of subunits with extremely high amino acid similarity have been shown to be expressed reciprocally in response to γ-IFN (Akiyama *et al.*, 1994b; Belich *et al.*, 1994; Früh *et al.*, 1994). This γ-IFN-induced subunit replacement in proteasomes is presumably responsible for changes in the functional diversity of proteasomes, as observed in cells treated with γ-IFN (Gaczynska *et al.*, 1993; Driscoll *et al.*, 1993; Aki *et al.*, 1993).

This alteration of the subunit pattern was presumably due to changes in the biosynthesis of X and Y because γ-IFN had no effect on the levels of X and Y synthesized before its addition. Thus, it is unlikely that exchange of posttranslationally modified subunits with subunits of preexisting proteasomes is involved in the formation of γ-IFN-induced proteasomes (Aki *et al.*, 1993). Possibly, transcriptional regulation of MHC-encoded proteasome genes participates in construction of the altered proteasomes because the levels of mRNAs encoding LMP2 and LMP7 were in apparent accord with their protein amounts. Moreover, no free pools of LMP2/7 proteins were found in cells, indicating that the large amounts of LMP2/7 synthe-

sized in response to γ-IFN were all incorporated into the proteasome complex. Accordingly, the most probable explanation for the mechanism of subunit substitution is the preferential incorporation of γ-IFN-inducible LMP subunits and the possibly rapid degradation of unassembled X and Y subunits. This possibility is further supported by the finding that transfection with cDNA of individual subunits, *LMP2* and *LMP7*, resulted in preferential incorporation of these subunits into the proteasome complex (Gaczynska *et al.*, 1994; Kuckelkorn *et al.*, 1995); nevertheless, the synthesis of their homologous counterparts, X and Y subunits, remained unchanged.

Gaczynska *et al.* (1996) found that transfection with X cDNA in cells resulted in a reduction in proteasomal chymotryptic and tryptic activities. In contrast, when the expression of Y was increased by transfection, the postglutamyl activity of proteasomes increased. These findings strongly suggest that subunits X and Y apparently cause the opposite effect of LMP2 and LMP7 mentioned previously. We have proposed that the proteasomes containing these γ-IFN-inducible subunits should be named "immunoproteasomes" to emphasize their specialized functions in antigen presentation (Akiyama *et al.*, 1994a; Tanaka, 1994).

E. Third Pair of Subunits, Z and MECL1, Regulated Reciprocally by γ-IFN

As described previously, the defect of antigen presentation in mutant cells bearing deletions of not only the *TAP1/2* genes but also the *LMP2/LMP7* genes can be suppressed almost completely by transfections with *TAP1/2* genes, suggesting that *LMP2/7* do not have much effect on the antigen processing pathway (Arnold *et al.*, 1992; Momburg *et al.*, 1992; Yewdell *et al.*, 1994; Zhou *et al.*, 1994). In addition, the effect of γ-IFN on the functions of the proteasome is quite controversial (Gaczynska *et al.*, 1993; Driscoll *et al.*, 1993; Aki *et al.*, 1993; Boes *et al.*, 1994; Ustrell *et al.*, 1995). Another interpretation of such apparently conflicting observations is that there may be an additional γ-IFN-inducible proteasome gene(s) responsible for antigen processing mediated by the proteasome. Therefore, for clarification of the exact role of γ-IFN in affecting the structure of the proteasome, a careful reinvestigation of whether γ-IFN alters the subunit pattern of proteasomes in human HeLa cells was made. The existence of a third pair of proteasome subunits reciprocally regulated by γ-IFN was found (Hisamatsu *et al.*, 1996). From the N-terminal sequence of the protein upregulated by γ-IFN, distinct from LMP2 and LMP7, this protein was found to be identical to that encoded by *MECL1*, which was cloned as a unique gene within a tight cluster of five unrelated human genes on chromosome 16q22.1 (Larsen *et al.*, 1993). Similar sequencing showed that the protein downregulated by γ-IFN was not identi-

cal to known proteasomal subunits, and it was named subunit Z (Hisamatsu *et al.*, 1996). Molecular cloning of a cDNA-encoding subunit Z, downregulated by γ-IFN, showed that this subunit Z is a novel proteasomal subunit with a high homology to MECL1 protein, which is markedly upregulated in response to γ-IFN. Therefore, γ-IFN induces subunit replacements of not only X and Y by LMP7 and LMP2, respectively, but also of Z by MECL1, producing proteasomes with an alteration of the proteolytic specificity and that are perhaps more appropriate for the immunological processing of endogenous antigens (see hypothetical model in Fig. 3). However, the functional consequences of incorporation of MECL1 and Z remain to be investigated.

Knowledge of the existence of this third pair of proteasome subunits, Z and MECL1, regulated reciprocally by γ-IFN is of considerable importance in clarifying the role of the proteasome in the antigen processing pathway. Initially, the term immunoproteasomes for functionally distinct proteasomes in which subunits X and Y are replaced by subunits LMP2 and LMP7 in response to γ-IFN was proposed (Tanaka, 1994). However, it should be noted that immunoproteasomes are present as very heterogeneous populations in cells, which would modulate processing of endoge-

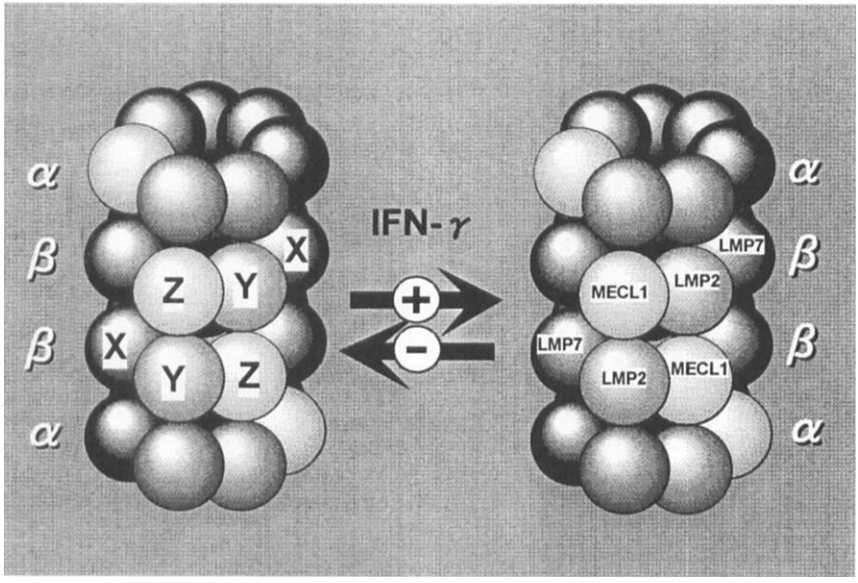

FIG. 3. Hypothetical model for the housekeeping-type 20S X/Y/Z-type proteasome and the γ-IFN-induced LMP2/LMP7/MECL1-type immunoproteasome. The locations of the γ-IFN-regulated subunits are only tentative because their exact topology is currently unknown.

nous antigens variously. Thus, the altered molecular organization of the proteasome induced by γ-IFN may be responsible for the acquisition of its functional change as a variety of heterogenous complexes.

γ-IFN greatly increased the level of the mRNA-encoding MECL1 protein in various types of cells (Hisamatsu *et al.*, 1996), as it did in the case of LMP2 and LMP7 (Akiyama *et al.*, 1994b). Increases in the mRNAs for LMP2, LMP7, and MECL1 were observed after treatment with γ-IFN for about 12 hr, suggesting acute responses to γ-IFN by the biosynthesis of these three proteasomal subunits. In addition, β-IFN, unlike α-IFN, significantly enhanced the effect of γ-IFN on the expressions of LMP2 and LMP7 without affecting MECL1 expression (Hisamatsu *et al.*, 1996). This finding suggests that the mechanism regulating the expression of subunit MECL1 may differ somewhat from those mechanisms regulating subunits LMP2 and LMP7, although γ-IFN acts as a major inducer of all of them, and that the physiological role of proteasomes containing MECL1 may differ from that of proteasomes containing LMP2 and/or LMP7. The protein levels of LMP2, LMP7, and MECL1 increased in response to γ-IFN, which roughly correlated with the amounts of their mRNAs. However, the mRNA levels of X, Y, and Z were not greatly affected by γ-IFN, suggesting that the complete loss or marked decrease in the expressions of subunits X, Y, and Z induced by γ-IFN is due to posttranslational degradation of X, Y, and Z. The amounts of these three proteins were found to decrease gradually 2 or 3 days after stimulation by γ-IFN. Moreover, Aki *et al.* (1993) showed that γ-IFN has no effect on the subunit pattern of preexisting proteasomes. Probably, although the induction of proteasomes containing LMP7, LMP2, and MECL1 by γ-IFN is rapid, the overall change from preexisting X/Y/Z-type proteasomes is slow because of the large pool size and slow turnover of the latter constitutive proteasomes (Rivett, 1993; Coux *et al.*, 1996).

F. γ-IFN-Regulated Subunits and N-Terminal Threonine Catalytic Residues

It is noteworthy that most β-type proteasomal subunits are synthesized as proproteins and processed to the mature forms by removal of their N-terminal prosequences (Seemüller *et al.*, 1995), suggesting that proteasomes are a zymogen-type protease like other intracellular proteinases such as calpains and lysosomal cathepsins. Yeast genetical studies indicate that the β subunits are related to the active functions, but the catalytic mechanism remains unknown. Recently, it was demonstrated by X-ray crystallography (Löwe *et al.*, 1995) and by site-directed mutagenesis (Seemüller *et al.*, 1995) that the *Thermoplasma acidophilum* proteasome is a novel threonine protease, the N-terminal threonine residue of the β subunit

being essential for the proteolytic function. Thus, this proteasome is a threonine protease with a catalytic mechanism different from that of other proteases determined to date (Seemüller *et al.*, 1995). This assumption is consistent with a recent observation that the novel selective proteasome inhibitor lactacystin modifies the N-terminal threonine residue of subunit X and possibly that of Z specifically and covalently, resulting in almost complete inhibition of the proteolytic activity (Fenteany *et al.*, 1995). Several eubacterial putative β subunits, i.e., *Escherichia coli* HslV and *Rhodococcus* β1 and β2 gene products (Tamura *et al.*, 1995), have the same N-terminal structure (for review, see Lupas *et al.*, 1994). It is therefore of particular interest that the γ-IFN-induced subunits, X, Y, and Z, and the counterparts they replace, LMP7, LMP2, and MECL1, respectively, also have two N-terminal threonine residues, suggesting that all three pairs of homologous subunits appear to be catalytically active or are a part of the catalytic center. Therefore, all the active subunits with two N-terminal threonine residues, which have been conserved during evolution, seem to be regulated by γ-IFN, which perhaps accounts for the changes in the proteolytic functions of the proteasome. In contrast, the other four human β-type subunits, named HC5, HC7, HC10, and HN3, do not, suggesting that the latter four subunits may be inactive (Seemüller *et al.*, 1995). Otherwise, these four subunits may have unknown mechanisms for peptide cleavage reactions.

G. Evolution of Proteasomal Genes and Molecular Adaptation

During evolution, the α subunit family has been well conserved, whereas the β subunit family may have diverged at an earlier stage (Kuma *et al.*, personal communication). This divergence of β-type subunits may have been associated with the acquisition of specific functions for proteasomes. The three γ-IFN-dependent, downregulated subunits, X, Y, and Z, are remarkably similar to their respective γ-IFN-responsive subunits LMP7 (68% identity), LMP2 (57% identity), and MECL1 (63% identity, excluding the N-terminal region). The high identities of these three pairs of subunits differ markedly from those of all other β-type subfamily subunits, which are approximately 15%, suggesting that the pairs of their γ-IFN regulatory genes have evolved differently from other proteasome genes.

LMP2 and *LMP7* genes are located at p21 of human chromosome 6 (Monaco, 1992), which differs from that of the *MECL1* gene, i.e., 16q22.1 (Larsen *et al.*, 1993). In addition, X (MB1), Y (δ), and Z were localized to distinct loci on 14q11.2, 17p13, and 9q34.11–q34.12, respectively (Belich *et al.*, 1994; Hisamatsu *et al.*, 1996). It is therefore of interest to examine the mechanisms for how these genes are regulated in response to γ-IFN at the molecular level, particularly how the *MECL1* gene, located outside

the MHC cluster, is able to respond to γ-IFN treatment. The location of subunit Z gene on chromosome 9 is noteworthy, because inspection of human loci adjacement to the Z locus provides evidence that the paracentromeric region of chromosome 9 and the MHC region on chromosome 6 probably arose by duplication at an early stage of vertebrate evolution (Kasahara *et al.*, 1996). Traces of this duplication are also evident in homologous mouse chromosomal regions (chromosome 2 and chromosome 17). These findings have implications in understanding the genomic organization of present-day MHC and provide insights into the origin of MHC.

Molecular genetic studies on yeast have shown that most proteasome genes are essential for cell proliferation (Coux *et al.*, 1996; Tanaka, 1995). To date, all 14 (7α and 7β) proteasomal genes of *Saccharomyces cerevisiae* have been isolated (Hilt and Wolf, 1995). Three of them, named *PRE2, PRE3,* and *PUP1,* which are essential genes, were found to show high similarities with the three pairs of γ-IFN regulatory human homologous subunits, LMP7/Y, LMP2/X, and Z/MECL1, respectively. Intriguingly, the similarities between X amd PRE2, Y and PRE3, and Z and PUP1 are approximately 68, 54, and 55%, respectively, which are higher than the 54% similarity of LMP7 with PRE2, the 44% similarity of LMP2 with PRE3, and the 47% similarity of MECL1 with PUP1. Thus, the actual homologs of PRE2, PRE3, and PUP1 may be X, Y, and Z, and not LMP7, LMP2, and MECL1, respectively, suggesting that γ-IFN-inducible *LMP7, LMP2,* and *MECL1* genes may have been generated from the housekeeping-type X, Y, and Z genes by gene duplication. Thus, in response to extracellular signals such as γ-IFN, some β-type subunits appear to be replaced by very homologous, but different, gene products. Therefore, the total number of β-type subunits may have increased in multicellular organisms during evolution for acquisition of the MHC-restricted immune system.

Previously, we proposed the term molecular adaptation for the interesting new phenomenon that the proteasomal multisubunit complex acquires functional diversity by a change in its molecular composition in response to environmental stimuli (Tanaka, 1994). The subunit changes of proteasomes in various physiological conditions may be one reason why the proteasome has such a complex organization. If so, 20S proteasomes may exist in cells as heterogeneous populations even if they seemingly resemble one another. In line with this idea, Brown *et al.* (1993) reported various subsets of proteasomes with different subunit patterns, depending on the type of the cells. In addition, it was also reported that in the early development stage, the subunit pattern of proteasomes differs considerably, with the level of each subunit being altered during differentiation of the cells, and that in *Drosophila* isoforms of one subunit are expressed in different tissues at specific stages of development (Coux *et al.*, 1996), suggesting

that molecular adaptation may occur in response to not only events of the immune system but also other situations. Moreover, the p31 is the human 26S proteasome regulatory subunit and is a homolog of the yeast protein Nin1p (Kominami *et al.*, 1995), which was found to be required for both G1/S and G2/M transitions of the cell cycle based on genetic analysis of *nin1-1* ts mutants. Subsequently, the *su*ppressor of *n*in1-1 (*SUN1*) gene was isolated as a multicopy suppressor to restore the function lost due to the *nin1-1* ts mutation. Interestingly, when *SUN1* was overexpressed, the null mutation of *nin1-1* was also complemented, indicating that there are two types of 26S proteasomes in cells, one containing Nin1p and the other containing Sun1p (Kominami *et al.*, 1996). The functional consequence of these two types of 26S proteasomes is unknown, but the heterogeneity of the 26S proteasome may also be implicated as another type of molecular adaptation.

H. The Proteasome Activator PA28 and γ-IFN Regulation

Ma *et al.* (1992) and Dubiel *et al.* (1992) independently found an activator protein of the 20S proteasome and named it PA28 (or 11S regulator). The purified PA28 protein greatly stimulated multiple peptidase activities of the 20S proteasome without affecting destruction of large protein substrates, even though the proteins had already been ubiquitinated, suggesting that it may cooperate with the 26S proteasome in a sequential proteolytic pathway. PA28 association with the proteasome resulted in both an increase in the V_{max}, the maximal velocity of the hydrolytic reaction, and a decrease in K_m, the concentration of substrate required for half-maximal reaction velocity, of the peptidase activities of the 20S proteasome, suggesting that it functions as a positive allosteric effector.

PA28 is composed of subunits of 28–32 kDa, but the native molecule has a molecular weight of approximately 170–180 kDa, leading to the assumption that it is an oligomeric complex, perhaps hexameric or heptameric (Gray *et al.*, 1994). Electron microscopic investigations indicate that PA28, free of the proteasome, is a ring-shaped particle and that it associates with the 20S proteasome by forming a conical structure on both ends of the complex (Lupas *et al.*, 1993; Gray *et al.*, 1994), indicating that PA28 occupies the same site on the 20S core particle as the regulator complex does in the case of the 26S proteasome. An average projection image of the PA28 associated with the 20S proteasome is shown in Fig. 4 (top; Lupas *et al.*, 1993).

Although the PA28 activator was initially thought to be present as a multimeric complex of a single subunit (Ma *et al.*, 1992; Gray *et al.*, 1994), subsequent protein chemical analyses have revealed that another subunit (tentatively named PA28β for new species) is present in PA28 samples (Dubiel *et al.*, 1992; Mott *et al.*, 1994). PA28α (equivalent to the originally described

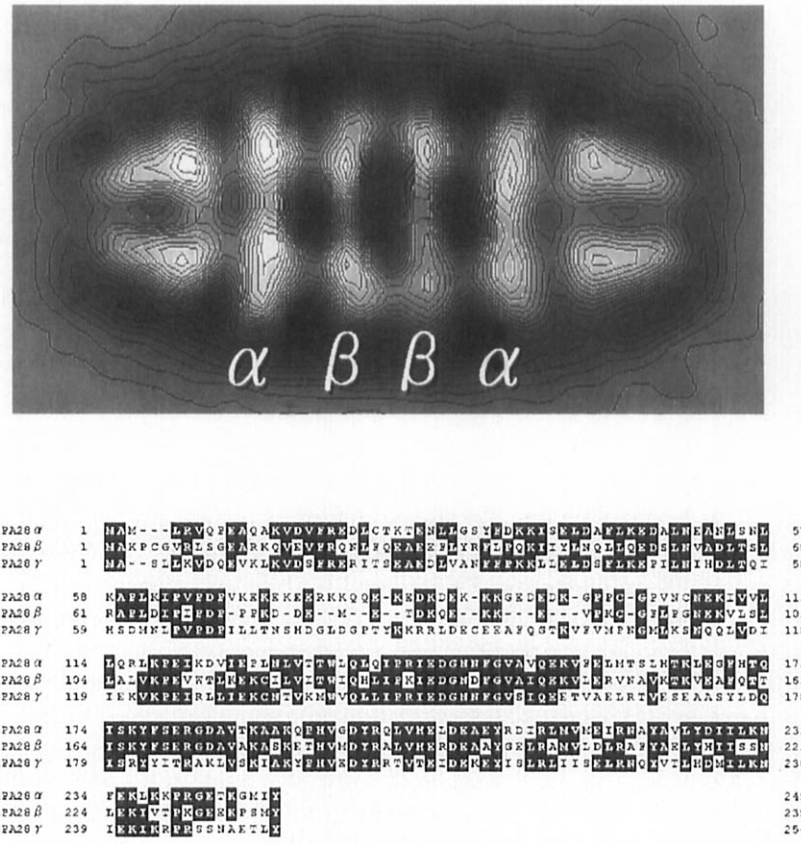

FIG. 4. Structural features of the 11S proteasome activator–20S proteasome complex. (Top) rat liver 20S proteasome–PA28 complex (photo taken by W. Baumesiter). (Bottom) sequence alignment of the human PA28 activator family proteins. Darkened blocks indicate identical amino acid residues. The sequences of human PA28α, PA28β, and PA28γ have been taken from published reports by Realini *et al.* (1994a), Ahn *et al.* (1995), and Nikaido *et al.* (1990), respectively.

PA28/11S regulator subunit), whose primary structure was deduced from the cDNA, is a novel 28-kDa protein unrelated to known proteins, including any of the 20S proteasomal subunits (Realini *et al.*, 1994a; Ahn *et al.*, 1995). Intriguingly, PA28α is identical to a protein whose gene was cloned previously, namely IGUP I-5111, which is one of the major gene products induced by γ-IFN in primary human keratinocytes (Honoré *et al.*, 1993). PA28α and PA28β are approximately 47% identical to each other and are highly conserved between rat and human (Ahn *et al.*, 1995).

Surprisingly, PA28α and PA28β are homologous to a previously described protein, Ki antigen, whose function is unknown (Nikaido *et al.*,

1990). Ki antigen was identified as a nuclear protein detected with autoantibodies found in sera of patients with systemic lupus erythematosus. The primary structures of these three proteins with similar sizes and charges are approximately 40% identical, suggesting that they constitute a new protein family generated by duplication of a common ancestral gene. The size of the mRNAs encoding PA28α and PA28β is about 0.9 kb, but the Ki antigen mRNA differs markedly, being approximately 2.5 kb long, with a long 3′-noncoding region. Chromosomal loci of PA28α and PA28β are closely linked to each other and are present on human chromosome 14q11.2, whereas the Ki antigen is located on a different chromosome, at 17q21.32–17q21.33 (Tanahashi *et al.*, 1997). Although it remains to be resolved whether Ki antigen is a genuine component of PA28, an antibody specific for Ki antigen reacted appreciably with purified PA28 preparations (Tanahashi *et al.*, 1997), suggesting that it is a genuine component of PA28. Ki antigen was thus renamed PA28γ. Thus, the PA28 activator is now thought to be present in cells as an heteropolymer composed of two or three nonidentical but homologous subunits (Fig. 4, bottom). However, the PA28α subunit expressed in *E. coli* is capable of greatly stimulating latent 20S proteasome activity, suggesting that the homopolymeric complex of PA28α is also active (Realini *et al.*, 1994a). On the contrary, PA28β alone has no notable effect. However, it reduces appreciably the K_m, but not the V_{max}, of the PA28α-activated proteasome activity when it is added simultaneously with PA28α, suggesting an important functional interaction between these two PA28 subunits (Tanahashi *et al.*, 1997).

The physiological relevance of the PA28–proteasome complex has not yet been established. It is of particular interest that PA28α is identical to γ-IFN-inducible IGUP I-5111. γ-IFN induces markedly the levels of the mRNAs coding for PA28α and PA28β without affecting the level of Ki antigen (PA28γ) (Honoré *et al.*, 1993). Interestingly, the PA28α and PA28β proteins are induced greatly, in rough accordance with their mRNA level, in response to γ-IFN, whereas γ-IFN treatment resulted in an almost complete and time-dependent loss of the Ki antigen (Tanahashi *et al.*, 1997). γ-IFN can thus alter the subunit composition of PA28 in the cells, a process similar to the replacement of three proteasomal subunits observed upon γ-IFN treatment. These data suggest that PA28 could play an important role in the generation by the 20S proteasome of antigenic peptides capable of being presented by the MHC class I molecules. Thus, these newly identified γ-IFN-regulated activator genes in combination with the three pairs of γ-IFN regulated proteasome genes perhaps act synergically to favor antigen presentation. Based on transfection analysis with PA28α cDNA, Groettrup *et al.* (1995) reported that PA28, together with MHC-encoded LMP subunits, increases the spectrum of peptides that can be generated in antigen-presenting cells. Consequently, involvement of the

PA28 in the antigen processing has been documented to show that it plays an important role in the induction of specific cytotoxic T cells *in vivo* (Groettrup *et al.*, 1996) and for production of dominant MHC ligand *in vitro* (Dick *et al.*, 1996). On the other hand, PA28γ may be responsible for functions unrelated to immunity, unlike PA28α and PA28β, because its mRNA level increased during the S-phase of the cell cycle (Nishida, personal communication); indeed, its protein level was found to be very high in some, but not all, tumor cells, implying its involvement in cell proliferation (Tanahashi *et al.*, 1997). However, the exact function of PA28γ is still unknown.

I. Cleavage Properties

MHC class I molecules consist of a membrane-anchored polymorphic class I heavy chain, soluble β_2-microglobulin (β_2-M), and a peptide ligand of 8–10 amino acids with an allele-specific sequence motif. Class I molecules hold such antigenic peptides with both amino and carboxyl termini tightly fixed in the groove (Engelhard, 1994; Rammensee *et al.*, 1995). If proteasomes act as a processing enzyme, they have to have the capability of producing peptides with a motif complementary to the peptide-binding cleft of class I molecules. However, few experiments have been done to explore this important function of proteasomes. Proteasomes reveal an unexpectedly broad cleavage activity capable of degrading peptide bonds on the carboxyl side of basic, hydrophobic, and acidic residues of target polypeptides (Orlowski, 1990; Rivett, 1993). Therefore, it is puzzling how the proteasome can generate accurately the antigenic peptides with appropriate size and sequence specificity. Although the mechanism is still unknown, Baumeister and colleagues (Wenzel *et al.*, 1994) proposed that proteasomes have a specialized function as a molecular ruler capable of determining the length of the cleavage products; the function may rely on the positions of the catalytic sites present inside the cavity of the proteasome complex (Löwe *et al.*, 1995; see Fig. 3).

Dick *et al.* (1994) have demonstrated that the pure 20S proteasome is capable of generating antigenic peptides from β-galactosidase and OVA for presentation by class I molecules. Moreover, Niedermann *et al.* (1995) also showed that the 20S proteasome contributed to the production of the hierarchy of epitopes presented by MHC class I molecules because it showed preferred cleavage sites directly adjacent to the N- and C-terminal ends of the immunodominant epitope of OVA. In contrast, Boes *et al.* (1994) reported that the 20S proteasome produced a naturally processed nonamer peptide from a synthetic 25-mer polypeptide substrate containing the epitopic peptide sequence derived from the murine cytomegalovirus IE pp89 protein as a minor cleavage product and suggested that the peptides generated by the proteasome could be trimmed to their final

peptide size. Interestingly, they also showed that γ-IFN-induced proteasomes created a different set of peptides compared with those not treated with γ-IFN. Similarly, Shimbara *et al.* (unpublished results) found that the 20S proteasome can produce a single tumor-antigenic 10-mer peptide from a synthetic 20- to 30-mer polypeptide derived from the *Akt* oncogene (Uenaka *et al.*, 1994), the peptide of which can be presented by MHC class I molecules to induce a specific CTL. Moreover, the purified PA28 activator increased several fold the generation of the antigenic peptides. In addition, this activator protein also acted to produce correctly the antigenic peptide (Shimbara *et al.*, 1997).

It is most important to test the cleavage specificity of the 26S proteasome because the 26S proteasome is an ATP-dependent protease catalyzing the initial cleavage reaction in cells for native proteins with a degradation signal such as a multiubiquitin chain (Hershko and Ciechanover, 1992; Rechsteiner *et al.*, 1993; Hochstrasser, 1997). However, the precise degradation mode employed by 26S proteasomes has not yet been examined for ubiquitinated proteins because of the difficulty of preparing a sufficiently large amount of substrate proteins for analyzing their products. Previously, we reported that ornithine decarboxylase (ODC) is degraded ATP dependently by the 26S proteasome in the presence of antizyme (AZ), an ODC inhibitor (Murakami *et al.*, 1992). The cleavage specificity for ODC by the 26S proteasome, tested with ODC purified from ODC-overproducing cells and an excess of AZ in the presence of ATP, was examined (Tokunaga *et al.*, 1994). Because ODC is degraded specifically without appreciable breakdown of AZ, most degradation products of ODC, which were separated by high-performance liquid chromatography (HPLC) on a reverse-phase column, were identified by N-terminal amino acid sequencing. The amino acid residues at the cleavage sites of peptide bonds, determined by aligning these peptides with the primary structure of ODC, showed that the major sites of ODC cleaved by the 26S proteasome were on the carboxyl side of neutral/hydrophobic amino acid residues but that a few were on that of acidic or basic amino acid residues. Thus, the 26S proteasome has an endoproteolytic action on the naturally occurring short-lived protein ODC that occurs in a multicatalytic- and ATP-dependent manner; however, it did not produce any free amino acid residue, indicating that the 26S proteasome has no exopeptidase activity. Most important, the major products produced by the 26S proteasome had lengths of 5–11 amino acid residues, which included a peptide with appropriate size and motif to be bound by class I molecules. However, these peptides did not cover the entire primary sequence of the ODC molecule, being only 15–20% of total amino acid residues. Presumably, the undetected peptides were di or tripeptides, which would have been too small for detection on a reverse-phase HPLC column. It is interesting

to question why such peptides were resistant to exhaustive degradation by the 26S proteasomes, which were added repeatedly. The 26S proteasome may be able to somehow recognize differences in peptide sequences that allow some peptides to escape from proteasome attack; however, the mechanism is not known. Thus, the 26S proteasome has the capability of generating peptides with appropriate sizes corresponding to the MHC class I-binding peptides.

However, it remains to be investigated how the immuno-26S proteasome is responsible for cleaving natural cellular and/or viral proteins and how the PA28 activator can contribute to the peptide generation. In addition, various other proteins including ubiquitinated proteins should also be examined by product analysis to clarify the cleavage specificity of the 26S proteasome.

V. Model for Generation of MHC Class I-Associated Peptides

In this article, we repeatedly addressed the importance of the multiple roles of γ-IFN in the antigen processing pathway mediated by proteasomes because this cytokine plays a central role in immunomodulatory functions through upregulation of the expression of many genes responsible for the immune response (Billiau, 1996). We discussed the specialized adaptations induced by γ-IFN that appear to have evolved to increase generation of peptides appropriate for antigen presentation. Based on the current knowledge, we propose that the basal rate of processing of intracellular antigens is catalyzed by constitutive X/Y/Z-type proteasomes. However, when large amounts of non-self-antigens invade the cells, as during viral infection, γ-IFN is secreted by activated T cells and/or natural killer cells. γ-IFN may induce subunit replacement of X, Y, and Z by LMP7, LMP2, and MECL1, respectively, and the altered molecular organization of the proteasome may be responsible for acquisition of its functional change. We propose the term immunoproteasomes for functionally distinct proteasomes induced by γ-IFN that are perhaps more appropriate for the immunological processing of endogenous antigens (for a hypothetical model, see Fig. 5). Because γ-IFN has no effect on the cellular content of proteasomes, it must therefore modify the subunit organization and functions of proteasomes resulting in accelerated processing of endogenous antigens. However, it is still unknown whether these cytosolic antigens are efficiently processed by γ-IFN-induced immunoproteasomes. Alternatively, the immunoproteasome may change the specificity of cleavage sites of target polypeptides and contribute to the generation of cryptic epitopes. The model in Fig. 5 is consistent with the finding of multiple defects in the immune response in mutant mice in which the *LMP2* gene had been disrupted by viral infection (Van Kaer *et al.*, 1994). This is a novel mecha-

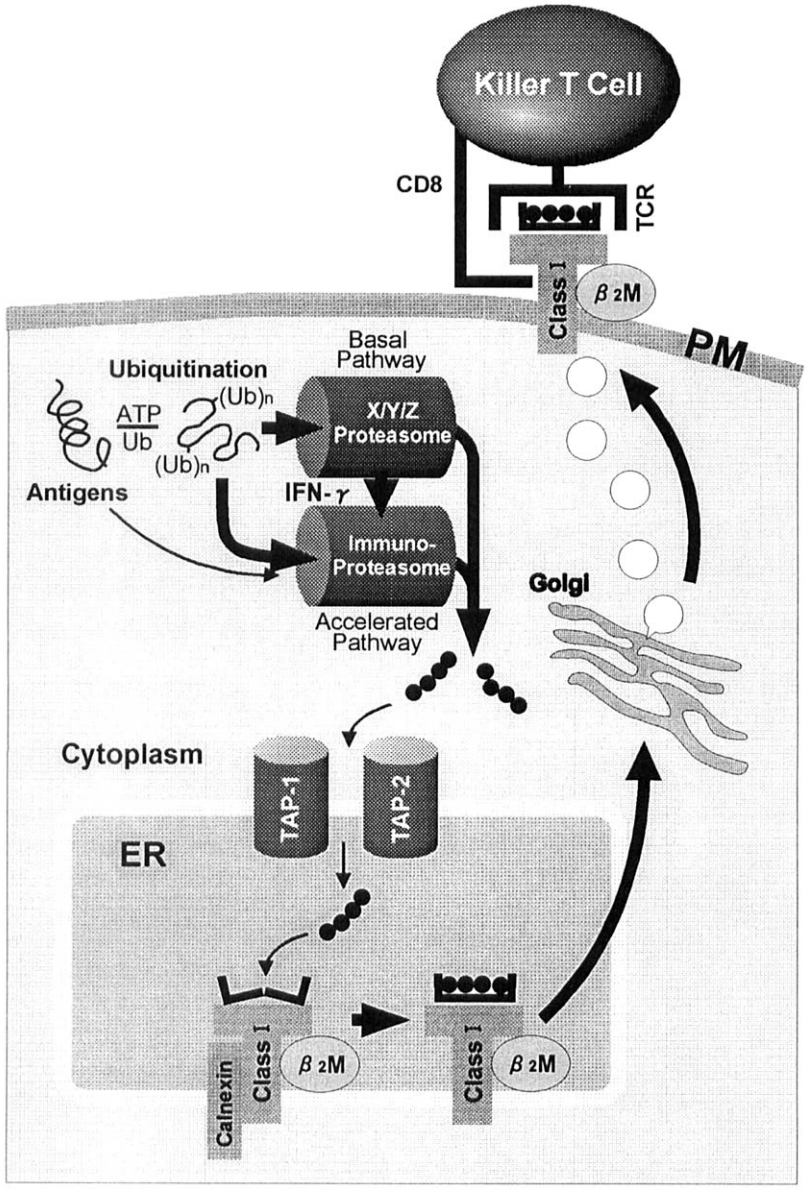

FIG. 5. A hypothetical overview of the MHC class I-restricted antigen presentation pathway. Ub, ubiquitin; (Ub)n, multi-Ub chain; TAP, transporter associated with antigen processing; PM, plasma membrane; ER, endoplasmic reticulum; β_2m, β2-microglobulin; Class I, MHC class I H chain; TCR, T cell receptor. For details, see text.

nism for the immunomodulatory action of γ-IFN, clearly differing from the previously known mechanism involving marked increases in the cellular concentrations of TAP transporter and MHC molecules responsible for enhanced antigen presentation by cells that is stimulated at the transcriptional level of these genes. Before generation of antigenic peptides, the modifications of certain antigens by ubiquitination are involved in cytosolic processing. Indeed, York and Rock (1996) suggested that the ubiquitination pathway is the major route for generation of MHC class I peptides because it is involved in production of antigenic peptides from intracellular proteins with both rapid and slow turnover rates. However, it is unknown whether processing of all proteins is mediated by the ubiquitin pathway. The peptides generated are transported into the ER through the TAP1/2 transporter system to be assembled with MHC class I molecules for presentation on the cell (see the model in Fig. 5).

Briefly, we again address the roles of γ-IFN, the cytokine of which may induce not only the immunoproteasome but also the PA28 proteasome activator family proteins. Thus, these newly identified γ-IFN-regulated activator genes in combination with the three pairs of γ-IFN-regulated proteasome genes may provide answers to unsolved fundamental questions about the antigen-processing pathway. Although the PA28 protein greatly stimulates the multiple peptidase activities of the 20S proteasome, it fails to enhance hydrolysis of large protein substrates with native or denatured structures. Thus, PA28 does not play a central role in regulating the initial cleavage of cell proteins. It perhaps has a stimulating effect on the degradation of polypeptides of intermediate size that are generated by other endoproteinases, such as the 26S proteasome, catalyzing the final cleavage reactions in a proteolytic pathway functioning sequentially or cooperatively (see the proposed model in Fig. 6). Preliminary evidence supporting the involvement of PA28 activator in the antigen-processing pathway was provided by Groettrup *et al.* (1995, 1996; Dick *et al.*, 1996) and Shimbara *et al.* (unpublished results) based on the findings that PA28 may be responsible for an increase in class I peptide generation. Currently, it remains unclear whether γ-IFN alters the composition of the regulator complex of the 26S proteasome. To determine this, further study is necessary.

Escape from attack by the many very active cytosolic exopeptidases and dipeptidyl peptidases that could hydrolyze them to free amino acids is necessary for the antigenic peptides produced by the proteasome pathway to be efficiently transferred to the TAP transporter located in the ER. Presumably, cytosolic peptides may be attached to other molecules that would protect them from degradation by such abundant peptidases present in the cytosol. The most likely candidates for such molecules with peptide-binding functions in cells are the heat-shock proteins (hsps). These molecu-

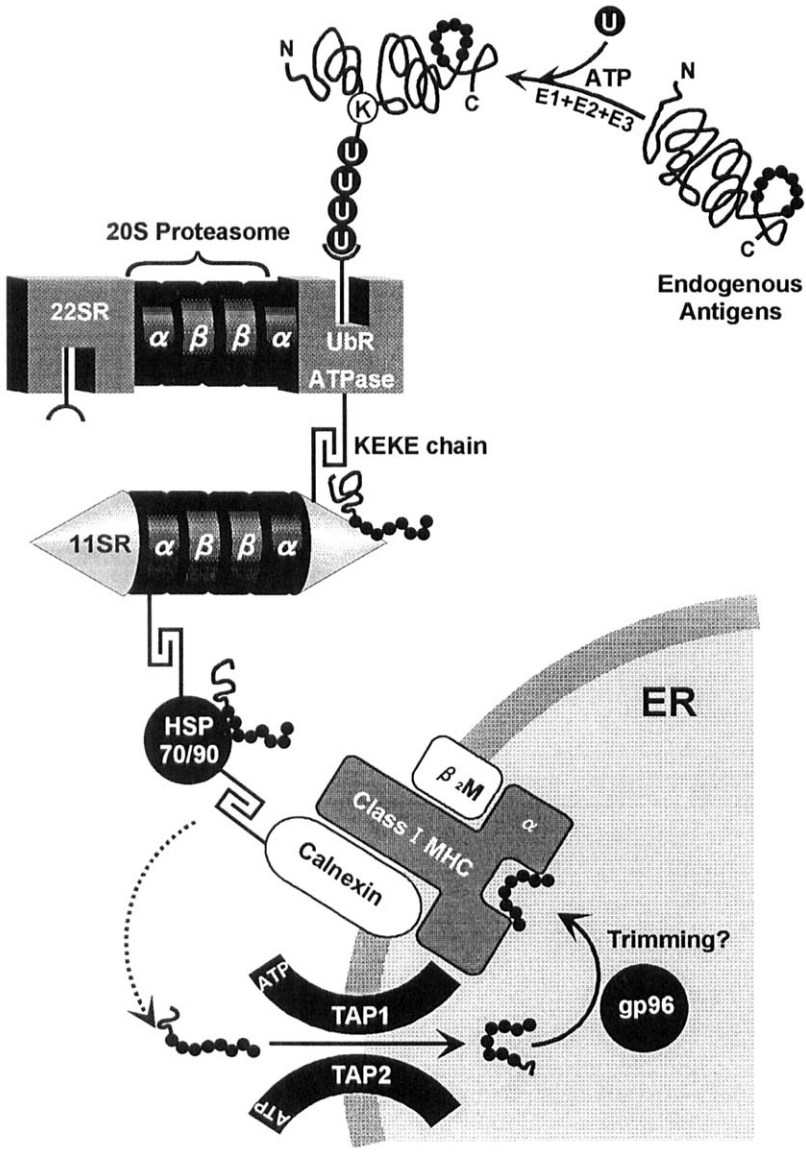

FIG. 6. A simplified and hypothetical model of the MHC class I-restricted antigen-processing pathway. hsp70/90, family of heat-shock proteins 70 and 90; UbR, Ub receptor (equivalent to multi-Ub binding subunit); 11SR, PA28 activator protein complex; 22SR, 26S proteasomal regulatory unit. For other symbols, see legends to Figs. 1, 2, and 5. For details, including an explanation of the KEKE motif and trimming, see text.

lar chaperones are associated with newly synthesized unfolded proteins in the cytosol and are also responsible for promoting the correct assembly of newly synthesized MHC molecules in the ER (for review, see Williams and Watts, 1995). Srivastava *et al.* (1994) proposed that hsps play an essential role in transfer of antigenic peptides from the proteasome to the TAP transporter (Fig. 6). A similar model was also proposed by Rammensee *et al.* (1995) and H. Udono *et al.* (personal communication). This proposal is based on the finding that peptides associated with hsp70 have tumor-rejecting activities (Udono and Srivastava, 1993; see review by Srivastava *et al.*, 1994, for details). This proposal is compatible with an interesting aspect of hsp70, which was suggested by computer-assisted modeling, of a putative peptide-binding domain such as the peptide-binding pocket of MHC class I molecules (Rippmann *et al.*, 1991; Flajnik *et al.*, 1991). Indeed, hsp70 was shown to have the ability to associate with various peptides containing 7–10 amino acid residues and release these peptides in an ATP-dependent fashion (Blond-Elguindi *et al.*, 1993). In addition, grp94/gp96, a family of hsp90s, has been shown to have a similar tumor-rejecting effect, demonstrating intrinsic ATPase activity and the ability to bind to several peptides (Li and Srivastava, 1993). Thus, grp94/gp96 may function as a carrier for peptide transfer from TAP to class I MHC in the ER (Srivastava *et al.*, 1994; Rammensee *et al.*, 1995). The proteasome appears to be responsible for the cleavages that determine the C termini of antigenic peptides, but there is no evidence to suggest that the proteasome also generates the N termini. In fact, some additional trimming of the N terminus by amino peptidases appears to occur in the ER (Roelse *et al.*, 1994; Elliott *et al.*, 1995). Alternatively, such N-terminal trimming may occur in the secretory vesicle or at the cell surface (Eisenlohr *et al.*, 1992; Link *et al.*, 1994). Molecular chaperones may also be involved in this trimming process in the ER, but the enzymes responsible for this trimming have not been characterized.

Intriguingly, several subunits of both the 20S and 26S proteasomes and PA28α have a unique segment, named a KEKE motif by Realini *et al.* (1994b); it consists of a very hydrophilic domain rich in "alternating" lysine (positive) and glutamate (negative) residues. A similar sequence is present in a variety of proteins such as heat-shock proteins and Ca^{2+}-binding ER proteins. The KEKE sequence has been proposed to promote protein–protein interactions and to play a role in antigen presentation (Realini *et al.*, 1994b). Therefore, the molecules responsible for processing and intracellular transportation of antigenic peptides may be associated physically through the KEKE motif, as shown in the hypothetical mode in Fig. 6, but there is as yet no direct evidence to support this possibility. However, in support of it, hsp70 was found to be associated with not only the cytosolic 20S proteasome but also TAP in the ER (Sato, personal communication),

although the functional significance of these observations remains to be investigated. Moreover, TAP proteins are directly associated with class I molecules in the ER, indicating direct transfer of antigenic peptides from the TAP transporter to class I molecules (Ortmann *et al.*, 1994; Suh *et al.*, 1994). However, it is still unknown whether all peptides would bind to MHC molecules through this direct transfer system because N-terminal trimming of certain peptides is necessary before their association with MHC molecules in the ER (Roelse *et al.*, 1994; Elliott *et al.*, 1995). This hypothetical physical association model may explain the efficient processing of endogenous antigens and transfer of the resulting ligand peptides from the cytoplasm into the receptor MHC class I molecules in the ER.

VI. Perspectives

In this review, we have briefly summarized the recent findings related to the roles of proteasomes in the MHC class I-restricted antigen processing pathway. It is now well established that proteasomes play a central role in various biological processes, one of which is the generation of the peptides presented by MHC class I molecules to the circulating T lymphocytes. In this process, small fragments generated during the breakdown of cellular proteins are transported into the ER through the specified peptide pump designated TAP where they become bound to MHC class I molecules and then are delivered to the cell surface in order to activate the cytotoxic T cells. The presentation of these peptides initiates the process whereby the cell-mediated immune system can destroy cells expressing extraordinary viral or other unusual polypeptides.

Proteasomes have been implicated as the processing enzyme for the generation of the ligand peptides for MHC class I receptors. The accumulating evidence supporting this possibility has been documented in this review, but it is apparent that many unresolved questions remain to be answered. Even if proteasomes work in this capacity, it is still unknown how they are able to recognize sizes and sequences in the given polypeptide antigens to generate the exact epitope peptides. These mechanisms need to be clarified at the molecular level. It is also important to accurately determine if immunoproteasomes indeed contribute to the accelerated antigen processing in response to γ-IFN stimulation, as was depicted in Fig. 5. Can they indeed be responsible for the production of cryptic epitopes? If the immunoproteasomes are present in cells as heterogenous populations according to the combination of multiple types of γ-IFN-induced β subunits in an up- and/or down-expressed fashion, the role of the individual proteasome is entirely unknown. The structure–function relationship of proteasomes variously organized should also be examined to clarify their significance under distinct pathophysiological conditions.

Particularly, the immunological role of the newly identified pair of proteasomal subunits MECL1 and Z is unknown. As in the case of LMP2 and LMP7, their roles must be tested by the molecular genetical approach used in the murine knockout system, which would be necessary for determining the functions of the genes for MECL1 and Z. Similar KO analysis in mice would help to answer unresolved questions about the functions of the family of PA28 activator genes. In addition, the processing mechanism in the ER should be studied more precisely by identifying the enzymes responsible for the trimming of precursor antigenic peptides. Recently, it was reported that treatment with proteasome inhibitors resulted in considerable protein breakdown of several proteins in the ER membranes. Accordingly, it is an open question as to whether this proteasome-mediated ER degradation system is a part of the antigen-processing pathway. In this review, we proposed a physical association model of molecules involved in antigen processing as was shown in Fig. 6. However, this attractive model is mostly speculative and further study is required to ascertain whether this model is accurate.

Finally, future studies should address how proteasome genes have evolved. Acquisition of the γ-IFN-responsive proteasomal and PA28 family genes may be related to that of multiple MHC and TAP genes during evolution, but the mechanisms underlying such coordinate evolution are not known. Studies on molecular evolution may provide new insight into the alternative roles of proteasome genes in immunity.

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ADVANCES IN IMMUNOLOGY, VOL. 64

Recent Advances in Understanding V(D)J Recombination

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I. Introduction

The immune system is unique in its requirements for the remodeling of DNA. Antigen receptor genes are assembled during lymphoid development by V(D)J recombination, the effector functions of functional immunoglobulin genes are later remodeled by switch recombination, and antigen affinity is refined by somatic hypermutation. In some species a further elaboration is introduced by gene conversion between nonexpressed and expressed copies of immunoglobulin genes. This multilayered complexity serves to allow the organism to mount an immune response with the greatest possible flexibility and efficiency.

This chapter will focus on V(D)J recombination. Of all these processes, it is the best understood and the only one for which any genetic factors have been identified. A thorough review of V(D)J joining was published in this series 2 years ago (Lewis, 1994a). This chapter will therefore provide background information to frame current problems and will then concentrate on more recent developments. The intervening period has seen significant advances in both the genetics and biochemistry of V(D)J recombination. The goal will be to cover these new observations and the resulting insights and to place them in the perspective of broader questions concerning the rearrangement of antigen receptor genes.

II. Basic Properties

The V(D)J recombination process is used for the task of assembling immunoglobulin and T cell receptor genes with both the requisite precision and variability (for reviews, see Gellert, 1992; Lewis, 1994a; Thompson 1995). In the arrays of V, J (and in some loci also D) segments, recombination is accurately targeted by the recombination signal sequences (RSS) that lie next to each fragment of coding region, even when these sites are separated by distances of 1 or 2 megabases. An RSS is made up of conserved heptamer and nonamer sequences separated by a nonconserved spacer whose length [typically 12 or 23 base pairs (bp)] is important. The consensus form of an RSS is 5′—(coding sequences)—CACAGTG—(12- or 23-bp spacer)—ACAAAAACC—3′. The rearrangement is limited to the proper

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pairs of sites (V to J or V to D to J regions) by the rule that joining only takes place between an RSS with a 12-bp spacer and one with a 23-bp spacer (Tonegawa, 1983). At each locus, the pattern of RSSs is uniform; for example, at the Igκ locus, all V regions adjoin a 12-spacer signal and all J regions adjoin a 23-spacer signal. Joining of V to J regions is thus directed toward making functionally useful genes, whereas the biologically futile joining of one V to another V, or a J to a J, is rare.

Recombination makes a junction of two coding segments (a coding joint) and in parallel produces a heptamer to heptamer fusion of the signals (a signal joint). Depending on the orientation of the signals, the rearrangement can lead to either a deletion or an inversion of the intervening DNA. In the most common arrays at the antigen receptor loci, the coding joint is retained in the chromosome and a circular DNA molecular containing the signal joint is excised and subsequently lost from the cells.

The alternative naturally occurring arrangement of signals leads to an inversion. This arrangement is also found at some Ig or TCR loci—most notably at the human Igκ locus, where about half of the V segments are found in inverted orientation with respect to Jκ (Weichhold *et al.*, 1990). These segments are used in expressed Igκ genes. There are also single inverted V segments at the mouse TCR-β and TCR-δ loci (Korman *et al.*, 1989; Malissen *et al.*, 1986).

The signal sequences are the only recognition sites strictly required for recombination; coding segments can be replaced by other DNA and joining still occurs at the signal border. This has made it possible to generate specially designed recombination substrates, either extrachromosomal or chromosomally integrated, for detailed studies or for the selection of recombinationally active cell lines.

Most coding sequences are utilized more or less equivalently, but in special cases the first few nucleotides of the coding flank adjoining the signal sequences can strongly influence the efficiency of recombination (Boubnov *et al.*, 1995; Ezekiel *et al.*, 1995; Gerstein and Lieber, 1993). In particular, a run of T's (reading 5′ to 3′ into the heptamer) greatly depresses recombination.

A. Signal Joints and Coding Joints

The processing of coding and signal sequences before joining is quite different. Signal joints made either in the antigen receptor loci or in designed substrates are usually precise fusions of the heptamer sequences. A small fraction of the joints contain nucleotide insertions, but loss of nucleotides is very rare (Lewis *et al.*, 1985; Lieber *et al.*, 1988). In contrast, coding joints are not usually simple junctions of the germline sequences. In most cases, several nucleotides are lost from one or both ends, and

nucleotide insertions are also common. The variability of coding joints contributes greatly to the diversity of antigen receptors because the junctional sequence is part of the antigen binding site. The variability leads to much waste among the recombined genes because the number of bases added or lost is essentially random, so many coding joints shift the reading frame and cause premature termination of the antigen receptor protein.

The nucleotide insertions in coding joints are of two types—nontemplated and templated. Insertions of nontemplated nucleotide tracts (N regions) up to 15 nucleotides in length are generated by the enzyme terminal deoxynucleotidyl transferase, as is discussed later in this chapter. Templated insertions arise by a process more closely coupled to the V(D)J reaction itself. These "P nucleotide" insertions (P for palindromic) consist of a few nucleotides (generally fewer than four) complementary to the last bases of the coding end (Lafaille *et al.*, 1989; McCormack *et al.*, 1989). It is generally accepted that they arise from the off-center opening of coding end intermediates with a hairpin structure, which are discussed extensively later in this chapter.

How nucleotides are lost in coding junctions is unknown. Coding junctions with some terminal bases deleted are found in all cell types in which V(D)J recombination is made to occur, including nonlymphoid cells induced to recombine by expressing RAG1 and RAG2 (see below). It is unclear whether the activity responsible is specific to the V(D)J system or a more general cellular activity. It is generally assumed that an exonuclease is responsible, but an endonucleolytic nick some distance inside the coding end would also be a possible source (Lewis *et al.*, 1985).

B. Hybrid and Open-and-Shut Joints

Unusual products of V(D)J recombination have been intensively studied as potential clues to the reaction mechanism. "Hybrid joints," in which a signal becomes joined to its partner signal's coding flank, have been found quite frequently in artificial substrates (Lewis *et al.*, 1988). For example, in a substrate designed to produce an inversion upon joining, the formation of a hybrid joint links the distal signal and coding DNA while deleting the intervening fragment. In some artificial substrates, hybrid joints can account for up to 30% of the total recombinants (Lewis *et al.*, 1988). More rarely, hybrid joints have also been observed in the antigen receptor loci. End processing is normal in hybrid junctions. The signal end is usually kept intact, but the coding end has often lost bases, and nucleotide insertions are also found.

Less frequently, the original pair of coding and signal ends is rejoined, after base loss and addition, to form an "open-and-shut" joint (Lewis and Hesse, 1991; Lewis *et al.*, 1988). These events are usually seen at only one

signal and may escape some of the normal rules of V(D)J recombination. For example, open-and-shut joints are still made in plasmid substrates containing only one signal (Lewis and Hesse, 1991). This raises the possibility that, even when two signals are present, cutting at one signal might under some conditions precede recognition of the other.

For these two types of aberrant junctions to be made, signal ends and coding ends must be able to locate each other in unusual combinations. This suggests that at one stage in the process, all four ends are held together in a complex, and joining takes place within that structure. With the ends close together in such a complex, misjoining could occasionally result.

III. Broken DNA Molecules in V(D)J Recombination

Work during the past few years has identified broken DNA species that are intermediates in the V(D)J recombination process. Such molecules were first found at the TCRδ locus in thymocytes of neonatal mice (Roth *et al.*, 1992a) and have since also been demonstrated at most of the other antigen receptor loci (Bogue *et al.*, 1996; Livak and Schatz, 1996; Ramsden and Gellert, 1995; Schlissel *et al.*, 1993). Recently, it has become possible to detect double-strand breaks in plasmid substrates undergoing recombination (Steen *et al.*, 1996). In all cases, specifically broken DNA is found only in cells actively performing V(D)J recombination. The broken signal ends are cut exactly at the border between the signal sequence and coding sequence and are blunt-ended with a 5′ phosphoryl and a 3′ hydroxyl group (Roth *et al.*, 1993; Schlissel *et al.*, 1993). Broken coding ends have also been identified and are not simply the broken counterparts of signal ends. Instead, they are always found to be hairpin structures, with the 5′ and 3′ ends covalently joined back on themselves (Roth *et al.*, 1992b). These hairpins have been shown to contain the full germline coding sequence on both strands (Zhu and Roth, 1995), indicating that they are the primary products of V(D)J cleavage, made in the same event that generates the broken signal end.

Broken signal ends can be remarkably abundant. At the TCR-δ locus, 10–20% of the total DNA hybridizing to a TCR-δ probe was found to contain a break at the Dδ2 element (Roth *et al.*, 1992a). This high level implies that broken molecules must have a long lifetime, a point that is discussed more fully below. Cell sorting experiments show that signal ends are restricted to the G1 phase of the cell cycle (Schlissel *et al.*, 1993), where V(D)J recombination is thought to occur (Lin and Desiderio, 1995).

Although signal ends are profuse, broken coding ends exist at far lower levels in normal cells. By a recent estimate, there are at least 1000-fold more signal ends than coding ends at TCR-δ in normal mouse thymocytes

(Zhu and Roth, 1995). Initially, coding ends were only found in thymus DNA from mice with the *scid* mutation, where completion of coding joints is blocked (Roth *et al.*, 1992b). This led to the suggestion that in normal cells coding ends are joined much faster than signal ends and do not survive long enough for detection (see below).

It is immediately clear that hairpins are the most likely precursors of P nucleotide insertions. Off-center nicking of a hairpin would lead to a short self-complementary single-stranded overhang, and if the opposite strand is filled in and the end incorporated into a coding joint, a self-complementary insert is the natural result. Of course, P nucleotide insertions are not always the result; sometimes a hairpin can be nicked exactly at its center, leaving a blunt end for processing and joining, or a self-complementary overhang can be trimmed off before the ends are joined.

Direct evidence that broken signal ends are intermediates in recombination has come from analysis of a pre-B cell line (103/BCL-2) in which a high level of recombination is temperature inducible (Chen *et al.*, 1994). Incubation of these cells at 40°C induces high expression of the *RAG1* and *RAG2* genes, greatly increases recombination of test substrates, and turns on rearrangement at the Igκ locus (Chen *et al.*, 1994). Concomitantly, many broken signal ends appear at Igκ (as much as 40% of the DNA is broken at Jκ1) (Ramsden and Gellert, 1995). Coding joints accumulate at about the same rate as signal ends; here again, the joining of signal and coding ends is uncoupled, and coding ends are joined much faster. The signal ends are quite stable at high temperature (for more than a day) but are efficiently joined when the cells are returned to low temperature. Thus, the signal ends are proper intermediates in recombination (Ramsden and Gellert, 1995). It is not clear what stabilizes the ends at high temperature (from degradation as well as joining) or conversely what allows them to be joined when the temperature is lowered. Two possibilities that have been suggested are that joining may require downregulation of the RAG genes or reentry of cells into the cell cycle from their G1 arrest at the high temperature (Ramsden and Gellert, 1995).

In the induced cells, broken coding ends can also be detected, but at a much lower level than signal ends. Presumably, only those few coding ends that have not yet been joined are being trapped. Most of the coding ends are once again hairpins, but some open coding ends that have lost bases can also be seen. A plausible intrepretation is that hairpins are once again the primary cleavage products, and that in this situation a later stage of processing can also be detected.

Taken together, these data begin to sketch out a molecular pathway of V(D)J recombination. In recombinationally active cells, the initial coding ends are hairpins, which are then opened and processed and quickly joined.

The blunt-cut signal ends are much more stable, and one can speculate that they may well be protected by bound proteins.

Are broken ends made singly or in pairs? An answer to this question could identify the stage at which the 12/23 rule is effective. It has been possible to ask this question in two intracellular situations. At the TCRδ locus, the Dδ2 and Jδ1 elements are separated by only 800 bp, so the products of coupled or uncoupled cleavage could easily be distinguished. Only DNA cut at both sites was found, suggesting that the two cuts are either directly coupled or at least closely linked in time (Roth *et al.*, 1992a). In plasmid substrates, it has also been shown that the large majority of cleavages are coupled at the 12-spacer and 23-spacer signals; coupled cutting was 30-fold higher than cutting at a single signal (Steen *et al.*, 1996). Because this ratio is close to that governing correct 12/23 coupling in overall V(D)J recombination (in plasmids, there was about 50 times as much recombination of a 12/23 signal pair as of 12/12 or 23/23 pairs), these results strongly indicate that the pairing specificity is determined at the very first stage of the recombination process. This topic will be discussed further in the context of cell-free reactions.

IV. Factors Involved in V(D)J Recombination

A. The RAG Proteins

The RAG1 and RAG2 proteins are the two factors most closely linked to the specific stages of V(D)J recombination. During lymphoid development, expression of both these proteins runs exactly parallel to gene rearrangement. Furthermore, coexpression of the RAG proteins confers V(D)J recombination activity on a variety of otherwise inactive cell lines (Oettinger *et al.*, 1990; Schatz *et al.*, 1992), implying that all other factors required for the process must be ubiquitously expressed. For example, fibroblast cell lines that express RAG1 and RAG2 perform V(D)J recombination at frequencies comparable to pre-B cell lines (Oettingeret *et al.*, 1990).

Mice with either the *RAG1* or *RAG2* gene inactivated by mutation contain no mature B or T cells, lack V(D)J recombination completely (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992), and do not even initiate recombination (Schlissel *et al.*, 1993). This fact and a variety of other data suggested that the RAG proteins act at the first step of recombination. For some time, it seemed equally possible that they acted even earlier to prime some factors directly operating on the DNA. However, the results described later in this article, obtained with the purified RAG proteins, now make it clear that RAG1 and RAG2 together carry out the first steps of V(D)J recombination unaided and thus constitute the site-specific "V(D)J recombinase."

The RAG genes themselves make up a very unusual unit. In all organisms tested to date, they are the nearest neighbors on the chromosome, convergently transcribed, and, in most cases (*Xenopus*, chicken, mouse, and human), devoid of introns in both structural genes. The only exceptions are in zebrafish (Willett *et al.*, 1996) and rainbow trout (Hansen and Kaattari, 1995) in which two and one introns respectively have been found in *RAG1*. It is not yet known whether introns were present in a primordial *RAG1* gene and were lost afterwards or whether the introns in the bony fish represent a later acquisition. Analysis of the RAG genes in more primitive animals (e.g., shark) should clarify this issue.

Both RAG genes display a high degree of conservation (between 50 and 90% at the protein level) among these various species. On the other hand, the RAG proteins are not closely related to any other proteins by sequence. RAG2 has no known relatives, and its only notable sequence landmark is a highly acidic region toward the C terminus, but this is not required for recombination (see below). RAG1 contains a potential zinc finger of the RING family, which could be involved in either protein–DNA or protein–protein interactions. However, the N-terminal domain containing this motif can be deleted without destroying the recombination activity, as is also discussed below. The RAG1 C terminus has some similarity to the HPR1 factor of yeast, which in turn has been suggested to have some homology to topoisomerases and related recombination enzymes. However, mutations of HPR1 (Fan and Klein, 1994) or RAG1 (Sadofsky *et al.*, 1993) that remove potential active-site tyrosines do not abolish the activity of either protein, and evidence cited below shows that the RAG proteins do not operate by a topoisomerase-like mechanism.

Mutational studies of murine *RAG1* and *RAG2* have been helpful in identifying the essential and dispensable portions of both genes (Cuomo and Oettinger, 1994; Kirch *et al.*, 1996; Sadofsky *et al.*, 1993, 1994; Silver *et al.*, 1993). These studies have all made use of cotransfection of fibroblasts with plasmid recombination substrates and expression vectors for the RAG variants. Surprisingly large portions of both the RAG proteins can be deleted while still retaining the ability to support recombination. In RAG1, the N-terminal 383 amino acids (out of 1040) are dispensable, and a small portion (32 amino acids) of the C terminus can also be removed (Sadofsky *et al.*, 1993). The N-terminal deletion removes the zinc-finger motif located around position 300. In the rest of the protein, local mutations generally abolish activity. Inspection of the sequence relationships among RAG1 proteins shows that the deletable region is not as well conserved as the essential "core" region downstream.

In RAG2, it is again possible to delete a large portion of the protein and retain activity. The dispensable part is at the C terminus and includes

almost all the highly acidic domain. Only the first 383 amino acids (out of 527) need to be retained for activity (Cuomo and Oettinger, 1994; Sadofsky *et al.*, 1994). Furthermore, there is no functional redundancy between the dispensable segments of RAG1 and RAG2 because cotransfection of the two truncated variants still supports normal recombination. Even for integrated substrates, the truncated proteins are sufficient for recombination (Kirch *et al.*, 1996). In RAG2, the deletable region is surprisingly well conserved among species. It should be emphasized that recombination has so far been tested only with artificial substrates, and it remains possible that the truncated RAG derivatives will not act normally in rearrangement of the endogenous loci.

Expression of both RAG proteins is normally limited to immature B and T cells and in general correlates exactly with the occurrence of V(D)J recombination. RAG expression in both B and T cells continues even after expression of a surface antigen receptor but then ceases after crosslinking of the membrane-bound Ig or TCR (Ma *et al.*, 1992; Turka *et al.*, 1991). In a pre-B cell line, the level of RAG mRNA was induced by chemicals that elevate cellular cAMP but was diminished by agents that enhance protein kinase C activity (Menetski and Gellert, 1990).

New evidence indicates that the control of RAG expression in the course of lymphoid development is more intricate than was previously suspected. It has been shown that there are two periods of high RAG expression spanning the period of gene rearrangement in B cells, corresponding to the times of heavy and light chain rearrangement, separated by a period of lower expression (Grawunder *et al.*, 1995). However, except in these general terms, little is known about the transcriptional control of the RAG genes or about their promoter regions.

Posttranslational control of RAG2 protein may play a significant role in the cell cycle regulation of V(D)J recombination. RAG2 can be phosphorylated at several sites *in vitro*, and mutation of one of these sites (Thr 490) leads to a large increase in the steady-state amount of intracellular RAG2 protein (Lin and Desiderio, 1993). Furthermore, the amount of RAG2 protein in the HAFTL-1 pro-B cell line was found to vary greatly through the cell cycle, being high in G1 and then decreasing by a factor of 20 or more in the S, G2, and M phases, although *RAG2* RNA hardly changes (Lin and Desiderio, 1995). This variation can be correlated with evidence that double-strand breaks at signal sequences are found primarily in G1 cells (Schlissel *et al.*, 1993) and that V(D)J recombination occurs in the G1 phase (Chen and Rosenberg, 1992; Lin and Desiderio, 1995). In contrast, RAG1 protein levels appear to be rather constant across the cell cycle; however, RAG1 protein is quite unstable, with a half-life of 15–20 min (Sadofsky *et al.*, 1993; Spanopoulou *et al.*, 1995), so that disappear-

ance of RAG mRNA would lead to a rapid shutdown of V(D)J recombination even in noncycling cells.

In two situations, one of the RAG genes is expressed without the other. *RAG1* mRNA is found at a low level in the central nervous system of mice, without parallel expression of *RAG2* (Chun *et al.*, 1991). This *RAG1* mRNA is found in all neuronal cells, but its biological significance is unclear because mice with a disrupted *RAG1* gene appear to be normal in brain functions. Thus, the role of *RAG1* expression in the nervous system remains enigmatic.

Conversely, *RAG2* is expressed without *RAG1* in B cells in the bursa of Fabricius in chickens (Carlson *et al.*, 1991). At one time this observation appeared to be a promising lead for explaining the high level of immunoglobulin gene conversion that takes place in the bursa. The mechanism of this gene conversion is unknown, and it was suggested that *RAG2* could be involved. However, a bursa-derived cell line with the *RAG2* gene knocked out retains its gene-conversion activity (Takeda *et al.*, 1992). It has also been shown that, although there is a high level of *RAG2* mRNA in the bursa, there is little protein (Ferguson *et al.*, 1994). Overall, there is still no evidence that one of the RAG proteins has activity in the absence of the other.

Attention has also been focused on factors that might bind to the RAG proteins and modify their activities. Two closely related human proteins, named Rch1 and SRP1, that interact specifically with RAG1 in a yeast two-hybrid screen have been described (Cortes *et al.*, 1994; Cuomo *et al.*, 1994). These proteins fall into the importin-α family of factors required for transport of proteins into the nucleus (Görlich and Mattaj, 1996), and it is not yet clear whether they have any role other than nuclear transport in relation to the RAG system.

B. Double-Strand Break Repair Factors

It is now recognized that the later steps of V(D)J recombination have many aspects in common with general DNA double-strand break repair and share a number of factors with that process (for reviews, see Bogue *et al.*, 1996; Jackson and Jeggo, 1995; Weaver, 1995). This linkage first became apparent from the properties of the mouse *scid* mutation. Although this mutation was first recognized because it caused immunodeficiency and a defect in V(D)J recombination (Bosma *et al.*, 1983; Schuler *et al.*, 1986), later work showed that its effects were not restricted to lymphoid cells. Thus, fibroblast cell lines derived from *scid* mice are hypersensitive to X rays and are defective in repair of DNA double-strand breaks (Biedermann *et al.*, 1991; Fulop and Phillips, 1990; Hendrickson *et al.*, 1991). Also, V(D)J recombination in *scid* fibroblasts transfected with RAG expression

vectors displays the same defects as those found in *scid* lymphoid cells. This link between DNA repair and V(D)J recombination led to the screening of cell lines for other DNA repair defects with similar properties. Chinese hamster ovary (CHO) cell lines that had originally been isolated as being X-ray sensitive, and had defects in complementation groups XRCC4 or XRCC5, were again defective in V(D)J recombination. Unlike *scid* cells, they could not efficiently make either signal or coding joints (Pergola *et al.*, 1993; Taccioli *et al.*, 1993). In addition, the XRCC5 mutant cells were found to lack a DNA end-binding protein called Ku (Getts and Stamato, 1994; Rathmell and Chu, 1994; Smider *et al.*, 1994; Taccioli *et al.*, 1994). Ku, originally isolated as an autoantigen in human autoimmune disorders, is a dimer with 70- and 80-kDa subunits, and mutations in the XRCC5 complementation group affect the 80-kDa subunit. When the human *XRCC5* gene is expressed in XRCC5 mutant cells, DNA end binding, resistance to X rays, and V(D)J recombination are all restored (Boubnov and Weaver, 1995; Rathmell and Chu, 1994; Smider *et al.*, 1994; Taccioli *et al.*, 1994). The binding of Ku protein to DNA is known to require some kind of interruption in the regular DNA structure—broken ends, single-stranded gaps, double strand/single strand boundaries, or DNA hairpins are all possible sites for Ku binding. Ku is thus a plausible component in the repair of double-strand breaks.

Ku had also been shown to be the DNA-binding component of the DNA-dependent protein kinase (DNA-PK) (Dvir *et al.*, 1992; Gottlieb and Jackson, 1993). XRCC5 mutant cells have very low kinase activity presumably because the kinase cannot bind to DNA. The catalytic subunit of the kinase (DNA-PK_{CS}) is a protein with the very large molecular weight of 465,000 (Hartley *et al.*, 1995). Once Ku was linked to double-strand break repair and V(D)J recombination, one could ask whether DNA-PK_{CS} might have a related function. Work during the past 2 years has identified DNA-PK_{CS} as the factor defective in *scid* cells (and in CHO cells with the similar V3 mutation) and showed that expression of DNA-PK_{CS} overcame both types of defect in these cells (Blunt *et al.*, 1995; Kirchgessner *et al.*, 1995; Peterson *et al.*, 1995). Cloning of the gene for DNA-PK_{CS} has revealed that it shares some homology to the ATM kinase involved in the ataxia-telangiectasia defect (Hartley *et al.*, 1995) and the yeast kinase TEL1, among others. All these proteins have some functions, not yet well defined, in DNA repair.

A puzzling anomaly in understanding the roles of Ku and DNA-PK_{CS} is that they produce rather different defects in V(D)J recombination, with Ku80 mutations affecting both signal and coding joint formation, whereas the *scid* and V3 mutations mainly affect coding joints. If Ku and DNA-PK_{CS} operate only together, it is hard to see why the same defect should

not result from a defect in either one. A possible explanation is furnished by a newly described *scid* mutation in the Arabian horse, which also results in a defective DNA-PK_{CS} (Wiler *et al.*, 1995). This mutation affects the formation of coding joints and signal joints similarly, suggesting that the less complete defect of the mouse *scid* mutation may be due to its being leaky. This suggestion has some support: Low-level expression of Ku80 in cells carrying an *XRCC5* mutation also restores signal joint formation more effectively than coding joint formation (Errami *et al.*, 1996).

Although most of the later steps in V(D)J joining could plausibly overlap with similar steps in repair of double-strand break damage in DNA, the opening of hairpin ends is likely to be special to the V(D)J pathway because radiation damage will probably not result in hairpins. Hairpin ends must contain a few unpaired bases to allow the DNA to negotiate the 180° turn (e.g., Blommers *et al.*, 1991; Howard *et al.*, 1991). Thus, they can be targets for single-strand-specific nucleases (Kabotyanski *et al.*, 1995), but it is not yet known whether *in vivo* they are targeted by a special factor or opened by such nonspecific nucleases.

In this context, the exact relation between the *scid* defect and V(D)J recombination has not yet been clarified. The accumulation of hairpin coding ends in *scid* cells might mean that DNA-PK has a special role in hairpin cleavage, but plasmids with hairpin ends are efficiently cut and recircularized in *scid* cells (Lewis, 1994b; Staunton and Weaver, 1994). Any *scid*-related defect in the opening of hairpins would thus have to be specific for the particular complex resulting from V(D)J cleavage.

It is possible that in certain circumstances the *scid* defect can be bypassed by another pathway for repairing breaks. When newborn mice are irradiated with X rays, they briefly become competent to carry out V(D)J recombination and produce apparently functional TCR-β chains (Danska *et al.*, 1994; Murphy *et al.*, 1994). This recombination is dependent on a functional *p53* gene, whereas V(D)J recombination in normal mice is not (Bogue *et al.*, 1996).

Other genes in the repair pathway are not as well understood. The human *XRCC4* gene has recently been cloned, and codes for a protein of 38 kDa, but the sequence does not yet offer any clue as to its function (Li *et al.*, 1995). No mutations affecting mammalian Ku70 protein have been found.

The linkage to DNA double-strand break repair provided by these studies leads to a picture of the V(D)J recombination reaction that is divided into distinct stages (Fig. 1). The first stage contains all that is specific to V(D)J recombination. In this stage, which is directed by the RAG1 and RAG2 proteins, the RSSs are recognized, DNA is broken, and hairpins are made on the coding ends. In the second nonspecific stage, the broken ends are

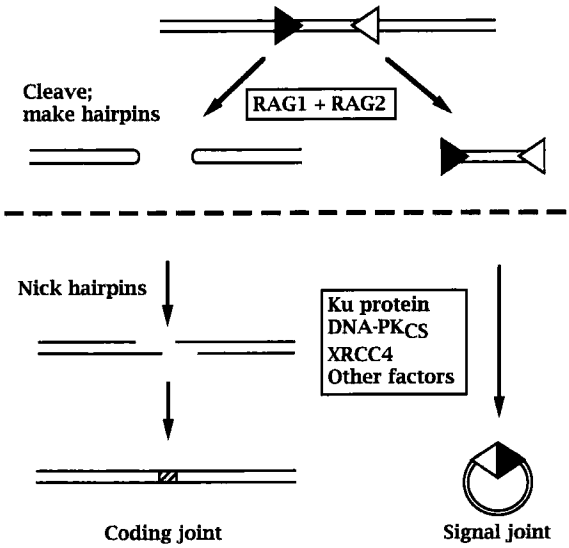

FIG. 1. The separate stages of V(D)J recombination. The triangles represent the recombination signal sequences; the vertical edge of each triangle is the border between the RSS and coding sequence. The first stage is the site-specific part of the process. The RAG1 and RAG2 proteins recognize and cleave at the signal/coding border, simultaneously converting the coding ends to hairpins. The heavy dashed line separates this part of the reaction from the less specific later stages in which the hairpins are opened (by unknown factors) and proteins involved in DNA double-strand break repair come into action. The coding ends are usually modified by loss and addition of nucleotides; the hatched box in the coding joint symbolizes these sequence variations. In parallel, the signal ends are precisely joined with the use of the same double-strand break repair factors.

then identified and joined by a process closely related to the repair of X-ray-induced breaks. The catalog of factors utilized in this second stage is undoubtedly still very incomplete (Fig. 1).

C. Terminal Deoxynucleotidyl Transferase

Terminal deoxynucleotidyl transferase (TdT) is responsible for the quasi-random insertions found in many coding joints (N regions). It is expressed only in early lymphoid cells, approximately at the stage where V(D)J joining is active, and probably has no other function than to introduce additional variability into Ig and TCR proteins. TdT is not essential for V(D)J recombination because its activity is low in newborn mice, in which antigen receptor genes are actively rearranged. Furthermore, TdT is completely lacking in fibroblasts, although these cells can be recombinationally active when expressing RAG1 and RAG2. If fibroblasts are made to express TdT in

addition to the RAG proteins, the only noticeable change in their recombinational properties is the appearance of N regions in their coding joints (Kallenbach *et al.*, 1992).

Mice with their TdT gene knocked out are not significantly immunodeficient (Gilfillan *et al.*, 1993; Komori *et al.*, 1993), although their immune repertoire, even as adults, is similar to that of normal newborn mice in lacking N regions. TdT is thus an accessory factor with significance for immune diversity but not for the intrinsic V(D)J recombination reaction itself.

V. Biochemistry of the First Steps of V(D)J Recombination

A. Cleavage at an RSS

Evidence cited previously suggests that the specific initial phase of V(D)J recombination can be separated from the nonspecific later stages and that this first stage is tightly coupled to the activities of RAG1 and RAG2. A reasonable strategy to developing a cell-free system was thus to search for double-strand breakage at RSSs. Broken signal ends were first detected by incubating plasmid substrates in extracts of the same 103/BCL-2 hyperrecombinational cell line described previously (van Gent *et al.*, 1995). The extent of breakage was low and was detectable only by a sensitive ligation-mediated PCR assay. However, the structure of the ends was the same as that of ends previously found *in vivo*. They were once again blunt, 5′-phosphorylated ends cut exactly at the RSS border and thus were authentic products of V(D)J cleavage. In another AMuLV-transformed pre-B cell line (204-1-8) that has been widely used in work on V(D)J recombination, breaks were barely detectable. A pre-B cell line from a RAG1−/− mouse was inactive but could be complemented by purified recombinant RAG1 protein (van Gent *et al.*, 1995).

Further progress was dependent on a more systematic study of the protein chemistry of RAG1 and -2. The full-length of RAG1 and RAG2 proteins had always been found to be frustrating to work with because their expression was very low in most recombinationally active cells, and attempts at expression in *Escherichia coli* or insect cells failed due to the insolubility of the recombinant proteins. However, the truncated recombinationally active proteins described previously proved to have much better solubility properties. These core proteins (RAG1, amino acids 384–1008; RAG2, amino acids 1–383 or 1–387) could be purified from insect cells infected with suitable baculovirus vectors or from HeLa cells infected with recombinant vaccinia virus.

With suitable protein preparations, it then became apparent that only the RAG1 and RAG2 proteins are required for cleavage (McBlane *et al.*,

1995). In the presence of Mn^{2+}, a DNA fragment containing a single RSS with either a 12 spacer or a 23 spacer is efficiently cut by RAG1 and RAG2 to yield exactly the products made by cleavage *in vivo*: blunt, 5′-phosphorylated signal ends and hairpin coding ends that retain the full coding sequence. Thus, the surprising result is that the RAG proteins by themselves are sufficient to both recognize and cleave an RSS. In this highly simplified reaction, they carry out the site-specific part of the V(D)J recombination process unaided. Significantly, there is no requirement for an energy source such as ATP.

Cleavage can be shown to occur in two steps. First, a nick is made at the 5′ end of the signal heptamer, leaving a 5′ phosphoryl group on the signal and a 3′ hydroxyl on the coding end. A distinct second step then joins this 3′ hydroxyl to the opposite phosphate on the other strand, resulting in a DNA hairpin and a blunt signal end (McBlane *et al.*, 1995). In each step, an RSS and the RAG1 and RAG2 proteins are needed. These reactions are diagrammed in Fig. 2. The two reactions can be separated. For example, an oligonucleotide with a premade nick at the coding signal border is readily converted to a hairpin and a cleaved signal end.

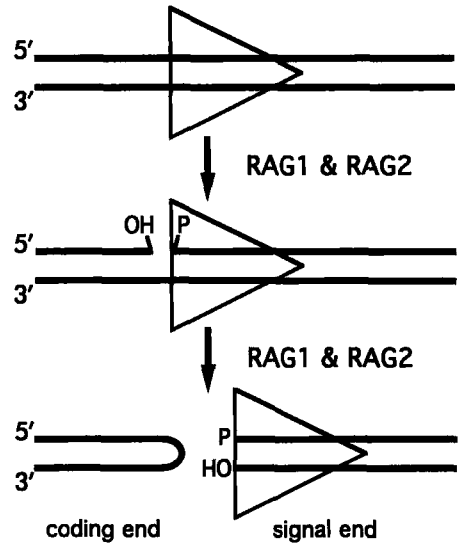

FIG. 2. An outline of the first steps of V(D)J recombination. The reaction takes place precisely at the border between the RSS and the neighboring coding DNA. A strand-specific nick is first made at this site by the RAG1 and RAG2 proteins. Then the nick is converted to a double-strand break with a hairpin structure on the end of the coding DNA; this reaction also requires the RAG1 and RAG2 proteins and the RSS.

It should be emphasized that in all these reactions, and in the more extensive biochemical analysis described below, both the RAG proteins are required at every step. There is no evidence so far of their individual activities, for example, whether one of them contains the active site and the other is a cofactor or whether the active site is shared between both proteins.

B. Coupled Cleavage

Cleavage at an isolated RSS is useful for many of the studies described later in this chapter, but it does not fully represent the biological situation. Complete recombination utilizes a 12/23 RSS pair and, as described previously, the same requirement holds for *in vivo* cleavage. What is different in the reaction with the purified RAG proteins? One-signal cleavage works efficiently with Mn^{2+} as the divalent ion but not with Mg^{2+}. However, Mn^{2+} is known to "loosen" substrate requirements for many other nucleic acid enzymes, and Mg^{2+} is present at much higher concentrations in cells. It was thus reasonable to search for cleavage that required a 12 and a 23 RSS and cut them pairwise in a cell-free reaction with Mg^{2+}. This coupled cleavage has recently been demonstrated both in cell extracts (Eastman *et al.*, 1996) and with the purified RAG1 and RAG2 proteins (van Gent *et al.*, 1996b). The results obtained with the RAG proteins are described below. A DNA with 12- and 23-spacer signals is cleaved in Mg^{2+} much better at both signals than at either one alone, whereas in Mn^{2+} the two cuts are independent. Also, DNA with a single RSS is cut well in Mn^{2+} (better at a 12 signal than a 23 signal) but very poorly in Mg^{2+}. Cleavage in all cases generates a blunt signal end and a hairpin coding end, either in coupled or in uncoupled reactions. Coupled cleavage requires only the core RAG1 and RAG2 proteins; the portions not needed for recombination are also dispensable in this reaction with the purified proteins. Among signal pairs, a 12/23 pair is cleaved more effectively in Mg^{2+} than either a 23/23 pair or a 12/12 pair, and coupled cleavage is abolished by mutation of one signal. However, cutting of the 12/12 pair is only diminished about fourfold from the 12/23 pair; this is the only feature of the reaction that lacks some of the specificity of the *in vivo* situation.

One noteworthy feature of these reactions is that in Mg^{2+}, much of the DNA is nicked at the signal border, regardless of whether the DNA contains one RSS or two (van Gent *et al.*, 1996b). Evidently, the limitation to a correct signal pair is enforced only at the double-strand cleavage/hairpin step. Because coupled cutting presumably requires the two signals, with bound RAG proteins, to be brought together in a "synaptic complex," this complex must only be required after the initial nick is made. It will be interesting to test whether nicking at RSSs is similarly permissive *in vivo*.

The first stage of V(D)J recombination requires that individual RSSs be recognized and also that the correct 12/23 coupling and cleavage are established. Both these aspects of the specificity of V(D)J recombination have now been shown to be determined by the RAG1 and RAG2 proteins.

C. Signal Sequence Recognition

The rules of RSS sequence utilization have been the subject of considerable work in the past (reviewed in Gellert, 1992; Lewis, 1994a) because they help to map out the relative efficiency of recombination of various antigen receptor genes and to distinguish functional from pseudo-RSSs. For example, a previous study of recombination in plasmid substrates (Hesse *et al.*, 1989) had shown that the heptamer is the essential part of the RSS; without a recognizable heptamer, there is no recombination. On the other hand, without a nonamer there is still a residual level of joining. These results have parallels in recombination at the Ig and TCR loci, where an isolated heptamer sequence occasionally recombines with an RSS. An example is the "V gene replacement" at the IgH locus, in which a heptamer within the C-terminal end of the VH coding sequence allows an already rearranged IgH gene to recombine again with an upstream VH region (Kleinfield *et al.*, 1986; Reth *et al.*, 1986).

The plasmid studies also showed that within the heptamer, the three bases closest to the recombination site are invariant, but other mutations in the heptamer are remarkably well tolerated. The nonamer sequence can also be widely varied, with only positions 6 and 7 (reading from the direction of the heptamer) particularly significant.

These requirements have their analogies in the cleavage of an oligonucleotide by RAG1 and RAG2 (Cuomo *et al.*, 1996; Ramsden *et al.*, 1996). For convenience, these experiments were done with oligonucleotides containing only one RSS (therefore in Mn^{2+}). Complete cleavage, with the formation of a double-strand break and hairpin, again requires a functional heptamer and is very sensitive to mutations in the first three bases. If only the heptamer is retained, cleavage still occurs at a reduced level. The initial nicking step is not nearly as demanding as complete cutting. Mutation of any of the first three heptamer positions still allows an essentially normal amount of nicking. Similarly, retention of the nonamer alone still yields some imprecise nicking centered around the position where the heptamer boundary would be (i.e., 19 bp from the nonamer). It appears that double-strand breakage has essentially the same requirements as recombination, but that nicking is more tolerant.

Changes in the spacer length of oligonucleotides are also informative. It has often been pointed out that the normal 12- and 23-bp spacer lengths differ by almost exactly one turn of the DNA double helix so that proteins

bound "in register" to the two parts of one RSS type would again be in register on the other. Variations of the oligonucleotide spacer length support this picture. When the spacer is changed by half-integral turns, to 18 or 29 bp, cleavage is distinctly worse than with 12 or 23 bp, with the 29-bp spacer worse than having no nonamer at all. Further lengthening to 34 bp restores some cooperation between heptamer and nonamer.

Taken together, these results indicate that the heptamer and nonamer to some extent function independently. A heptamer alone can target a certain amount of cleavage and, more remarkably, an isolated nonamer directs a low level of nicking to approximately the position where the heptamer border would be. Nevertheless, the two elements must be in the proper helical phase for synergistic stimulation of cleavage, and if they are wrongly spaced they can actually clash.

DNA structure, as distinct from sequence, also appears to be significant. Quite remarkably, a substrate in which the RSS is single stranded (the bottom strand of Figure 2) and only the coding flank is double helical can still be efficiently converted to a hairpin. The only abnormalities are that in this case the exposed coding end attaches to either end of the heptamer, yielding two different hairpins, and that only the heptamer of the RSS is required. This and other results described below suggest that unpairing in and near the heptamer may be a normal part of the cleavage reaction. Two possible sources of unpairing have been suggested: either the RAG proteins could disrupt the structure or partial unpairing could be intrinsic to the heptamer sequence. In this context, it has been pointed out that the CACA/GTGT motif in the heptamer is known from NMR and X-ray crystallographic studies to have an abnormal structure, with disruption of the usual base pairing (Cheung *et al.*, 1984; Patel *et al.*, 1987; Timsit *et al.*, 1991). The functionality of the heptamer may thus involve both sequence recognition and some DNA unwinding.

The effects of certain coding flank variations are in agreement with this idea. This line of work began with the observation that mutations in one small region of RAG1 (amino acids 605–610) rendered plasmid recombination extremely sensitive to the first few bases of coding sequence flanking the heptamer (Sadofsky *et al.*, 1995; Roman and Baltimore, 1996). The strongest effects were with a mutant designated RAG1-D32 (Sadofsky *et al.*, 1995). Thus, with TA or TG flanks (reading 5′ to 3′ into the heptamer), D32 gave essentially wild-type recombination levels, whereas if "bad flanks", such as AC, GG, TC, or GA, were attached to both RSSs, recombination with D32 decreased 100- to 1000-fold while recombination with wild-type *RAG1* remained normal. It was then surprising that cleavage with the purified RAG proteins displayed exactly the same pattern of sensitivity to coding flanks, even in the absence of the D32 mutation

(Cuomo *et al.*, 1996; Ramsden *et al.*, 1996). In all cases, these bad flank sequences supported efficient nicking, but hairpin formation was severely inhibited.

However, the block could be overcome by unpairing in the coding flank. If the two bases nearest the heptamer were mispaired (e.g., TC paired with CC), hairpinning was restored to normal even if both sequences were of the bad flank type. It was noted that good flanks were those that continued the pattern of purine–pyrimidine alternation in the heptamer (and thus might allow a spreading of base unpairing), whereas bad flanks broke this pattern. Unpairing at the coding/signal border may be helpful to allow the large rotation of the nicked coding end needed to make a hairpin, and this unpairing may be aided either by the local sequence (such as a good flank) or by the introduction of deliberate mismatches.

These coding flanks appeared to have different effects *in vivo* and in cleavage by the purified RAG proteins, but the discrepancy was resolved when the coupled cleavage reaction was analyzed. In coupled cleavage, the inhibition by bad flanks disappeared (van Gent *et al.*, 1996b). The most reasonable explanation, though currently unproved, is that interaction of RAG proteins in a synaptic complex opens the DNA for more efficient reaction even with unfavorable flanking sequence, possibly by RAG1 bound to one RSS acting on the other. The RAG1-D32 mutant would then be disabled from providing this *trans*-acting help.

D. Transposase-like Mechanism for Hairpin Formation

Formation of the new phosphodiester bond in the hairpin is an energy-requiring reaction, but it takes place without any external source of energy; as mentioned previously, no ATP or other high-energy cofactor is present. The energy source must be the breakage of a preexisting phosphodiester bond. Two types of such "conservative" DNA strand transfers are known in the DNA recombination field. One type is used by the site-specific recombinases such as the Int protein of phage λ, the bacterial Cre, Hin and Gin proteins, and the yeast Flp protein (Craig, 1988). These proteins all operate by breaking one DNA strand of each of two recombination partners while attaching themselves covalently to one end of the break. By doing so, they transform the energy of the original phosphodiester bond into that of a protein–DNA bond. In the second step of the reaction, the attached DNA ends are then exchanged to the partner DNA and the proteins are released. This mechanism is closely related to the action of topoisomerases, which go through similar DNA attachment–release cycles, except that the protein-bound DNA strand attaches back to the same end from which it was broken.

The second type of conservative breakage–rejoining, which has been found only with the proteins responsible for genetic transposition (transposases), is quite different. In this mechanism, the 3′ hydroxyl exposed by the first nick is activated by the transposase to attack a phosphodiester bond in the target DNA (into which the transposon will be inserted), resulting in a new bond between this 3′ OH and the second DNA (Mizuuchi, 1992). This "direct trans-esterification" reaction, which takes place in a single chemical step, has been demonstrated for the bacteriophage Mu transposase (MuA) and the integrases (IN) of HIV and other retroviruses, which are responsible for inserting DNA copies of the viral genome into the cellular chromosomes (Engelman *et al.*, 1991; Mizuuchi and Adzuma, 1991). In all these cases, the same transposase makes the first nick and carries out the subsequent strand transfer.

A standard method to distinguish these two very different mechanisms relies on a stereochemical analysis of the new phosphodiester bond. The method effectively counts the number of chemical steps in the reaction. Direct transesterification is expected to take place in a single step, but the topoisomerase-like reaction would require two steps—one for making the protein–DNA bond and one for transferring the bound DNA end to its new partner. Because each attack on a phosphodiester bond inverts the chemical chirality (handedness) at the phosphate, a two-step reaction will result in overall retention of the initial chirality, i.e., two inversions restore the initial configuration. On the other hand, a one-step direct transesterification will invert the chirality.

To allow the two chiralities to be distinguished, all four groups bound to the phosphate must be different; therefore, one of the two nonbonded oxygens in the phosphodiester linkage has to be substituted, usually by a sulfur. Such an experiment on the hairpin formation mediated by the RAG proteins has shown that the hairpins are made by direct transesterification, thus linking the RAG-mediated reaction chemically to the transposase/integrase family (van Gent *et al.*, 1996a).

RAG cleavage also resembles the processing of DNA by HIV-IN in another way. A nick is made in the first step of IN processing, releasing a dinucleotide from the end of the viral DNA. Although this reaction is normally a hydrolysis, in the presence of moderate concentrations of an alcohol, such as glycerol or 1,2-ethanediol, a dinucleotide with a covalently attached alcohol can result (Vink *et al.*, 1991). This reaction, called alcoholysis, demands a flexibility in the use of attacking groups that is very unusual for DNA nucleases. It is striking that in the nicking step of RAG cleavage these same alcohols can again replace water as the substituent (van Gent *et al.*, 1996a). This result strengthens the similarity of RAG cleavage to the transposase/integrase reactions.

A relationship between V(D)J recombination and transposable elements had been proposed previously on the bases of two different types of evidence. It was first pointed out that the most usual arrangement in the Ig and TCR loci, with the RSSs facing in opposite directions, is reminiscent of the ends of transposons, many of which are inverted repeats (Sakano *et al.*, 1979). Later, it was noted that the structure of the RAG locus, with the two genes extremely near each other and both lacking introns, is also reminiscent of mobile genetic elements (Oettinger *et al.*, 1990). The relationship of the chemical mechanism now adds support to a possible link with transposition.

However, the outcome of the RAG-mediated reaction is quite distinct from transposition and can be explained by one notable difference between the chemical pathways of the RAG proteins and those of the HIV-IN/MuA family of transposons (see Fig. 3). The initial nick made by MuA and IN leaves a 3′ hydroxyl on the transposon end; transfer of the 3′ OH

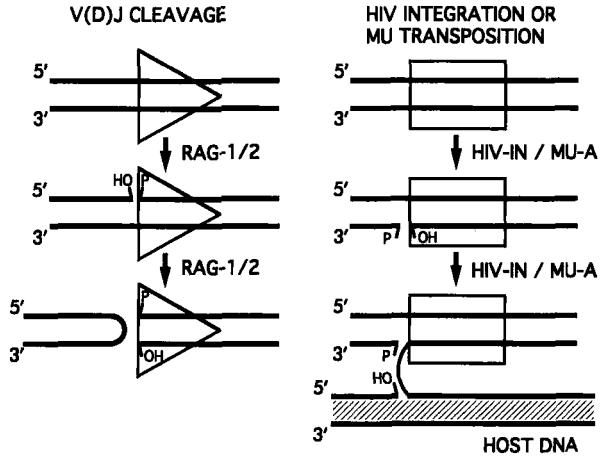

FIG. 3. Comparison of V(D)J cleavage with HIV integration (or Mu transposition). The RSS is once again depicted as a triangle and the HIV recognition sequence as a box. The reactions are shown for only one recognition sequence (the left end of HIV and the left member of an RSS pair). Cleavage at the RSS by the RAG proteins takes place in two steps as shown in Fig. 2, with a nick preceding formation of a hairpin. HIV integration similarly begins with a nick, exposing a 3′ hydroxyl end on the viral DNA. In the second step, this hydroxyl then attacks the host DNA (represented by a hatched bar) into which the HIV DNA integrates. The strand transfer process in Mu transposition is very similar. Note that the strand polarities are opposite in the two families so that the 3′ hydroxyl is inside the HIV (or Mu) recognition element but outside the V(D)J signal. The recognition element of HIV or Mu thus becomes covalently linked to the new host DNA, but the RSS cannot be similarly transferred.

to a second DNA then leads to integration of the transposon at a new site. In V(D)J cleavage, the strand polarity is reversed; the nick puts the 3′ OH in the coding flank and not in the recognition element (the RSS). Transposition by a mechanism similar to Mu or retroviral integration is thus not possible because any attempt would separate the transferred end from the RSS. If V(D)J cleavage was in fact derived from a primordial transposable element, the strand transfer has now been diverted into the intramolecular reaction responsible for hairpin formation.

VI. Biological Consequences

The increased biochemical understanding of V(D)J cleavage helps to clarify the picture of overall V(D)J recombination and its regulation. At the simplest level, it is now obvious why coexpression of RAG1 and RAG2 is completely correlated with V(D)J recombination. Because these proteins are the essential agents for initiating V(D)J recombination, and because they can act without assistance, their joint expression will render any cell capable of initiating recombination, and conversely there will be no pathway for bypassing them. As the factors for later steps of recombination are ubiquitously expressed, any (nonmutant) vertebrate cell type in which V(D)J cleavage is triggered should then be competent to complete the process. Beyond that, it is becoming increasingly clear that the precision of V(D)J recombination is mainly controlled at the initial cleavage step. The 12/23 rule is already in place at cleavage, both *in vivo* and in the RAG-mediated reaction. Thus, alternative models that could once have been considered, for instance, of a cut at one signal followed by a search for a suitable partner signal, are no longer reasonable. In addition, the effects of signal and (at least some) coding flank mutations on cleavage by the RAG proteins are so similar to their effects on recombination *in vivo* that there is no need to search further for an explanation of specificity at this level.

Nevertheless, V(D)J recombination is not extremely precise. The 12/23 rule is violated in a small fraction of either recombination events or *in vivo* cleavages (12/12 or 23/23 events are perhaps 30- to 50-fold less frequent than 12/23 events) and somewhat more often in cell-free RAG-mediated cleavage. RSS usage is also relatively permissive, and misplaced junctions in the antigen receptor loci may be relatively frequent. It is still an open question how this imprecision is dealt with. Presumably, many cells with incorrect junctions die. Cells with oncogenic translocations may represent a small residue of incorrect rearrangements that are strongly selected to survive, but in many cases only one end of the translocation junction appears to be derived from a V(D)J-related event so that an end

made initially by V(D)J cleavage may occasionally be diverted to another type of joining. It is also conceivable that some translocations may start from a nick at an RSS rather than from a double-strand break.

Another question that can now be approached in a different way is that of locus accessibility. It is well known that in recombinationally active cells, many RSSs are unavailable for rearrangement at any given time. Despite the use of the same recombinase activity, TCR loci generally stay unrearranged in B cells, as do Ig loci in T cells (except for some IgH D-J joining in T cells and some TCR-β D-J joining in B cells). Within each lineage, rearrangements are further temporally and developmentally staged (e.g., at the IgH locus, D to J joining precedes V to DJ joining).

It has now become apparent that regulation at the level of site accessibility must be focused on the cleavage step; once broken, the ends will presumably go on to complete recombination. It should thus be possible to attack the accessibility problem at this step by detecting cleavage instead of waiting for the completion of joining. This should facilitate a search for factors that block access to RSSs. Because DNA unwinding of the RSS seems to be important for allowing RAG cleavage, it is possible that accessibility factors could operate in part by modulating this DNA opening. If binding of a negative regulatory factor also inhibits unwinding, this could work as an amplification device to make the transition between closed and accessible states more abrupt.

As for the V(D)J recombination process itself, the major gaps in understanding now concern the later steps. The opening of hairpin ends is an essential but unknown step, and it is not clear whether it happens before or during engagement of the double-strand break repair machinery. It is also unknown how the system usually chooses correct pairs of ends to join to produce coding and signal joints rather than hybrid junctions.

Despite the identification of DNA-PK_{CS} and its associated Ku cofactor as essential components, their role in recombination is not yet clear. Is it the protein kinase activity that is important or does the very large kinase protein have other functions (e.g., as an organizing center for a repair complex) that have not yet come to light? Similarly for Ku protein, binding to DNA ends may be the beginning of the story, but it is certainly not the end. It is not clear whether the ATPase or helicase activities play any part in recombination or what effects the interaction of Ku with factors other than DNA-PK_{CS} may have. The function of the XRCC4 protein is still unknown, and there are undoubtedly other factors required for double-strand break repair (nuclease, DNA polymerase, and ligase) that have not been identified. Even at this basic biochemical level of identifying factors and their functions, the recent advances leave a large number of unanswered questions.

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The Role of Ets Transcription Factors in the Development and Function of the Mammalian Immune System

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Introduction

The Ets (*E*26-*t*ransformation *s*pecific or *E*-*t*wenty-six-*s*pecific) family of eukaryotic transcription factors is a large (and rapidly growing) set of winged helix–loop–helix DNA-binding proteins all of which recognize a purine-rich sequence motif centered around the core sequence, GGA/T (Karim *et al.*, 1990; Ho *et al.*, 1990; Leprince *et al.*, 1983; Nunn *et al.*, 1983; Nye *et al.*, 1992). Ets proteins are found in animals across the phylogenetic spectrum, from flies and worms to man. Invertebrate Ets proteins are both positive and negative regulators of a wide variety of important developmental events including vulval cell-fate determination in *Caen elegans* (Beitel *et al.*, 1995) and steroid hormone-induced metamorphosis (Burtis *et al.*, 1990; Fletcher and Thummel, 1995; Urness and Thummel, 1990), photoreceptor development (Lai and Rubin, 1992; O'Neill *et al.*, 1994; Tei *et al.*, 1992), and oogenesis (Pribyl *et al.*, 1991; Schulz *et al.*, 1993) in *Drosophila.* In *Xenopus,* Ets transcription factors regulate oocyte pattern formation (Chen *et al.*, 1990), and Ets proteins play an important role in regulating hematopoiesis in birds (Dieterlen *et al.*, 1990). To date, at least 17 different mammalian Ets transcription factors have been described. These include Ets-1, Ets-2, PU.1 (Spi-1), Spi-B, Fli-1 (ErgB), Elf-1, GABPα (E4TF1-60), Elk-1, Sap-1, Sap-2 (Erp/Net), PEA3, ER81, ER71, ERM, Tel, Erg, and Erf. These proteins display widely different patterns of expression. However, it is noteworthy that many of them are preferentially expressed in hematopoietic and immune cell lineages.

In addition to regulating the development and function of a number of mammalian cell lineages, several Ets proteins, including Ets-1, Ets-2, PU.1, Fli-1, Tel, and Erg, display oncogenic potential. For example, expression of a mutant form of Ets-1 (along with c-myb) in the E26 avian retrovirus causes erythroblastosis and myeloblastosis in birds (Leprince *et al.*, 1983; Nunn *et al.*, 1983). Activation of Fli-1 expression by insertion of the Friend Leukemia virus leads to erythroleukemias in mice (Ben *et al.*, 1990, 1991). And dysregulation of Fli-1 or Erg by chromosomal translocation leads to Ewing's Sarcoma in man (Delattre *et al.*, 1992).

A large number of studies during the past 10 years have provided strong evidence supporting an important role for Ets proteins in regulating both

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the normal development and the function of the mammalian immune system. Binding sites for Ets proteins have been described in a large number of genes involved in immune system function, and at least 11 Ets proteins are known to be expressed in immune cells. Of equal importance, the transcriptional activities of different Ets proteins have been shown to be highly regulated at both the transcriptional and posttranslational levels during hematopoietic development and following immune cell activation. During the past 3 years, nuclear magnetic resonance (NMR) and X-ray crystallographic studies have provided us with the first structures of Ets proteins bound to DNA, biochemical studies have revealed the importance of direct physical interactions between Ets proteins and other transcription factors, and transgenic and gene targeting studies have begun to elucidate the roles of individual Ets proteins in regulating hematopoietic cell development and function. In this review, we summarize our current understanding of the role of specific Ets proteins in immune cell function. We have focused on recent structural, biochemical, and genetic studies that have revealed the patterns of expression, function, and regulation of specific immunologically important Ets proteins. The rapidly expanding nature of this field makes it impossible to cite all of the important work on Ets proteins reported during the past decade. We apologize for any inadvertent omissions of specific references. The reader is also referred to several reviews for a summary of the evolutionary analysis and sequence comparisons of the different Ets proteins (Degnan *et al.*, 1993; Laudet *et al.*, 1993; Lautenberger *et al.*, 1992; Wasylyk *et al.*, 1993), the role of Ets proteins in oncogenesis (Hromas and Klemsz, 1994; Papas, 1992; Papas *et al.*, 1990a,b), and the role of Ets proteins in regulating diverse developmental processes (Beitel *et al.*, 1995; Burtis *et al.*, 1990; Crepieux *et al.*, 1994; Fletcher and Thummel, 1995; Janknecht *et al.*, 1993; Janknecht and Nordheim, 1993; Leiden, 1992, 1993; Leiden and Thompson, 1994; Leiden *et al.*, 1992; Macleod *et al.*, 1992; Moreau, 1994; Seth *et al.*, 1992; Treisman, 1994; Urness and Thummel, 1990; Wasylyk *et al.*, 1993).

II. Structure of the Ets DNA-Binding Domain

Each of the Ets proteins binds to a purine-rich sequence element, GGA, via a conserved 82-amino-acid motif termed the Ets domain (Karim *et al.*, 1990). In addition, PU.1 has been reported to bind a noncanonical purine-rich sequence element, GCAGAA (Shin and Koshland, 1993). With the exception of GABPα, which binds to Ets sites only as a heterodimer with the ankryin repeat containing protein GABPβ, Ets proteins bind to their recognition sites as monomers. However, in

many cases, the affinity of the Ets–DNA interaction is increased by the association of Ets proteins with other transcription factors. Within the past 2 years, NMR solution structures have been described for both the Fli-1–Ets domain bound to DNA (Liang *et al.*, 1994a,b) and the Ets-1–Ets domain bound to DNA (Werner *et al.*, 1995). Recently, the crystal structure of the PU.1–Ets domain bound to DNA has been reported (Kodandapani *et al.*, 1996). Taken together, these studies have significantly advanced our understanding of the structure–function relationships of the Ets DNA-binding domain. Remarkably, the predicted structure, based on biochemical and mutagenesis analyses, proved quite accurate (Bosselut *et al.*, 1993; Mavrothalassitis *et al.*, 1994; Wang *et al.*, 1992), although the high-resolution NMR and crystal structures have yielded several new and surprising insights. As predicted previously, the DNA-binding domain of the Ets proteins is formed by three α-helices (H1–H3). However, the presence of a four-stranded antiparallel β-sheet between the first and second helices and C terminal to the third helix was not predicted by previous studies. This topology yields a folding pattern for the Ets domain that is similar to the $\alpha + \beta$ helix–turn–helix (HTH) DNA-binding domains of the bacterial catabolite gene activator protein and the "winged" HTH proteins such as histone H5, HNF-3γ/forkhead, and heat shock transcription factors (Landsman and Wolffe, 1995). However, the PU.1-Ets structure reveals a longer turn (perhaps more appropriately termed a loop) between helices 1 and 2 than is found in the HTH proteins. A second loop between B3 and B4 of the β-sheet constitutes a "wing" structure. Thus, the Ets domain binds to DNA as a "winged helix–loop–helix." Interestingly, the amino acids in the wing and in the loop of the helix–loop–helix are not highly conserved among Ets family members, suggesting that these regions may confer differences in DNA-binding specificities between different Ets proteins or that they may be available to make specific protein–protein contacts with other transcription factors (see below).

All three structural studies indicate that amino acids in H3 make direct contacts with the major groove of the DNA helix. Despite this similarity, there are significant and somewhat surprising differences between the three structures. First, although the four amino acids shown to make direct DNA contacts in the PU.1 crystal structure are highly conserved among different Ets proteins, it is not clear that the corresponding four amino acids contact the DNA in the Ets-1 and Fli-1 structures. Second, unlike the Fli-1– and PU.1–Ets domains, the Ets-1–Ets domain makes minor groove contacts via intercalation of a tryptophan residue. These differences between the three structures may be explained in at least four ways. First, they may simply reflect artifacts

of the renaturation and/or crystallization process. Second, because Ets proteins have been shown to bind DNA nonspecifically prior to high-affinity docking (Petersen *et al.*, 1995), structures such as DNA intercalation may represent transient conformations. Third, the discrepancies may reflect the fact that different target sites were used for each structure. Because sequences flanking the GGA core affect the DNA-binding affinities of Ets proteins (Wang *et al.*, 1992), it is not inconceivable that different binding sites might induce structural changes in the Ets domain. In this regard, it will be interesting to determine the solution structures of the same Ets proteins bound to different DNA target sites. Finally, the observed variations may reflect true differences in the structures of the three Ets domains. However, this explanation seems somewhat less likely given the high degree of sequence conservation between both the Ets domains of the different proteins and the binding sites for these proteins. These explanations are, of course, not mutually exclusive.

As described in detail below, many Ets proteins bind to DNA cooperatively with other transcription factors. For example, direct physical interactions between Ets and AP1 proteins increase the affinity of Ets protein binding to the GM-CSF and polyoma virus enhancers resulting in synergistic transcriptional activation of these enhancers by these transcription factors (Bassuk and Leiden, 1995; Wasylyk *et al.*, 1990). Similarly, PU.1 and NF-EM5/Pip form a ternary complex with the Igκ (Hiramatsu *et al.*, 1995; Pongubala *et al.*, 1992, 1993) and Igλ (Eisenbeis *et al.*, 1993, 1995) enhancers, whereas Ets-1 and ATF-2 appear to cooperatively regulate the TCRα enhancer (Giese *et al.*, 1995). In this light, it will be interesting to determine if the structure of the Ets domain bound to its target site as a monomer is distinct from its structure when bound to DNA as a multiprotein complex. The solution structures demonstrate that several amino acids are not involved in making DNA contacts. These amino acids may be free to interact with other DNA-binding proteins or with TAFs, members of the transcription complex that do not bind directly to DNA. Mutational studies of the Ets domain, structural analyses of cooperative DNA binding, and additional solution structures for other Ets family members will be important in enhancing our understanding of the structure–function relationships of Ets transcription factors.

III. Ets Binding Sites in Immune/Immune-Related Genes

In the past 5 years, Ets binding sites have been identified in the regulatory elements of more than 80 genes that are involved directly or indirectly

in the development and/or function of the immune system. These are summarized in Table I and described briefly below.

Ets transcription factors bind to regulatory sites in a large number of T cell-specific or -restricted genes including adenosine deaminase, TCRα, TCR Vβ2, TCR γ/δ V3, CD2, CD3ε, CD8α, CD4, IL-2, *p56^{lck}*, and perforin. Functionally important Ets binding sites have also been identified in the promoters and/or enhancers of several B cell-restricted genes including Igλ, Igκ, IgH, Ig J chain, *mb-1, B29,* and the btk and blk protein tyrosine kinases. Important Ets binding sites are also found in the regulatory elements of several myeloid-specific genes including c-*fes*/c-*fps,* the CSF-1 receptor, the CD11b integrin, the class A scavenger receptor (SR A), the γ-interferon high-affinity receptor (FcγRI), CD13/APN, and the α subunit of the GM-CSF receptor.

Ets binding sites have also been reported in the regulatory elements of many genes that play a more generalized function in immune cells: for example, the SCl/TAL-1 bHLH transcription factor that is expressed in multipotent hematopoietic precursors prior to lineage commitment, both MHC I-Aβ and subunits of MHC class II, the *Ets-1, Ets-2,* and *PU.1* genes themselves, terminal deoxynucleotidetransferase, MIP-1α, IL-1β, IL-3, IL-4, TNF, the α, β, and γ subunits of the IL-2 receptor, the α subunit of the IL-3 receptor, lymphotoxin β, the α4 and αV integrins, the α subunit of the vitronectin receptor, osteopontin, CD18, CD10, CD34, c-*kit,* VCAM-1, and GM-CSF. Ets proteins are also important regulators of genes involved in cell cycle progression and programmed cell death including *JunB,* c-*fos,* c-*myc, egr-1, cdc2, p53, Rb, nur77,* and *bcl-2.*

As is true of many cellular transcription factors, Ets proteins have been co-opted by lymphotropic viruses as part of their regulatory apparatus. For example, functionally important Ets binding sites have been described in the MoMLV, HIV-1, HIV-2, and HTLV-1 LTRs. Finally, Ets binding sites have been identified in the regulatory elements of many housekeeping genes including cyclooxegenase, thymidylate synthetase and the alpha subunit of DNA polymerase.

In summary, a careful analysis of the published Ets binding sites suggests a large number of potentially important but distinct roles for Ets proteins in the development and function of the immune system. These include the regulation of lineage-specific gene expression during lymphoid ontogeny, the regulation of inducible or activation-specific gene expression during inflammation, the regulation of cell cycle progression and programmed cell death during both lymphoid development and activation, the regulation of expression of cellular housekeeping genes, and the regulation of viral gene expression. The complexity of these functions underscores the need for carefully designed genetic experiments to dissect the specific roles of

TABLE I

Ets BINDING SITES IN THE IMMUNE SYSTEM[a]

Gene regulated/site	Ets protein(s)	Other proteins	TA	Reference
m Pentraxin	ETS	AP1, AP2, NF-κB, SP1, Hox1.3	−	Altmeyer (1992)
m Cox	Ets-1	C/EBP, AP2, SP1, NF-κB	+	Appleby *et al.* (1994)
h ADA 1st intron enhancer	Ets-1	AP-1, TCF-1α, μe	+	Aronow *et al.* (1992)
m SCL/TAL-1 promoter 1b	ETS	C/EBP, MAZ	−	Bockamp *et al.* (1995)
h IA-β promoter	PU.1	NF-YA, NF-YB	−	Borras *et al.* (1995)
HTLV-LTR	Ets-1, Ets-2, Elf-1	SP1, PRDII-BFI	+	Bosselut *et al.* (1990), Clark *et al.* (1993), Gegonne *et al.* (1993), Gitlin *et al.* (1991), Seeler *et al.* (1993)
h CD18 promoter	PU.1, GABPα		−	Bottinger *et al.* (1994)
h IL-1β promoter	PU.1		+	Buras *et al.* (1995), Kominato *et al.* (1995)
m COXIV	GABPα	SP1	−	Carter *et al.* (1992)
BKV late gene	ETS		−	Cassil and Subramani (1988)
h *ets-1* enhancer/ promoter	Ets-1, PEA3	AP1, AP2, Oct, SP1	+	Chen and Wright (1993),Chen *et al.* (1993), Majerus *et al.* (1992), Oka *et al.* (1991)
h *bcl-2* 5′ and 3′	ETS		−	Chen *et al.* (1995)
m γδ TCR-Vγ3 promoter	ETS	SP1, CTF/NF-1	−	Clausell and Tucker (1994)
CD-3-ε	ETS		−	Clevers *et al.* (1988)
m *JunB* promoter	Ets-1, Ets-2	CRE	+	de Coffer *et al.* (1994), Nakajima *et al.* (1993)
m VCAM-1 promoter	ETS	NF-κB, GATA	−	Cybulsky *et al.* (1993)
h Integrin αV promoter	ETS	SP1, GATA	−	Donahue *et al.* (1994)
h TF VII/VIIa	ETS	AP1, NF-κB	−	Donovan-Peluso *et al.* (1994)
m Igλ B 2-4 3′ enhancer	PU.1	PIP	−	Eisenbeis *et al.* (1993)
m TdT promoter	Ets-1, Fli-1	Lyf-1	−	Ernst *et al.* (1993)
m and h Surf-1/Surf-2 promoter	ETS-2, PEA3	YY-1	+	Gaston and Fried (1995a,b)

h TCRα enhancer	Ets-1	LEF-1, ATF/CREB, CBFα	−	Giese *et al.* (1995), Ho *et al.* (1990)
h IL-3	Elf-1	AP1	−	Gottschalk *et al.* (1993)
m IgH 3′ enhancer	Elf-1	AP1	+	Grant *et al.* (1992, 1995)
m MIP-1α	ETS	C/EBP, NF-κB	−	Grove and Plumb (1993)
Mo-MuLV enhancer	Ets-1, Ets-2, GABPα, Elf-1	CBF	+	Gunther and Graves (1994), Gunther *et al.* (1990), Sun *et al.* (1995)
m OPN	ETS		−	Guo *et al.* (1995)
mb-1 promoter	Ets-1, PU.1		−	Hagman and Grosschedl (1992)
h CD8α intronic enhancer	Ets-1	TCF-1, CRE, GATA-3, LyF-1, bHLH	−	Hambor *et al.* (1993)
h GP Ib α promoter	ETS	GATA	−	Hashimoto and Ware (1995)
m and h CD34 enhancer	ETS	myb, myc	−	He *et al.* (1992)
m IL-4 2nd intronic enhancer	PU.1	GATA	−	Henkel and Brown (1994)
h c-*fes*/c-*fps* promoter	PU.1, Elf-1, SpiB	SP1	+	Heydemann *et al.* (1996), Ray-Gallet *et al.* (1995)
h GPIX promoter	ETS	GATA	−	Hickey and Roth (1993)
h CD11b enhancer	PU.1	SP1, AP2	−	Hickstein *et al.* (1992)
HIV-2LTR	Elf-1, Fli-1, Ets-2	NF-κB	−	Hilfinger *et al.* (1993), Leiden *et al.* (1992), Markovitz *et al.* (1992)
h and m c-*fos* SRE	TCFs	SRF	+	Hipskind *et al.* (1991), Janknecht and Nordheim (1992), Price *et al.* (1995)
m Igκ chain E3′	PU.1	Pip	−	Hiramitsu *et al.* (1995), Pongubala *et al.* (1992)
h GM-CSF R α	PU.1	C/EBPα	+	Hohaus *et al.* (1995)
HIV-1LTR	Ets-1	LEF-1, TFE-3, NF-κB, SP1	+	Holzmeister *et al.* (1993), Pazin *et al.* (1996), Seth *et al.* (1993), Sheridan *et al.* (1995)
h SR A enhancer	PU.1, Ets-2	AP1	+	Horvai *et al.* (1995), Moulton *et al.* (1994), Wu *et al.* (1994)
h CD10 promoter	PU.1		−	Ishimaru and Shipp (1995)
h MHCII DRA promoter	Ets-1	CREB, C/EBP, AP1	+	Jabrane and Peterlin (1994), Peterlin (1991)
h IL-2Rα enhancer	Elf-1	NF-κB, HMG-I(Y)	.+	John *et al.* (1995)
h PDGF β chain core promoter	ETS	AP1, SP1	−	Khachigian *et al.* (1994)
m PU.1 promoter	PU.1	Oct, SP1	+	Kistler *et al.* (1995)

(*continued*)

TABLE I—*Continued*

Gene regulated/site	Ets protein(s)	Other proteins	TA	Reference
m PFP promoter	ETS		−	Koizumi *et al.* (1993)
h TNF promoter	Ets-1	AP1	+	Kramer *et al.* (1995)
HSV-1 IE gene enhancer	GABPα	GABPβ	+	LaMarco and McKnight (1989), LaMarco *et al.* (1991), Thompson *et al.* (1992)
h GPV promoter	Ets-1	SP1, GATA	−	Lanza *et al.* (1993)
LMP/TP2	PU.1, Spi-B		+	Laux *et al.* (1994)
h GPIIB promoter	Ets-1, Ets-2	GATA-1	+	Lemarchandel *et al.* (1993)
Polyoma virus enhancer	PEA3, Ets-1	AP1, PEA3	−	Leprince *et al.* (1992), Wasylyk *et al.* (1990)
h *lck* promoter	Ets-1, Ets-2	Myb	+	Leung *et al.* (1993), McCracken *et al.* (1994)
m TS promoter	ETS	SP1	−	Liao *et al.* (1994)
m IgH enhancer π element	Erg-3, Fli-1, PU.1	E12	+	Libermann and Baltimore (1993), Rivera *et al.* (1993)
h IL-2Rβ promoter	Ets-1	GATA	−	Lin *et al.* (1993)
h *blk* promoter	ETS		−	Lin *et al.* (1995)
m TIMP-1 promoter	Ets-1	AP1	+	Logan *et al.* (1996)
h *Ets-2* promoter	Erf		+	Mavrothalassitis *et al.* (1990), Sgouras *et al.* (1995)
egr-1 promoter	TCF	SRF	−	McMahon and Monroe (1995)
m Vβ2 promoter	ETS	AP1	−	Messier (1992)
h and m c-*mpl* promoter	ETS	GATA	−	Mignotte *et al.* (1994)
m IL-3Rα	ETS	GATA, c-myb, SP1, AP2	−	Miyajima *et al.* (1995)
IgH μB and μA enhancer elements	PU.1, Ets-1		+	Nelsen *et al.* (1993)
h IgH 3′ enhancer	ETS			Neurath *et al.* (1995)
h IL-2Rγ promoter	ETS		−	Ohbo *et al.* (1995)
B29 promoter	ETS	Oct, LyF1, SP1	−	Omori and Wall (1993)
DNA Polα	ETS		−	Pearson *et al.* (1991)
h FcγRI promoter	PU.1		+	Perez *et al.* (1994)
m LT-β promoter	ETS		−	Pokholok *et al.* (1995)
h TCRβ enhancer	Ets-1, Ets-2, GABPα	CBF	+	Prosser *et al.* (1992), Gottschalk *et al.* (1993), Sun *et al.* (1995), Wotton *et al.* (1994)

m Vγ1TCR promoter	ETS	CRE, Ebox	−	Punturieri *et al.* (1993)
m NVL3 enhancer	ETS	AP1	−	Reddy *et al.* (1992)
h and m c-*fms* promoter	ets-1, Ets-2, PU.1	c-myb	+	Reddy *et al.* (1994)
h α4 Integrin promoter	GABPα, Ets-1		−	Rosen *et al.* (1994)
h CD18/CD11a promoter	PU.1, GABPα		+	Rosmarin *et al.* (1995), Shelly *et al.* (1993)
h and m *myc* promoter	Ets-1, Ets-2	E2F	+	Roussel (1994)
m and h CD4 enhancer	Elf-1, Ets-1, Ets-2	AP1, GATA, SP1	−	Salmon *et al.* (1993), Wurster *et al.* (1994)
h Rb promoter	GABPα	SP1, ATF	−	Savoysky *et al.* (1994)
IgVκ19 light chain promoter	PU.1	Oct-1, Oct-2	+	Schwarzenbach *et al.* (1995)
CD13/APN enhancer	Ets-1, Ets-2	Myb	+	Shapiro (1995)
IgJ chain promoter	PU.1		+	Shin and Koshland (1993)
m and h *Btk* promoter	ETS	zeste, PuF	−	Sideras *et al.* (1994)
h IL-2 enhancer	Elf-1	AP1, NFAT	−	Thompson *et al.* (1992)
m *p53*	ts-1, Ets-2		+	Venanzoni *et al.* (1996)
h ATPsynβ promoter	Ets-1, Ets-2		+	Villena *et al.* (1994)
m GM-CSF promoter	Elf-1	AP1	−	Wang *et al.* (1994)
E4 promoter	GABPα		−	Watanabe *et al.* (1993)
cdc2	Ets-2		+	Wen *et al.* (1995)
nur77 promoter	ETS	AP-1	−	Williams and Lau (1993)
h CD2 3′ enhancer	Elf-1	SOX4	−	Wotton *et al.* (1995)
DNA Pol β	ETS		−	Yamaguchi *et al.* (1988)
m and h c-*kit* promoter	ETS	SP1, AP-2, bHLH, Myb, GATA-1	−	Yamamoto *et al.* (1993), Yasuda *et al.* (1993)

[a] Genes regulated by Ets proteins are listed in alphabetical order according to the last name of the first author in the Reference column. The genes are listed in the leftmost column. "m" or "h" indicate whether the site is from a murine gene or human gene, respectively. The particular Ets proteins shown to bind to the specific regulatory element are indicated under Ets protein(s). Cases in which an Ets site was identified but no specific Ets protein was shown to bind are indicated by "ETS." Functional binding sites for non-Ets transcription factors adjacent to or near the identified Ets binding site are indicated under Other Proteins. A "+" or "−" under the TA column indicates whether or not a cotransfection experiment was performed demonstrating transcriptional transactivation by the specific Ets protein.

individual Ets proteins in regulating immune cell development and function.

IV. Individual Ets Proteins and Immune Function

A. Introduction

The fact that most, if not all, cells of the immune system express multiple Ets proteins, and that each of these proteins can recognize a large number of structurally related Ets binding sites, raises important questions about the molecular mechanisms underlying the specificity of gene regulation by different Ets transcription factors. Although there may be some functional redundancy between the different proteins, the existing data support a model in which individual Ets family members perform largely distinct functions in the immune system (see below). There are several nonmutually exclusive mechanisms that may account for this specificity. First, many Ets proteins are expressed in a tissue-specific manner, and specific Ets factors are expressed at different times during development, cell cycle progression, and/or activation. Second, Ets proteins are subject to multiple posttranslational modifications that can both positively and negatively regulate their transcriptional activity. Finally, combinatorial interactions between Ets proteins and other members of the transcription complex play important roles in the fine regulation of immune cell gene expression. In this section, we explore each of these regulatory mechanisms for the Ets proteins expressed in cells of the immune system including Ets-1, Ets-2, PU.1, Spi-B, Fli-1, Elf-1, GABPα, and the ternary complex factors (TCFs) (Table II). We focus on the lineage-specific and temporally restricted patterns of expression of each protein, the known roles of posttranslational modification and protein–protein interactions, and the results of gene targeting and transgenic studies in defining the function of each family member.

B. Ets-1

1. Expression

Ets-1 is preferentially expressed in the immune systems of adult chickens, mice, and men (Bhat *et al.*, 1987, 1989; Chen, 1985; Ghysdael *et al.*, 1986). However, its expression is more widespread during early vertebrate development. Ets-1 expression is first detected by Northern blot analysis at Embryonic Day 8 in the mouse (Kola *et al.*, 1993). *In situ* hybridization analysis shows Ets-1 expression at murine Embryonic Day 15.5 (Kola *et al.*, 1993; Maroulakou *et al.*, 1994). During murine development, Ets-1 expression is observed in lymphoid tissues and in several other organs including the developing nervous system, vascular structures, lung, gut,

and skin (Kola *et al.*, 1993; Maroulakou *et al.*, 1994). Ets-1 expression is maximal at Postnatal Days 1 or 2 (Bhat *et al.*, 1989). Beginning in neonatal life, Ets-1 is expressed at highest levels in lymphoid tissues and brain (Kola *et al.*, 1993). However, expression has also been detected in the lung, vascular smooth muscle, gut mesynchyme, and in the theca interna cells of the ovaries of adult mice (Hultgardhnilsson *et al.*, 1996; Kola *et al.*, 1993; Maroulakou *et al.*, 1994; Rowe and Propst, 1992). Several nonlymphoid cell lines have also been shown to express Ets-1, including mesynchymal lines (Grevin *et al.*, 1993; Kola *et al.*, 1993), astrocytomas (Fleischman *et al.*, 1995), and megakaryocyte lines (Lemarchandel *et al.*, 1993). The significance of Ets-1 expression in such cell lines remains unclear.

Ets-1 is first expressed at high levels in the thymus on Embryonic Day 17, at the same time that mature, single-positive ($CD4^+$ or $CD8^+$) T cells begin to accumulate in significant numbers (Bhat *et al.*, 1987, 1989). Within the thymus, Ets-1 expression is restricted largely to such mature single-positive cells. There is some evidence that Ets-1 levels may be highest in $CD4^+$ single-positive cells (Bhat *et al.*, 1989). Ets-1 expression is maintained in mature peripheral T cells. However, the levels of expression are less than those seen in single-positive thymocytes. Interestingly, both *Ets-1* mRNA and protein levels, as well as *Ets-1* DNA binding activity, decrease rapidly (and markedly) following T cell activation (Fisher *et al.*, 1991; Hodge *et al.*, 1996; Rabault and Ghysdael, 1994). As described below, T cell activation results in the Ser phosphorylation of *Ets-1*, which in turn decreases both the DNA binding activity and half-life of the Ets-1 protein. It remains unclear if the decrease in *Ets-1* mRNA upon T cell activation is due to diminished transcription, increased mRNA turnover, or both.

Ets-1 gene expression has also been detected in splenic B cells and in several immature B cell lines (Faust *et al.*, 1993; Maroulakou *et al.*, 1994). However, little is currently known about the patterns of Ets-1 expression during B cell development or the regulation of Ets-1 expression and function following B cell activation.

Many studies have attempted to implicate chromosomal translocations involving the *Ets-1* gene and/or dysregulated Ets-1 gene expression in mammalian oncogenesis. However, the *Ets-1* gene has never been assigned to a tumor-specific chromosomal breakpoint and viral insertion into regulatory regions of the Ets-1 gene has not been shown to modify Ets-1 expression patterns. Although the MoMuLV virus has been shown to integrate into the first exon of the rat *Ets-1* gene, this integration event does not appear to alter Ets-1 gene expression (Bear *et al.*, 1989; Bellacosa *et al.*, 1994). Thus, the *Ets-1* gene, discovered as the cellular homolog of an oncogenic inducing virus in birds, has yet to be definitively classified as a mammalian protooncogene.

TABLE II

EXPRESSION PATTERNS, FUNCTIONAL DOMAINS, MODIFICATIONS, AND BINDING SITES OF IMMUNE CELL Ets PROTEINS[a]

	Protein							
	Ets-1	Ets-2	PU.1	Spi-B	Fli-1	Elf-1	TCFs	GABPα
Expression patterns								
Predominant lineages	Lymphoid	Ubiquitous	Hematopoietic restricted	Lymphoid restricted	Hematopoietic	Hematopoietic	Ubiquitous	Ubiquitous
Peripheral T cells	++++	++++	−	±	+	+	+	+
Quiescent T cells	++++	+	−	±	+	+	?	?
Activated T cells	+	++++	−	±	+	+	?	?
Thymocytes	++++	++++	+/− Developing thymocytes	+, Developing thymocytes	+	+	+	+
SP $CD4^+$	++++	++++	−	±	?	+	?	?
SP $CD8^+$	++	++	−	±	?	+	?	?
B cells	+	+	++++	++++	+	+	+	+
Myeloid cells	+	+	++++	−	+	+	+	+
Functional domains/ modifications								
Transcriptional activation domain	N terminus	N terminus	7–118	N terminus	106–201, 401–452	1–100	C terminus, C box	in GABPβ
Inhibitory domain	280–331, 423–441	N-terminal homo ets-1?, 453–459	119–160, PEST-may inhibit	PEST-may inhibit	?	?	SRF assoc reg/B box	?
Phosphorylation	+	+	+	+	?	+	+	?
Site(s)	Ser 251, 257, 282, 285; T 38	Thr 72	Ser 148, in PEST	Thr 56, and other S/T		?	various S/T in activation domain	

Kinase pathway(s)	Ca^{2+} calmodulin/Ras	Ras	Casein kinase II	ERKI, JNKI		?	ERK, JNK	
Associated proteins	Jun, ATF-2	?	PIP, Rb, and TFIID; Jun and NFIL-6b; HMG I/Y; SSRP; MKP1	Rb	Jun	Rb, Jun, NF-κB, HMG I/Y	SRF, p44 MAPK	GABPβ
Binding sites								
Association sites	ETS, N terminus	?	PEST, activation domain, Ets domain, ?	Activation domain	Ets domain	Activation domain, Ets domain	Box B, box C	316–437
Consensus binding site	G/C A **G G A** A/T G/C T/C G	C C A **G G A** A G T G	A A A A A/T G/C A C G **G G A** A G/C T A/G G/C	A A A A A AA/T G/C A/C G **G A** A G/C T A/TT N		A/C **G G A** A A/G	A/C Y C/A C/A **G G A** W Y R	C **G G A** A/T
Selected binding site(s)	Pu C/G C/A **G G A** A G T/C Py A/G C C/A **G G A** A/T G/A C/T N A C C/A **G G A** A/T G/A T T		A C C **G G A** A G/A T/C	A A/T C/A C C **G G A** A G T A/S				

[a] Expression in various cell lineages is indicated by "+" or "−." Relative amounts of the individual protein expressed in different cell lineages are indicated by + to ++++ amounts of +. This is not meant to reflect the relative levels of expression of the different Ets proteins in a single cell lineage. SP, single positive T cell. The consensus Ets-1 binding site is from Wang *et al.* (1992). Selected Ets-1 sites are from Nye *et al.* (1992) and Woods *et al.* (1992). The consensus Ets-2 site is from Woods *et al.* (1992). The consensus PU.1 and Spi-B binding sites are from Ray-Gallet *et al.* (1995). The selected Fli-1 binding site is from Mao *et al.* (1994). The consensus Elf-1 binding site is from Wang *et al.* (1992), and the selected Elf-1 binding site is from John *et al.* (1996). The TCF consensus binding site is the Elk-1 consensus binding site (John *et al.*, 1996). The GABPα consensus site is from Brown and McKnight (1992).

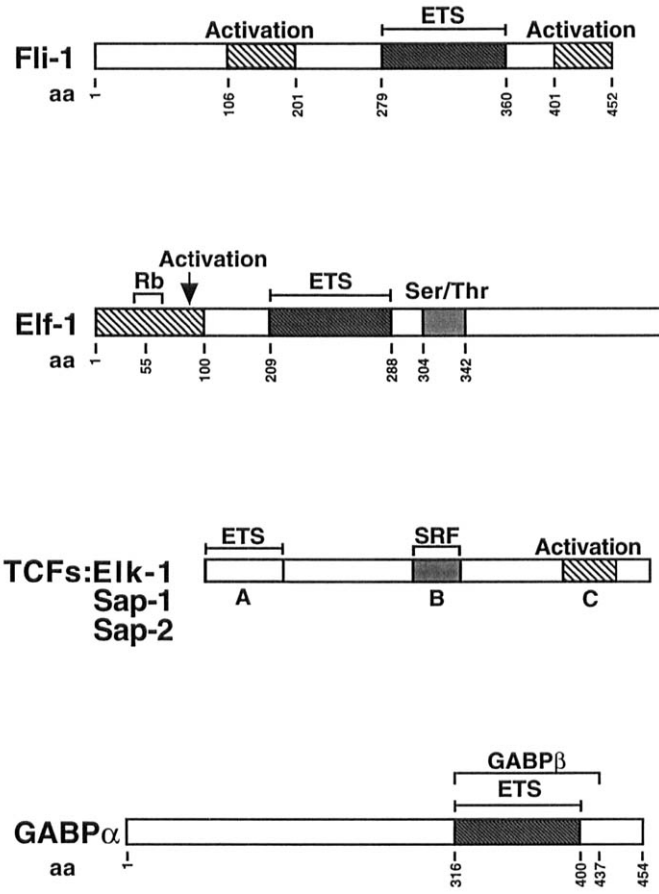

FIG. 1. Schematic illustrations of the structures of immune cell Ets proteins. For a complete description of the functional domains of each protein see the accompanying text. Amino acid (aa) numbers are indicated below each protein. Phosphorylated threonine and serine residues are indicated by a "P." Transcriptional activation domains (Activation) are indicated by stripes. For Ets-1 and Ets-2, domains inhibiting DNA binding (Inhib.) are shaded. The DNA-binding domain of the Ets proteins (ETS) are indicated by hatching. Regions that are required for binding the retinoblastoma gene product (Rb), the RNA polymerase II basal transcription multiprotein complex (TFIID), the serum response factor (SRF), Pip, and GABPβ are also indicated. The serine and threonine (Ser/Thr)-rich region of Elf-1 is indicated by hatching. The acidic (Acidic) and glutamine-rich (Glut.) subdomains of the PU.1 transcriptional transactivation domain are also indicated. A, B, and C represent conserved regions of the TCF family (Crepieux *et al.*, 1994).

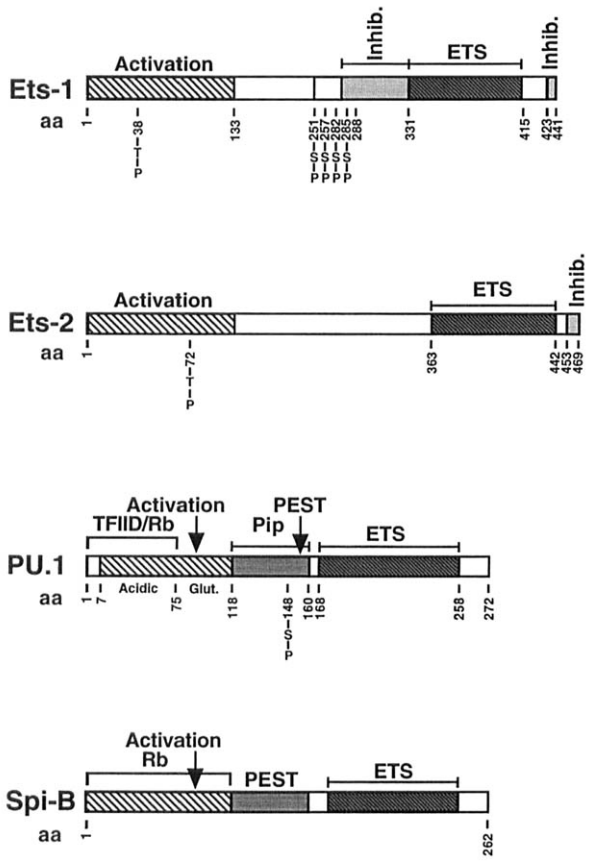

FIG. 1—*Continued*

2. *Structure–Function Studies*

Although Ets-1 activity is highly regulated at the level of gene expression, the Ets-1 protein itself is also subject to several types of posttranslational regulation. The trancriptional transactivation activity of Ets-1 maps to an acidic domain between amino- acids 60 and 133 (Gegonne *et al.*, 1992) (*Fig.* 1). This domain may be positively regulated by Ras-dependent phosphorylation (Yang *et al.*, 1996). In addition, the DNA-binding affinity of the Ets-1–Ets domain is negatively regulated by at least two intramolecular inhibitory sequences; an N-terminal region (amino acids 280–331) (Lim *et al.*, 1992) and a C-terminal region (amino acids 423–441) (Hagman and Grosschedl, 1992; Hahn and Wasylyk, 1994; Lim *et al.*, 1992). Interestingly, the N-terminal inhibitory domain (which is also subject to functionally

important phosphorylation—see below) is deleted in a naturally occurring splice variant of Ets-1 found in both mice and men, indicating that the activity of the Ets-1 protein may also be regulated by alternative splicing (Koizumi *et al.*, 1990; Pognonec *et al.*, 1990). Several recent studies have revealed that a direct physical interaction between the N-terminal Ets-1 inhibitory sequence and both the Ets domain and the C-terminal inhibitory sequence are responsible for the intramolecular inhibition of Ets-1 DNA-binding activity (Jonsen *et al.*, 1996; Petersen *et al.*, 1995; Skalicky *et al.*, 1996). Specific or nonspecific DNA binding induces the unfolding of an α-helix in the N-terminal inhibitory region, thereby relieving inhibition and rendering the Ets-1 protein competent to bind DNA.

Ets-1 is rapidly phosphorylated following T and B cell activation by antigen receptor cross-linking or pharmacological stimulation (Fisher *et al.*, 1991; Koizumi *et al.*, 1990; Pognonec *et al.*, 1988). This phosphorylation is mediated by a calcium-dependent signaling pathway and can be mimicked by treatment with calcium ionophore and inhibited by Ca^{2+} chelating agents (Pognonec *et al.*, 1988). Interestingly, the calcium/calmodulin-dependent Cam kinase II seems to phosphorylate Ets-1 *in vitro* and *in vivo* (Rabault and Ghysdael, 1994; Valentine *et al.*, 1995). However, it is not yet clear whether this enzyme is responsible for the phosphorylation of Ets-1 *in vivo*.

Following T cell activation, Ets-1 is phosphorylated predominantly on Ser residues (Pognonec *et al.*, 1988). Ser 251, 257, 282, and 285, which all lie within the N-terminal inhibitory domain, appear to be the specific targets of the activation-induced phosphorylation of Ets-1 (Rabault and Ghysdael, 1994). Phosphorylation of these residues inhibits both the specific and nonspecific DNA-binding activities of Ets-1 and decreases the half-life of the protein (Fisher *et al.*, 1991; Hodge *et al.*, 1996; Rabault and Ghysdael, 1994). Thus, the mitogen-activated, calcium-dependent phosphorylation of Ets-1 is a negative signal that both decreases the level and DNA-binding activity of the protein.

Several recent studies have placed Ets-1 in the Ras signaling pathway Wasylyk *et al.*, 1994; Yang *et al.*, 1996). Deletion mutants of Ets-1 encoding only the Ets domain have been shown to cause reversion of Ras transformation in some cell types (Wasylyk *et al.*, 1994). Moreover, reporter constructs containing Ras responsive elements can be highly activated by the combination of Ets-1 and Ras but not by an Ets-1 protein containing a Thr 38 to Ala mutation (Yang *et al.*, 1996). Because Thr 38 maps within the transactivation domain of Ets-1, Ras-induced Thr phosphorylation at this site may serve to regulate the transcriptional transactivation capacity of the Ets-1 protein.

Protein–protein interactions also appear to play an important role in regulating the activity of Ets-1. Several recent studies have demonstrated functionally significant direct physical interactions between Ets-1 and Jun proteins (Bassuk and Leiden, 1995; Logan *et al.*, 1996; Sieweke *et al.*, 1996). These interactions, which can occur in the absence of DNA, are mediated by the DNA-binding domains of each protein; the Ets domain of Ets-1 and the basic domain of Jun family members. The ability of Ets proteins to interact with Jun factors represents an evolutionarily conserved property of both families because all Ets and Jun proteins tested are capable of associating (Bassuk and Leiden, 1995). Ets-1 (via its Ets domain) has also been shown to intereact with MafB (via its basic domain), a Jun-related protein expressed in myelomonocytic cells, indicating that the functional consequences of the Ets-1–Jun interaction may be regulated, in part, by lineage-restricted expression of particular Jun (and Ets) family members (Sieweke *et al.*, 1996). The Ets-1–c-Jun interaction has been shown to be functionally significant in that the direct physical interaction between the two proteins is required for their ability to synergistically activate appropriate reporter constructs (Bassuk and Leiden, 1995). In other studies using different reporter constructs, the interaction appears to inhibit the activity of both the Ets and Jun proteins (Goldberg *et al.*, 1994; Sieweke *et al.*, 1996). Thus, AP1–Ets interactions may function to both positively and negatively regulate transcription depending both on the patterns of expression of the different proteins and on the specific target genes analyzed.

Ets-1 has also been shown to physically associate *in vitro* with ATF-2 (Giese *et al.*, 1995). This interaction is mediated by the N-terminal region of Ets-1. In addition, LEF-1 and an ATF/CREB stabilize the DNA binding of both Ets-1 and core binding factor (CBFα or PEP2α) on the TCRα enhancer, in agreement with previous studies that demonstrated the importance of multiple protein–protein interactions in regulating TCRα enhancer function in T cells (Ho *et al.*, 1990).

3. *Transgenic/Gene Targeting Studies*

The role of Ets-1 in regulating lymphoid development and function has recently been addressed by two different groups who have introduced homozygous Ets-1-deficient (Ets-1$^{-/-}$) embryonic stem (ES) cells produced by standard gene targeting strategies into RAG-2-deficient blastocysts (Bories *et al.*, 1995; Muthusamy *et al.*, 1995). In the resulting Ets-1$^{-/-}$/RAG-2$^{-/-}$ chimeric mice, all peripheral B and T cells are derived from the Ets-1$^{-/-}$ ES cells (Chen *et al.*, 1993; Shinkai *et al.*, 1992) thus allowing an accurate assessment of the role of Ets-1 in lymphoid development *in vivo*, in the absence of other Ets-1-related defects. The

Ets-1$^{-/-}$/Rag-2$^{-/-}$ chimeric mice displayed several defects in immune function: (i) Total numbers of thymocytes were severely reduced in the chimeric mice; (ii) although all thymocyte subsets were present, there was an increased percentage of double-negative ($CD4^-CD8^-$) cells and a decreased population of double-positive ($CD4^+CD8^+$) cells; (iii) there was a decrease in the number of splenic and lymph node T cells. These cells had a normal cell surface profile (i.e., there were normal percentages of single-positive $CD4^+$ and $CD8^+$ cells). However, they displayed an increased rate of spontaneous apoptosis when cultured in the absence of activating signals *in vitro;* (iv) splenic T cells from the Ets-1$^{-/-}$/RAG-2$^{-/-}$ chimeric mice displayed a proliferative defect in response to activation following treatment with an α-CD3 mAb or Con A; and (v) there was an increased population of $IgM^+/B220^{dull}$ B cells in the spleens of the chimeric animals that correlated with an increased number of splenic plasma cells and a 10-fold increase in serum IgM levels. These results have several important implications with regard to the function of Ets-1 in lymphocytes. First, the finding that normal B and T cell development occurred in these animals suggests either that Ets-1 is not required for the expression of genes, such as TCRα and -β, CD4 and CD8, and IgH, Igκ, and Igλ (each of which contain putative Ets-1 binding sites; see Table I), or that other Ets proteins can assume the function of Ets-1 in regulating these genes in the Ets-1$^{-/-}$ lymphocytes. Taken together with the patterns of expression of Ets-1 described previously, these studies were also consistent with a model in which Ets-1, which is preferentially expressed in resting mature single-positive T cells, is required to maintain such mature T (and B) cells in a resting state. In such a model, Ets-1 deficiency might favor spontaneous B cell differentiation into plasma cells (accounting for the increased plasma cell populations in these mice) and spontaneous T cell cycle progression resulting in apoptotic cell death (thereby accounting for the decreased numbers of mature T cells in these animals). Alternatively, Ets-1 might regulate genes involved in T cell apoptosis, in which case Ets-1 deficiency might directly activate apoptosis in T cells. Ongoing studies investigating the phenotype of mice containing germline mutations of Ets-1, and elucidating the targets of Ets-1 in resting T cells, should increase our understanding of the function of Ets-1 both within the immune system and in nonimmune cell lineages.

C. Ets-2

1. Expression

In contrast to the largely lymphoid-restricted pattern of expression of the closely related Ets-1 protein, Ets-2 is expressed ubiquitously in the

embryo and the adult (Baffico *et al.*, 1989; Bhat *et al.*, 1987; Kola *et al.*, 1993; Maroulakou *et al.*, 1994). In early murine embryonic development, Ets-2 is expressed in the developing limb buds and distal tail (Maroulakou *et al.*, 1994). In later development, it is expressed in developing bone, tooth buds, epithelial layers of the lung, gut, skin, nasal sinus, uterus, and several regions of the brain (Maroulakou *et al.*, 1994). In the adult mouse, Ets-2 is expressed at low levels in most tissues and at higher levels in particular regions of the brain, thymus, and ovaries (Bhat *et al.*, 1987; Maroulakou *et al.*, 1994; Rowe and Propst, 1992).

Ets-2 is first detected in murine thymocytes on Embryonic Day 17, coinciding with the appearance of significant numbers of single-positive thymocytes. Levels of Ets-2 are maximal at Embryonic Day 20 (Bhat *et al.*, 1989). Like Ets-1, Ets-2 expression is highest in single-positive T cells. Also, as is the case with Ets-1 expression, lymph node T cells contain relatively lower amounts of Ets-2 than single-positive thymocytes (Bhat *et al.*, 1989). Ets-2 has also been detected at low levels in adult murine splenocytes (Maroulakou *et al.*, 1994). In contrast to Ets-1, Ets-2 protein levels increase upon T cell activation (Fujiwara *et al.*, 1988).

2. *Structure–Function Studies*

The structures of Ets-1 and Ets-2 are quite similar. The transcriptional transactivation domain of the human Ets-2 protein lies within the N-terminal 185 amino acids of the protein (Chumakov *et al.*, 1993) and is structurally homologous to the transactivating domain of human Ets-1. Interestingly, within this region of homology, both proteins can be phosphorylated at a conserved Thr residue (see below). Thus, the transactivating activity of Ets-2, like that of Ets-1, may be regulated by phosphorylation (Yang *et al.*, 1996). The C-terminal inhibitory domain present in Ets-1 has also been shown to be conserved in Ets-2 (Hagman and Grosschedl, 1992). Given the recent structural determination of the mechanism of Ets-1 intramolecular inhibition of DNA binding (Petersen *et al.*, 1995), it is reasonable to assume that the mechanisms of intramolecular inhibition described for Ets-1 have been conserved in Ets-2, although this will require independent experimental confirmation.

Like Ets-1, Ets-2 can be phosphorylated in response to stimulation through the TCR or through the release of intracellular calcium by calcium ionophore (Fujiwara *et al.*, 1990; Pognonec *et al.*, 1988). However, in contrast to Ets-1, whose protein levels and DNA binding activity are both reduced by antigen receptor cross-linking, *Ets-2* mRNA and protein levels are increased markedly following T cell activation. Moreover, phosphorylation of Ets-2 has been shown to increase the stability of the protein in T cells. (Fujiwara *et al.*, 1988). As is the case with Ets-1, several recent

studies have placed Ets-2 in the Ras signaling pathway (Wasylyk *et al.*, 1994; Yang *et al.*, 1996). Deletion mutants of Ets-2 encoding only the Ets domain have been shown to cause reversion of Ras transformation in some cell types (Langer *et al.*, 1992; Wasylyk *et al.*, 1994). Conversely, transactivation through tandem Ets-2 binding sites can be activated by Ras (Galang *et al.*, 1994). Moreover, reporter constructs containing Ras-responsive elements could be highly activated by the combination of Ets-2 and Ras but not by a mutant Ets-2 protein in which Thr 72 (the Ets-2 homolog of Thr 38 in Ets-1, which is phosphorylated upon Ras-induced activation) is changed to Ala.

Protein–protein interactions have not yet been reported for Ets-2. However, the high degree of structural similarity between Ets-2 and Ets-1 and the finding that multiple Ets proteins including Ets-1 interact directly with Jun proteins (Bassuk and Leiden, 1995) suggest that Ets-2 will also be shown to interact with Jun proteins.

In summary, although the Ets-1 and Ets-2 proteins are structurally quite similar, their distinct patterns of expression and DNA-binding activity with respect to T cell activation (Ets-1 is active in resting T cells, whereas Ets-2 is induced in activated T cells) is most consistent with a model in which the two proteins play distinct roles in regulating T cell function. As described previously, gene targeting studies have suggested an important role for Ets-1 in maintaining T and B cells in the resting state. Thus, it is tempting to speculate that Ets-2 may be important in regulating cell cycle progression and/or activation-specific gene expression in T cells.

3. *Transgenic/Gene Targeting Studies*

To date, there has been a single *in vivo* transgenic study examining overexpression of Ets-2 in mice (Sumarsono *et al.*, 1996). In this study, the *Ets-2* gene was expressed from the ubiquitously active mmt-1 and smt-1 promoters. Interestingly, the *Ets-2* gene is located on human chromosome 21 and is overexpressed in Down's syndrome (trisomy 21). Remarkably, mice with less than twofold overexpression of Ets-2 developed skeletal abnormalities similar to those found in trisomy 16 mice and in humans with Down's syndrome. The Ets-2 transgenic animals also displayed abnormal thymic histology similar to that seen in Down's syndrome. However, a detailed analysis of T cell development and function was not presented in this initial study. Further studies addressing the immune phenotype of these mice, or mice overexpressing dominant negative mutant forms of Ets-2, will help to elucidate the role of Ets-2 in the immune system. Because Ets-2 is ubiquitously expressed and appears to play an important role in the development of many different tissues, one might expect that a homozygous mutation of the *Ets-2* gene would result in an early embry-

onic lethal phenotype. Nevertheless, it will be important to produce both Ets-2-deficient mice and Ets-$2^{-/-}$/RAG-$2^{-/-}$ chimeric mice, which will allow the analysis of the specific function of Ets-2 in lymphocyte development and activation.

D. PU.1

1. Expression

PU.1 expression is limited to cells of hematopoietic origin (Hromas *et al.*, 1993; Klemsz *et al.*, 1990; Pettersson *et al.*, 1995; Tenen *et al.*, 1995). In adult animals, PU.1 is expressed at high levels in the spleen and at very low levels in the thymus (Klemsz *et al.*, 1990). Little is known about the developmental pattern of expression of PU.1. Purified $CD34^+$ cells (multipotent hematopoietic precursors) from both mouse and man express high levels of PU.1 as do mature human monocytes and peripheral blood neutrophils (Chen *et al.*, 1995; Chen and Boxer, 1995). PU.1 can also be detected in early granulocytic and erythroid cells and megakaryocytes but not in mature erythroid cells (Hromas *et al.*, 1993). Interestingly, expression of PU.1 in vascular smooth muscle cells has recently been reported, representing the first demonstration of PU.1 expression outside of the hematopoietic system (Inaba *et al.*, 1996). Several differentiated and undifferentiated myeloid cell lines (monocytes, macrophages, and neutrophils), pro-pre- and mature B cell lines, and undifferentiated erythroid cells also express varying levels of PU.1. However, PU.1 is not expressed in T cell lines (Chen *et al.*, 1995; Chen and Boxer, 1995; Hromas *et al.*, 1993; Klemsz *et al.*, 1990; Pettersson *et al.*, 1995). The highly restricted expression pattern of PU.1 suggests its potential role as a genetic determinant of hematopoietic lineage commitment and differentiation (see below).

2. Structure–Function Studies

The activation domain of PU.1 has been analyzed in detail (Hagemeier *et al.*, 1993; Klemsz and Maki, 1996). Activation is mediated by an N-terminal domain between amino acids 7 and 118. This domain is composed of three acidic subdomains and one glutamine-rich subdomain. The deletion of any of these subdomains results in a significant decrease in PU.1 transactivation potential.

PU.1 also contains a potentially protease-sensitive PEST region spanning amino acids 118–160 (Klemsz *et al.*, 1990). Because PU.1 can interact with at least one protein via this domain (see below), protease sensitivity of the PU.1 PEST region may be regulated via protein interactions within this region (Rogers *et al.*, 1986).

Protein–protein interactions appear to play an important role in modulating the activity of PU.1. PU.1 has been shown to interact with Pip (pre-

viously called NF-EM5; Eisenbeis *et al.*, 1995); a lymphoid-restricted, PU.1-dependent factor, to cooperatively transactivate the Igκ and λ enhancer elements (Eisenbeis *et al.*, 1993; Hiramatsu *et al.*, 1995; Pongubala *et al.*, 1992, 1993). The formation of a ternary complex is dependent on previous DNA binding of PU.1 and on phosphorylation of PU.1 at Ser 148 (Pongubala *et al.*, 1993). Interestingly, PU.1 can also bind to DNA independently of Pip (Pongubala *et al.*, 1993). This cell type-restricted and phosphorylation-dependent interaction between PU.1 and Pip illustrates another mechanism by which Ets proteins can achieve target gene selectivity. PU.1 has also been shown to associate *in vitro* with Rb and TFIID via the activation domain of PU.1 (Hagemeier *et al.*, 1993). It will be interesting to determine if PU.1 and Rb interact in a cell cycle-dependent manner. The interaction of the acidic domain of PU.1 with TFIID is consistent with molecular genetic studies in yeast that suggest that the function of acidic transactivating regions is to stabilize the binding of the TBP subunit of TFIID (Cormack *et al.*, 1995; Lee and Struhl, 1995; Stargell and Struhl, 1995).

The Ets domain of PU.1, like the Ets domains of Fli-1, Ets-1, and Elf-1, has been shown to interact directly with Jun family members (Bassuk and Leiden, 1995), reflecting the conserved nature of the Ets–Jun interaction. Recently, PU.1 was also shown to interact by λgt11 expression screening with the NF-IL6β transcription factor, two HMG proteins (HMG I/Y and SSRP), MKP1 (a phosphatase that can utilize MAP kinase as a substrate), and the 90-kDa heat shock protein, hsp90 (Nagulapalli *et al.*, 1995). It will be interesting and important to demonstrate these interactions in mammalian cells and to elucidate their functional significance.

PU.1 can be Ser phosphorylated both *in vitro* and *in vivo*. *In vitro*, Ser 148 can be phosphorylated by casein kinase II (Mao *et al.*, 1996; Pongubala *et al.*, 1993). JNK1 has also been shown to phosphorylate PU.1 *in vitro* (Mao *et al.*, 1996). Several other Ser residues have also been shown to be phosphorylated in B cells (Pongubala *et al.*, 1993). However, to date, the functional significance of these phosphorylation events remains unclear.

3. *Transgenic/Gene Targeting Studies*

Transgenic mice that overexpress the PU.1 protein have recently been shown to develop multistep erythroleukemias (Moreau-Gachelin *et al.*, 1996). This phenotype is consistent with the earlier finding of erythroleukemias caused by the proviral integration of the Friend leukemia virus into the 5′ region of the *PU.1* gene (Moreau-Gachelin *et al.*, 1996; Paul *et al.*, 1991). Taken together, these studies demonstrate that dysregulated PU.1 expression results in the persistence of an undifferentiated erythroid state, ultimately leading to erythroleukemia.

Recent gene targeting studies have demonstrated a critical role for PU.1 in the differentiation of multiple hematopoietic lineages (Scott *et al.*, 1994a). Mice containing homozygous disruptions of the *PU.1* gene die between Embryonic Days 16 and 18. Such PU.1$^{-/-}$ embryos produce normal numbers of megakaryocytes and erythroid progenitors but show variably impaired erythroblast maturation resulting in embryonic anemia. In addition, these mice display a severe defect in the generation of progenitors for B and T lymphocytes, monocytes, and granulocytes, resulting in a complete absence of mature myeloid and lymphoid cells. These defects in hematopoiesis were shown to be cell autonomous in experiments in which embryonic PU.1$^{-/-}$ fetal liver cells were transplanted into irradiated PU.1$^{+/+}$ hosts (E. Scott and H. Singh, personal communication). Taken together, these results demonstrated a critical role for PU.1 in the development of multiple hematopoietic lineages including myeloid cells and both B and T lymphocytes. In addition, they suggested the existence of a novel PU.1-dependent multipotent progenitor for cells of both lymphoid and myeloid lineages. Alternatively, PU.1 may be required for the differentiation of multiple distinct myeloid and lymphoid progenitor cells.

To more precisely define the role of PU.1 in myeloid development, Olson *et al.* (1995) examined hematopoietic lineage-specific gene expression in both PU.1$^{-/-}$ embryos and in hematopoietic colonies differentiated *in vitro* from PU.1$^{-/-}$ ES cells. Early markers of myeloid development, such as GM-CSFR, G-CSFR, and myeloperoxidase, are expressed normally in both PU.1$^{-/-}$ embryos and in hematopoietic colonies derived *in vitro* from PU.1$^{-/-}$ ES cells. In contrast, expression of genes associated with terminal myeloid differentiation, such as CD11b, CD64, and M-CSFR, is completely lacking in hematopoietic colonies derived from differentiated PU.1$^{-/-}$ ES cells. The capacity for myeloid development can be restored in PU.1$^{-/-}$ ES cells by transfection of a *PU.1* cDNA under the transcriptional control of its own promoter. These results suggest that PU.1 is not required for early myeloid gene expression but is absolutely essential for terminal myeloid differentiation. Future studies designed to more precisely study the target genes for PU.1 in the erythroid, myeloid, and lymphoid lineages should improve our understanding of the mechanisms by which PU.1 regulates the development of multiple hematopoietic lineages *in vivo*.

E. Spi-B

1. Expression

The recently characterized Spi-B protein is closely related to PU.1 (43% overall identity at the amino acid level and 67% identity within the Ets domain) (Ray *et al.*, 1992). Spi-B can bind to all known PU.1 binding sites

(Su *et al.*, 1996). During mouse development, Spi-B is expressed exclusively in lymphoid cells and can be detected in both the early fetal thymus and spleen (Su *et al.*, 1996). In adult mice, Spi-B is expressed in the medulla of the thymus, the white pulp of the spleen, and the germinal centers of lymph nodes. Spi-B expression is abundant in most B cell lines and detected at low levels in some T cell lines. Spi-B expression appears to increase during B cell maturation and to decrease during T cell activation (Su *et al.*, 1996).

2. *Structure–Function Studies*

The structural domains of Spi-B have not yet been fully characterized. However, the protein displays significant sequence similarity to PU.1, both in the Ets DNA-binding domain and in the N-terminal acidic region that is known to function as a transcriptional activating domain in PU.1 (Klemsz and Maki, 1996; Ray *et al.*, 1992). Spi-B also contains a central PEST sequence that is similar to the PEST domain of PU.1 (Ray *et al.*, 1992).

To date, only one report has addressed the issue of Spi-B phosphorylation (Mao *et al.*, 1996). Like the similar region of PU.1, the PEST domain of Spi-B can be phosphorylated on Ser residues *in vitro* by casein kinase II. The functional consequences of this phosphorylation event are unknown. Both ERK1 and JNK1 can phosphorylate the activation domain of Spi-B *in vitro*, with ERK1 phosphorylating Spi-B at Thr 56. Interestingly, the Rb protein is able to bind to the transactivation domain of Spi-B (again paralleling the situation with PU.1), but binding is abrogated when the Spi-B transactivation domain is phosphorylated *in vitro* by ERK1. Although these *in vitro* studies have not yet been confirmed *in vivo*, they are strikingly reminiscent of the known interactions between the transcription factor E2F and Rb, in which cell cycle-dependent phosphorylation of each protein regulates both the ability of the proteins to interact and the transcriptional activity of each protein (Fagan *et al.*, 1994).

3. *Transgenic/Gene Targeting Studies*

Spi-B-deficient mice have recently been produced using standard gene targeting strategies (Su *et al.*, 1996). Interestingly, these mice appear to develop normally and, thus far, do not display any gross abnormalities in hematopoietic development. They do, however, appear to display a defect in B cell proliferation, a finding that is consistent with the B-cell-restricted pattern of expression of Spi-B. A detailed understanding of the role of Spi-B in immune cell function will await the further characterization of these mice.

F. Fli-1

1. Expression

Fli-1 is preferentially expressed in lymphoid organs with high levels of expression observed in the adult murine spleen, lung, and thymus and lower levels of expression seen in adult skeletal muscle (Ben *et al.*, 1991). Recent *in situ* hybridization analysis has elucidated the expression pattern of Fli-1 during mouse development (Melet *et al.*, 1996). In the mouse embryo, Fli-1 expression is first detected at Embryonic Day 8, when it is mainly restricted to mesodermal cells. Beginning at Embryonic Day 11.5, Fli-1 expression can be detected in the liver. By Embryonic Day 14.5, endothelial cells throughout the embryo express Fli-1, and in later embryonic states Fli-1 is expressed predominantly in these cells. Low but uniform levels of Fli-1 were observed in the developing spleen and thymus. Similarly, low-level Fli-1 expression was observed in the adult spleen and thymus. Fli-1 is expressed more abundantly in quiescent peripheral T cells but is downregulated upon T cell activation (Mao *et al.*, 1994). In bone marrow macrophages, Fli-1 expression is highest in undifferentiated cells and decreases upon LPS, TPA, retinoic acid, or interferon-γ treatment (Klemsz *et al.*, 1993). Fli-1 is also highly expressed in B cell lines (Faust *et al.*, 1993), T cell lines (Mao *et al.*, 1994; Prasad *et al.*, 1992), and in a subtype of human erythroleukemia cell lines (HEL, KU812, TMK, and JK-1) (Klemsz *et al.*, 1993).

The expression of Fli-1, like PU.1, can be activated by viral insertion of a component of the murine Friend erythroleukemia virus. In neonatal mice infected with the Friend virus, the resulting erythroleukemias are not the result of proviral insertion upstream of the PU.1 locus, but rather they are induced by the integration of the F-MuLV component upstream of the *Fli-1* gene (Ben *et al.*, 1990, 1991). Fli-1 has also been shown to be dysregulated by chromosomal translocations. Human Ewing's sarcoma cells contain a reciprocal translocation between chromosomes 11 and 22 resulting in the synthesis of an EWS fusion protein containing an N-terminal region homologous to the RNA polymerase II C terminus and a C-terminal region containing the Ets domain of Fli-1 (Delattre *et al.*, 1992). Interestingly, a functional Fli-1–Ets domain is required for the oncogenecity of the fusion product (Zucman *et al.*, 1993), suggesting that transformation may be mediated by genes containing Fli-1 binding sites (Bailly *et al.*, 1994).

2. Structure–Function Studies

Fli-1 contains autonomous N-terminal (amino acids 106–201) and C-terminal (amino acids 401–452) transcriptional activation domains (Klemsz

et al., 1993; Rao *et al.*, 1993; Rao and Reddy, 1993a,b). To date, phosphorylation of the Fli-1 protein has not been reported. The Ets domain of Fli-1, like the Ets domains of Elf-1, Ets-1, and PU.1, was shown to interact directly with the basic domain of multiple Jun family members (Bassuk and Leiden, 1995).

3. *Transgenic/Gene Targeting Studies*

A report by Zhang *et al.* (1993) describes the phenotype of transgenic mice overexpressing the *Fli-1* gene under the control of the ubiquitously active H-2K^k promoter. Although dysregulation of the *Fli-1* gene by retroviral insertion or chromosomal translocation results in erythroleukemia and Ewing's sarcoma, respectively, the *Fli-1* transgenic mice do not display an increased incidence of malignancy. The most interesting phenotypic feature of these mice is the development of a progressive lethal autoimmune renal disease characterized by tubulointertstitial nephritis and immune complex glomerulonephritis. This phenotype was seen specifically in mice expression high levels of the transgene in lymphoid tissue. Diseased mice displayed hypergammaglobulinemia, splenomegaly, and B cell hyperplasia. They also accumulated abnormal CD3$^+$B220$^+$ T cells and CD5$^+$B220$^+$ B cells in peripheral lymphoid tissues and expressed a variety of autoantibodies. Moreover, splenic B cells from the transgenic mice exhibited increased proliferation and prolonged survival *in vitro* in response to various mitogenic signals. Taken together, these results suggested that the nephritis observed in these animals is a result of immune cell dysfunction rather than an intrinsic renal defect and were consistent with a model in which overexpression of Fli-1 in lymphocuyes leads to dysregulated lymphocyte proliferation (and terminal differentiation) and decreased programmed cell death, thereby causing autoimmune nephritis.

Recently, gene targeting of the *Fli-1* locus has been described (Melet *et al.*, 1996). However, the mice described in this study do not contain homozygous null mutations of *Fli-1*. Rather, they produce a truncated Fli-1 protein missing the N-terminal 76 amino acids but containing both the transactivation and Ets domains of Fli-1. Interestingly, despite this minor mutation, these mice exhibit thymic hypocellularity that is not related to a defect in a specific subpopulation of thymocytes nor to increased apoptosis, suggesting that Fli-1 may be a regulator of a prethymic T cell progenitor. Also, homozygous mutant mice are still susceptible to erythroleukemia induction by Friend murine erythroleukemia virus, but the latency period following infection is increased. Given the interesting phenotypes associated with overexpression of Fli-1 and the mutation of the Fli-1 N terminus, it will be important to determine the phenotype of mice containing homozygous null mutations of the *Fli-1* gene.

G. Elf-1

1. Expression

A complete analysis of Elf-1 expression in murine development has recently been completed. (A. Bassuk and J. Leiden, manuscript submitted). In the murine embryonic thymus, Elf-1 expression can be detected by *in situ* hybridization as early as Embryonic Day 15 and expression levels remain high throughout thymic ontogeny. Elf-1 expression can be detected in a variety of other organs during embryonic development, particularly in the epithelial linings of the nose, mouth, stomach, gut, and lungs. The Elf-1 protein is highly expressed in lymphoid organs (thymus, spleen, and bone marrow) in the adult. Elf-1 protein is seen in double-negative, double-positive, and single-positive thymocytes. Moderate levels of Elf-1 protein expression can be detected in the kidney and gut and much lower levels are found in all other tissues tested. The Elf-1 protein is expressed abundantly in peripheral B cells, T cells, macrophages, and monocytes. Interestingly, unlike Ets-1, Ets-2, and Fli-1, levels of Elf-1 protein do not change significantly during T cell activation.

The Elf-1 protein is expressed highly in immortalized human and murine cell lines of hematopoietic origin including K562, HL-60, U937, Bcl-2, CEM, Clone 13, Jurkat, EL4, FCS12, Mk315, and Ctl2. Interestingly, Elf-1 is also highly expressed in some prostate carcinoma cell lines. Elf-1 protein levels are very low but detectable by immunoblot analysis in nonhematopoietic cell lines including HeLa, U20S, SAOS, P815, MEL, 3T3, C2C12, STO, CME, H19-15, H19-17, and HML 1031 (A. Bassuk and J. Leiden, manuscript submitted).

2. Structure–Function Studies

Elf-1 contains a potent acidic transcriptional transactivation domain located between amino acids 1 and 100 (Wang *et al.*, 1993). This N-terminal region of the protein also contains a high-affinity binding site for the pocket region of Rb that partially overlaps the acidic transactivation domain. Elf-1 has been shown to bind to the nonphosphorylated form of Rb in normal resting peripheral T cells. Overexpression of a constituitively active form of Rb inhibits Elf-1-dependent transcriptional activation in T cell lines. Taken together, these findings are consistent with a model in which the acidic transcriptional activation domain of Elf-1 is "masked" by binding to unphosphorylated Rb in resting T cells. Following T cell activation, phosphorylation of Rb causes its dissociation from Elf-1, thereby activating its transcriptional activity. This pattern of regulation suggests that Elf-1 plays an important role in coordinating activation-specific gene expression and cell cycle progression in T cells.

The phosphorylation of the Elf-1 protein is currently under investigation (Bassuk and Leiden, 1995). Elf-1 is unphosphorylated in resting T cells but is phosphorylated on multiple Ser residues during the first 8 hr of T cell activation. Interestingly, many of these Ser residues correspond to consensus MAP kinase sites. Phosphorylation of Elf-1 appears to be required for its DNA-binding activity. Thus, the activity of Elf-1 is posttranslationally regulated in a cell cycle-dependent manner. Although resting peripheral T cells contain abundant amounts of unphosphorylated Elf-1, the protein is inactive in these cells both because it is bound to Rb and because it is unphosphorylated. T cell activation results in both Rb phosphorylation (and its subsequent dissociation from Elf-1) and Elf-1 phosphorylation, which in turn activates its DNA-binding activity (Gottschalk *et al.*, 1993; Thompson *et al.*, 1991; Leiden *et al.*, 1992). It will be of interest to more precisely characterize the signaling pathways responsible for Elf-1 phosphorylation and to determine the functional significance of individual phosphorylation events.

Interactions between Elf-1 and AP1 are important in the regulation of multiple hematopoietic genes including IL-3 and GM-CSF, which both contain adjacent functionally important Elf-1 and AP1 binding sites (Gottschalk *et al.*, 1993; Wang *et al.*, 1994). The cooperative activity of Elf-1 and AP1 appears to be mediated both by the geometry of their adjacent binding sites and by direct physical interactions between Elf-1 and AP1 proteins that can occur in the absence of DNA (Bassuk and Leiden, 1995). Elf-1 interacts directly with multiple Jun proteins (c-Jun, JunB, and JunD) via the Ets domain of Elf-1 and the basic domain of the Jun proteins. Elf-1 bound to Jun can recruit Fos proteins into a trimolecular protein complex via leucine zipper-mediated interactions between Jun and Fos.

The Ets domain of Elf-1 has also been shown to interact *in vitro* with members of the NF-κB/c-rel family of proteins (John *et al.*, 1995). Although the functional consequences of this interaction have not been fully explored, this finding is intriguing because many genes contain adjacent binding sites for Ets and Rel proteins (see Table I). Elf-1, like PU.1, can interact with HMG I/Y via the Ets domain of Elf-1. Cotransfection of Elf-1 and HMG I/Y additively increases the transcriptional activity of the IL-2Rα enhancer (John *et al.*, 1995).

3. *Transgenic/Gene Targeting Studies*

The phenotypes of transgenic mice overexpressing Elf-1 and Elf-1-deficient mice have not yet been reported. Such studies should help to more precisely define the role of Elf-1 in the development and function of the immune system.

H. TCFs

1. Expression

The TCF family currently contains three members, Elk-1, Sap-1, and Sap-2 (ERP/NET), all of which are expressed ubiquitously (Dalton and Treisman, 1992; Giovane *et al.*, 1994; Hipskind *et al.*, 1991; Lopez *et al.*, 1994; Price *et al.*, 1995; Rao and Reddy, 1992a,b). B and T cell lines express relatively high levels of TCFs (Giovane *et al.*, 1994; Lopez *et al.*, 1994). Although the TCFs seem to play an important role in the regulation of the immediate early gene, c-*fos* (Treisman, 1994; Treisman *et al.*, 1992), TCF binding sites have not yet been identified in immune cell-specific genes.

2. Structure–Function Studies

The TCFs contain three regions of sequence homology termed A, B, and C boxes (Dalton and Treisman, 1992; Hipskind *et al.*, 1991; Janknecht *et al.*, 1993, 1994; Janknecht and Nordheim, 1993). Box A comprises the TCF–Ets domain (Janknecht and Nordheim, 1992). The C-terminal C box mediates transcriptional transactivation (Marais *et al.*, 1993) and is subject to several phosphorylation events (see below). Interestingly, the Elk-1 protein contains a second N-terminal transcriptional activation domain that overlaps with the Ets domain and that is absent in the Δelk-1 splice variant (Bhattacharya *et al.*, 1993; Rao *et al.*, 1993; Rao and Reddy, 1993a,b). TCF box B is a 30-amino acid region that is both necessary and sufficient to mediate protein–protein interactions between the TCFs and the serum response factor (SRF), a 62-kDa MADS box transcription factor that binds cooperatively with TCFs to many serum responsive sequences elements (SREs) including one in the c-*fos* promoter (Shore and Sharrocks, 1994). The Δelk-1 protein does not contain box B and thus does not bind SRF or form ternary complexes at the c-*fos* SRE. However, Δelk-1 is able to bind Ets sites not accessible to full-length TCFs (Rao *et al.*, 1993; Rao and Reddy, 1993a,b).

TCFs have been shown to be modified by multiple phosphorylation events mediated by the ERK group of MAP kinases (Janknecht *et al.*, 1993, 1995; Janknecht and Nordheim, 1993). Phosphorylation on Ser and Thr residues in the C box transcriptional transactivation domain upregulates the transcriptional activity of the TCFs (Gille *et al.*, 1992; Hill *et al.*, 1993; Marais *et al.*, 1993). The N-terminal activation region present in Elk-1 is also positively regulated by MAPK-mediated phosphorylation (Janknecht *et al.*, 1993; Janknecht and Nordheim, 1993). MAPK-mediated phosphorylation can serve to increase both autonomous and SRF-dependent DNA binding by the TCFs (Sharrocks, 1995; Sharrocks

and Shore, 1995). TCFs can also be phosphorylated by the JNK group of MAP kinases. Phosphorylation by JNK kinases both increases ternary complex formation with SRF and induces the transcriptional activation potential of TCFs (Whitmarch *et al.*, 1995).

3. *Transgenic/Gene Targeting Studies*

To date, transgenic and gene targeting studies of the TCFs have not been reported. Given the multiplicity of TCF family members, and their ubiquitous patterns of expression, it may be particularly useful to assess the role of TCFs in lymphocyte development and function using the RAG-2 chimeric mouse system described previously.

I. GABPα

1. *Expression*

GABPα and its heterodimerization partner, GABPβ, are expressed in all tissues tested, including embryonic spleen and thymus. B and T cell lines also express GABPα (Brown and McKnight, 1992; LaMarco *et al.*, 1991).

2. *Structure–Function Studies*

The transactivation domain of the $GABP\alpha_4/GABP\beta_4$ heterotetrameric protein complex is thought to reside in the β subunit (Brown and McKnight, 1992). Unlike all other known Ets proteins that can bind to DNA as monomers, GABPα only binds DNA with high affinity as a heterotetramer with GABPβ (LaMarco and McKnight, 1989; LaMarco *et al.*, 1991; Thompson *et al.*, 1992; Triezenberg *et al.*, 1988). GABPβ homodimerizes via its C terminus and then heterodimerizes with DNA-bound GABPα dimers via amino acids 316–437 (which include the Ets domain) of GABPα and the N-terminal half of GABPβ (Thompson *et al.*, 1991). GABPβ is unable to dimerize with other known Ets protein (Brown and McKnight, 1992). Therefore, the specificity of GABPα transcriptional activation is achieved by its unique ability to interact with GABPβ. To date, phosphorylation of GABPα has not been reported.

3. *Transgenic/Gene Targeting Studies*

To date, genetic studies of GABPα have not been reported.

V. Conclusions and Future Directions

During the past 10 years, we have learned a great deal about the structure, function, and mechanisms of regulation of mammalian Ets transcrip-

tion factors. When one considers that Ets proteins were recognized as transcription factors only in 1990 (Ho *et al.*, 1990; Karim *et al.*, 1990), and that 9 of 17 known mammalian Ets proteins have been described in the past 5 years, the progress in this area has been quite remarkable. Several themes have emerged from these studies. First, different sets of Ets proteins are expressed in different hematopoietic lineages. Second, despite the fact that several Ets proteins may be expressed simultaneously in a given immune cell lineage, Ets proteins play different and, in some cases, opposite role in immune cell development and function. This finding is quite reminiscent of the situation in *Drosophila* photoreceptor development in which two invertebrate Ets proteins, Pointed (a positive transcriptional regulator) and Yan (a negative transcriptional regulator), play opposite roles in the development of the R7 photoreceptor cells (O'Neill *et al.*, 1994). Third, it is becoming clear that in many systems, Ets proteins may function as molecular scaffolds, coordinating the proper assembly of multiprotein enhancer/DNA complexes. Finally, it is clear that the transcriptional activities of Ets proteins are highly regulated at both the transcriptional and posttranslational levels. Such regulation includes the lineage-specific expression of specific Ets proteins, the phosphorylation of Ets proteins in response to diverse extracellular signals, and regulated interactions between Ets proteins and other transcription factors and cell cycle regulatory molecules.

Despite these advances, much remains to be learned about the role of Ets proteins in the development and function of the mammalian immune system. In the area of structure–function, it will be important to resolve the issue of whether the structures of different Ets domains bound to DNA are different or identical. Moreover, it will be of great interest to determine the structures of the non-Ets domains of the different Ets proteins, both alone and in conjunction with associated transcription factors such as AP1. Similarly, it will be important to elucidate the structural bases for the intramolecular inhibition of activity of proteins such as Ets-1 and the structural consequences of the phosphorylation of proteins such as Elf-1. The fine mapping of Ets phosphorylation sites and an understanding of the role of individual sites in regulating specific functions, such as DNA binding and protein–protein interactions, will also be essential for understanding the regulation of Ets proteins, as will the elucidation of the signaling pathways that lead to the phosphorylation of Ets proteins following immune cell activation. Finally, it will be important to identify additional partners that interact with Ets proteins to regulate their transcriptional activities.

In the area of genetics, the targeted mutation of each of the Ets proteins expressed in immune cells and the cross-breeding of individual knockout

mice will yield important insights into the functions of each of the proteins as well as the level(s) of redundancy in this system. These results will be complemented by the production of transgenic mice overexpressing specific mutants of individual Ets proteins: for example, mutants lacking DNA-binding activity or the ability to interact with other transcription factors or to be phosphorylated following immune cell activation. Mice (and ES cells) containing targeted mutations of individual Ets genes will also be useful for identifying the important targets of these genes. Finally, it will be important to characterize the transcriptional regulatory elements that control the lineage-specific and developmentally regulated expression of specific Ets proteins. Given the recent rapid progress in this field, it seems safe to predict that the next 5 years will represent a second revolution in our understanding of the role of Ets transcription factors in immune cell function. In addition to increasing our basic understanding of the functions of this important family of transcription factors, such studies may also lead to the development of novel therapeutic strategies for a variety of diseases of the immune system including immunodeficiency states, autoimmune disorders, immune cell neoplasms, and lymphotropic viral infections.

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Mechanism of Class I Assembly with β_2 Microglobulin and Loading with Peptide

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I. Background

Townsend and colleagues (1985) initially discovered that proteins lacking signals that target the secretory compartment can contribute peptide epitopes for presentation to the immune system via class I molecules at the cell surface. Furthermore, studies by Nuchtern *et al.* (1989) and Yewdell and Bennink (1989) using the antimetabolic drug, Brefeldin A, indicated that functional peptide loading of class I occurs in the endoplasmic reticulum (ER), the initial secretory compartment containing nascent, membrane-spanning class I molecules. These combined results led to a search for a novel biological pathway that would permit cytosolic peptides to be processed, translocated into the lumen of the ER, and loaded onto nascent class I molecules. Using both chemical and genetic approaches, many of the details of peptide loading onto class I have been resolved. For example, recent studies have made it patently clear that class I molecules are heterotrimers. Stable complexes exist only after the heavy (H) chain has assembled with β_2 microtubulin (β_2m) and bound a peptide ligand. In this review, we discuss recent evidence elucidating the mechanism of β_2m assembly and peptide loading and the accessory molecules involved in these processes.

II. Proteasome

The 26S proteasome complex present in the cytoplasm consists of a 20S proteasome catalytic core and a 19S regulatory particle (Goldberg, 1995). This structure is thought to be primarily responsible for the degradation of cytosolic proteins and has been implicated in the generation of peptides for the class I antigen presentation system. The structure of the 20S proteasome from the archaebacterium *Thermoplasma acidophilum* was examined by X-ray crystallography and shown to be a cylindrical structure consisted of four stacked rings and a total of 28 subunits (Lowe *et al.*, 1995). Each of the top and bottom rings consists of 7 α subunits, whereas each of the two central rings consists of 7 β subunits. The catalytically

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active sites of the β subunits are located within the interior of the barrel-shaped structure, and only unfolded proteins can enter proteasome through the 13 Å opening of the structure, limiting accessibility of cytoplasmic proteins for proteolytic degradation (Lowe *et al.*, 1995; Wenzel and Baumeister, 1995). Although only one type of β subunit was found in *T. acidophilum,* seven families of different but structurally similar β subunits were revealed in eukaryotic cells (Monaco and McDevitt, 1986). Each of the different types of subunits probably occupies a particular site within the 7-subunit ring. The overall composition of the central (β subunit) rings within the 20S proteasome complex is controlled by the cytokine interferon-γ IFN-γ). This cytokine upregulates the transcription of the *LMP-2* and *LMP-7* genes, located within the MHC class II region adjacent to the *TAP-1* and *TAP-2* genes. *LMP-10* (apparently the mouse homolog of the human *MECL-1* gene), a non-MHC-linked gene, is also transcriptionally induced by IFN-γ (Nandi *et al.*, 1996). The increased incorporation of the LMP-2, LMP-7, and LMP-10 β subunits into proteasomes by IFN-γ results in the reduced presence of 3 other constitutively produced β subunits in the complex: LMP-19 (identical to δ or Y), LMP-17 (identical to X or MB-1), and LMP-9 (identical to Z). The partial and complete replacement of the constitutively synthesized LMP-19, LMP-17, and LMP-9 β subunits with the respective IFN-γ-inducible LMP-2, LMP-7, and LMP-10 β subunits could result in heterogeneous proteasome structures with different catalytic specificities (Nandi *et al.*, 1996).

In eukaryotes, only 6 of the 10 mature β subunits possess the two conserved amino terminal threonine residues thought to be essential for proteolytic activity—the 3 IFN-γ-inducible subunits, LMP-2, LMP-7, and LMP-10, and the subunits that they replace, LMP-19, LMP-17, and LMP-9, respectively (Monaco and Nandi, 1995; Nandi *et al.*, 1996; Belich *et al.*, 1994; Fruh *et al.*, 1994). Therefore, IFN-γ induction of cells could have a major impact on the types of peptides produced. Several reports suggested that IFN-γ induction changes the specificity of the proteolytic cleavage particularly with regard to the specificity of the carboxyl-terminal residues. More specifically, IFN-γ was reported to decrease the production of peptides with acidic carboxyl-terminal residues and to increase that of peptides with hydrophobic and basic carboxyl-terminal residues (Gaczynska *et al.*, 1993; Driscoll *et al.*, 1993). The latter profile correlates better with the types of peptides that bind to MHC class I molecules. However, Boes *et al.* (1994) observed that IFN-γ induction yielded proteasomes with decreased chymotryptic activity and no changes in tryptic activity. Furthermore, their studies revealed no differences in the generated amount of a class I-restricted peptide with a hydrophobic carboxyl terminus from a 25-residue peptide. The discrepancy in these studies may result from the isolation of

proteasomes from different cell types with different levels of the aforementioned β subunits, the substrate used in the assays, and the access to downstream elements of the class I antigen presentation system. In addition, the recent finding that LMP-10 is also induced by IFN-γ complicates the interpretation of studies in which only LMP-2 and LMP-7 were introduced to replace their homologs in the proteasome compared to proteasomes from IFN-γ-induced cells. As a final note, IFN-γ also induces the production of two other proteins that comprise the 11S regulator. This regulator competes for binding to the end of the 20S proteasome core with the 19S particle that is involved in ubiquitin-dependent proteolysis (Realini *et al.*, 1994; Grag *et al.*, 1994). Currently, it is difficult to discern whether the IFN-γ-induced changes in the regulator or the β subunit ring's composition or both alter the specificity of proteolysis by proteasomes. Furthermore, IFN-γ-induced changes in the composition of the proteasome and its regulator may also increase the kinetics of the processing (Ustrell *et al.*, 1995; Groettrup *et al.*, 1995).

Several studies provide strong evidence that proteasomes are important contributors to the MHC class I antigen presentation pathway. First, the 26S proteasome is integrally involved in ubiquitin-dependent processing of polypeptides (Goldberg, 1995), and ubiquination of intact antigens enhances their efficiency of presentation (Townsend *et al.*, 1988). Second, peptide aldehyde inhibitors inhibit both 20S and 26S proteasomes as well as MHC class I antigen presentation (Vinitsky *et al.*, 1994; Rock *et al.*, 1994). However, these inhibitors also affect other proteases. Third, the *in vivo* functions of LMP-2 and LMP-7 have been studied in mice lacking either of these activities. LMP-2 deficiency adversely affects the selection of $CD8^+$ lymphocytes (Van Kaer *et al.* 1994), whereas LMP-7 deficiency decreases surface expression of MHC class I molecules. Both deficiencies adversely affect the presentation of particular antigenic peptides (Fehling *et al.*, 1994). Finally, the *in vitro* processing of a natural influenza nucleoprotein epitope that binds to HLA-B27 by isolated proteasomes provides strong evidence that these structures are sufficient for processing of some antigens (Svensson *et al.*, 1996). Cumulatively, these reports implicate the proteasome as the predominant cytoplasmic structure responsible for the generation of class I-restricted peptides.

III. Calnexin

Calnexin is an ER resident type I transmembrane protein that associates transiently with a large number of glycoproteins such as newly synthesized subunits of the T cell receptor, immunoglobulin, and MHC class I and class II complexes. Indeed, several studies suggest that calnexin preferentially

associates with misfolded or incompletely assembled oligomeric glycoproteins. Based on these findings, calnexin has been proposed to be a key component of a ER quality control system that facilitates the assembly and expression of oligomers with which it associates (Ou *et al.*, 1993; Hammond *et al.*, 1994). Although there is little doubt that calnexin is a molecular chaperone, its precise role in the expression of class I molecules is unclear. Although previous evidence led to the speculation that calnexin was the predominant class I chaperone (Williams and Watts, 1995), recent studies raise questions with regard to the specific role of calnexin in class I expression. Two general chaperone functions have been previously ascribed to calnexin: ER retention of H chain subunits and facilitation of their assembly.

A. Calnexin's Proposed Role in H Chain Assembly with β_2m

In a study by Jackson *et al.* (1994), mouse class I subunits were expressed in *Drosophila melanogaster* Schneider cells. Coexpression of calnexin in this system was found to enhance the efficiency of assembly of H chain/β_2m complexes. Moreover, in cells lacking calnexin only 20% of the K^b H chains were associated with β_2m, whereas 100% of the H chains were associated with β_2m in cells that expressed calnexin (Williams and Watts, 1995). Although these data are consistent with calnexin having a direct role in promoting class I assembly, its role may rather be indirect. Coexpression of calnexin with free H chains was found to protect them from rapid intracellular degradation. In fact, the half-lives of K^b and D^b were extended four- or fivefold (Jackson *et al.*, 1994). This dramatic protection of H chains could be the cause, at least in part, of the aforementioned increase in the detection of assembled β_2m/H chains. Thus, calnexin clearly stabilizes unassembled subunits, but whether it has a direct role in assembly remains unclear.

An additional obfuscation in defining the role of calnexin in class I assembly is a reported species difference with regard to which class I folding intermediates are associated with calnexin. In human cells, several studies were unable to detect calnexin in association with H chains after they were assembled with β_2m (Ortman *et al.*, 1994; Sugita and Brenner, 1994; Nossner and Parham, 1995). Therefore, it was proposed that β_2m binding to human H chains induced the release of calnexin. This widely accepted model (Fig. 1) is quite attractive because it implies a direct and precise role for calnexin in class I assembly. However, there are conflicting observations that must be considered. First, mouse class I H chain/β_2m complexes are readily detected in association with calnexin (Jackson *et al.*, 1994; Carreno *et al.*, 1995a), and the addition of β_2m to cell lysates lacking β_2m did not induce class I dissociation from calnexin. Second, human H

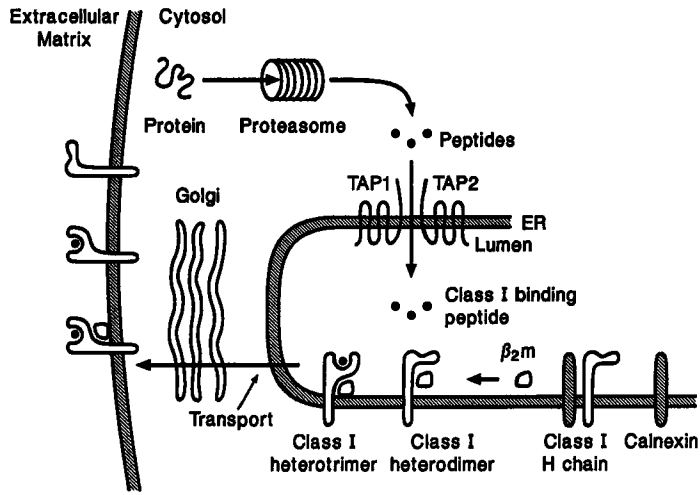

FIG. 1. Calnexin association with human class I molecules. The model in this figure depicts a proposed role of calnexin in the assembly of class I molecules. In this model calnexin associates with free class I H chains prior to their assembly with β_2m. Although there is considerable evidence in human cells that calnexin preferentially associates with free H chains, in the mouse this is certainly not the case. In the mouse, and more recently also in humans, calnexin has been detected in association with H chain/β_2m heterodimers. Thus, the model depicted here should be considered simplistic, such that more complex models (e.g., Fig. 3) must also be considered.

chain/β_2m complexes were recently also detected in association with calnexin using Western blotting (Carreno *et al.*, 1995b). Thus, the binding of β_2m versus calnexin to H chains is clearly not mutually exclusive in either mouse or human cells. Alternatively, in both mouse and human cells calnexin appears to be associated with both free and β_2m-assembled H chains. However, the levels of calnexin found in association with β_2m-assembled forms of various H chain alleles/loci were found to differ markedly (Carreno *et al.*, 1995b). These latter differences could explain why earlier studies of human class I molecules failed to detect calnexin associated with β_2m-assembled human H chains. In any case, it remains unclear whether calnexin promotes β_2m assembly with H chains and whether β_2m assembly has any influence on calnexin dissociation.

B. Calnexin's Proposed Role in ER Retention of Assembly Intermediates of Class I Molecules

The initial evidence that calnexin may be involved in the ER retention of incompletely assembled class I molecules was correlative data (Degen

et al., 1992). For example, in β_2m-deficient cells, calnexin was found to remain in association with free H chains that accumulate intracellularly. Similarly, in peptide-deficient [*t*ransporter *a*ssociated with antigen *p*rocessing-negative (TAP$^-$)] cells, calnexin dissociates very slowly in parallel with the slow transport of H chain/β_2m dimers in these cells. Thus, a correlation was observed between calnexin association and ER retention, consistent with the possibility of a cause and effect relationship. Furthermore, there are two experimental findings that implicate calnexin in ER retention of assembly intermediates. In a study by Jackson *et al.* (1994) using class I subunits expressed in *Drosophila* cells, free mouse H chains and H chain/β_2m dimers were found to be rapidly transported to the cell surface. Interestingly, coexpression of mouse calnexin in these cells was found to significantly retard the transport of both free H chains and H chain/β_2m intermediates, thus directly implicating calnexin in ER retention. A second experiment implicating calnexin in ER retention used F0-1 cells, a β_2m-deficient human cell line (Rajagopalan and Brenner, 1994). Comparisons were made between untransfected F0-1 cells and F0-1 cells transfected with a cDNA-encoding calnexin lacking its ER retention signal. In the untransfected cells, free H chains were retained in the ER in association with the intact endogenous calnexin. By contrast, in the cells expressing calnexin lacking its ER retention signal, class I H chains were expressed at significant levels at the cell surface. This finding thus corroborated the role for calnexin in ER retention of class I assembly intermediates. It is important to note that all of the previously described evidence implicating calnexin in ER retention was obtained in cells either lacking TAP or in cells in which H chains are unable to associate with TAP. More specifically, *Drosophila* cells and RMA-S cells lack functional TAP complexes and, in β_2m-deficient cells, H chains fail to physically associate with TAP. The fact that the cells used for these studies lack TAP, or class I association with TAP, is of particular importance. This conclusion stems from the fact that TAP, as discussed below, has also been implicated in ER retention of assembly intermediates.

C. Site of Interaction on Class I for Calnexin Association

The structural nature of the interaction between calnexin and several different secretory and viral glycoproteins has been studied. Reports by Ou *et al.* (1993) and Hammond *et al.* (1994) demonstrated that the binding of glycoproteins to calnexin is blocked by inhibitors of ER glycosylation. Indeed, it was recently proposed that calnexin is a lectin that binds to N-linked oligosaccharides. More specifically, in elegant studies of *in vitro* translated influenza hemagglutinin, Hebert *et al.* (1995) demonstrated that calnexin association was dependent on the presence of monoglucosylated

N-linked glycans, created by trimming and reglucosylation of terminal glucose residues. Consistent with this conclusion, there have been several reports that show class I association with calnexin is diminished by drugs that inhibit glycosylation (Balow *et al.*, 1995; Ware *et al.*, 1995; Zhang *et al.*, 1995). By contrast, mutant L^d class I molecules lacking N-linked glycosylation sites retained high levels of association with calnexin (Carreno *et al.*, 1995a). Thus, the nature of the interaction of class I with calnexin would appear to entail more than glycans. In this regard, cross-linking studies have demonstrated close proximity between calnexin and the transmembrane domain of the class I H chain (Margolese *et al.*, 1993). The fact that class I molecules are not solely dependent on N-linked glycans for their association with calnexin is consistent with the finding that aglycosylated forms bind β_2m and peptide, are transported to the cell surface, and appear functionally normal compared to glycosylated class I molecules. By contrast, proteins such as influenza hemagllutinin are completely dependent on glycosylation for assembly and expression.

D. Phenotype of Calnexin-Negative Cells

Based on the chaperone functions attributed to calnexin, it was very surprising that a cell line lacking calnexin also lacked a phenotype distinguishing it from wild type. The CEM.NK^R cell line is natural killer resistant and lacks detectable calnexin protein. Despite this apparent complete lack of calnexin, these cells were found to have normal assembly with β_2m, normal peptide loading of H chain/β_2m dimers, normal TAP association, and the same rate of egress from the ER as seen in calnexin$^+$ control cells (Scott and Dawson, 1995; Sadasivan *et al.*, 1995). Overall, these data argue that calnexin has no essential functions in class I assembly and transport. However, it remains very possible that calnexin has important functions in class I expression, but that these functions are included among those of related chaperone proteins. Such redundancies perhaps should have been expected for functions so vital to the immune system as class I assembly and expression. We furthermore speculate that if calnexin does have important chaperone functions for class I assembly, they may be relatively nonspecific and difficult to precisely define. After all, calnexin is not a specific chaperone for class I, but is known to associate with several different oligomers of various structures and functions. Together, these recent observations reinforce the lack of knowledge of a specific function for calnexin in class I synthesis. What is now required is to define proteins with overlapping functions with calnexin and produce sequential genetic knockouts to better define their contributions. In regard to defining proteins with overlapping functions with calnexin, the structurally homologous heme-binding protein calreticulin may have similar chaperones functions

(Nauseef *et al.*, 1995; Wada *et al.*, 1995). As detailed below, it is also very intriguing that TAP appears to be involved in both of the chaperone functions previously ascribed to calnexin, namely, retaining class I subunits prior to assembly and directly facilitating the assembly process.

IV. TAP

The characterization of mutant cell lines expressing class I molecules that are unstable unless loaded with exogenous peptides led to the identification of two genes that are required for proper peptide loading of class I molecules (Townsend *et al.*, 1989). Characterization of these genes found them to be MHC-linked genes encoding products termed TAP1 and TAP2. Both TAP1 and TAP2 are membrane-spanning proteins with an ATP-binding cassette, and TAP1 and TAP2 form a heterodimer in the membrane of the ER. It has now been well documented that TAP1/TAP2 heterodimers (TAP) translocate peptides into the lumen of the ER and that this process requires ATP hydrolysis (Androlewicz *et al.*, 1993; Neefjes *et al.*, 1993). Indeed, the function of TAP was elegantly demonstrated using lymphoblastoid cells permeabilized by Streptolysin O and test peptides containing a consensus N-linked glycosylation site (Neefjes *et al.*, 1993b). The inclusion within the peptide of an N-linked glycosylation site was critical for the success of this approach because it prevented efflux of the peptide back through the ER membrane, and it allowed easy detection of the test peptide with Con A sepharose. This assay system not only demonstrated the ATP-dependent function of TAP in the translocation of peptides but also was used to define the peptide specificity of this transport.

A. Peptide Length and Sequence Specificity of TAP Transport

Peptides of various lengths were tested for their ability to inhibit the transport of labeled test peptide using *in vitro* assays such as the one described previously (Momburg *et al.*, 1994a; Androlewicz and Cresswell, 1994). It should be noted that in these assays inhibition probably occurs at the putative peptide binding site on the cytosolic face of TAP. Interestingly, TAP has been found to display a dramatic preference for peptides of 7–13 amino acids in length, closely matching the 8- to 11-amino acid length typically preferred by class I molecules. Whereas shorter peptides appeared not to be transported, longer peptides appeared to be transported but less efficiently. Certain of these longer peptides may either bind to class I molecules in novel ways (Collins *et al.*, 1994; Frumento *et al.*, 1993; Urban *et al.*, 1994; Joyce *et al.*, 1994) or return to the cytosol for further trimming. Peptides not trapped by class I molecules in the ER were found to have very short half-lives. Rapid turnover of these peptides could result

either from a putative ATP-independent pump that returns peptides to the cytosol or from ER-degradation pathways. The rapid turnover of peptides in the ER is consistent with failure to detect certain peptides in the ER of cells lacking the appropriate class I molecules that can bind the peptide (Falk *et al.*, 1990).

In addition to this marked preference of TAP for peptides of specific lengths, TAP also displays some sequence preference. The carboxyl (C)-terminal amino acids of peptides have been found to influence TAP transport, but precise specificity varies among species and alleles (Momburg *et al.*, 1994). More specifically, in humans, TAP preferentially excludes peptides with C-terminal proline and possibly glycine residues. In mice, TAP is more restrictive and only transports peptides with hydrophobic C-terminal residues (Heemels *et al.*, 1993). Although allelic forms of both mouse and human TAP have been reported, differences appear not to alter the specificity of peptide interaction (Colonna *et al.*, 1992). However, in the rat, two allelic forms of TAP coexist in the population and differ in their peptide specificity (Powis *et al.*, 1992; Joly *et al.*, 1994). Furthermore, the binding specificity of one rat allelic product corresponds with that of the permissive human TAP, whereas the other rat allele corresponds with that of the more restrictive mouse TAP. Thus, in the rat, a given class I molecule can be loaded with collections of peptides with very different motifs depending on which TAP allele is present (Powis *et al.*, 1992). Although the significance of the species differences in TAP is unclear, there is evidence that they have coevolved with the class I molecules of the species that they serve. For example, in the mouse, peptides with hydrophobic C-terminal amino acids are transported by TAP, and all known mouse classical class I molecules bind peptides with hydrophobic C-terminal amino acids. By contrast, in humans, the more permissive TAP accommodates several HLA alleles that preferentially bind peptides with positively charged C termini. The matched peptide specificity of TAP and class I within a species is a likely factor explaining why certain human class I molecules exhibit poor peptide loading when expressed in mouse cells (Carreno and Hansen, 1994).

B. Physical Association of TAP with Class I Molecules

Recent studies of human (Ortmann *et al.*, 1994) and mouse (Suh *et al.*, 1994; Carreno *et al.*, 1995b) have shown that antibodies to TAP coprecipitate class I molecules. This finding demonstrated that class I molecules are physically associated with TAP in the ER. Importantly, class I molecules were not found in association with TAP in β_2m-deficient cells. This finding implies that TAP is associated with H chain/β_2m heterodimers and not free H chains. Peptide binding to the H chain/β_2m heterodimers was found

to release the class I molecules from TAP. Thus, when all three subunits of class I (H chain, β_2m, and peptide) are assembled, this completed heterotrimer is released from TAP.

To better define the changes in the conformation of the H chain ligand binding site that induce class I dissociation from TAP, we have taken a serological approach (Carreno *et al.*, 1995b). Our findings with the mouse class I molecules L^d are particularly informative. Previous studies showed that monoclonal antibodies (mAbs 64-3-7 and 30-5-7 serologically distinguish open versus folded forms of L^d, respectively (Lie *et al.*, 1991; Smith *et al.*, 1992, 1993). Furthermore, using *in vitro* folding assays in cell lysates, open forms of L^d can be converted into folded L^d by the addition of specific peptide ligands. Importantly, β_2m-associated open L^d forms were preferentially induced by peptides to undergo folding. This implied that β_2m can bind to H chain prior to peptide and that β_2m association alone is insufficient to induce folding of the class I ligand binding site at 37°C. Thus, these findings are in complete accordance with the previous findings on TAP-associated class I molecules. Both approaches suggest that the typical order of events is β_2m assembly with H chain prior to peptide binding (Fig. 2).

In the L^d system, when peptide binding occurs the L^d ligand binding site is converted from its open to its folded conformation as distinguished serologically by mAbs 64-3-7 and 30-5-7, respectively. Dramatically, TAP

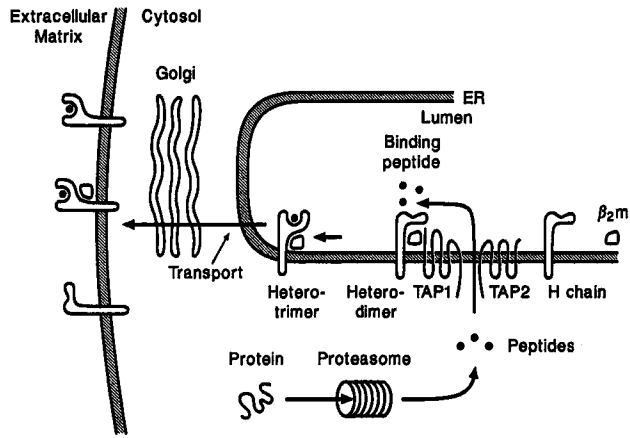

FIG. 2. TAP association with class I molecules before peptide binding. The model shown here shows β_2m-assembled class I H chains in physical association with TAP. Once peptide binds the H chain/β_2m heterodimer, the finished class I heterotrimer of H chain/β_2m/peptide is released from TAP to transit to the cell surface. There is considerable evidence using both mouse and human cells that supports the validity of this model.

was exclusively found in association with the open L^d form (Carreno *et al.*, 1995b). Thus, the change in L^d H chain structure coincident with peptide binding and TAP dissociation is uniquely distinguished by these two mAbs. Similar serological approaches using human mAb that distinguish open ($HC10^+$) versus assembled ($W6/32^+$) HLA molecules have found that open human H chains are uniquely associated with TAP (Neisig *et al.*, 1996). Thus, in both humans and mice, β_2m-assembled open H chains are found in association with TAP, and peptide ligands can cause the dissociation of finished class I heterotrimers from TAP.

C. TAP Association with β_2m in HLA-Deficient Cells

Because β_2m is required for efficient H chain association with TAP, we asked whether the reciprocal were also true. To test whether β_2m can bind to TAP in the absence of H chain, the HLA-deficient cell line LCL 721.221 was used. Due to chromosomal deletions, .221 cells fail to express HLA-A, -B, and -C, but they express normal levels of TAP and β_2m (Shimizu and DeMars, 1989). Interestingly, β_2m showed high levels of association with TAP in .221 cells (Carreno *et al.*, 1995b). This result clearly demonstrated that β_2m can bind to TAP in the absence of classical class I molecules, but it should be noted that these cells do contain very low levels of nonclassical class I molecules. More specifically, .221 cells have been reported to contain low levels of HLA-E and -F mRNA (a quantity less than 1% of the normal HLA mRNA level). However, preclearance of nonclassical class I protein from a .221 lysate did not affect the amount of β_2m associated with TAP. It should also be noted that class I protein was not coprecipitated with anti-TAP antibody in the .221 lysates (Ortmann *et al.*, 1994; Carreno *et al.*, 1995b). Thus, even if .221 cells have a low level of nonclassical class I protein, it would appear to be insufficient to account for the high level of β_2m associated with TAP in these cells. These findings thus strongly suggest that β_2m binding can occur in the absence of H chain.

Thus, we proposed the model that β_2m is docked on TAP in the absence of H chain and that this docking of β_2m prior to H chain serves as a mechanism to concentrate the amount of β_2m in the ER. In H chain-positive cells, the order of binding would be first β_2m to TAP, followed by H chain and peptide to complete the complex. Following peptide binding, the completed class I heterotrimeric complex is released from TAP and is free to transit to the cell surface (Fig. 2).

D. Temporal Role of β_2m in the Folding of the H Chain Ligand Binding Site

With β_2m-deficient cell lines such as RIE/D^b, the surface expression of class I molecules is greatly impaired, and the class I molecules that are

found on the cell surface are misfolded (Allen *et al.*, 1986). This conclusion is based on the serological observation that most D^b molecules expressed by R1E/D^b cells are only detectable with mAb to the $\alpha 3$ domain and not with a mAb to the $\alpha 1/\alpha 2$ domains. To determine whether β_2m must be continually present with the class I H chain for the class I molecule to reach the cell surface in a folded conformation, a sequence encoding an ER retention signal (KDEL) was attached onto the 3′ end of a β_2m cDNA (Solheim *et al.*, 1995b). After this chimeric cDNA was transfected into R1E/D^b cells, β_2m-KDEL protein was detectable by an anti-β_2m serum within a cell lysate but not at the cell surface. Interestingly, R1E/D^b cells transfected with β_2m-KDEL were found to express a high level of conformationally correct D^b molecules at the cell surface. This observation implies that β_2m has a critical and temporal role in the *de novo* folding of the H chain ligand binding site. We speculate that the critical time for β_2m association is when the H chain is docked with TAP and first interacts with peptide. It is important to note that others have shown that the presence of β_2m during H chain folding aids formation or maintenance of proper disulfide bonds (Ribaudo and Margulies, 1992; Wang *et al.*, 1994). Furthermore, serological studies by several groups have suggested that the initial interaction of β_2m with H chain leads to H chain conformations that are fixed at the cell surface (Myers *et al.*, 1989; Tatake *et al.*, 1992; Danliczk and Delovitch, 1994). Future crystallographic data may be able to confirm the precise structural changes imparted temporally by β_2m on the H chain ligand binding site.

E. An Additional MHC-Encoded Factor (gp 48) Involved in Class I Assembly

Remarkably, yet another MHC-encoded factor appears to be required for class I assembly, and this factor too was originally defined using yet another mutant cell line from the collection of DeMars *et al.* (1985). The cell line defining this new factor, LCL 721.220, was isolated after repeated mutagenesis of an MHC hemizygous cell line followed by selection for deficient class I expression. In transfection studies, Grandea *et al.* (1995) demonstrated that these .220 cells have impaired surface expression of diverse class I molecules. In addition, these .220 cells were shown to express functional β_2m and TAP. Furthermore, H chains derived from .220 cells were found to associate normally with calnexin, but H chain/β_2m dimers from .220 cells failed to efficiently capture peptides. This latter conclusion was based on their observation (Grandea *et al.*, 1995) that class I molecules from .220 cells are unstable *in vitro* unless peptide ligands were added. Interestingly, four different H chain/β_2m dimers failed to associate with TAP in the .220 transfected cell lines. Grandea *et al.* (1995)

concluded that the factor missing in .220 cells is required for class I association with TAP and that class I/TAP association is required for normal class I peptide loading and surface expression. Consistent with the conclusion that the factor missing in .220 cells is required for class I association with TAP, we found no β_2m bound to TAP in lysates from .220 cells. It is thus of paramount importance to define the structure and function of this missing gene product(s) in .220 cells. Based on its size in gels this missing factor has been termed gp48.

F. Structures on Class I Molecules That Influence Their Association with TAP

Two different groups have addressed the question of whether soluble class I forms are associated with TAP. In studies by Carreno *et al.* (1995b), chimeric L^d/Q10 that are soluble and expressed at high levels were clearly found to be TAP associated. By contrast, Lee *et al.* (1995) compared membrane-bound and soluble forms of HLA-G and found that only the membrane-bound forms were associated with TAP. Interestingly, in the absence of detectable TAP association, soluble HLA-G molecules were found to show high levels of expression and comparable peptide loading relative to membrane-bound HLA-G molecules. These findings with the two different soluble class I molecules thus yield conflicting conclusions. Analyses of additional soluble class IB molecules will be require to determine whether membrane association is typically required for class I association with TAP.

To define the precise structural site of interaction on class I for TAP, several groups have looked for H chain mutations or polymorphisms that influence class I association with TAP. Studies by two groups (Carreno *et al.*, 1995b; Suh *et al.*, 1995) suggested that mutations in the α3 class I domain ablate class I association with TAP. When comparing the levels of TAP associated with various class I alleles, Neisig *et al.* (1996) reported that class I polymorphism within the ligand binding site influenced TAP association. Finally, Peace-Brewer *et al.* (1996) characterized an HLA-A*0201 mutant (T134K) with a substitution at position 134 and found a lack of TAP association. Position 134 is on a β-chain loop outside the ligand binding site. This study of the T134K mutant is particularly interesting because it assessed functional efficacy. A minigene approach was used to demonstrate that, without TAP association, class I antigen presentation by T134K mutant molecules is clearly impaired relative to wild type. The conclusion that TAP association is required for optimal antigen presentation by class I molecules is based on the assumption that the T134K mutation directly and exclusively affects H chain interaction with TAP. Alternatively, certain of these mutations could affect the binding of select peptides or

the ability to assemble with β_2m or, associate with some other molecule involved in class I expression. As mentioned previously, impaired *in vivo* β_2m assembly would be predicted to result in lack of H chain association with TAP. Thus, interpretation of the phenotypes of the previously mentioned H chain mutations requires rigorous demonstrations that the structural changes induced by the mutation affect the ability of the mutant class I to associate with TAP but not peptide or β_2m. Definition of the direct site of interaction between class I and TAP should help in determining the physiological significance of this association.

G. Functional Significance of TAP/Class I Association

It is intriguing to speculate on the functional significance of the physical binding of class I molecules to TAP prior to peptide loading. However, at the time of the writing of this review, this question remains open. Although the findings with .220 cells imply that class I association with TAP is critical for peptide loading and surface expression, the factor (gp 48) missing in these cells has yet to be characterized (Grandea *et al.*, 1995). Similarly, the results with the HLA-A*0201 T134K mutant suggest that class I association with TAP is critical for class I antigen presentation and surface expression. However, it remains unclear whether position 134 is the site on the H chain that directly interacts with TAP. Indeed, the primary defect of T134K mutant molecules may be in their impaired ability to interact with peptide of β_2m. Alternatively, position 134 could be the site of interaction with gp 48, the putative factor missing in .220 cells. Until all the factors involved in class I antigen presentation are identified, it will be difficult to make definitive conclusions from the previous observations regarding the importance of the class I/TAP association.

It is perplexing why certain class I molecules lack TAP association but appear to have normal peptide loading or expression. As mentioned previously, soluble HLA-G and mouse L^d and D^d α3 mutants have been reported to lack TAP association but appear to have high levels of expression and equivalent peptide loading compared with their TAP-associated partner molecules. Although this issue is currently unresolved, it is reasonable to assume that the specific interaction of TAP with class I is maintained to serve some evolutionarily important function. In speculation of what this function might be, we offer four putative roles. As discussed below, TAP would appear to perform two chaperone functions for class I molecules: the facilitation of subunit assembly and ER retention of empty H chain/β_2m dimers. In addition to these two functions, a third role of TAP association may be to concentrate β_2m in the ER, given the previous evidence that TAP binds free β_2m. A fourth function of TAP association may be to facilitate the efficient trapping of peptides that enter the ER.

Such trapping may be required to prevent ER degradation of peptides or peptide removal via a specific pump yet to be defined. If this model is correct, then class I molecules may be loaded shortly after peptide entry into the lumen of the ER. The consequence of this for antigen presentation is that peptides may not have to compete for access to the class I ligand binding site. In other words, peptide competition may not occur and which peptides get loaded may be more a reflection of peptide abundance than peptide affinity. Consistent with this conclusion, it is noteworthy that antigenic immunodominant peptides have been described that bind to class I with poor affinities yet are derived from abundant proteins (Connolly, 1994).

V. Proposed Function of TAP Versus Calnexin: Might TAP Be the Predominant Class I Chaperone?

Although TAP has not been considered a chaperone, it has been implicated in chaperone-like functions for class I molecules. TAP would appear to (a) concentrate β_2m in the ER at the site of H chain assembly, (b) juxtapose peptide-receptive H chain/β_2m dimers with peptide upon entry of peptide into the ER, and (c) retain H chain/β_2m dimers in the ER prior to peptide binding and then release completed H chains/β_2m/peptide heterotrimers for transit to the cell surface. All these functions are not only chaperone-like functions but also have previously been ascribed to calnexin. Thus, we suggest that TAP may be the predominant class I chaperone. This hypothesis would make sense in light of the fact that the TAP has evolved presumably to service only class I molecules and therefore may have more clearly defined functions in class I expression.

The previously described observations raise questions in regard to the relative contributions of calnexin versus TAP in class I expression. We propose a general model (Fig. 3) of class I interaction with TAP and calnexin based on all the previously mentioned information including the detection of both free H chains and H chain/β_2m dimers in association with calnexin. In this model there are alternative pathways for chaperone interaction involving a terminal association with either TAP or calnexin. Free class I H chains would start out in association with calnexin and then switch to TAP when a TAP docking site is available (preferred pathway). Presumably, a high-affinity TAP docking site would include a prebound β_2m. The obligatory role of β_2m for optimal class I interaction with TAP could explain the different kinetics of intracellular transport and class I expression observed with certain class I molecules. For example, L^d molecules have a weak affinity for β_2m that could result in an impaired interaction with TAP and suboptimal peptide loading. Such an impaired interac-

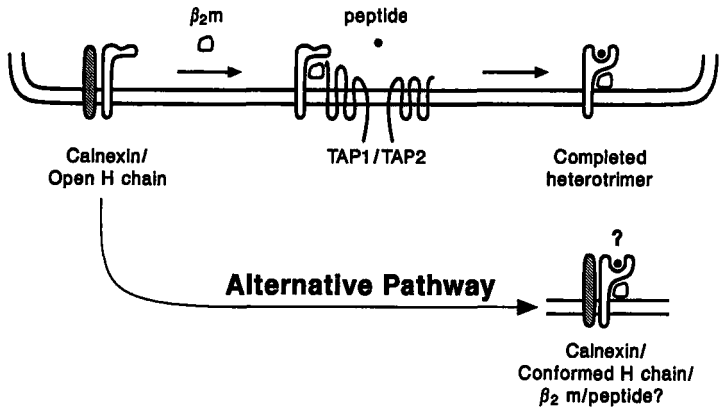

FIG. 3. Proposed model for the interaction of class I molecules with calnexin or TAP. A two-pathway model for the interaction of class I folding intermediates with calnexin and TAP is shown. In the preferred pathway, free class I H chains initially associate with calnexin. Subsequently, β_2m-assembled H chains will bind TAP if a TAP complex is available. Peptide binding to these TAP-associated class I molecules results in a change in the H chain conformation inducing release from TAP and egress from the ER. If TAP molecules are not available (e.g., in TAP-negative cells), then class I molecules proceed via an alternative pathway in which class I H chains remain calnexin associated even after β_2m assembly and gain of reactivity with conformation-dependent antibodies. Peptide occupancy of these folded class I molecules is unclear, and their questionable occupancy may result in their ER retention (adapted from Carreno *et al.*, 1995b).

tion with TAP could ultimately be responsible for the slower intracellular transport and lower surface expression of L^d compared to D^d or K^d (Beck *et al.*, 1986). Studies of both human and mouse indicate that peptide ligand binding dissociates class I molecules from TAP (Ortmann *et al.*, 1994; Suh *et al.*, 1994; Carreno *et al.*, 1995b). Once peptide has bound, the trimeric complexes of β_2m/H chain/peptide should be unimpeded in their egress from the ER. It should be noted that ER retention of unloaded class I/β_2m complexes in this pathway is a function of TAP and not calnexin.

Alternatively, if class I molecules do not proceed by the preferred pathway by docking with TAP, we propose that they remain associated with calnexin even after β_2m assembly. This backup pathway (Fig. 3) would be exclusively utilized in either TAP- or β_2m-deficient cells and would be utilized more in cell lines that overexpress H chains and thus limit TAP interactions. Indeed, these are precisely the situations in which we detect the most class I/β_2m associated with calnexin (Carreno *et al.*, 1995b). TAP- and β_2m-deficient cells have been shown to contain large pools of intracellular class I molecules detectable with conformation-dependent

mAb (Neefjes *et al.*, 1993a; Smith *et al.*, 1993). Although some of these molecules are peptide loaded based on functional studies, most appear thermally labile, which suggests they are empty (Ljunggren *et al.*, 1990). In this alternative pathway, assembled and conformed class I molecules remain calnexin associated and thus would be predicted to be retarded in their egress from the ER. Indeed, published data demonstrate that, in the absence of β_2m or TAP, class I expression is significantly reduced at the cell surface (Williams *et al.*, 1989; Ljunggren *et al.*, 1990). In *Drosophila* cells, which load peptides poorly onto mouse class I molecules, calnexin has been shown to retard surface expression of conformed class I/β_2m complexes (Jackson *et al.*, 1994). Thus, we speculate that the physiologic role of the terminal calnexin association with class I molecules in the alternative pathway is to retain these molecules in the ER due to their questionable peptide occupancy.

VI. ER versus Cytosolic Processing

A. ER Processing

Falk *et al.* (1990) suggested that the final processing of some class I ligands may occur in the lumen of the ER; this prediction was based on the fact that the cellular composition of peptides is regulated by the alleles of MHC class I molecules present. Subsequently, several other studies have provided support for the processing of peptides in the ER. First, the studies of Henderson and colleagues (1992) and Wei and Cresswell (1992) revealed the presence of processed signal peptides in association with HLA-A2 molecules in TAP-deficient cell lines; these processed signal peptides were derived from proteins initially targeted to the ER. Most of these peptides were longer than the reported optimal length for A2-restricted peptides but were truncated versions of signal sequences, suggesting the presence of proteases other than the signal peptidase in the ER. Second, other studies have shown limited trimming of longer peptides for class I presentation in TAP-deficient cell lines when the peptides are targeted to the ER by signal sequences (Snyder *et al.*, 1994; Roelse *et al.*, 1994). Furthermore, Elliott *et al.* (1995) found that a 170-amino acid partial influenza nucleoprotein polypeptide could be processed into a peptide ligand that is presented by D^b in TAP-deficient cells when the polypeptide is targeted to the lumen of the ER via a signal sequence. In addition, Hammond *et al.* (1995) demonstrated that a class I-restricted epitope found on the HIV-1 envelope protein (normally targeted to the ER via its signal sequence) can be presented in a TAP-deficient cell line. These latter two studies provide the strongest evidence that epitopes can be fully processed from comparatively large polypeptides by proteolytic activities

in the lumen of the ER. However, Elliott *et al.* (1995) also found that the D^b-restricted epitope was not processed and presented when the full-length influenza nucleoprotein, instead of a shorter 170-amino acid polypeptide derived from nucleoprotein, was introduced into the lumen of the ER via a signal sequence. This result suggested that processing in the ER is limited compared to that in the cytoplasm. Moreover, subsequent studies on the processing of the HIV-1 envelope protein into class I ligands revealed the presence of other class I-restricted CTL epitopes within this protein that are TAP dependent for their presentation (Hammond *et al.*, 1995). In addition, Aldrich *et al.* (1994) showed that the presentation of a signal sequence peptide by the class Ib molecule Qa-1 is TAP dependent. Thus, clearly not all class I-restricted CTL epitopes in ER-localized proteins can be presented efficiently in a TAP-independent manner.

Although the previously mentioned studies suggest the presence of different proteolytic activities in the lumen of the ER, only one protease, the signal peptidase, has been localized to this compartment (Blobel and Dobberstein, 1975). Despite this, other investigators have reported the proteolytic degradation of different ER-localized proteins within this compartment (Bonifacino *et al.*, 1989; Wikstrom and Lodish, 1991; Tsao *et al.*, 1992; Wassler and Fries, 1993). Furthermore, cysteine and serine proteases have been suggested to be present in the ER because the degradation of target proteins by these activities is sensitive to agents that inhibit these types of proteases (Wikstrom and Lodish, 1991; Wileman *et al.*, 1993).

B. TAP-Dependent Presentation of Epitopes on ER-Localized Proteins

Two different pathways of processing and peptide transport have been postulated by Siliciano and Soloski (1995) to explain why some epitopes on ER-localized proteins are presented in a TAP-dependent manner. In the first pathway, they hypothesized that ER-localized proteins may be partially degraded in the ER, but that further processing in the cytoplasm is required to generate the class I ligands (see Fig. 4, model I).Thus, this model necessarily invokes retrograde transport of the partially processed peptides from the lumen of the ER to the cytoplasm. Such a recycling system that appeared to operate independently of TAP was reported by Roelse *et al.* (1994). Once the partially processed peptide is recycled to the cytoplasm and further processed, its transport back to the lumen of the ER for assembly with class I molecules is TAP dependent. A second alternative pathway was also proposed in which a small portion of the protein containing the signal sequence (for localization to the lumen of the ER) fails to be targeted correctly and remains in the cytoplasm (see Fig. 4, model II). This inappropriately targeted protein can then be pro-

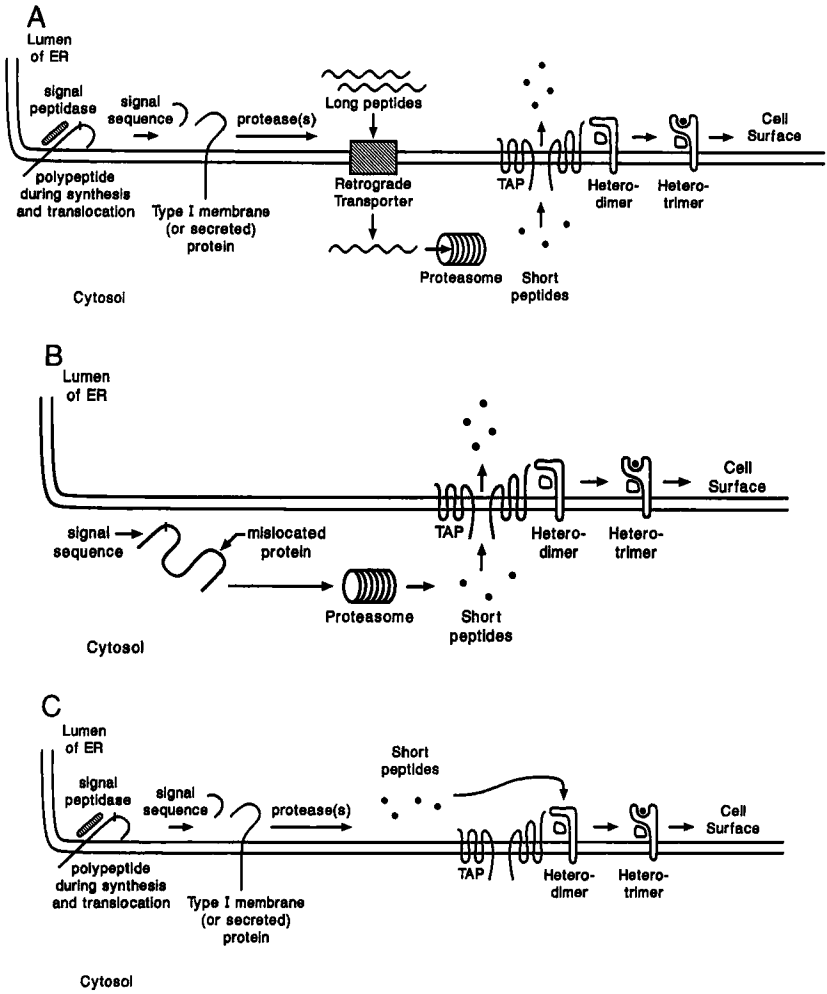

FIG. 4. Three different models to explain the TAP-dependent presentation of antigenic peptide derived from ER-localized proteins. Model I requires recycling of partially degraded peptides derived from ER-localized proteins into the cytosol for entry into the default class I antigen presentation pathway. Model II involves the mislocation of normally ER-localized proteins to the cytosol for entry into the default class I antigen presentation pathway. Because these first two pathways utilize the default pathway, TAP would be required for transport of peptides into the ER. In model III, peptides are generated from ER-localized proteins in the lumen of the ER, and TAP would be required as a chaperone to assist in the assembly of class I/β_2m heterodimers and peptides. These models are not mutually exclusive and TAP is likely to have chaperone functions regardless of the validity of each of the proposed models.

cessed like a cytosolic protein into peptides that can then be loaded onto MHC class I molecules after TAP-dependent transport into the lumen of the ER. In support of this model, Ferris *et al.* (1996) observed that an HIV-1 envelope epitope, which contains a site normally glycosylated in the intact protein, is presented as a nonglycosylated peptide but not as a glycosylated nor as a deglycosylated form. However, the support of this model by this study assumes that none of the protein can be degraded in the ER and recycled to the cytoplasm before it is glycosylated.

A third distinct model that would explain the TAP-dependent presentation of ER-localized peptides is one in which the TAP-dependent presentation of such peptides derives from their reliance on class I/β_2m heterodimers associated with the TAP complex for efficient assembly (see Fig. 4, model III). This model correlates well with the predicted chaperone function of TAP as discussed under Section V. Furthermore, the TAP-dependent presentation of many epitopes derived from cytosolic proteins may in part also be due to this putative chaperone function in addition to the TAP-dependent transport process. Clearly, the association of class I/β_2m with TAP is not entirely essential for the assembly with peptide ligands as evident in the studies cited previously in which epitopes are localized to the ER via signal sequences for assembly with class Iβ_2m heterodimers in TAP-deficient cells. However, these studies may represent situations in which the peptide ligands are supplied for assembly at higher levels than found physiologically. Alternatively, the peptide ligands that are TAP independent in their assembly (but not their transport) may be high-affinity peptides. Thus, the proposed requirement for TAP in the assembly process, in addition to the transport process, may only be manifested for low-affinity ligands or for ligands that are present at low concentrations. Taken together, the studies described previously with ER-localized peptides and polypeptides that were performed in TAP-deficient cell lines indicate that ER processing can occur and that class I assembly can occur in the absence of TAP association. However, to what extent these ER processes take place in the presence of a potentially more efficient system of cytosolic processing, TAP-dependent transport, and TAP-induced assembly is a critical but as yet unanswered question.

VII. Peptide Anchoring

The primary considerations for the binding of peptides to MHC class I molecules have generally been elucidated. Several different publications review the concept that residues at certain positions or registries within peptides serve as anchors in that they provide sites of interactions within pockets of the MHC class I molecules (Matsumura *et al.*, 1992; Falk *et*

al., 1991; Engelhard, 1994; Rammensee, 1995). These class I pockets are composed of both monomorphic and polymorphic residues and, thus, different class I molecules possess different residues within each pocket that allow them to bind peptides with different anchor motifs (Bjorkman *et al.*, 1987; Matsumura *et al.*, 1992; Saper and Wiley, 1991; Falk *et al.*, 1991, 1993; Fremont *et al.*, 1992; Engelhard, 1994; Rammensee, 1995). These motifs can differ with regard to either the position of the anchors within the peptide or the chemical nature of the residue at a position that acts as an anchor. Almost all class I peptide ligands are 8–10 amino acids in length. However, examples of longer peptides that can be presented by class I molecules have been demonstrated (Chen *et al.*, 1994; Henderson *et al.*, 1992; Urbasn *et al.*, 1994; Parker *et al.*, 1992). Most of the class I ligands studied possess a carboxyl-terminal anchor (P_n) that is usually of a hydrophobic nature. However, the position of other anchors and the chemical nature of the anchor residues are determined by the particular class I molecule to which the ligand can bind. Most class I molecules require an anchor at position 2 (P2) of the ligand. For instance, HLA-A2 and -A3 molecules preferentially bind peptides with a P2 Leu residue, whereas HLA-B7, -B35, and -B53 and the murine H-2L^d molecules prefer peptides with a P2 Pro residue. Other class I molecules preferentially bind peptides with P3 or P5 anchors. Thus, the specificity of different class I molecules for a combination of a P2, P3, or P5 along with a P_n anchor usually results in substantial differences in the sets of peptides bound by each class I molecule. It is our intention to discuss several interesting features of peptide binding to class I molecules, including (a) the effect of nonanchor residues on the binding of peptides bearing the consensus motif to a class I molecule, (b) the definition of alternative anchors on ligands lacking a consensus anchor, and (c) the ability of peptides to bind to closely related class I molecules.

A. Effect of Nonanchor Residues on Peptide Binding to Class I Molecules

Several studies have scanned protein sequences for peptides that will bind to and be presented by particular class I molecules on the basis of the consensus anchor motif. Due to the prevalence of the HLA-A2 allele in the human population, several investigators have concentrated their search for peptides bearing the anchor motif essential for binding to the HLA-A*0201 molecule (Sette *et al.*, 1994; Merson, 1993; Wentworth *et al.*, 1996; Ressing *et al.*, 1995). In these studies, proteins derived from several human viruses (hepatitis B and C viruses and human papillomavirus), tumor antigens, and *Plasmodium faciparum* were scanned for peptides bearing the consensus A*0201 motif (P2 Leu and a P9/P10 Val, Leu, or

Ile), and these peptides were tested for their ability to bind to A*0201. Although all the peptides tested contained the aforementioned P2/P_n consensus A*0201 anchor motif, vast differences existed among them in their affinity for A*0201. Thus, these results suggest that other residues besides the consensus anchors play important roles in the binding of peptide ligands to class I molecules. This model is further supported by a similar analysis of the peptide ligands that bind to other HLA-A and HLA-B alleles (DiBrino *et al.*, 1993; Sette *et al.*, 1994; Ressing *et al.*, 1995; Kast *et al.*, 1994). Furthermore, prominent roles for secondary anchors at nonconsensus anchor positions have been demonstrated (Ruppert *et al.*, 1993). In fact, some class I molecules have been demonstrated to bind peptides that possess two of three possible anchors; furthermore, these anchors act cumulatively to stabilize binding to the class I molecule (Parker *et al.*, 1994b; DiBrino *et al.*, 1994).

In addition to nonconsensus anchor residues within a peptide serving as secondary and alternative anchors, the nonanchor positions can also adversely affect peptide binding to class I molecules. Thus, although the residue at a particular position may not serve as a primary or secondary anchor, particular amino acids at that position may negatively impact on the capacity of a particular peptide to bind to a particular class I molecule. For instance, P1 acidic residues inhibit the binding of peptides to A*0201 (Parker *et al.*, 1994a) presumably because of an adverse ionic interaction with the A*0201 Glu 63 known to be located in pocket A; pocket A is known to interact with the amino terminus of full-length A*0201 ligands. Furthermore, the wild-type version of the aforementioned L^d-restricted tum^- P91A 14–22 peptide contains a P4 Arg instead of P4 His (Lurquin *et al.*, 1989). The wild-type version of the peptide has a considerably reduced capacity to bind to L^d compared to the P4 His version or other P4-substituted versions of the peptide (Robinson and Lee, 1996). Because this position is thought to be a TCR contact site (Alexander-Miller *et al.*, 1994) and not an anchor (Robinson and Lee, 1996), the failure of the P4 Arg-substituted tum^- peptide to bind to L^d could be due to steric hindrance. A similar explanation for the failure of the P6 Ala → Asp-substituted tum^- peptide to bind to L^d was invoked (Robinson and Lee, 1996). Thus, although it is not always clear why certain residues at nonanchor positions adversely affect the binding between class I molecules and their ligands, models are currently being tested to consider both the positive and negative influence of both anchor and nonanchor positions on the class I/peptide interaction (van Kuyk *et al.*, 1994; DiBrino *et al.*, 1994; Sidney *et al.*, 1996).

B. Definition of Alternative Anchors on Class I Ligands

The use of an alternative anchor instead of the consensus anchor for peptide binding to class I molecules is exemplified by the mouse L^d ligands,

p2Ca and tum$^-$ P91A 14–22. The former is an immunodominant peptide recognized in allogeneic responses against the L^d molecule (Udaka *et al.*, 1992; Connolly, 1994), whereas the latter is a tumor antigen present on variants of the P815 mastocytoma (Lurquin *et al.*, 1989; Solheim *et al.*, 1993). Neither ligand possesses the consensus P2 Pro anchor. Furthermore, substitution of the P2 residue of either ligand does not adversely affect binding to L^d, whereas substitution of the P6 Phe in the p2Ca octamer and the P8 Asp in the tum$^-$ nonamer drastically reduces the capacity of the respective peptides to bind to L^d (Al-Ramadi *et al.*, 1995; Robinson and Lee, 1996). Thus, both these peptides bear alternative anchors that otherwise would have been missed by sequencing peptide pools eluted from class I molecules (Corr *et al.*, 1992). The ability of L^d to present ligands bearing alternative anchors suggests that other class I molecules may have similar capacities, thus expanding the potential self and antigenic repertoires presented via the class I pathway.

C. Peptide Binding to Closely Related Class I Molecules

Although initial studies demonstrated that different class I molecules differ significantly in the sets of peptides that they bind, further analyses of more closely related class I molecules have defined groups of class I molecules that may be able to bind overlapping sets of peptides. Again, due to its prevalence in the population, HLA-A2 has been heavily studied and consists of at least 16 closely related alleles, including A*0201, A*0202, A*0205, A*0206, A*0207, A*0214, A*6801, and A*6901, that differ by as many as five amino acids (Arnett and Parham, 1995; Barouch *et al.*, 1995). Although some members bind overlapping sets of peptides, single amino acid differences in pocket B of some members of this family can significantly alter their peptide specificity (Barouch *et al.*, 1995; Sudo *et al.*, 1995; Rotzchke *et al.*, 1992). Similar findings were demonstrated for the binding of peptides to different HLA-B27 subtypes (Tanigaki *et al.*, 1994; Colbert *et al.*, 1994). Despite these differences in the peptides bound by A2 and B27 subtypes, considerable overlap exists in the peptides bound by most of the members of each family of class I molecules. Furthermore, several other families of related class I molecules that bind overlapping sets of peptides have been defined, including the L^d family consisting of L^d, L^q, and L^{w16} (Lie *et al.*, 1990; Talken *et al.*, 1994; Oldstone *et al.*, 1992; Schulz *et al.*, 1991), the HLA-B7 supertype consisting of B*0701, B*3501, B*3502, B*3503, and B*5401 (Sidney *et al.*, 1995), and the HLA-A3-like supertype composed of A*0301, A*1101, A*3101, A*3301, and A*6801 (Sidney *et al.*, 1996). The value of these families of related class I molecules for the design of peptide vaccines against tumors, viruses, and other intracellular pathogens is being investigated. Because the A2, A3, and B7 supertypes

would cover more than 90% of the human population, this concept is critical to the success of the use of CTL epitopes as peptide vaccines.

VIII. Final Remarks

There has been remarkable progress in the elucidation of many of the key processes of class I peptide loading. For example, as detailed previously, many of the key properties of proteasomes, calnexin, and TAP in the assembly of the class I heterotrimer have been defined. However, there remain other molecules whose role in class I assembly has yet to be resolved, such as heat shock protein HSP (which may assist peptide loading in the ER) or calreticulin (which appears to have chaperone functions similar to calnexin). In addition, gp48, the factor missing in the .220 cell line, promises to be a critical contributor to class I assembly with β_2m and loading with peptide. Thus, there are clearly more contributors whose role in class I expression remains to be defined. Future studies should define the stoichiometric relationship and precise individual contributions of each of these accessory molecules. We can then more fully appreciate how the concerted efforts of these molecules combine to effectively load peptides for presentation to T lymphocytes.

Overshadowing the progress scientists have made in understanding class I expression are the sophisticated mechanisms that viruses have devised to hinder antigen presentation by class I molecules. It is now clear that viruses have evolved several different molecular strategies to prevent presentation of their peptides and thus avoid detection by T cells. Interestingly, many of these mechanisms appear to act posttranslationly at various steps of class heterotrimer assembly. For example, proteins encoded by adenovirus (Burgert and Kvist, 1985) and mouse cytomegalovirus (del Val *et al.*, 1992) induce ER retention of class I molecules, thus preventing surface expression of class I/peptide complexes. Alternatively, herpes simplex virus produces a protein that blocks class I surface expression by preventing TAP transport of peptides (Fruh *et al.*, 1995; Hill *et al.*, 1995). Finally, human cytomegalovirus produces a protein that dislocates H chains from the ER to the cytosol where they are rapidly degraded (Wiertz *et al.*, 1996). A future challenge of the MHC biologist is to be as clever as the virus and learn how to advantageously intervene in class I antigen presentation during immune responses to tumors and intracellular pathogens or to prevent unwanted autoimmune responses.

IX. Note Added in Proof

Subsequent to our writing of this review, two papers of particular relevance were published. In a paper by Lewis *et al.* (1996), the HLA-

A2.1 mutant T134K was shown to bind peptide antigens and assemble with β_2m as efficiently as wild-type HLA-A2. This and other data in this report further support the claim that TAP association is obligatory for class I antigen presentation. In a study by Sadasivan *et al.* (1996), the factor missing in .220 cells (gp 48) was named tapasin and shown to be a key intermediary in the assembly of calreticulin and H chain/ β_2-m with TAP.

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How Do Lymphocytes Know Where to Go: Current Concepts and Enigmas of Lymphocyte Homing

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I. Introduction

The immune system is anatomically and functionally divided into three distinct compartments. In the primary lymphoid organs, lymphocytes are continuously produced in enormous numbers and their antigen specificity is determined. Secondary lymphoid organs, i.e., lymph nodes and spleen, are specialized for offering a suitable microenvironment for efficient interactions between lymphocytes and antigens. In these organs, antigens are locally concentrated, processed, and presented to B and T lymphocytes. The progeny of the activated cells then mainly exert their effector functions in extralymphoid or tertiary lymphatic tissues.

An elaborate system of continuous lymphocyte recirculation has evolved to connect the three types of discrete lymphoid tissues. Thus, after release from the thymus and bone marrow, each naive lymphocyte can freely migrate between the blood and different lymphatic tissues to maximize the probability of the rare event that a lymphocyte with a single antigenic specificity encounters its cognate antigen introduced anywhere in the body. This migration is called lymphocyte recirculation—a process that encompasses binding of the blood-borne lymphocyte to the vascular endothelial lining, emigration of the lymphocyte between the endothelial cells into the tissue side, movement through the lymphoid organ, and finally exit via the lymphatic vessels back to the systemic circulation. After activation by an antigen, the recirculatory capacity of a lymphocyte changes dramatically. It no longer randomly patrols through different tissues but instead specifically extravasates at sites similar to those where it initially became activated. Thus, lymphocyte trafficking disperses effector cells to sites where they are most useful for defense of the host. At the molecular level, surface receptors on lymphocytes and their counterparts on endothelial cells determine the specificity and magnitude of lymphocyte recirculation. Regulation of the expression and function of these adhesion molecules plays a pivotal

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role in controlling both physiological immunosurveillance and all types of inflammatory processes. A detailed understanding of lymphocyte–endothelial cell interactions allows one to intervene with the recirculation to enhance, redirect, or suppress lymphocyte accumulation and, hence, establishes a rational basis for medical antiadhesion therapy.

II. Lymphocyte–Endothelial Cell Interactions: Historical Aspects and the Conceptual Framework

In human there are approximately 1×10^{12} lymphocytes, of which $\sim 5 \times 10^{9}$ are simultaneously in the blood volume of ~5 liters. It has been estimated that in an adult man the total length of vasculature exceeds 100,000,000 m and the speed of blood flow varies from 0.5 m/s (in aorta) to 0.5 mm/s (in postcapillary venules) (Ganong, 1995; Roitt *et al.*, 1996; Zweifach, 1961). Therefore, the pertinent question is how can a single lymphocyte find the right site to extravasate from the blood circulation.

In the early 1960s it was shown for the first time that lymphocytes actually leave the blood in peripheral lymphoid organs and eventually return back to the systemic circulation via the efferent lymph. In the pioneering experiments, Gowans and Knight (1964) isolated lymphocytes from the efferent lymph (thoracic duct), labeled them radioactively, injected the cells back into rats, and followed their fate by scintillography and autoradiography. They showed that small lymphocytes distribute themselves to all secondary lymphoid tissues, whereas activated immunoblasts home selectively back to mucosal sites (Gowans and Knight, 1964). Subsequent *in vivo* studies with several animal species and lymphocytes from different anatomical origins (Cahill *et al.*, 1977; Chin and Hay, 1980; Ford and Simmonds, 1972; Goldschneider and McGregor, 1968; Griscelli *et al.*, 1969; Guy-Grand *et al.*, 1974; Hall *et al.*, 1979; Husband and Gowans, 1978; Issekutz *et al.*, 1982; Lance and Taub, 1969; Parrott and Ferguson, 1974; Rose *et al.*, 1976) led to the emergence of a general concept of lymphocyte recirculation (Fig. 1). In lymphatic tissues, blood-borne lymphocytes bind to vascular endothelium, are arrested, and transmigrate through the vessel wall. Thereafter, lymphocytes slowly migrate through the tissue parenchyma where they search for their cognate antigens presented by professional antigen-presenting cells. If a lymphocyte does not encounter its specific antigen it leaves the lymphoid organ by penetrating the endothelium of lymphatic vessels in medullary sinuses and is carried via the efferent lymphatic system to the thoracic duct and finally back into the circulation. This naive lymphocyte can then reenter the same or a different lymphatic tissue and the recirculation cycle, taking 12–24 hr on average, will be repeated over and over again until the cell finds its antigen

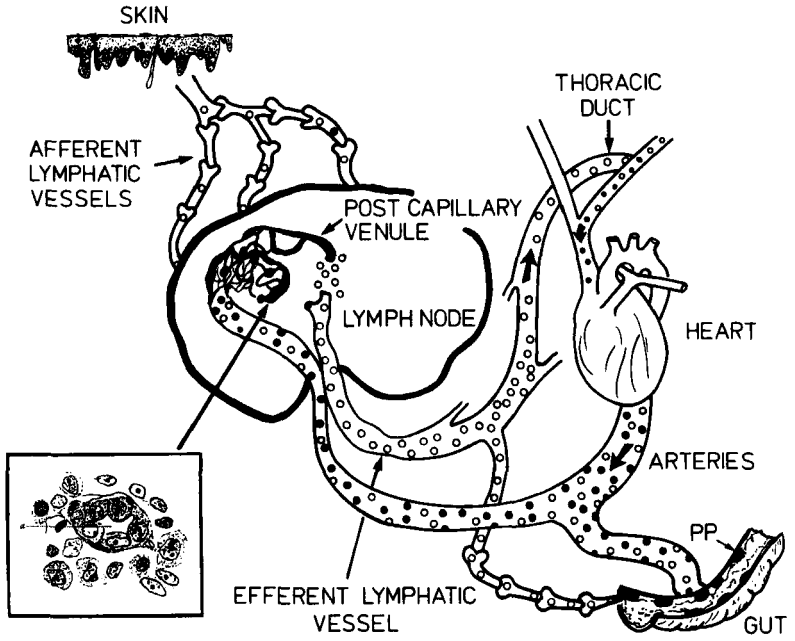


FIG. 1. Lymphocyte recirculation routes under physiologic conditions. A low level of continuous antigenic transport into lymphoid tissues takes place via afferent lymphatics draining the skin and through the epithelium of the gut. Blood-borne lymphocytes enter the organized lymphatic tissues (PLNs and Peyer's patches; PP) from the circulation via the arterial tree, flow through the capillary bed, and extravasate in the postcapillary HEVs. Then, the lymphocytes percolate through the tissue parenchyma, enter the lymphatic vessels, and are carried via the efferent lymphatics back to the systemic circulation. Most of the venous circulation has been omitted from the figure. The inset is a reproduction of a figure illustrating an HEV as it was depicted in 1898 by a microscopic examination of monkey tissues (Thomé, 1898).

or dies. When a lymphocyte encounters its antigen it halts in the secondary lymphatic organ and starts clonal, antigen-driven expansion and differentiation. Thereafter, the progeny of the effector cells follow the same route as the naive cells back to the blood, but their recirculation is then restricted to the sites of the original antigenic insult and related lymphoid tissues. Currently, four such functionally distinct lymphocyte recirculation pathways are known: peripheral lymph nodes (PLNs), mucosa-associated lymphatic tissues, inflamed skin, and synovium (Chin and Hay, 1980; Griscelli *et al.*, 1969; Jalkanen *et al.*, 1986a). On the other hand, not all lymphocytes recirculate. For instance, germinal center cells, CD5-positive peritoneal B1 B cells, and intraepithelial lymphocytes remain sessile for extended periods or indefinitely (reviewed in Picker and Butcher, 1992).

In lymph nodes, most cells enter the tissue by binding to and transmigrating between endothelial cells of specialized postcapillary venules called high endothelial venules (HEVs) (Marchesi and Gowans, 1964). These vessels have metabolically active cuboidal endothelial cells that protrude into the lumen (Anderson *et al.*, 1976; Freemont and Jones, 1983). The shape and voluminous glycocalyx of high endothelial cells, together with sluggish blood flow, maximize the potential for collisions between blood-borne lymphocytes and the vascular lining (Girard and Springer, 1995). Originally, it was proposed that lymphocytes transmigrate through the endothelial cells (Marchesi and Gowans, 1964), but careful examination of serial electronmicroscopic sections revealed that lymphocytes were, in fact, always found between the endothelial cells (Schoefl, 1972). Hence, lymphocytes insinuate themselves between endothelial cells during the process of extravasation. The discontinuous spot-welded-like interendothelial cell junctions in HEVs promote lymphocyte binding and transmigration (Anderson and Shaw, 1993; Anderson *et al.*, 1976). Thus, up to 25% of peripheral blood lymphocytes (PBLs) can bind to endothelial cells (Bjerknes *et al.*, 1986) and be extracted via paracortical HEVs during a single pass through lymph nodes (in other words, approximately 15,000 cells extravasate each second in a typical sheep node) (Cahill *et al.*, 1976; Hay and Hobbs, 1977). Although naive cells almost exclusively leave the blood via HEV, the effector cells mainly enter lymph nodes by extravasating through the flat-walled endothelium in peripheral tissues and by traveling to nodes in afferent lymph (Smith *et al.*, 1970).

The next milestone in lymphocyte–endothelial cell interaction studies was the development of *in vitro* binding assays. Stamper and Woodruff (1976) showed that lymphocytes adhere specifically and reproducibly to HEVs when incubated in suspension under rotatory conditions on frozen tissue sections. This HEV-binding assay accurately reproduces the specificity of the *in vivo* homing pattern of different normal and malignant lymphocytes (Butcher *et al.*, 1980; Stevens *et al.*, 1982). Earlier techniques were developed to isolate and propagate human umbilical vein endothelial cells (HUVECs) *in vitro* (Jaffe *et al.*, 1973). An alternative binding assay became possible when monolayers of these cells were adopted for the adhesion studies.

A new era in lymphocyte homing studies arrived in 1983, when Weissman and colleagues defined the first molecule that mediated tissue-selective lymphocyte homing. They raised monoclonal antibodies (mAbs) against a lymphoma cell line that only binds to PLN HEVs *in vitro*. One mAb, MEL-14, almost completely inhibited binding of these cells to PLN HEVs in the HEV assay and homing to PLNs in *in vivo* transfusion studies. Notably, MEL-14 had no effect on lymphocyte localization to mucosa-

associated lymphatic tissues (Gallatin *et al.*, 1983). Hence, the MEL-14 antigen was the first lymphocyte surface adhesion molecule, termed homing receptor, that at the molecular level could explain the tissue-selective lymphocyte recirculation patterns that had been known for 20 years. Thereafter, intense efforts were launched to isolate the endothelial cell counterparts of lymphocyte homing receptors, and in 1988 the first such molecule was reported (Streeter *et al.*, 1988a). The term vascular addressin was introduced to describe the postal address-like properties of these tissue-specific endothelial ligands of lymphocyte homing receptors.

The characterization of homing receptors and addressins led to the hypothesis that lymphocyte recirculation is regulated by simple key and lock-type responses in different tissues (Butcher, 1986). However, the emergence of ever increasing numbers of new adhesion-related molecules, detailed structure–function analyses under static and flow conditions, and integration of *in vivo* observations have replaced this theory by much more complicated multistep adhesion models (Butcher, 1991; Springer, 1994). Currently, the leukocyte–endothelial cell binding is envisioned to be composed of at least four distinct but overlapping steps: (i) initial transient interactions between the two types of cells (tethering and rolling), (ii) activation, (iii) stable adhesion, and (iv) transmigration (see Section V,H).

III. Molecules and Signals Mediating Lymphocyte–Endothelial Cell Interactions

During the past few years numerous molecules that play a pivotal role at different stages of lymphocyte emigration have been identified in several animal species. Other important, or even primary, functions of these molecules serve diverse processes like cell–extracellular matrix interactions, lymphocyte activation, tumor metastasis, blood clotting, microbial adherence, and organogenesis. Moreover, certain adhesion-related molecules are mainly utilized by leukocyte types other than lymphocytes, although fundamentally all leukocyte–endothelial cell interactions appear to be variations on the same theme. The most pronounced difference is that whereas lymphocytes travel continuously between the blood and tissues, macrophages and granulocytes mainly or solely extravasate into sites of inflammation. Due to the scope of this review, emphasis will be put on molecules involved in lymphocyte–endothelial cell interactions. The expression and functions of these molecules outside the lymphocyte–endothelial system and molecules mediating nonlymphocyte adhesion will only be referred to when relevant to illustrate general concepts. In this review we will first briefly discuss these adhesion molecules in a systematic way and then illustrate with a few examples how the expression and function of these

interplayers are regulated. Finally, we will deal with the issue of how the organ-selective lymphocyte recirculation under physiological conditions and in the setting of inflammation is determined.

Most adhesion molecules involved in lymphocyte–endothelial cell interactions can be grouped into five distinct molecular superfamilies (selectins, sialomucins, integrins, immunoglobulin like, and proteoglycans) that serve as a useful basis for their classification (Tables 1A and 1B).

A. Selectins

The selectin superfamily includes three adhesion molecules: L (leukocyte)-, P (platelet)-, and E (endothelial)-selectin. L-selectin is expressed only on leukocytes, whereas the two other selectins are found on endothelial cells (Bevilacqua and Nelson, 1993; Lasky, 1995; Ley and Tedder, 1995; McEver *et al.*, 1995; Rosen and Bertozzi, 1994; Vestweber, 1993).

L-selectin is the principal PLN homing receptor that was initially defined by the mAb MEL-14 (Gallatin *et al.*, 1983). It is expressed on lymphocytes as a 80- to 90-kDa N-glycosylated transmembrane protein (Van de Rijn *et al.*, 1990). Cloning of L-selectin revealed that it is a new type of adhesion molecule with a chimeric architecture (Lasky *et al.*, 1989; Siegelman *et al.*, 1989; Tedder *et al.*, 1989). The N-terminal domain showing homology to calcium-dependent (C-type) lectins is followed by an epidermal growth factor (EGF)-like area and two consensus repeats similar to those found in complement regulatory (CR) proteins. Functionally, the lectin domain is the most critical one because it mediates binding to the ligands of L-selectin (GlyCAM-1, CD34, PPME, fucoidin, etc.) and most function-inhibiting mAbs, including MEL-14, map to this domain (Bowen *et al.*, 1990; Kansas *et al.*, 1991; Spertini *et al.*, 1991b). However, the EGF module has also been directly implicated in adhesion (Siegelman *et al.*, 1990). The CR elements of L-selectin can also contribute to the ligand binding because a mAb recognizing CR domains of both L- and E-selectin inhibits lymphocyte adhesion to venules (Jutila *et al.*, 1992). The cytoplasmic tail of L-selectin is absolutely required for a functional molecule (Kansas *et al.*, 1993), and it appears to regulate the cytoskeletal interactions or the avidity of the receptor (Pavalko *et al.*, 1995). L-selectin is present on all naive mature lymphocytes, whereas memory cells show bimodal expression: A subpopulation expresses higher levels than naive cells, whereas the rest are negative (Picker *et al.*, 1993b). Expression of L-selectin is controlled by several cytokines (Griffin *et al.*, 1990), but only interferon-α (IFN-α) has been shown to clearly increase its synthesis (Evans *et al.*, 1993). Activation of lymphocytes may lead to a transient increase in L-selectin activity (Spertini *et al.*, 1991a) before the receptor is shed from the lymphocyte surface (Jung and Dailey, 1990). In addition to peripheral node homing,

L-selectin also mediates lymphocyte binding to other lymphatic organs (e.g., mucosa-associated lymphatic tissues; Hamann *et al.*, 1991) and to activated systemic microvascular endothelium (Dawson *et al.*, 1992; Ley *et al.*, 1993; Piczueta and Luscinskas, 1994).

E-selectin is an inducible endothelial molecule that was initially characterized on HUVECs mediating adherence of granulocytes (Bevilacqua *et al.*, 1987; Pober *et al.*, 1986). Later, a subpopulation of lymphocytes was found to bind to E-selectin as well (Shimizu *et al.*, 1991). Upregulation of E-selectin by proinflammatory mediators, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and lipopolysaccharide (LPS), requires new protein synthesis, is maximal at 4–6 hr, and is short lived, at least in HUVEC models (Pober *et al.*, 1986; Wellicome *et al.*, 1990). The rapid clearance of E-selectin from the plasma membrane is possibly due to active internalization (Subramaniam *et al.*, 1993; von Asmuth *et al.*, 1992). However, differential utilization of three different polyadenylation sites can generate multiple forms of transcripts with different turnover rates (Chu *et al.*, 1994). E-selectin expression *in vivo* is largely restricted to activated endothelial cells, but it is not endothelial cell specific because induced hepatocytes and developing astrocytes synthesize E-selectin as well (Essani *et al.*, 1996; Hurwitz *et al.*, 1992). Molecular cloning of E-selectin revealed a similar overall structure as was seen on L-selectin (Bevilacqua *et al.*, 1989; Hession *et al.*, 1990). The adhesive motifs of E-selectin have been mapped into the lectin domain (Erbe *et al.*, 1992). In addition, a role for the CR repeats has been suggested in enhancing the avidity of ligand binding (Li *et al.*, 1994). The crystal structure of the two outermost domains of E-selectin reveals that the lectin domain contains a calcium-binding site and a positively charged hydrophilic region (Graves *et al.*, 1994), which reinforces the ligand-binding requirements of this molecule. In lymphocyte homing, E-selectin has been proposed to function as a skin-selective vascular addressin that preferentially binds skin-seeking memory lymphocytes (Picker *et al.*, 1991a). It should be pointed out, however, that under conditions of inflammation induction of E-selectin is by no means restricted to the skin.

P-selectin synthesized in endothelial cells is sorted to specialized granules called Weibel–Palade bodies by virtue of signals in its cytoplasmic tail (Bonfanti *et al.*, 1989; Disdier *et al.*, 1992; McEver *et al.*, 1989). From these storage organelles P-selectin is, upon stimulation, translocated to the cell surface within minutes (Hattori *et al.*, 1989; McEver *et al.*, 1989). This rapid, protein synthesis-independent induction is mediated by different mediators (histamine, thrombin, complement components, etc.) than is the slower induction of E-selectin. Surface P-selectin has a short half-life due to shedding and/or reinternalization (Hattori *et al.*, 1989; McEver *et*

TABLE IA

LYMPHOCYTE ADHESION MOLECULES MEDIATING ENDOTHELIAL BINDING[a]

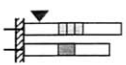
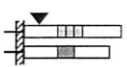
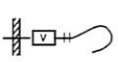
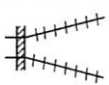
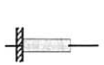
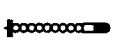
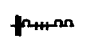
	Structure								
								?	
Molecule	L-selectin	$\alpha 4\beta 1$	$\alpha L\beta 2$	$\alpha 4\beta 7$	CD44	PSGL-1	ESL-1	CLA	CD73
Other names	CD62L, LECAM-1, LAM-1, MEL-14 ag, Leu-8, TQ-1	CD49d/CD29, VLA-4, LPAM-2	CD11a/CD18, LFA-1	LPAM-1, $\alpha 4\beta P$, VLA-4 alt	Hermes, Pgp-1, ECMRIII			HECA-452 ag	5′-ectonucleotidase
Superfamily	Selectin	Integrin	Integrin	Integrin	Proteoglycan	Sialomucin		?	
Isoforms[b]	1	1	1	1	>18	1	1	?	1
Modifications[c]	55%	30%/30%	30%/15%	30%/15%	60%, N- and O-glycans	75%, O-glycans, Fuc, Sia	15%, N-glycans, Fuc	? Fuc, Sia	10%
MW[d]	80	150/120	180/95	150/110	90	120	150	200	70
Ligands[e]	PNAd (CD34, GlyCAM-1), MAdCAM-1	VCAM-1, (MAdCAM-1)	ICAM-1, -2	MAdCAM-1, VCAM-1	Hyaluronate	P-selectin E-selectin	E-selectin	E-selectin	?

TABLE IB

ENDOTHELIAL ADHESION MOLECULES MEDIATING LYMPHOCYTE BINDING[a]

	Structure									
										
Molecule	P-selectin	E-selectin	ICAM-1	ICAM-2	VCAM-1	CD31	MAdCAM-1	GlyCAM-1	PNAd CD34	VAP-1
Other names	CD62P, PADGEM, GMP-140	CD62E, ELAM-1, INCAM-110	CD54	CD102	CD106	PECAM-1	MECA-367 ag	Sgp50	Sgp90	
Superfamily	Selectin	Selectin	Ig	Ig	Ig	Ig	Ig	Sialomucin	Sialomucin	
Isoforms[b]	3	1	2	1	2	4	2	1	1	1
Modifications[c]	40%	55%	40%	55%	35%	40%	35% O-glycans, Sia	75% O-glycans, Sia, Fuc, Sulf	60% O-glycans, Sia, Fuc, Sulf	15%, Sia
MW[d]	140	110	90	65	120	130	65	50	90–105	90/170–180
Ligands[e]	PSGL-1	CLA ESL-1 PSGL-1	LFA-1	LFA-1	$\alpha4\beta1$, $\alpha4\beta7$	CD31	$\alpha4\beta7$ ($\alpha4\beta7$), ($\alpha4\beta1$) L-selectin	L-selectin	L-selectin	Non-L-selectin

[a] Data from human adhesion molecules are shown except for ESL-1, MAdCAM-1, and GlyCAM-1 (mouse).

- o CR-type module
- ▪ Cysteine-rich domain
- ▫ EGF-repeat
- ▾ Cleavage site
- ● Lectin-like domain
- ▣ Variant insertion site
- ▥ Metal-binding domains
- ıı Mucin-like domain
- ▨ Inserted domain
- ∩ Ig repeat
- ▭▭ Chicken fibroblast growth factor Receptor-like region

[b] Number of alternatively spliced isoforms reported so far.

[c] Percentage of posttranslational modifications = predicted MW of the protein core/observed MW. Functionally relevant oligosaccharides are indicated.

[d] Molecular weight of the predominant form under reducing conditions.

[e] Physiologically relevant glycoprotein species.

al., 1989; Subramaniam *et al.*, 1993). A longer lasting P-selectin induction is detectable *in vivo* and *in vitro* after cytokine (like TNF-α) stimulation (Hahne *et al.*, 1993; Weller *et al.*, 1992) and, at sites of chronic inflammation, sustained P-selectin expression is also seen (Grober *et al.*, 1993). These data indicate that different pools of P-selectin may exist in endothelial cells. P-selectin cloning revealed the characteristic lectin-like EGF and CR modules (Johnston *et al.*, 1989). The lectin domain of P-selectin contains the binding sites for carbohydrate ligands (Erbe *et al.*, 1993), although the exact face of this domain interacting with sialyl-Lewis^x (sLe^x) remains controversial (Revelle *et al.*, 1996). The lectin part has to be sufficiently extended from the plasma membrane to be functionally intact (Patel *et al.*, 1995). The EGF domain of P-selectin has also been proposed to directly determine the ligand-binding specificity (Kansas *et al.*, 1994). Among selectins P-selectin has the largest number of CR modules and their number differs between animal species (nine in man). Two variant forms of P-selectin have been reported: one lacks a CR-type repeat and the other lacks the transmembrane domain (Johnston *et al.*, 1990). P-selectin plays a prominent role in granulocyte tethering but lymphocytes can also adhere to it (Damle *et al.*, 1992; Moore and Thompson, 1992).

These three molecules, which all contain a similar domain organization and share 60–70% amino acid identity in the lectin domains, thus constitute a distinct adhesion molecule family. The genes of each selectin have been mapped into chromosome 1 (Watson *et al.*, 1990) and their exon–intron structure corresponds closely with the domain organization (Collins *et al.*, 1991; Johnston *et al.*, 1990; Ord *et al.*, 1990) suggesting that they may have arisen by gene duplication and exon shuffling. A colorful nomenclature of these molecules (see Tables 1A and 1B) was unified when they were designated selectins, which highlights their selective carbohydrate-binding properties (Bevilacqua *et al.*, 1991). They all play a critical role in initial binding (tethering) and subsequent rolling of cells under conditions of flow (Bevilacqua and Nelson, 1993; Lasky, 1995; McEver *et al.*, 1995; Rosen and Bertozzi, 1994; Ley and Tedder, 1995; Vestweber, 1993). Functionally, the most important domain of all selectins is the outermost lectin-like domain. However, the EGF- and CR-like modules not only serve as a stalk to extend the lectin domain from the plasma membrane, but they can also directly (by providing adhesive motifs) and indirectly (by affecting the conformation of the lectin domain) contribute to the ligand binding.

B. Sialomucins, Oligosaccharides, and Other Selectin Ligands

The lectin-like nature of selectins predicts that they have carbohydrate ligands. Several studies have demonstrated that lactosamine structures, phosphorylated mono- and polysaccharides, and sulfated polysaccharides

are recognition elements for selectins. Proteinaceous ligands of the selectins have been searched for concurrently, and only recently have these two lines of investigation started to converge on a unifying model of selectin–ligand interactions (Bevilacqua and Nelson, 1993; Feizi, 1993; Lasky, 1995; McEver *et al.*, 1995; Rosen and Bertozzi, 1994; Tedder *et al.*, 1995a; Varki, 1994; Vestweber, 1993).

PLN-specific vascular addressins (PNAds) defined by the MECA-79 mAb were the first characterized counterreceptors for lymphocyte L-selectin (Streeter *et al.*, 1988b). In normal adults, PNAd is predominantly expressed in HEVs in PLNs, whereas it is absent or abluminal at mucosal and other sites. During inflammation, however, PNAd is inducible at nonPLN tissues (Michie *et al.*, 1993; Salmi *et al.*, 1994). mAb MECA-79 defines an oligosaccharide-dependent epitope that can decorate at least six different protein scaffolds (Berg *et al.*, 1991a; Hemmerich *et al.*, 1994). PNAds are sialylated, fucosylated, and sulfated glycoproteins, and all these oligosaccharide modifications are required for efficient interaction with L-selectin (Berg *et al.*, 1991a; Hemmerich *et al.*, 1994; Imai *et al.*, 1991, 1992, 1993). The 50-kDa species of MECA-79 antigens incorporates avidly inorganic sulfate, which aided its purification from the conditioned medium from *in vitro*-labeled slices of mouse lymph nodes. Cloning and sequencing revealed that this PNAd species contains two mucin-like domains and approximately 75% of its mass is composed of O-linked oligosaccharides and, thus, it was designated glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) (Lasky *et al.*, 1992). Surprisingly, the GlyCAM-1 cDNA had no apparent consensus sequence enabling binding to plasma membrane and it actually appears to be a secreted molecule. The protein core of the 90-kDa form of the MECA-79 antigens proved to be identical with CD34 (Baumhueter *et al.*, 1993). mAb MECA-79 detects only specialized glycosylation variants of CD34 because CD34 is present in many types of endothelia and also on several nonendothelial cells (Baumhueter *et al.*, 1994; Krause *et al.*, 1996). On HUVECs, expression of CD34 is diminished by the same stimulators (e.g., IL-1, TNF-α), which cause upregulation of most other endothelial adhesion molecules (Delia *et al.*, 1993), but the relevance of this finding to the MECA-79 decorated forms of CD34 remains to be established. CD34 isolated from HEVs is the main component of PNAds that supports leukocyte rolling *in vitro* (Puri *et al.*, 1995). The largest 200-kDa sulfated glycoprotein of MECA-79 antigens appears to be the most important inducible species of PNAd at sites of inflammation (Hoke *et al.*, 1995). The molecular identity of the other PNAd species remains to be determined. The 60-kDa form of PNAd antigens, however, may be identical to mucosal cell adhesion molecule-1 (MAdCAM-1), which

has been shown to display MECA-79 epitopes (Berg *et al.*, 1993) (see Section III,E).

There are also multiple other glycoproteins and oligosaccharides that bind L-selectin. In HUVECs, an inducible, surface-expressed (Spertini *et al.*, 1991c) and another intracellular heparin-like (Norgard-Sumnicht *et al.*, 1993) L-selectin ligand have been proposed. In a rat HEV-like cell line multiple sulfated glycoprotein ligands of L-selectin have been defined (Tamatani *et al.*, 1993), but their identity with the mouse molecules remains to be determined. Furthermore, L-selectin binds to polylactosamine (sLe^x [NeuAcα2–3Galβ1-3(Fucα1-4)GlcNAc] and its isomer sLe^a [NeuAcα2–3Galβ1-4(Fucα1-3)GlcNAc], mannose-6-phosphate, fucoidan (sulfated polymer of fucose), heparin (sulfated polymer of GlcA-GlcNAc disaccharide), and certain sulfated lipids (e.g., sulfatide), although the affinities of these interactions are rather low (Berg *et al.*, 1992; Green *et al.*, 1992; Moore *et al.*, 1994; Nelson *et al.*, 1993; Yednock *et al.*, 1987; reviewed in Feizi, 1993; Rosen and Bertozzi, 1994; Varki, 1994).

E- and P-selectin have both shared and unique ligands. *In vitro*, both selectins bind to sLe^x, sLe^a, and their modifications, but interactions with E-selectin are of higher affinity and their requirements for a specific position and linkage of fucose and a negatively charged group (sialic acid or sulfate) are more stringent (Brandley *et al.*, 1993; Feizi, 1993; Larsen *et al.*, 1990; Lowe *et al.*, 1990; Phillips *et al.*, 1990; Polley *et al.*, 1991; Rosen and Bertozzi, 1994; Varki, 1994; Walz *et al.*, 1990). Both P-selectin and E-selectin bind in a carbohydrate-dependent manner to P-selectin glycoprotein ligand-1 (PSGL-1). It is a novel 240-kDa homodimeric transmembrane protein that was cloned by using an ingenious strategy of cotransfection of a fucosyltransferase with a cDNA expression library from a PSGL-1-positive cell line and screening for the P-selectin-dependent cell adherence (Sako *et al.*, 1993). PSGL-1 is a mucin-type molecule that contains 16 (or 15) repeats of short threonine-rich motifs in its extracellular part (Sako *et al.*, 1993; Veldman *et al.*, 1995). P- and E-selectin bind to different but partly overlapping areas in the N-terminal part of PSGL-1, but the affinity of the interaction between PSGL-1 and P-selectin is significantly higher (Larsen *et al.*, 1992; Moore *et al.*, 1994; Pouyani and Seed, 1995; Sako *et al.*, 1995; Wilkins *et al.*, 1995). Most of the P-selectin-dependent binding can be attributed to uncommon tyrosine sulfate modifications of PSGL-1 (Pouyani and Seed, 1995; Sako *et al.*, 1995; Wilkins *et al.*, 1995). PSGL-1 mediates leukocyte rolling both *in vitro* and *in vivo* (Alon *et al.*, 1994; Moore *et al.*, 1995; Norman *et al.*, 1995). Although most leukocytes including lymphocytes constitutively express PSGL-1, its affinity toward the selectin ligands is regulated because only a minor subpopulation

of total cell surface PSGL-1 can bind leukocytes (Moore *et al.*, 1995; Vachino *et al.*, 1995).

On the other hand, P-selectin and E-selectin also have independent ligands. E-selectin recognizes a glycoprotein called cutaneous lymphocyte antigen (CLA), which is found on all HEVs and a subpopulation of PBLs (Duijvestijn *et al.*, 1988; Picker *et al.*, 1991a). These memory PBLs are believed to be skin-seeking lymphocytes (Picker *et al.*, 1993a). The expression of CLA can be induced via an IL-12-dependent mechanism by bacterial superantigens (Leung *et al.*, 1995). CLA has oligosaccharide decorations related to, but not identical with, sLe^x that are crucial for its interaction with E-selectin (Berg *et al.*, 1991b). In keeping with its role as a selectin counterreceptor, CLA has been suggested to be involved in the rolling phase of lymphocyte–endothelial cell interaction (Alon *et al.*, 1994), but evidence for its participation in transmigration of lymphocytes through the HUVEC monolayer has also been presented (Santamaria-Babi *et al.*, 1995). At least on granulocytes, E-selectin also interacts with a 150-kDa variant of a fibroblast growth factor receptor (E-selectin ligand-1; ESL-1) in an N-linked glycan-dependent manner (Lenter *et al.*, 1994; Levinovitz *et al.*, 1993). ESL-1 is expressed on most cell types, but only properly fucosylated forms are functionally competent E-selectin ligands (Steegmaier *et al.*, 1995). On $\gamma\delta$ T cells another 250-kDa glycoprotein, whose possible similarity with PSGL-1 needs to be addressed, has been identified. It mediates binding to E-selectin in a sialic acid- and cation-dependent manner (Walcheck *et al.*, 1993). In contrast to granulocyte L-selectin, lymphocyte L-selectin does not appear to carry appropriate sLe^x-like modifications to be a ligand of E-selectin (Picker *et al.*, 1991b). The crystal model of E-selectin suggests that the sialic acid of its ligands binds to the cationic hydrophilic pocket and the fucose interacts with the calcium binding site (Graves *et al.*, 1994). The crystal structure also implies that the lectin domain contains only enough exposed critical residues for binding to a single sLe^x counterreceptor.

P-selectin has a unique 160-kDa myeloid glycoprotein ligand to which it binds in an N-linked oligosaccharide- and sialic acid-dependent manner (Lenter *et al.*, 1994). Finally, P-selectin, but not E-selectin, has been reported to interact with sulfated glycolipids (*O*-sulfoglucuronyl ceramide and sulfatides or 3-sulfated galactosyl ceramides) and sulfated polysaccharides (Aruffo *et al.*, 1991; Needham and Schaar, 1993; revieved in Feizi, 1993; Rosen and Bertozzi, 1994; Varki, 1994).

The physiological significance of different selectin ligands has remained unclear. The common feature appears to be a type 1 or 2 lactosamine backbone and, when present, sialic acid is in an α2–3 linkage and fucose in an α1–3 or α1–4 linkage. The location of fucose to sialic acid is also

critical (Feizi, 1993; Rosen and Bertozzi, 1994; Varki, 1994). It is evident that the presence of a suitable oligosaccharide as such is not enough for conferring binding capacity. sLe^x, for example, is expressed on most leukocytes on several different protein or lipid backbones, but all P-selectin binding activity resides on a small subpopulation of these molecules (Norgard *et al.*, 1993). The existence of these common sugar constituents in special clustered oligosaccharide patches (such as within sialomucin-like domains), however, can yield unique complex arrays of sugars from simple components. Physiologically relevant ligands of selectins are therefore likely to be glycoprotein molecules that contain abundant oligosaccharide decorations. The protein core can merely serve as a scaffold for sugars or make additional contacts with the ligand. In very general terms it appears that lymphocyte CLA interacts with E-selectin, lymphocyte PSGL-1 with P-selectin, and endothelial cell PNAds (and MAdCAM-1) bind to lymphocyte L-selectin. It also appears that unique as well as common receptors for each selectin exist and the avidity of each interaction will be decisive in enabling fine-tuning of the selectin-mediated phase of adhesion in different settings.

C. Chemoattractants and Their Receptors

After their initial binding to the vascular lining, lymphocytes need to undergo an activation step before they can firmly adhere to endothelial cells and penetrate the vessel wall (Butcher, 1991; Springer, 1994). The activation signals can come from chemoattractant molecules that are synthesized locally, trapped from the blood, or transported from the tissue parenchyma via a specialized fibroblastic reticular cell conduit system to the HEV (Anderson and Shaw, 1993). The ability of many chemoattractants to bind to endothelial cell proteoglycans may solve the problem of how these secreted molecules with short half-lives can be concentrated in effective amounts at the sites of lymphocyte exit (Tanaka *et al.*, 1993b).

A novel class of chemoattractive cytokines, called chemokines, is most relevant to lymphocyte adhesion (Baggiolini and Dahinden, 1994; Ben-Baruch *et al.*, 1995; Butcher, 1991; Oppenheim *et al.*, 1991; Schall and Bacon, 1994). These molecules are small (8–10 kDa) inducible heparin-binding proteins synthesized by several types of cells including leukocytes and endothelial cells. Three subgroups of chemokines can be distinguished on the basis of spacing of conserved cysteins. So called C–C chemokines (containing two conserved adjacent cysteine residues) are, in general, active toward mononuclear cells. In chemotactic assays certain C–C chemokines attract lymphocytes in a subpopulation-specific way. For example, RANTES (Schall *et al.*, 1990) and monocyte chemoattractant peptide-1 (MCP-1) (Carr *et al.*, 1994) preferentially enhance migration of memory

lymphocytes, and macrophage inflammatory peptide-1α (MIP-1α) enhances migration of B- and $CD8^+$ cells and MIP-1β that of CD4 helper cells (Schall *et al.*, 1993; Taub *et al.*, 1993a). In addition to MCP-1, MCP-2 and MCP-3 are also chemotactic for human lymphocytes (Taub *et al.*, 1995). The C–X–C chemokines (containing two cysteines separated by one amino acid) can be further divided into two branches based on the presence or absence of an ELR motif. These chemokines preferentially act on neutrophils, but interferon-inducible protein-10 (Taub *et al.*, 1993b) and IL-8 (Larsen *et al.*, 1989) have also been reported to be chemotactic for lymphocytes. However, their effects on T cells have been questioned (Roth *et al.*, 1995). A novel C chemokine called lymphotactin is a lymphocyte-specific chemoattractant (Kelner *et al.*, 1994), but it mainly affects the migration of immature cells such as thymocytes.

In addition to chemokines a variety of other molecules can modulate the migration of lymphocytes. Hepatic growth factor has been shown to cause directional migration of memory T cells (Adams *et al.*, 1994) and lymphocyte chemoattractant factor that of naive CD4-positive cells (Cruikshank *et al.*, 1994). In addition, several neuropeptides, such as vasoactive intestinal peptide and substance P, and metabolites of prostaglandin pathway can modulate lymphocyte migration (Oppenheimer-Marks *et al.*, 1994; Ottaway and Husband, 1994; Smith *et al.*, 1993; To and Schrieber, 1990). Also, acute-phase proteins such as serum amyloid A can be chemotactic for lymphocytes (Xu *et al.*, 1995). In contrast, lymphocytes lack surface receptors for classical chemoattractants, such as complement component C5a and platelet-activating factor, that play an important role in the activation of other types of leukocytes (reviewed in Springer, 1994).

Chemokines bind to seven transmembrane domain receptors expressed on lymphocytes (Ben-Baruch *et al.*, 1995; Kelvin *et al.*, 1993; Murphy, 1994). The third intracellular loop of these serpentine receptors interact with G-proteins. Ligand binding to these receptors is coupled to guanosine nucleotide exchange and results in dissociation of α subunit from $\beta\gamma$ G-protein subunits, which results in activation of phospholipase C with subsequent signaling effects. These data reconcile with the long known fact that treatment of lymphocytes with pertussis toxin does not interfere with their binding to HEVs but totally prevents lymphocyte emigration into the tissue parenchyma because $G\alpha i$ subunits are irreversibly inactivated by this toxin (Braaten *et al.*, 1984; Morse and Barron, 1970). Chemokine receptors can be found on HEVs as well. At least the Duffy blood group antigen, which can bind several chemokines, is expressed on endothelia (Hadley *et al.*, 1994), implying that it may immobilize these mediators on the vascular lining.

The functions of chemokines have mainly been defined in *in vitro* chemotaxis assays. There is only limited evidence that they indeed enhance lymphocyte binding to endothelial cells or cause accumulation of lymphocytes to sites of inflammation *in vivo* (Murphy *et al.*, 1994; Taub *et al.*, 1993a,b, 1996). Even in these cases, the formal proof that they function by modulating the activity of integrins has been lacking. Only recently has activation of leukocyte integrin function by formyl-methionyl-leucyl-phenylalanine (FMLP) in plasma cell transfectants expressing FMLP receptor been demonstrated. This integrin activation was mediated by a small GTP-binding protein ρ as a cytosolic signal transducer (Laudanna *et al.*, 1996), and ρ has been reported to mediate phorbol myristate acetate-induced lymphocyte function associated antigen-1 (LFA-1)-dependent lymphocyte aggregation (Tominaga *et al.*, 1993). On the other hand, it has been suggested that chemokines such as MCP-1 and RANTES are more important for the triggering of lymphocyte very late activation antigen-4 (VLA-4) and VLA-5 interactions with extracellular matrix component fibronectin than in the triggering of the stable binding to endothelial ligands vascular cell adhesion molecule-1 (VCAM-1) or intercellular cell adhesion molecule-1 (ICAM-1) (Carr *et al.*, 1996; Gilat *et al.*, 1994; Lloyd *et al.*, 1996). Furthermore, because the activation kinetics of integrins is extremely rapid and many lymphocytes express only low levels of chemokine receptors, other mechanisms of triggering of stable adhesion remain to be found. Thus, the role of chemokines may prove to be more critical to the later steps of the extravasation cascade, in particular to chemotactic and chemokinetic migration within the tissue stroma, than to the rapid activation of rolling lymphocytes on the endothelial lining.

D. Integrins

Integrins are versatile heterodimeric cell surface adhesion molecules that are composed of noncovalently associated α and β chains (Hynes, 1992). More than 20 integrins are currently known and they have been traditionally divided into subfamilies according to their β chains. The β1-integrin VLA-4, leukocyte integrins of the β2 family (LFA-1, Mac-1, and p150.95), and the β7 integrin $\alpha 4\beta 7$ have major functions in leukocyte–endothelial cell interactions (Arnaout, 1990b; Figdor *et al.*, 1990; Hemler *et al.*, 1990; Hu *et al.*, 1993; Larson and Springer, 1990; Lobb and Hemler, 1994; Stewart *et al.*, 1995). The α chains of all these integrins contain seven repeats of a homologous sequence, three of which are EF hand loop-like putative divalent cation-binding domains. In the extracellular part of the α chains of β_2 integrins there is an inserted I domain, an ~200-amino-acid motif homologous to those found, e.g., in von Willebrand factor and in certain collagens. The β chains of these integrins contain cysteine-

rich repeats that are probably involved in formation of internal disulfide bonds. Divalent cations, intact heterodimers, and an activation-dependent conformational shift are usually required to render integrins functionally competent (Hynes, 1992).

Of the β2 integrins, LFA-1 is most important for lymphocyte homing. LFA-1 is almost ubiquitously expressed on all normal lymphocytes, and the highest levels are seen on activated cells (Krensky *et al.*, 1983; Kürzinger *et al.*, 1981). It consists of a specific αL and a shared β2 chain (Kishimoto *et al.*, 1987b; Larson *et al.*, 1989; Law *et al.*, 1987; Sánchez-Madrid *et al.*, 1983). Ligand binding apparently occurs at the interface of α and β subunits at the globular head of LFA-1 (reviewed in Hynes, 1992). Both the I domain and cation-binding domains V and VI are involved in ICAM-1 binding (Randi and Hogg, 1994; Stanley *et al.*, 1994). Crystal structure of isolated I domain from Mac-1, and later from LFA-1, revealed that it contains a novel metal ion-dependent adhesion site (MIDAS motif), which corroborates well with the divalent cation dependency of the integrin function (Lee *et al.*, 1995; Qu and Leahy, 1995). Further sequence comparisons revealed that there may also be a MIDAS-like motif in the β2 chain. Ligation of LFA-1 can convey signals from the extracellular environment to the interior of the cells (outside in signaling) that result in cell activation (Keizer *et al.*, 1988; Pircher *et al.*, 1986; van Noesel *et al.*, 1988). Equally important, intracellular signals can alter LFA-1 function and thus cellular behavior (inside out signaling) (Patarroyo *et al.*, 1985; Rothlein and Springer, 1986). Activation of LFA-1 is accompanied by a conformational shift that results in the exposure of cryptic activation-dependent epitopes, as exemplified by mAb 24 (Dransfield and Hogg, 1989), and an increase in the binding affinity (Dustin and Springer, 1989; van Kooyk *et al.*, 1989). The cytoplasmic tail of LFA-1 is linked to actin filaments of the cell cytoskeleton (Sharma *et al.*, 1995) and its phosphorylation can regulate the activity of the integrin (Hibbs *et al.*, 1991b). Lymphocytes express only low levels of Mac-1 and p150.95 (Landay *et al.*, 1983; Uciechowski and Schmidt, 1989); hence, it is likely that these β2 integrins do not play a major role in the lymphocyte–endothelial cell adherence. It should be remembered, nevertheless, that on granulocytes, 10% of all surface Mac-1 molecules harbor more than 90% of the functional activity (Diamond and Springer, 1993). The most recent member of the β2 family, αDβ2 (Danilenko *et al.*, 1995), is expressed at low levels on most PBLs in man and it can bind ICAM-1 but it appears to be more critically involved in macrophage functions (Van der Vieren *et al.*, 1995).

The α4 integrin subunit can pair with two different β chains. Initially, an α4 chain associated with an unknown non-β1 subunit (designated βP) was shown to constitute an integrin called murine lymphocyte Peyer's

patch HEV adhesion molecule-1, which directs lymphocyte trafficking to mucosal sites (Holzmann *et al.*, 1989). Cloning of the β chain revealed that it is a mouse homolog of human β7 (Hu *et al.*, 1992). α4β7 integrin is present at low levels on resting PBLs, although significantly increased synthesis is seen in activated cells, in lymphocytes within mucosa-associated tissues, and in PBLs with hallmarks of gut tropism (Erle *et al.*, 1994; Lazarovits *et al.*, 1984; Postigo *et al.*, 1993a; Schweighoffer *et al.*, 1993). β7 integrins are constitutively expressed in a partially active state that is dependent on the terminal residues of the cytoplasmic tail of β7 (Crowe *et al.*, 1994). The alternative pairing of α4 with β1 (Hemler *et al.*, 1987a; Takada *et al.*, 1989) also produces an integrin that has been implicated in mucosal homing (Holzmann and Weissman, 1989). α4β1 is the most abundant β1 integrin on PBLs and, notably, it is absent from neutrophils (Hemler *et al.*, 1987b; Sánchez-Madrid *et al.*, 1986). Expression of α4β1 integrin is upregulated on activated lymphocytes. In biochemical analyses α4 is present as a predominant 150-kDa chain (Hemler *et al.*, 1987b), but it appears to be physiologically synthesized as a 180-kDa form, which binds best to its ligands (Pujades *et al.*, 1996). α4 chain also has a unique intramolecular cleavage site that results in the cleavage of the 150-kDa chain into 70- and 80-kDa fragments but does not affect its adhesive functions (Teixidó *et al.*, 1992). The functionally critical domains of α4 are the divalent cation binding sites (Masumoto and Hemler, 1993) and a short, more N-terminal sequence to which most inhibitory anti-α4 mAbs map (Schiffer *et al.*, 1995). α4 has also been found on endothelial cells (Altevogt *et al.*, 1995). Therefore, it may be relevant that the 80-kDa α4 fragment of α4β1 and α4β7 can bind in a homotypic manner to α4 via an area that overlaps the other ligand recognition motifs (Altevogt *et al.*, 1995).

αEβ7, α5β1, α6β1, and αvβ3 are other integrins that have been implicated in lymphocyte homing. αEβ7 heterodimer is prominently expressed on intraepithelial lymphocytes but also on other mucosal lymphocytes and it is present on a minor subpopulation of PBLs as well (Cerf-Bensussan *et al.*, 1987, 1992; Kilshaw and Murant, 1990; Shaw *et al.*, 1994). It is the first integrin that has been found to bind to a cadherin (E-cadherin) counterreceptor (Cepek *et al.*, 1994). It is intriguing to speculate that αEβ7 may be involved in lymphocyte targeting to mucosal sites, but no experimental evidence is available to support this hypothesis. In fact, the inability of αEβ7 to bind to mucosal addressin MAdCAM-1 and lack of correlation between αEβ7 expression and mucosal homing argue against this possibility (Austrup *et al.*, 1995; Strauch *et al.*, 1994). More likely, αEβ7 secures the binding of intraepithelial lymphocytes to epithelial cells after they have first found their way to the epithelium via other adhesion

mechanisms. Lymphocyte $\alpha5\beta1$ has been suggested to participate in binding to endothelial cells because an anti-$\alpha5$ function-blocking mAb diminishes the adherence between human lymphocytes and rat-derived high endothelial type cells (Szekanecz *et al.*, 1992). Evidence for the $\alpha6\beta1$ as an endothelial cell adhesion molecule stems from the fact that an anti-$\alpha6$ mAb inhibits lymphocyte binding to mouse endothelioma cells (Imhof *et al.*, 1991; Lenter *et al.*, 1993; Ruiz *et al.*, 1995). Finally, $\alpha v\beta3$, which is expressed on activated lymphocytes (as well as on endothelial cells), may be involved in heterophilic recognition of the endothelial cell adhesion molecule CD31 (Piali *et al.*, 1995b).

E. Immunoglobulin Superfamily

Four intercellular adhesion molecules, ICAM-1, -2, -3, and -4, have been defined at the molecular level (Bailly *et al.*, 1994; Carlos and Harlan, 1994; Hogg and Landis, 1993; Springer, 1994), and telencephalin (Yoshihara *et al.*, 1992) may well be the fifth member of this family. ICAM-1 is constitutively expressed on different types of endothelia and its synthesis is markedly induced 12–20 hr after stimulation by proinflammatory cytokines such as IL-1 and TNF-α (Dustin *et al.*, 1986). ICAM-1 contains five C2-type Ig domains in the extracellular part (Simmons *et al.*, 1988; Staunton *et al.*, 1988). ICAM-1 was first shown to bind to LFA-1 (Marlin and Springer, 1987). The N-terminal domains are critical in adhesion: LFA-1 binds predominantly to the first domain of ICAM-1 and Mac-1 to the third domain, although residues from other nearby domains also contribute to the active sites (Diamond *et al.*, 1991). Glycosylation of ICAM-1 affects the ligand-binding specificity because deglycosylated forms show enhanced interaction with Mac-1 but not with LFA-1 (Diamond *et al.*, 1991). Dimerization of ICAM-1 is also critical to the high-avidity binding to LFA-1 (Miller *et al.*, 1995; Reilly *et al.*, 1995). The function of ICAM-1 can be further modulated by the secretion of a tailless soluble splice variant of the molecule (Wakatsuki *et al.*, 1995). ICAM-1 has also been reported to bind CD43 (Rosenstein *et al.*, 1991) (but this observation has not been confirmed by others [de Fougerolles *et al.*, 1994]) and hyaluronan (McCourt *et al.*, 1994).

ICAM-2, on the other hand, is constitutively expressed on endothelial cells and its expression cannot be upregulated *in vitro* with inflammatory mediators (de Fougerolles *et al.*, 1991; Nortamo *et al.*, 1991a,b). It contains only two Ig-like domains that are closely homologous to the two most N-terminal domains of ICAM-1 (Staunton *et al.*, 1989). Consequently, ICAM-2 can also bind to LFA-1 (de Fougerolles *et al.*, 1991). Evidence has been presented both for and against interaction of ICAM-2 with Mac-1 (Diamond *et al.*, 1990; Li *et al.*, 1993). Binding of LFA-1 to ICAM-2 is

probably important in the basal level of adhesion of lymphocytes to endothelial lining, whereas the relative contribution of the LFA-1–ICAM-1 interaction is more pronounced at sites of inflammation. Both ICAM-1 and -2 are synthesized by activated lymphocytes (de Fougerolles *et al.*, 1991; Rothlein *et al.*, 1986), but their possible roles in binding to endothelium remain to be determined.

ICAM-3 (CD50) is predominantly expressed on leukocytes (de Fougerolles and Springer, 1992; Juan *et al.*, 1993) and it may modulate the activity of LFA-1 toward ICAM-1 (Campanero *et al.*, 1994). In contrast to ICAM-1 and -2, ICAM-3 is not present on HEV, is very rarely found in certain inflamed endothelium, and is conspicuously expressed only on tumor endothelium (de Fougerolles and Springer, 1992; Patey *et al.*, 1996). ICAM-4 is identical to Landsteiner–Wiener blood group antigen and is only present on erythrocytes (Bailly *et al.*, 1994, 1995). Because it can bind LFA-1, it can theoretically modulate interaction of LFA-1 with endothelial cell ICAMs, but its role, if any, in lymphocyte recirculation has not been determined.

VCAM-1 is a ligand of the $\alpha 4$ integrins (Elices *et al.*, 1990; Postigo *et al.*, 1993b). Although VCAM-1 was initially reported to be expressed in HEV and in dendritic cells (Rice *et al.*, 1990), subsequent analyses have revealed very low levels of endothelial expression *in vivo* at any sites studied under normal or inflammatory settings (Rice *et al.*, 1991). In contrast, VCAM-1 can be readily induced by cytokines on HUVECs and maximal expression is seen 20 hr after stimulation (Osborn *et al.*, 1989; Wellicome *et al.*, 1990). VCAM-1 has also been found on certain lymphocytes (Leca *et al.*, 1995). A six-domain form of VCAM-1 was first cloned in man (Osborn *et al.*, 1989), but the most prevalent form of VCAM-1 has seven Ig-like domains (Cybulski *et al.*, 1991; Polte *et al.*, 1990). Additional eight-domain and glycophosphatidylinositol-anchored three-domain VCAM-1 variants have been found in animals (Cybulski *et al.*, 1991; Moy *et al.*, 1993; Terry *et al.*, 1993). Domains 1 and 4 of VCAM-1 (seven-domain form) are highly homologous and constitute the binding site for $\alpha 4$ integrins (Osborn *et al.*, 1992; Pepinsky *et al.*, 1992), suggesting that two $\alpha 4$ integrins can bind to one VCAM-1 molecule. The crystal structure of the first two domains of VCAM-1 confirms that the predicted integrin binding area is well exposed on the surface of domain 1 (Wang *et al.*, 1995).

$\alpha 4$ integrins also bind to MAdCAM-1. MAdCAM-1 was the first tissue-specific endothelial addressin reported and it mediates lymphocyte binding to gut-associated lymphatic tissues (Nakache *et al.*, 1989; Streeter *et al.*, 1988a). The extracellular part of mouse MAdCAM-1 is constituted of two C2-type Ig-like domains, a sialomucin-type domain, and a third Ig-like motif that is homologous to the constant region of IgA1 (Briskin *et al.*,

1993). MAdCAM-1 also exists as a shorter variant that lacks the mucin-like and IgA-like domains but retains the two most N-terminal domains important for ligand binding (Briskin *et al.*, 1996; Sampaio *et al.*, 1995). Human MAdCAM-1 resembles its mouse homolog but it lacks the IgA-like motif, has much longer mucin-like sequence in the extracellular part of the molecule, and has a longer cytoplasmic tail (Shyjan *et al.*, 1996). MAdCAM-1 is a glycosylated ~60-kDa molecule that is preferentially expressed in HEVs in Peyer's patches and in flat-walled vessels in lamina propria (Streeter *et al.*, 1988a). In cultured brain endothelioma cells it is inducible with TNF-α, IL-1, and LPS (Sikorski *et al.*, 1993). $\alpha 4\beta 7$ binding to MAdCAM-1 does not require prior activation of the integrin (Berlin *et al.*, 1993; Briskin *et al.*, 1996). $\alpha 4\beta 1$ has been variously reported either not to interact or to interact weakly with MAdCAM-1 (Berlin *et al.*, 1993; Strauch *et al.*, 1994). In addition, MAdCAM-1 isolated from mucosal tissue, but not from transfectants, can also display functionally relevant MECA-79 epitopes (Berg *et al.*, 1993), although this has not been confirmed in all studies (Hemmerich *et al.*, 1994). These findings indicate that both $\alpha 4$-containing integrins and L-selectin can mediate initial contacts between lymphocytes and MAdCAM-1 in gut-associated lymphatic tissues. At least four distinct sites on the extracellular matrix molecule fibronectin can also interact with integrin $\alpha 4$. Binding to the connecting segment-1 (CS-1)-containing form has the highest affinity, but the lumenal location of fibronectin isoforms and the physiological significance of these interactions remain elusive (Elices *et al.*, 1990, 1994; Lobb and Hemler, 1994).

CD31 is an Ig family member that is constitutively expressed on a subpopulation of T lymphocytes (predominantly naive $CD8^+$ cells) and in continuous endothelium of all vessel types, thus serving as a useful endothelial marker (DeLisser *et al.*, 1994b; Muller *et al.*, 1989). Evidence has been presented that CD31 expression on endothelial cells can be downregulated by inflammatory cytokines TNF-α and IFN-γ (Stewart *et al.*, 1996). Expression of CD31 is conspicuously concentrated at the intercellular junctions of endothelial cells (Albelda *et al.*, 1990; Muller *et al.*, 1989). Evidence has accumulated that CD31 is critical to the extravasation of leukocytes. At least in monocyte models, CD31 on both the leukocyte and the endothelial surface is required for successful transmigration through the endothelial cell layer *in vitro* and *in vivo* (Bogen *et al.*, 1994; Muller *et al.*, 1993). These findings have been reproduced with lymphocytes (Zocchi *et al.*, 1996). CD31 has six extracellular Ig-like domains (Newman *et al.*, 1990; Simmons *et al.*, 1990; Stockinger *et al.*, 1990). Homotypic cation-independent interaction has been reported to be dependent on the Ig-like domains of 2–3 or 5–6 of CD31 (Fawcett *et al.*, 1995). Other areas, like the glycosaminoglycan binding region in the second Ig domain,

contribute to the heterophilic cation-dependent binding (DeLisser *et al.*, 1993). In leukocyte migration, the two N-terminal Ig domains mediate the transendothelial migration, whereas the membrane-proximal domain 6 is important for subsequent movement in the extracellular matrix (Liao *et al.*, 1995). CD31 may also interact in a cation-dependent heterotypic manner with $\alpha v\beta 3$ (Piali *et al.*, 1995b).[2] The cytoplasmic tail further modulates the ligand specificity because it governs the intercellular localization of CD31 and can regulate the homophilic/heterophilic nature of CD31-mediated adhesion (DeLisser *et al.*, 1994a). Several shorter variants of CD31 have been described: In man two isoforms are lacking one exon (exon 13 or 14) from the cytoplasmic tail (Kirschbaum *et al.*, 1994; Newman *et al.*, 1990) and one is missing the transmembrane domain (Goldberger *et al.*, 1994). In mouse, at least six other isoforms of CD31 have been described (Baldwin *et al.*, 1994). Moreover, CD31 can affect lymphocyte binding to endothelium indirectly because cross-linking of CD31 induces an increase in the ligand-binding activity of integrins VLA-4, -5, and -6 (Tanaka *et al.*, 1992).

Immunoglobulin superfamily member LFA-3 (CD58) is constitutively expressed on endothelium and HLA class II is induced in activated endothelial cells. Both these molecules have been implicated in lymphocyte–endothelial adherence (Hughes *et al.*, 1990; Manolios *et al.*, 1988; Masuyama *et al.*, 1986), but elucidation of their definite roles requires further studies.

F. Proteoglycans

Proteoglycans are defined as proteins to which large glycosaminoglycan side chains with repeating oligosaccharide sequences are covalently linked (Hardingham and Fosang, 1992; Ruoslahti, 1988). Although proteoglycans, especially heparan sulfate-containing species, largely cover the luminal surface of HEVs, the role of individual molecules in lymphocyte binding has remained poorly defined (Ihrcke *et al.*, 1993). Collectively, negatively charged proteoglycans render the apical surface of the endothelial cell antiadhesive due to repulsion. However, proteoglycans can also serve as a docking site for certain proadhesive cytokines or for soluble adhesion molecules and also possibly directly offer complex carbohydrate recognition motifs to blood-borne lymphocytes.

CD44 is the best studied proteoglycan in respect to lymphocyte recirculation (Haynes *et al.*, 1989; Koopman *et al.*, 1993; Lazaar and Puré, 1995;

[2] It appears that Ig domains 1 and 2 mediate the homophilic adhesion of CD31. It is also possible that CD31 only mediates homophilic binding and that the heterophilic adhesion patterns observed are due to triggering of other adhesion pathways via CD31 (Sun, J., *et al.*, 1996; Sun, Q.-H., *et al.*, 1996).

Lesley *et al.*, 1993). It is found at high levels on mature lymphocytes, on non-HEV endothelia, as well as on most other cells (Carter and Wayner, 1988; Dalchau *et al.*, 1980; Isacke *et al.*, 1986; Jalkanen *et al.*, 1986b; Letarte *et al.*, 1985; Picker *et al.*, 1989; Telen *et al.*, 1983). It had initially been implicated in directing lymphocyte binding to HEVs because a certain anti-CD44 mAb inhibits lymphocyte adherence in Stamper–Woodruff assay (Jalkanen *et al.*, 1986b). CD44 has also been reported to bind to MAdCAM-1 (Picker *et al.*, 1989), although the interpretation of this finding has been questioned (Berlin *et al.*, 1993). On the other hand, CD44 has been proposed to play a role in leukocyte trafficking to extralymphoid sites of inflammation or in serving a more general role in non-tissue-specific adhesion strengthening, lymphocyte activation, and/or signaling (Aruffo *et al.*, 1990; Camp *et al.*, 1993; Denning *et al.*, 1990; Koopman *et al.*, 1990; Miyake *et al.*, 1990). Evidence has been proposed indicating that CD44 may directly mediate lymphocyte rolling on endothelial hyaluronate (DeGrendele *et al.*, 1996). The cloning of CD44 revealed an extracellular distal domain homologous to the cartilage proteoglycan core and link proteins, and a membrane proximal mucin-type region (Goldstein *et al.*, 1989; Stamenkovic *et al.*, 1989a). The N-terminal domain of CD44 binds to hyaluronan, which is a widely distributed extracellular matrix molecule (Aruffo *et al.*, 1990). Both the abundant O- and N-linked oligosaccharide modifications of CD44 appear to modulate its capacity to bind to hyaluronan (Bennett *et al.*, 1995a,b; Lesley *et al.*, 1995). The membrane proximal region of lymphocyte CD44 can be decorated by heparan and chondroitin sulfate glycosaminoglycans that mediate binding to fibronectin (Jalkanen and Jalkanen, 1992; Jalkanen *et al.*, 1988). Collagen types I and IV are also recognized by CD44 (Carter and Wayner, 1988). In addition, CD44 binds to a 600-kDa heparin sulfate ligand, serglycin, which is secreted by leukocytic cells (Toyama-Sorimachi *et al.*, 1995). Chemokines (MIP-1β and osteopontin) and growth factors (bFGF) have also been demonstrated to interact with CD44 (Bennett *et al.*, 1995b; Tanaka *et al.*, 1993a; Weber *et al.*, 1995) presumably via the oligosaccharide-rich membrane proximal area. The enormous structural diversity of CD44 arises from the ability of cells to choose among a large number of mRNA splice options and from further modifications by glycosylation. At least 10 variant exons can be inserted into the membrane proximal part of CD44, and there are two different variants of the cytoplasmic tail as well (Günthert *et al.*, 1991; Screaton *et al.*, 1992). However, the effects of the different isoforms on adhesive functions largely remain to be resolved (see Section IV,C).

G. Other Homing-Associated Molecules

A number of other molecules have also been implicated in lymphocyte adherence to endothelial cells. Vascular adhesion protein-1 (VAP-1) is a

homodimeric sialoglycoprotein composed of two 90-kDa subunits. It is more dominantly expressed (Fig. 2) in peripheral lymph node-type than in mucosal HEVs (Salmi and Jalkanen, 1992). VAP-1 seems to be stored within intracellular granules, from which it is rapidly translocated to the endothelial cell surface at sites of inflammation (Salmi *et al.*, 1993). Such inflammation sites include skin, gut, tonsil, and synovium, where VAP-1 is functional in mediating lymphocyte binding to the vascular endothelium (Arvilommi *et al.*, 1996; Salmi *et al.*, 1993). Mediators responsible for the upregulation of VAP-1 are unknown. Sialic acids are of fundamental importance for the function of VAP-1 because the nonsialylated form of this molecule no longer supports lymphocyte binding (Salmi and Jalkanen, 1996). Based on this carbohydrate dependence of binding and function under conditions of shear, VAP-1 is most likely involved relatively early in the adhesion cascade. Thus, VAP-1 extends the role of carbohydrate-dependent lymphocyte–endothelial cell interactions beyond the selectins. The lymphocyte counterreceptor of VAP-1 is currently unknown. However, VAP-1 does not use L-selectin or $\alpha 4$ integrins as its counterreceptors. We have shown that VAP-1 is able to mediate HEV binding of L-selectin-negative cells (Salmi and Jalkanen, 1996).

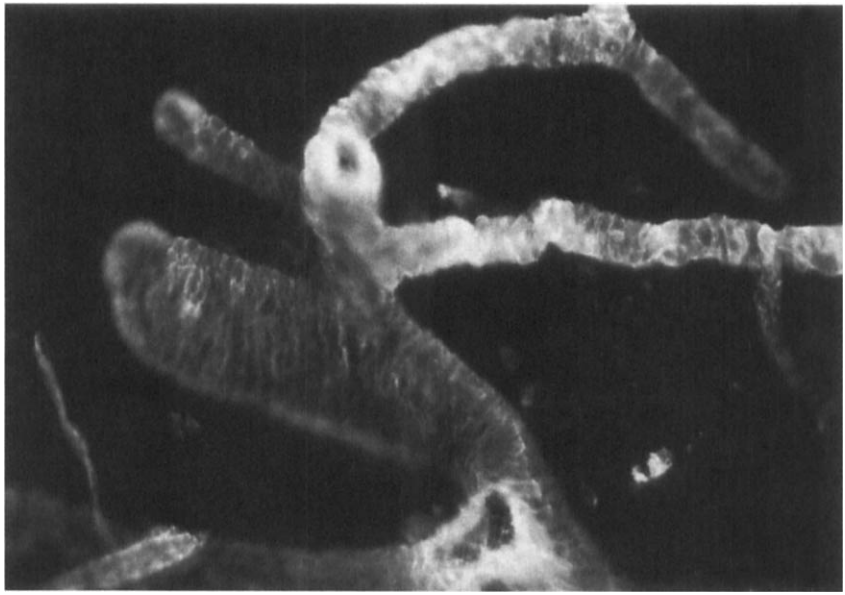


FIG. 2. Expression of VAP-1. Vessels were identified in a tonsil section using an immunofluorescence staining for VAP-1. Note the tortuous architecture of the vascular tree with many abrupt branchings.

CD73 or 5′-ectonucleotidase catalyzes conversion of extracellular adenosine monophosphate to adenosine (Resta *et al.*, 1993; Zimmerman, 1992). It was originally described as a differentiation marker for maturating B and T cells and later as an accessory molecule in T cell activation (Thompson *et al.*, 1989). On lymphocytes, it is preferentially expressed on $CD8^+$ T cells and B cells (Thompson *et al.*, 1987). Also, vascular endothelium in various organs expresses CD73 as do follicular dendritic cells in germinal centers (Airas *et al.*, 1993). It has been shown to mediate lymphocyte binding to cultured endothelial cells and to vascular endothelium in inflamed skin (Airas *et al.*, 1993; Arvilommi *et al.*, 1996). Whether the enzymatic activity of CD73 is involved in lymphocyte adherence to endothelium or whether the binding is a separate phenomenon independent of the enzymatic activity remains to be determined.

CD40, a member of the nerve growth factor receptor family, is expressed on several cell types (Durie *et al.*, 1994; Stamenkovic *et al.*, 1989b). Endothelial cells in many normal tissues express CD40 (Karmann *et al.*, 1995). The amount of endothelial CD40 has been shown to increase in response to several inflammatory mediators such as IFN-γ. Moreover, binding of endothelial CD40 to its ligand (CD40L) causes upregulation of E-selectin, VCAM-1, and ICAM-1; therefore, CD40 has been suggested to participate in T cell accumulation at sites of inflammation by acting as a signaling molecule (Hollenbaugh *et al.*, 1995; Karmann *et al.*, 1995; Yellin *et al.*, 1995).

Still other molecules have been indicated to mediate lymphocyte–endothelial cell interaction. One such molecule is CD38, which is a pleiotropic molecule with ADP-ribosyl cyclase activity (Howard *et al.*, 1993; Jackson and Bell, 1990). It has been shown to form an adhesion molecule pair with a 120-kDa endothelial cell ligand (Deaglio *et al.*, 1996; Dianzani *et al.*, 1994). Another example is a 85-kDa mAb HAL 1/13-defined molecule, which binds both neutrophils and lymphocytes. The binding takes place in normoxic conditions but it markedly increases in a hypoxic environment, which induces expression of this molecule (Ginis *et al.*, 1995).

IV. Regulation of Adhesion Molecule Expression and Function

During the past few years it has become increasingly evident that control of adhesion molecule activity takes place at several levels (Butcher, 1991; Carlos and Harlan, 1994; Springer, 1994). Although the expression of a given molecule is a prerequisite for its function, it is not always sufficient. In fact, many adhesion molecules appear to be physiologically expressed in inactive forms that require a particular activation step for induction of qualitative changes in affinity and avidity.

Elucidation of the genomic structures of many adhesion molecules has made it possible to study the transcriptional mechanisms that control the expression of these genes. These analyses start to cast light on the molecular mechanisms by which cytokines and other regulators modulate the synthesis of adhesion molecules. Moreover, they have revealed how adhesion molecules can be differentially regulated in different cell types. For instance, in resting endothelial cells the transcription of VCAM-1 is prevented by two octamer binding recognition elements that function as silencers. In TNF-α-stimulated endothelial cells two GATA elements and two NF-κB binding sites were shown to be important positive regulators of expression (Neish *et al.*, 1992). In contrast, in muscle cells a position-specific enhancer, which is not active in endothelial cells, overrides the activity of silencers and NF-κB sites leading to constitutive, TNF-α unresponsive expression of VCAM-1 (Iademarco *et al.*, 1993). Moreover, it has been demonstrated that even different types of endothelial cells utilize different transcriptional control mechanisms because in HUVEC binding of NF-κB to its binding site in response to IL-1α results in enhanced transcription of VCAM-1, whereas this effect is attenuated in dermal endothelial cells by an upstream regulatory element (Gille *et al.*, 1996).

Because analyses of the basic transcriptional and posttranscriptional gene regulatory mechanisms do not yet, in most cases, allow an understanding of cell type-specific regulation of adhesion molecule function, we will hereafter employ a more descriptive division to illustrate some of the best known examples.

A. Ontogenic Regulation

The expression of many adhesion molecules changes during the development of the host (Picker and Butcher, 1992). HEVs are first encountered in lymph nodes only shortly after small lymphocytes populate these organs during embryonic development (Miller, 1969). The phenotype of these early HEVs is, however, quite distinct from that of their adult counterparts. For example, HEVs in PLNs express MAdCAM-1 but lack PNAds during fetal development. During the first weeks of neonatal life, MAdCAM-1 disappears and PNAd appears in PLN HEVs. Thus, widespread MAdCAM-1 expression may serve to disperse neonatally acquired mucosal immunity for a more general use. Apparently, as yet unidentified ontogenically regulated tissue-specific factors turn on and off expression of addressins because, in transplantation studies, the phenotype of lymph node HEVs in adult animals is generally not affected by the new local microenvironment (Mebius *et al.*, 1993). Moreover, continuous lymph flow or exposure to antigens is a prerequisite for maintenance of HEV morphology as well as for expression and function of adhesion molecules (Mebius *et al.*, 1991).

A developmental shift in the expression of CD44 and L-selectin on T cells is another well-studied example of the periodical regulation of adhesion molecules. In man, $CD44^+$ and L-selectin-negative stem cells in bone marrow give rise to $CD44^+$, L-$selectin^+$ prothymocytes that lose these antigens upon arrival into thymus, gradually reaquire them during thymic maturation and T cells entering the blood display a mature $CD44^+$L-$selectin^+$ phenotype, which is further modulated by activation and memory cell transition (Horst *et al.*, 1990a,b; Lesley *et al.*, 1993; Picker *et al.*, 1993b; Terstappen *et al.*, 1992). These data have led to speculation on the contribution of CD44 and L-selectin in lymphocyte homing to all three categories of lymphoid tissues.

B. Upregulation and Induction

The expression of many endothelial adhesion molecules is mainly regulated by quantitative alterations in surface expression (Carlos and Harlan, 1994; Pober and Cotran, 1990). Most are sparse or absent under physiological conditions but can be readily induced or upregulated by inflammatory agonists. IL-1, TNF-α, and LPS are typical promiscuous inducers and elevate ICAM-1, VCAM-1, and E-selectin levels in endothelial cells. Although in each case *de novo* protein synthesis is required, the kinetics of the responses differ. E-selectin expression is upregulated in as little as 2 hr, whereas maximal ICAM-1 and VCAM-1 synthesis takes place after 10–20 hr when E-selectin expression is already declining (Wellicome *et al.*, 1990). In contrast, other cytokines display more "specific" effects. For example, IL-4 and IL-13 induce synthesis of VCAM-1, but not that of E-selectin or ICAM-1 (Masinovsky *et al.*, 1990; Sironi *et al.*, 1994; Thornhill and Haskard, 1990); IFN-γ upregulates ICAM-1 synthesis but has no effect on E-selectin or VCAM-1 (Carlos *et al.*, 1990); and IL-3 induces both E-selectin and P-selectin (Brizzi *et al.*, 1993; Khew-Goodall *et al.*, 1996), whereas C5a promotes only P-selectin expression (Foreman *et al.*, 1994). Moreover, combinations of cytokines may exert additive/synergistic or antagonistic effects. For instance, TNF-α + IFN-γ increases synthesis of E-selectin (Doukas and Pober, 1990; Leeuwenberg *et al.*, 1990) and TNF-α + IL-4 augments VCAM-1 but inhibits E-selectin and ICAM-1 expression (Masinovsky *et al.*, 1990; Thornhill and Haskard, 1990). These examples illustrate how the selectivity of cytokines in the induction of adhesion molecules varies and how the net effect of the myriad of cytokines released in the setting of inflammation is difficult to predict. Surprisingly, the effect of the cytokines involved in Th1 vs Th2 immune response on the regulation of lymphocyte homing has remained poorly characterized.

The regulatory effects of cytokines have been mostly studied in the HUVEC model, but inflammatory mediators have been shown to exert

local or systemic effects in *in vivo* models as well (Briscoe *et al.*, 1992; Silber *et al.*, 1994a; Weller *et al.*, 1992). However, caution in the interpretation of HUVEC studies is warranted because different microvascular beds seem to respond differently, emphasizing the marked heterogeneity of endothelial cells (Bicknell, 1993; Haraldsen *et al.*, 1996; Swerlick *et al.*, 1992b). On isolated dermal endothelial cells, for example, TNF-α, but not IL-1, induces VCAM-1 synthesis (Swerlick *et al.*, 1992a), and on synovial endothelial cells TNF-α and IL-1 poorly upregulate ICAM-1 expression (Gerritsen *et al.*, 1993). Moreover, although, e.g., both VCAM-1 and E-selectin are readily upregulated by IL-1 on HUVECs, only E-selectin corresponds analogously after an intradermal injection of IL-1, whereas only modest induction of VCAM-1 is detectable *in situ* (Fries *et al.*, 1993; Munro *et al.*, 1989, 1991). Also, arterial and venous endothelium appear to regulate the expression of adhesion molecules in a partially different manner because, for instance, TNF-α induces VCAM-1 expression in iliac venous but not on arterial cells (Hauser *et al.*, 1993).

Agonist-stimulated translocation of preformed adhesion molecules is a rapid way of altering the adhesive status of a cell. For instance, P-selectin stored in granules within the endothelial cell can be redistributed onto the cell surface within a few minutes after stimulation with thrombin, histamine, calcium ionophores, and complement components (Hattori *et al.*, 1989; McEver *et al.*, 1989). Lymphocytes do not contain abundant storage granules, but in phagocytes Mac-1 can be rapidly mobilized onto the cell surface from intracellular storages (Bainton *et al.*, 1987), which, however, is neither necessary nor sufficient for Mac-1-dependent adhesion (Vedder and Harlan, 1988).

In addition to mitogens and cytokines, oxygen radicals and lipids can also modulate the expression of endothelial adhesion molecules. For instance, superoxide induces P-selectin expression (Gaboury *et al.*, 1994) and lysophospahtidylcholine can upregulate the synthesis of VCAM-1 (Kume *et al.*, 1992). These compounds may explain the altered leukocyte trafficking observed in vascular diseases such as ischemia–reperfusion and atherosclerosis.

Mechanostimulation of endothelial cells regulates adhesion protein expression as well. For example, ICAM-1 is inducible by applying a constant shear stress to HUVEC monolayers (Nagel *et al.*, 1994). This mode of regulation may prove particularly relevant to the vascular system, which is continuously exposed to shear forces generated by the flowing blood. Due to the heterogeneity of flow conditions within the vascular tree caused by changes in turbulence and vessel caliber, mechanostimulation may also contribute to the differential phenotype of venous and arterial endothelium (Hauser *et al.*, 1993; Ley and Gaehtgens, 1991).

Expression of a given adhesion molecule can be regulated in a complex manner. In P-selectin, for example, there appears to be three distinct modes of enhanced expression. A very rapid translocation of preformed P-selectin from Weibel–Palade bodies is induced by, e.g., histamine and thrombin and is maximal at 10 min (Hattori *et al.*, 1989; McEver *et al.*, 1989). A slower upregulation peaking at 2–5 hr is seen after stimulation of endothelial cells by oxygen radicals, TNF-α, and LPS (Gaboury and Kubes, 1994; Hahne *et al.*, 1993; Luscinskas *et al.*, 1995; Weller *et al.*, 1992). A third, long-lasting peak of P-selectin expression that only starts 18 hr after stimulation is seen by IL-3 stimulation of HUVECs and is dependent on new protein synthesis (Khew-Goodall *et al.*, 1996).

C. Alternative Splicing

Enormous structural heterogeneity can be achieved from a single gene by alternative splicing of the set of exons available (Singer and Berg, 1991). Apart from the requirement for acceptor and donor sites, little restriction appears to limit the alignment of any combination of exons. By adding or deleting pieces of amino acid sequence, the functional properties of the protein can be cost-effectively modulated. The CD44 gene, for instance, contains 20 exons, from which at least 12 can be alternatively spliced (Screaton *et al.*, 1992). In theory, this could yield more than 1000 different CD44 isoforms. The addition of extra exons into the standard form of CD44 appears to impair its capacity to bind hyaluronan but, on the other hand, it can produce proteoglycan isoforms with novel functions. In particular, it has been shown that inclusion of epithelial-specific exons v8–10 in CD44 abrogate its hyaluronan-binding capacity (Jackson *et al.*, 1995; Stamenkovic *et al.*, 1991), although contradictory results have also been reported (Droll *et al.*, 1995; He *et al.*, 1992). The addition of v3 exon with glycosaminoglycan modifications endows CD44 with an ability to bind growth factors such as basic fibroblast growth factor (Bennett *et al.*, 1995b). Activated lymphocytes also express v6-containing variants of CD44 (Arch *et al.*, 1992), which in other cells have been implicated in metastatic spread (Günthert *et al.*, 1991). Therefore, it will be interesting to determine the effects of these variants on migration of normal lymphocytes. Deletions of the membrane-spanning domain and alterations in the length of the cytoplasmic tail are also commonly achieved using alternative splicing (e.g., CD31). The cell- and stimulus-specific factors that govern alternative splicing mostly remain to be unraveled. However, transfection of cells with SR cDNAs, nuclear phosphoproteins that can control the alternative splicing of pre-mRNAs, have been shown to explain, at least in part, the cell-specific splicing mechanisms of CD44 (Screaton *et al.*, 1995).

D. Posttranslational Modifications

After translation the protein core is subjected to extensive modifications, notably glycosylation, within the cell before the mature molecule is displayed on the cell surface. Cell type-specific posttranslational modifications probably account for most of the microheterogeneity of a given adhesion molecule on different cell types. For example, the amino acid core of ICAM-1 has a predicted molecular weight (MW) of 55 kDa (Staunton *et al.*, 1988), and yet it is physiologically found as a 70- to 120-kDa molecule on different cell types depending on the extent of N- and O-linked substitutions (Dustin *et al.*, 1986). Functionally, these modifications can mask certain adhesive motifs of the protein backbone; on the other hand, they can create new recognition specificities superimposed on the amino acid scaffold.

Ligands of selectins represent the best examples of the crucial importance of appropriate glycosylation. In addition to specific glycoprotein ligands, such as PNAd, MAdCAM-1, and CLA (see Section III,B), several cell surface molecules such as LFA-1 and CD66 can be decorated with sLe^x-related moieties to which selectins have been reported to adhere (Kotovuori *et al.*, 1993; Kuijpers *et al.*, 1992). Many natural selectin ligands appear to be molecules with mucin-like domains in which adjacent or successive O-linked glycans branch from the protein core. Selectins bind very poorly, if at all, to unglycosylated or underglycosylated ligands. PNAd, for instance, contains abundant oligosaccharide decorations containing fucose, sialic acid, and sulfated carbohydrates (Berg *et al.*, 1991a; Hemmerich *et al.*, 1994; Imai *et al.*, 1991, 1993). In GlyCAM-1, the L-selectin binding carbohydrate moiety has been shown to consist of a special 6-sulfo-sLe^x structure (Hemmerich and Rosen, 1994). Undersulfated GlyCAM-1 in endothelial cells fails to bind L-selectin (Imai *et al.*, 1993) and *in vivo* GlyCAM-1 with exactly the same amino acid sequence is found in milk, but a lack of sulfate modifications renders it incapable of interacting with L-selectin (Dowbenko *et al.*, 1991). The critical role of sialic acid is also evident because L-selectin does not bind to sialidase-treated HEV or isolated PNAd *in vitro* (Berg *et al.*, 1991a; Rosen *et al.*, 1985) and lymphocytes do not home to PLNs in animals injected with sialidase intravenously (Rosen *et al.*, 1989). Fucosidase treatment of GlyCAM-1 also adversely affects its interaction with L-selectin (Imai *et al.*, 1992). Moreover, naturally occurring polymeric fucose inhibits lymphocyte binding to PLN HEVs (Yednock *et al.*, 1987). Therefore, sialyltransferases, fucosyltransferases, and sulfotransferases can be expected to be found that can modify protein backbones in a tissue-selective manner and that therefore may prove to be of fundamental importance in the guidance of lymphocyte migration. In fact, one such enzyme, fucosyltransferase VII, has been identified (Nat-

suka *et al.*, 1994). Mice, which lack this fucosyltransferase activity, show profoundly diminished lymphocyte trafficking to PLNs.[3]

Protein phosphorylation is another widely used posttranslational modification among adhesion molecules. For example, the α subunit of LFA-1 is constitutively phosphorylated, whereas the phosphorylation of the β subunit is evoked by activation with protein kinase C (Buyon *et al.*, 1990; Chatila *et al.*, 1989). Although the cytoplasmic domain of CD18 is a prerequisite for ICAM-1 binding (Hibbs *et al.*, 1991b), mutation of the principal phosphorylated serine residue did not abolish ligand-binding capacity, which suggests that phosphorylation of CD18 was not required for adhesion (Hibbs *et al.*, 1991a). It appears, however, that the phosphorylation of threonine rather than serine residues may control the activation and hence ligand-binding properties of CD18 (Valmu and Gahmberg, 1995). Because phosphorylation/dephosphorylation events are extremely rapid, they may well play a crucial role in an instantaneous and short-lived activation of leukocyte integrins (Dustin and Springer, 1989), which is necessary in the control of the avidity of these adhesion molecules.

E. Conformational Effects

Integrins are a prototype of adhesion molecules that require a conformational switch to be functionally active. For example, $\alpha 4\beta 1$ is known to have

[3] Several new adhesion molecule knockouts have been produced lately. CD34 knockout mice display no alteration in lymphocyte recirculation or hematopoiesis, and in fact the only defect was seen in eosinophil accumulation in allergen-induced lung inflammation. Importantly, the lower part of the MECA-79 mAb cross-reactive 90-kDa band was still seen in these animals, which strongly suggests the existence of another, functionally important PNAd species of this size (Suzuki *et al.*, 1996).

In mice chimeric for α4-integrin T cell homing to Peyer's patches, but not to other secondary lymphoid organs, is dramatically reduced (Arroyo *et al.*, 1996). In β7 knockout animals the formation of mucosa-associated lymphatic tissues is severely impaired. β7-/- lymphocytes failed to stably arrest and transmigrate in Peyer's patch HEV, but no defect in initial rolling was observed. (Wagner *et al.*, 1996). Together, these data reinforce the critical role of $\alpha 4/\beta 7$ as a mucosal homing receptor.

CD11a knockout mice manifest with splenomegaly, reduced size of peripheral lymph nodes, impaired delayed type hypersensitivity reactions and defect in rejection of immunogenic tumors. However, many other immune responses are intact, indicating a differential requirement of LFA-1 for lymphocyte activation and proliferation under different circumstances (Schmits *et al.*, 1996).

The fundamental importance of a glycosyltransferease enzyme in regulating lymphocyte recirculation was for the first time shown directly in α(1,3)fucosyltransferase Fuc-TVII knockout mice. These animals lack E- and P-selectin ligand activity on leukocytes and have deficient L-selectin ligand activity in HEV. Intriguingly, lymphocyte homing to PLN was reduced by 80%, but the MECA-79 antigens were intact, indicating that this epitope does not fully recapitulate the function of L-selectin ligands (Maly *et al.*, 1996).

Taken together, all these new knockout animals are viable and apparently healthy which reinforces the redundancy of lymphocyte recirculation mechanisms.

at least four distinct activation stages that affect the ligand-binding specificity without discernable changes in the expression level (Masumoto and Hemler, 1993; Strauch *et al.*, 1994). $\alpha 4\beta 1$ can be expressed on the cell surface in a relatively inactive form that only binds in a cation-independent manner to invasin, a bacterial adherence structure. Divalent cations induce binding to VCAM-1. Further increases in the affinity status (mimicked by addition of optimal concentrations of divalent cations, manganese in particular, or stimulatory anti-$\beta 1$ antibodies) leads to binding to fibronectin, and a fully activated $\alpha 4\beta 1$ heterodimer is required for MAdCAM-1 binding. These changes in avidity are at least partially reflected by changes in the structure of the integrin heterodimer as evidenced by the concomitant exposure of activation-dependent neoepitopes on the molecules (Yednock *et al.*, 1995). Dissociation constants for LFA-1-ICAM-1 interaction have been measured by competition measurements. These data convincingly show that the activated LFA-1 integrin has a 200× higher affinity toward monomeric ICAM-1 than the resting LFA-1, and that only 15–20% of LFA-1 molecules convert to the high-affinity fraction by PMA treatment (Lollo *et al.*, 1993).

Several mAbs against conformation-dependent epitopes of integrins have been described and they should prove useful in dissecting integrin activation. By inside to outside signaling, extracellular factors (ligation of other surface receptors such as CD2, CD3, CD44, and T cell receptor, changes in divalent cation concentration, etc.) or activation of signal transduction pathways directly (e.g., with PMA or lipid modulators) induce a change in integrin conformation resulting in integrin activation (reviewed in Diamond and Springer, 1994; Hynes, 1992; Stewart *et al.*, 1995). The mechansim of the activation is still unclear, but theoretically it may result in a modification of an integrin subunit or binding to an additional molecule that increases the affinity of the ligand binding site of the integrin. However, it is also possible that a conformation shift of LFA-1 is not necessary for binding to ICAM-1 because lymphocyte activation with phorbol esters or TCR may not result in a high-affinity form of LFA-1 (Stewart *et al.*, 1996). Instead, the cells become activated and may form microaggregates of LFA-1 that can have sufficiently increased avidity for the ligand. The initial weak interaction of an integrin with its ligand may be a primary event in activation leading to exposure of additional sites (ligand-induced binding sites) to which ligands can then bind with higher affinity (Cabañas and Hogg, 1993).

F. Shedding, Secretion, and Internalization

A recirculating lymphocyte needs to undergo continuous and quick changes between the nonadhesive (within the blood) and adhesive (binding to the endothelial lining, locomotion, and cell–cell interactions within lymphatic organs) phenotypes. Thus, equally important as the induction

of adhesion molecule expression and activity is the rapid downmodulation of these molecules when they become unnecessary or deleterious.

Shedding is a rapid way of getting rid of a surface receptor. Many adhesion molecules actually contain protease-sensitive sites close to the membrane spanning domains that allow the cleavage of the ligand-binding extracellular part by endogenous proteases (Bazil, 1995). L-selectin, for example, is shed within minutes after cell activation. This shedding involves a novel nonclassical endogenous protease called secretase that has a very relaxed recognition sequence in the juxtamembrane sequence of L-selectin (Chen *et al.*, 1995; Migaki *et al.*, 1995; Walcheck *et al.*, 1996). In granulocytes the shedding of L-selectin diminishes the velocity of rolling on endothelial cells under shear conditions (Walcheck *et al.*, 1996). Alternatively, variants of adhesion molecules that lack the transmembrane domain and attach to the cell surface via an easily cleavable phosphatidyl-inositol linkage allow instantaneous shedding by the action of phospholipases. An increasing number of adhesion molecules, such as P-selectin, VCAM-1, and CD31, have been described to express such variant forms (Goldberger *et al.*, 1994; Johnston *et al.*, 1989; Moy *et al.*, 1993; Terry *et al.*, 1993). Other molecules, such as CD73, involved in lymphocyte adhesion have been described that are primarily phosphatidyl-inositol linked to the plasma membrane (Airas *et al.*, 1995; Resta *et al.*, 1993).

The balance in lymphocyte–endothelial interaction can also be skewed toward the nonadhesive side by secretion of soluble adhesion molecules (Gearing and Newman, 1993). GlyCAM-1, for instance, appears to be primarily a secreted molecule because it has no known consensus sequence enabling binding to the plasma membrane (Lasky *et al.*, 1992).

Both shedding and secretion produce soluble adhesion molecules that can circulate relatively freely in the blood or in other body fluids (Gearing and Newman, 1993). Most such molecules have intact ligand-binding domains and they therefore compete with the surface-bound receptors for available ligands. In effect, soluble adhesion molecules can function as antiadhesive compounds. For example, soluble dimeric ICAM-1 may bind to LFA-1 with sufficiently high avidity to block its interaction with the endothelial ICAM-1 (Meyer *et al.*, 1995). On the other hand, soluble isoforms can trigger a biological response on ligand-bearing cells. Soluble GlyCAM-1, for instance, appears to activate lymphocyte integrins by binding to L-selectin (Hwang *et al.*, 1996). Other serum components can modulate lymphocyte–endothelial adhesion as well. For example, soluble fibrinogen has been reported to enhance the attachment of leukocytes to endothelium and their subsequent transmigration by acting as a molecular bridge between Mac-1 and ICAM-1 molecules of the two interacting cells (Languino *et al.*, 1993, 1995). Interestingly, the fibrinogen binding site

of ICAM-1 appears to be distinct from the site mediating binding to leukocyte integrins.

Most adhesion molecules involved in lymphocyte–endothelial cell interactions are present in normal sera at detectable levels (reviewed in Gearing and Newman, 1993). Soluble L-selectin is found at the highest concentration (up to 1 μg/ml), whereas others circulate at levels that are only $\sim\frac{1}{10}$–$\frac{1}{100}$ that of L-selectin. It has been shown that under physiological conditions soluble L-selectin can occupy up to 40% of the available receptors and can inhibit lymphocyte adherence to endothelium (Schleiffenbaum *et al.*, 1992). Moreover, in disease, the levels of soluble molecules can fluctuate and further modulate lymphocyte trafficking. For example, elevated levels of soluble E-selectin have been found in sepsis (20-fold increment), in which it may correlate to the disease severity (Newman *et al.*, 1993), and in diabetes and vasculitides (2- or 3-fold increase), in which it only appears to be a sign of endothelial activation (Carson *et al.*, 1993; Gearing *et al.*, 1992).

Downregulation of adhesion molecules is also enhanced by a process of active internalization leading to restorage into granules or direction to protein degradation pathways. The short half-lives of P- and E-selectin can be largely accounted for by these two mechanisms (Subramaniam *et al.*, 1993).

Few physiological mediators have been defined that diminish leukocyte adhesion to endothelial cells. TGF-β renders the endothelial surface nonadhesive by mechanisms that may involve downregulation of E-selectin expression (Gamble and Vadas, 1991). Nitric oxide has also been implicated in the maintenance of the nonadhesive phenotype of normal endothelium (Kubes *et al.*, 1991). Also, some well-known anti-inflammatory drugs seem to exert their effect, at least in part, by downregulating the expression or function of adhesion molecules (reviewed in Cronstein and Weissmann, 1993). For example, glucocorticoids diminish E-selectin, VCAM-1, and ICAM-1 expression on HUVECs (Cronstein *et al.*, 1992) and indomethacin and aspirin downregulate L-selectin on leukocytes (Díaz-González *et al.*, 1995).

G. Oligomerization and Concentration on Membrane Microdomains

Conceivably, the avidity of binding can be increased if a receptor binds to its ligand via two or more sites instead of one. Many consensus repeats contained in adhesion molecules may have evolved via gene duplication and therefore it is not unexpected to see that some of them support binding to the same ligand. For instance, the first and fourth domains of VCAM-1 can both independently interact with $\alpha4\beta1$ and, hence, avidity of the binding is significantly higher in the full-length version of VCAM-1 than in the six-domain form that lacks the fourth domain (Osborn *et al.*, 1992; Vonderheide and Springer, 1992). Dimerization of an adhesion receptor

can also effectively improve the ligand recognition. For instance, most physiologically active ICAM-1 may be expressed as dimers on endothelial cells, and they bind to LFA-1 much better than the predominant monomeric forms of ICAM-1 (Miller *et al.*, 1995; Reilly *et al.*, 1995).

Clustering of the carbohydrate moieties in selectin ligands is still another example how functional oligomers are created (reviewed in Varki, 1994). Presentation of oligosaccharides on adjacent side chains within the mucin-like domains can greatly enhance the avidity of interaction with their receptors. Indeed, synthetic tetravalent sLe^x is a much better inhibitor of lymphocyte–endothelial cell binding than monovalent sLe^x (Turunen *et al.*, 1995). However, because the crystal structure of E-selectin revealed that there is just enough space for one sLe^x structure to interact with one E-selectin lectin domain (Graves *et al.*, 1994), the physiological significance of multimeric carbohydrate presentation in the absence of evidence from multimeric organization of selectins remains to be established.

Physical constraints introduced by the masking effects of the carbohydrate-rich cell surface can be alleviated by concentrating the adhesion molecules onto exposed parts of the cell surface. For example L-selectin, PSGL-1, and $\alpha 4\beta 7$ appear to predominantly exist in tips of villous plasma membrane projections that make the initial contacts with endothelial surfaces (Berlin *et al.*, 1995; Moore *et al.*, 1995; Picker *et al.*, 1991b). In fact, it has been shown that artificial targeting of these receptors to the planar domain of the cell surface abolishes their ligand-binding capacity under conditions of shear stress (von Andrian *et al.*, 1995). The other primary contact initiating ligands such as ESL-1 might therefore also be predicted to be found on the microvillous projections of the cell surface. Furthermore, selectins are known to exist as elongated monomeric molecules in solution (Hensley *et al.*, 1994; Ushiyama *et al.*, 1993), and the mucin-like selectin ligands are extended, rigid, rod-like structures that project well above the plasma membrane (Jentoft, 1990; Shimizu and Shaw, 1993). It has been estimated that the glycocalyx effectively covers 20 nm from the cell surface, P-selectin is 38 nm long, and its ligand PSGL-1 extends approximately 50–60 nm above the cell surface, making the interaction of these two molecule physically feasible (Patel *et al.*, 1995).

Clustering of adhesion molecules also effectively produces chances for multimeric binding. Although clear evidence for selectin clustering is lacking, integrins and members of the immunoglobulin superfamily are known to redistribute to the area of ligand binding (Campanero *et al.*, 1994; Detmers *et al.*, 1987; van Kooyk *et al.*, 1994). Cocapping of adhesion molecules also produces, in effect, multimolecular complexes that may facilitate contacts during different phases of the adhesion cascade. For example, ligation of lymphocyte CD44 has been shown to result in a concentration of LFA-1 and L-selectin into the same pole with CD44 by

a unidirectional process called syn-capping (Rosenman *et al.*, 1993). The cytoplasmic associations are vital to correct localization of most adhesion molecules within the appropriate areas of the plasma membrane, and the direct or indirect contacts with actin-based cortical cytoskeleton have already been established for several lymphocyte adhesion molecules (Pavalko *et al.*, 1995; Sharma *et al.*, 1995; Yoshida *et al.*, 1996).

V. Regulation of Tissue-Selective Homing

Despite the widespread expression of adhesion molecules, lymphocyte trafficking exhibits remarkable tissue specificity (Picker and Butcher, 1992). The migration of embryonic hematopoietic precursor cells from the allantoic membrane to the liver and later to bone marrow as well as the population of the thymus with bone marrow-derived prothymocytes can be regarded as tissue-specific homing, but very little is known about the molecular mechanisms governing these events (Weissman, 1994). In contrast, interactions controlling the patrolling of naive cells between secondary lymphatic organs and the changes in migratory pathways accompanying the naive to effector cell transition are relatively well understood, although unanswered questions and controversial issues still remain.

A. Lymphocyte Migration into PLN

L-selectin was originally described as the tissue-specific homing receptor that targets lymphocytes into peripheral nodes (Gallatin *et al.*, 1983). *In vitro* HEV-binding assays show that functional anti-L-selectin mAbs abolish 70–95% of PBL binding to PLN HEVs, and in short-term *in vivo* assays such antibodies almost completely prevent lymphocyte migration into PLNs. When compared to B cells, T cells show preferential tendency to home to PLNs (Stevens *et al.*, 1982). Among PBLs, the PLN-seeking subpopulation is generally thought to consist of L-selectin positive, CLA negative, $\alpha 4\beta 7$ low naive cells (Andrew *et al.*, 1996; Picker *et al.*, 1993a,b). The expression of L-selectin and PLN HEVs binding capacity also correlate well on several lymphoma lines (Bargatze *et al.*, 1987). Finally, the findings that transfection of L-selectin into a L-selectin-negative gut-seeking cell line is sufficient to confer PLN HEVs binding capacity to that cell line (Berg *et al.*, 1991a) and that L-selectin knockout mice show significantly diminished trafficking into PLNs confirm the crucial role of L-selectin in PLN homing (Arbones *et al.*, 1994).

The main ligands of L-selectin in peripheral nodes are PNAds. PNAds are selectively (Streeter *et al.*, 1988b), but not exclusively (Hänninen *et al.*, 1993b; Michie *et al.*, 1993; Salmi *et al.*, 1994), expressed in peripheral nodes. *In vitro* and *in vivo*, mAb MECA-79 against PNAd decreases lymphocyte adherence to PLN HEVs by 60–90% (Streeter *et al.*, 1988b).

However, the two cloned species of PNAd cannot explain its function as an addressin because GlyCAM-1 is a secreted molecule and CD34 is by no means limited in its expression to PLN HEVs. Rather, the MECA-79 epitope seems to be uniquely decorating common protein backbones in PLNs, suggesting that PLN-specific glycosyltransferases are decisive in PNAd synthesis (see footnote 3).

Although there is no doubt that L-selectin–PNAd interaction has a major impact in PLN homing, L-selectin is not a prerequisite for it. T cell lines, recently activated PBLs, and a subpopulation of memory cells lack L-selectin and yet both *in vitro* and *in vivo* binding experiments have unambiguously demonstrated that these cells can adhere to PLN HEVs and home into the tissue (Bookman *et al.*, 1986; Hamann *et al.*, 1988b; Jung *et al.*, 1988; Picker *et al.*, 1993b; Salmi *et al.*, 1992). Binding of these cell types may be partially mediated by LFA-1 because inhibition of this integrin on PBLs leads to diminished PLN adherence (Hamann *et al.*, 1988a; Pals *et al.*, 1988). However, function-inhibiting antibodies against ICAM-1 have failed to prevent lymphocyte binding to PLN HEVs (Duijvestijn and Hamann, 1989). Therefore, ICAM-2, yet unidentified adhesion molecules, or tissue-specific chemoattractants may prove to be critical for the binding of L-selectin-negative cells at least to PLN HEVs. One possible ligand is VAP-1, which is expressed in PLN HEVs and mediates binding of not only L-selectin-positive but also L-selectin-negative cells to these vessels (Salmi and Jalkanen, 1992, 1996). It may be that L-selectin-positive cells first bind to PNAd and then proceed to interact with VAP-1 before stable arrest on PLN HEVs. L-selectin-negative cells, including recently activated immunoblasts as well as half of memory cells, can perhaps omit the selectin-mediated adhesion and directly rely on VAP-1 in PLN HEV adherence. It is also worth noting that L-selectin expression as such is not enough for PLN homing. After the initial report, L-selectin has been described on virtually all types of leukocytes, but L-selectin-positive granulocytes, for example, bind to PLN HEVs but fail to migrate into the normal node (Lewinsohn *et al.*, 1987).

B. Lymphocyte Homing into Mucosa

Gut-associated lymphatic tissues (Peyer's patches and lamina propria) along the whole length of the intestine, lung-associated lymphatic tissues, the mammary gland, and the mucosal surfaces of reproductive organs form one functionally defined recirculation system (McDermott and Bienenstock, 1979). However, only lymphocyte homing to gut-associated tissues in the mouse has been well dissected at the molecular level (Hu *et al.*, 1993; Picker and Butcher, 1992; Springer, 1994). Although the experimental evidence is limited, it is generally thought that naive lymphocytes home into the gut via Peyer's patch HEV, whereas effector cells preferentially

transmigrate between the endothelial cells of flat-walled venules into the lamina propria where a low level of continuous immunostimulation occurs. On lymphocytes, $\alpha 4\beta 7$ integrin is the main determinant directing migration into gut (Hu *et al.*, 1992). mAbs against it inhibit approximately 70–90% of PBL binding to Peyer's patch HEV in *in vitro* assays and a similar inhibitory effect is seen in short-term homing experiments *in vivo* (Hamann *et al.*, 1994; Holzmann *et al.*, 1989). B lymphocytes bind about twofold better to mucosal HEV than T cells, albeit on an absolute basis equal numbers of T and B cells home into the gut in short-term *in vivo* homing assays (Stevens *et al.*, 1982). Most activated immunoblasts and memory cells found in thoracic duct lymph and blood have been activated in the gut and are therefore on their route back to the intestine (Gowans and Knight, 1964). Among PBL memory cells, the majority of the gut-seeking lymphocytes are thought to display an L-selectin-negative, CLA-negative, $\alpha 4\beta 7$-high, $\alpha 4\beta 1$-positive phenotype (Schweighoffer *et al.*, 1993). There is some evidence that $\alpha 4\beta 1$ can also target lymphocytes to mucosa, whereas $\alpha E\beta 7$, despite selective expression on the mucosa-seeking PBL population, seems to be involved only in interactions between intraepithelial lymphocytes and epithelial cells and it cannot bind to MAdCAM-1 (Cepek *et al.*, 1994; Strauch *et al.*, 1994). Currently, the most compelling evidence for the role of $\alpha 4\beta 7$ in gut tropism comes from experiments in which transfection of $\alpha 4\beta 7$ into a nonmucosa binding lymphoma line enables these cells to bind to Peyer's patch HEV and from the demonstration of direct binding of $\alpha 4\beta 7$ to MAdCAM-1 (Berlin *et al.*, 1993; Hu *et al.*, 1992; see footnote 3).

MAdCAM-1 is the principal mucosal addressin (Nakache *et al.*, 1989; Streeter *et al.*, 1988a). It is expressed at high levels in Peyer's patch HEV and it is also present in flat-walled venules in lamina propria. Inhibition of MAdCAM-1 function yields 70–95% inhibition in lymphocyte trafficking into Peyer's patches but only a 50% reduction in immunoblast homing into lamina propria (Hamann *et al.*, 1994; Picker and Butcher, 1992; Streeter *et al.*, 1988a). MAdCAM-1 appears to be more heavily decorated with MECA-79 epitopes in Peyer's patches than in lamina propria (Berg *et al.*, 1993). Therefore, L-selectin can probably tether lymphocytes to the endothelial lining in mucosa under conditions of flow and, in fact, *in vivo* experiments have shown 50% inhibition of lymphocyte localization into Peyer's patches after blocking of L-selectin (Hamann *et al.*, 1991). Thus, the original molecular dichotomy between PLN and mucosal migration pathways needs to be reevaluated. It may well be that naive L-selectin-high, $\alpha 4\beta 7$-intermediate lymphocytes use both receptors to interact with densely expressed MAdCAM-1 molecules decorated with MECA-79 epitopes in Peyer's patches, whereas increased expression and activation of $\alpha 4\beta 7$ on L-selectin-negative mucosal memory cells and immunoblasts can be suf-

ficient to guide the binding of these cells to lamina propria MAdCAM-1 expressed without the MECA-79 epitope. Moreover, on activated cells, $\alpha 4\beta 7$ can totally omit the selectin-dependent initial phase of adhesion because $\alpha 4\beta 7$ mediates lymphocyte binding to MAdCAM-1 and VCAM-1 under physiological shear stress (Berlin *et al.*, 1995). The significance of VCAM-1 as an $\alpha 4$ ligand at mucosal sites remains questionable, however, because VCAM-1 is practically absent from normal as well as inflamed gut (Rice *et al.*, 1991; Salmi *et al.*, 1994).

Mesenteric lymph nodes present a mixed-type lymphatic tissue having both mucosal- and PLN-binding specificities. Most of the HEVs in mesenteric nodes are both PNAd positive and MAdCAM-1 positive, and even individual HEV cells can express these antigens simultaneously (Streeter *et al.*, 1988b). On lymphocytes, both L-selectin and $\alpha 4\beta 7$ contribute equally to mesenterial binding (Hamann *et al.*, 1994).

C. Lymphocyte Homing to Thymus and Bone Marrow

A critical step controlling thymic colonization of immature lymphocytes committed to the T cell lineage is the interaction between T cell progenitors and the perithymic vascular endothelium. To date, two molecules participating in lymphocyte homing into the thymus have been characterized: CD44 on the progenitor cells and $\alpha 6$ integrins on vascular endothelium (Dunon and Imhof, 1993; Ruiz *et al.*, 1995; Wu *et al.*, 1993). In this process, CD44 utilizes an unknown ligand instead of hyaluronan (Wu *et al.*, 1993). It is obvious that other molecules involved in thymic homing will be found in the future. However, it seems unlikely that VCAM-1, ICAM-1, or endothelial selectins contribute significantly because of their undetectable level of expression on thymic endothelium (Dunon and Imhof, 1993; Ruiz *et al.*, 1995).

Significant lymphocyte trafficking is also directed into the bone marrow. Antibodies against VLA-4 and VCAM-1 cause mobilization of lymphocytes from the bone marrow and they also reduce homing of hemopoietic progenitors of the bone marrow when injected into lethally irradiated recipients, indicating that this receptor–ligand pair controls bone marrow lodging of lymphocytes (Papayannopoulou *et al.*, 1995; Papayannopoulou and Nakamoto, 1993). The experimental setup, however, does not allow one to conclude whether inhibition of bone marrow homing is due to diminished adherence of hemopoietic progenitors to the luminal surface of endothelium, their impaired transmigration, or deficient interaction with the stromal elements. Furthermore, these experiments by no means exclude the possibility of involvement of other adhesion molecules in lymphocyte homing into the bone marrow.

D. Lymphocyte Recirculation through the Spleen

Spleen is an important site for immunoglobulin production and has an essential role in immune defense against bacterial diseases. Spleen harbors massive numbers of recirculating B and T cells that have several-fold shorter transit time through the spleen than through the lymph nodes (reviewed in Pabst and Binns, 1989). Despite the extensive lymphocyte trafficking very little is known about the molecular mechanisms involved in lymphocyte entrance into the spleen. Lymphocytes leave the blood in the marginal zone and subsequently localize to specific splenic compartments, T cells preferentially to periarteriolar lymphatic sheath, and B cells to the marginal zone and corona (Pabst and Binns, 1989). Because the spleen lacks HEVs, an alternative port of entry for lymphocytes has been searched for and it appears that marginal zone macrophages that surround the white pulp may serve the function of HEVs in this organ (Lyons and Parish, 1995).

Certain homing-associated adhesion molecules are present in the spleen and theoretically could also control the lymphocyte trafficking into this site. MAdCAM-1 is expressed in sinus-lining cells closest to the lymphoid white pulp and, thus, blood flow to the white pulp is passing by the MAdCAM-1-positive cells. However, in *in vivo* short-term homing assays functional antibodies against MAdCAM-1 and its counterreceptor, $\alpha 4\beta 7$, could not block lymphocyte homing into the spleen, ruling out the role of this receptor–ligand pair in lymphocyte entry into the spleen (Kraal *et al.*, 1995). Moreover, results of other experiments suggest novel mechanisms to be involved in splenic homing of lymphocytes: When antibodies against VLA-4, VCAM-1, and L-selectin known to block lymphocyte homing to sites of inflammation and lymph nodes have been injected into mice, lymphocyte trafficking into the spleen has paradoxically increased (Hamann *et al.*, 1991, 1994). Similarly, enhanced lymphocyte homing into the spleen is also detected in L-selectin knockout mice (Arbones *et al.*, 1994).

Evidence that carbohydrate structures are also important in lymphocyte trafficking into the spleen come from experiments in which neuraminidase treatment has been found to inhibit lymphocyte entrance into the spleen (Kraal *et al.*, 1994). Moreover, intravenous administration of fucoidan disrupts lymphocyte interaction with marginal zone macrophages (Lyons and Parish, 1995). In contrast to lymphocyte homing into the lymph nodes that is blocked by pertussis toxin, lymphocyte migration into the spleen is not inhibited by this toxin as such (Cyster and Goodnow, 1995; Lyons and Parish, 1995). It, however, profoundly affects the lymphocyte localization within the spleen because B cells fail to enter the follicles and migration of T cells into the white pulp is blocked (Lyons and Parish, 1995). These findings suggest that a G-protein-coupled receptor is of fundamental impor-

tance in lymphocyte migration into the splenic white pulp. Based on the rather limited data gathered so far lymphocyte trafficking into the spleen seems to be regulated by unique mechanisms distinct from those controlling lymphocyte entry to other secondary lymphoid organs.

E. Lymphocyte Trafficking to Liver and Lung

A large portion of intravenously injected lymphocytes position in the liver (Pabst and Binns, 1989). This is not simply due to removal of damaged cells but rather represents physiological lymphocyte circulation. However, the great majority of the lymphocytes do not enter the liver parenchyma. Instead, they join the hepatic marginal pool, from which they can be easily mobilized by perfusion (reviewed in Pabst and Binns, 1989). In the normal liver, the sinusoidal endothelium expresses adhesion molecules such as ICAM-1 and ICAM-2. In contrast, normal noninflamed portal endothelium lacks ICAM-1 and only contains low levels of ICAM-2. Moreover, in some normal individuals low levels of VCAM-1 and E-selectin are found in portal vein endothelia (Steinhoff *et al.*, 1993). The liver-associated lymphocyte population expresses receptors for these endothelial ligand molecules, namely LFA-1 and VLA-4 (García-Barcina *et al.*, 1995). However, although it is highly likely that these conventional adhesion molecules are able to recruit lymphocytes into the liver, their involvement in this process has not been directly proven. Prominent expression of VAP-1 was described in hepatic sinusoidal and vascular endothelium (McNab *et al.*, 1996). Functional importance of VAP-1 in supporting lymphocyte binding to sinusoidal endothelium was also demonstrated (McNab *et al.*, 1996). Thus, although some potential participants mediating lymphocyte traffic into the liver have been identified, future studies are required to elucidate the detailed mechanisms operating in hepatic homing of lymphocytes.

Like liver, lung is also a significant accumulation site for intravenously injected lymphocytes (Pabst and Binns, 1989). Pulmonary lumphocytes seem to form a massive marginating pool while failing to migrate into the interstitial and bronchoalveolar spaces. Pulmonary memory/effector T cells have been reported to express low levels of CLA and lack L-selectin, thus possessing a strikingly distinct phenotype from the skin-seeking memory/effector T cells (Picker *et al.*, 1994). The characteristic pulmonary phenotype of T cells is not influenced by lung inflammation. In sheep, lymphocytes emigrating from the lung express low levels of both $\alpha 4\beta 7$ and L-selectin and likely utilize molecules other than these homing receptors for migration to the lung and associated lymphoid tissues (Abitorabi *et al.*, 1996). It has been suggested that neutrophils enter the lung parenchyma via capillaries. Because the diameter of the capillary is the same or less than that of a neutrophil, leukocytes are not capable of tethering or rolling in this vascular bed. Therefore, the process of extravasation into lung

cannot occur sequentially. Instead, the homing-associated molecules need to act simultaneously. This mode of action has been proposed to explain some of the results obtained from knockout animals (Bullard *et al.*, 1995), but there is no direct evidence for the applicability of this model to lymphocytes.

F. Interaction between Tumor-Infiltrating Lymphocytes and Tumor Endothelium: A Unique Specificity?

Practically all tumors contain variable numbers of normal lymphocytes that supposedly have entered the tumor to kill the malignant cells. The normal lymphocytes can be successfully isolated and expanded in *in vitro* culture conditions with the help of IL-2 (Topalian *et al.*, 1987). When sufficient number of tumor-infiltrating lymphocytes (TILs) have been grown, they can be intravenously injected back to the patient. In favorable conditions, TILs preferentially leave the blood circulation within the tumor vasculature and subsequently migrate into the tumor tissue to destroy the malignancy (Rosenberg, 1992). As a matter of fact, several reports of *in vivo* animal and human studies have described preferential accumulation of TILs in tumors (Ames *et al.*, 1989; Fisher *et al.*, 1989; Griffith *et al.*, 1989), but contradictory results have also been presented (Economou *et al.*, 1996).

Endothelium of tumor vasculature expresses several adhesion molecules and the expression varies both qualitatively and quantitatively between individual tumors. Frequently observed induction of E-selectin, VCAM-1, PNAd, and upregulation of ICAM-1 in tumor endothelium reflect the inflammatory nature of the tumor microenvironment (Salmi *et al.*, 1995b). Autonomous regulation of adhesion molecule expression within the tumor is demonstrated with animal models and human cancers. For example, in a mouse pancreatic tumor model the tumor vasculature was devoid of L-selectin ligands, including PNAd and MAdCAM-1, whereas adjacent vessels within the inflammatory infiltrates in the pancreatic islets expressed these molecules. In contrast, vascular lining in both types of lesions expressed CD31, ICAM-1, and VCAM-1 (Onrust *et al.*, 1996). Moreover, melanoma and carcinoma metastases in the lung have been shown to suppress endogenous expression of VCAM-1 within the metastatic lesions (Piali *et al.*, 1995a). It has also been demonstrated that tumor endothelium preferentially supports binding of TILs over the endothelium in lymph nodes, mucosa-associated lymphatic tissue, inflamed synovium (expressing several inflammation-induced adhesion molecules), and normal tissue of the same origin as the tumor (Salmi *et al.*, 1995b). These results suggest that interaction between TILs and tumor endothelium, a prerequisite for

successful immunotherapy, has some unique characteristics at least partially distinct from those operating at other locations of inflammation.

G. Different Recirculation Pathways of Naive and Memory Lymphocytes

Elegant *in vivo* studies with a sheep model have led to the emergence of a hypothesis that virgin lymphocytes and their progeny of memory lymphocytes utilize distinct trafficking pathways (Mackay, 1993). By analyzing the phenotypes of cells in blood, lymphoid tissues, and afferent and efferent lymph, it appears that naive lymphocytes are mainly extracted from the blood via HEVs in organized lymphoid tissues. The antigenic specificity of a naive lymphocyte does not restrict its recirculation pathways to certain tissues and foreign antigens may not be needed to support baseline continuous lymphocyte trafficking (Picker and Butcher, 1992). On the other hand, in afferent lymph almost all small lymphocytes have the characteristics of memory cells, indicating that most memory cells leave the circulation in the peripheral vascular beds via flat-walled vessels (Mackay *et al.*, 1988). Memory cells display very low levels of constitutive recirculation through thin-walled venules in epithelium-associated tissues, but after antigenic stimulation the rate of trafficking is dramatically enhanced (Hay *et al.*, 1980). Obviously, the effector cells get some imprint from the microenvironment of the activating site because they later preferentially home back to the tissues belonging to the same functional recirculation system rather than randomly to all secondary and tertiary lymphatic tissues (Cahill *et al.*, 1977; Griscelli *et al.*, 1969; Hall *et al.*, 1979). The different migration pathways are mirrored in the adhesion molecule phenotype of cells: $CD45RA^+RO^-$ naive lymphocytes are L-selectin intermediate, LFA-1 intermediate, $\alpha 4\beta 1$ low, $\alpha 4\beta 7$ low, and CD44 intermediate, whereas RA^-RO^+ memory-type cells typically express two- to fourfold higher levels of these adhesion molecules (Picker *et al.*, 1993b; Schweighoffer *et al.*, 1993). The notable exception is the subpopulation of L-selectin-negative memory cells that may represent the gut-seeking cells (Picker *et al.*, 1993b). In addition, there may be differences in the extravasation efficiency of the lymphocyte subpopulations. For example, γ/δ T cells have been shown to have a slightly greater ability to adhere to P-selectin than α/β T cells (Diacovo *et al.*, 1996). As a consequence, γ/δ T cells may enter sites of inflammation more vigorously than α/β T cells.

Recently, the concept of different recirculation pathways between naive and memory cells has been challenged. In a rat model, no significant differences in the tissue localization of the two cell types were found (Westermann *et al.*, 1994). The reasons for these discrepant observations are unknown but may reflect the different techniques or animal models

employed. Teleologically, dispersion of the effector cells to peripheral sites in the immediate vicinity of the entry ports of antigens would make sense, but obviously the recirculation patterns of memory cells remain to be confirmed.

H. Concept of the Multistep Adhesion Cascade

Lymphocyte homing receptors and addressins were once thought to act as tissue-specific labels that could explain the diverse and yet specific migration pathways employed by different types of lymphocytes. According to that hypothesis, a naive lymphocyte would carry a set of distinct receptors, one for each tissue, whereas only one type of receptor would be left on an activated immunoblast. Distinct tissue-specific endothelial molecules were also expected to be found in all organs (Butcher, 1986). However, this key and lock model (Fig. 3A) is clearly insufficient to explain the specificity and diversity of lymphocyte–endothelial interactions. For example, no endothelial adhesion molecule is strictly tissue specific in expression. In fact, endothelial cell-specific ligands do not exist because even the most endothelium-restricted molecules, PNAd, MAdCAM-1, and E-selectin, are expressed in other structures besides endothelium: PNAd in reticular fibers, MAdCAM-1 in germinal centers and sinus-lining cells of the spleen, and E-selectin in astrocytes and hepatocytes (Essani *et al.*, 1996; Hurwitz *et al.*, 1992; Kraal *et al.*, 1995; Streeter *et al.*, 1988b). Moreover, expression of a given lymphocyte adhesion molecule cannot absolutely predict a cell's capacity to bind to certain endothelial cell types (Salmi *et al.*, 1992).

Early observations showing that pertussis toxin does not interfere with HEV binding of lymphocytes but completely prevents diapedesis into the node suggested that lymphocyte emigration includes at least two distinct steps (Spangrude *et al.*, 1984). With the help of intravital microscopy and different flow chamber systems, the dynamic nature of leukocyte–endothelial cell interaction has become increasingly apparent (Arfors *et al.*, 1987; Atherton and Born, 1973; Bjerknes *et al.*, 1986; von Andrian *et al.*, 1991). In small venules of the exposed mesenterium of an experimental animal four distinct types of cellular interactions are readily discernible. Most leukocytes flow freely within the bloodstream, but some make transient contacts with the vascular lining. Rolling behavior of the leukocyte on the endothelial cells ensues from some of these transient interactions, whereas other cells are released back to the bloodstream and may or may not then reengage endothelial cells at a more distant site. A subpopulation of the rolling cells finally halts and sticks to the endothelial cells strongly enough to withstand the shear forces generated by the blood flow. These cells then ultimately flatten, seek interendothelial junctions, and diapedese between the endothelial cells into the underlying tissue (Fig. 3B). These

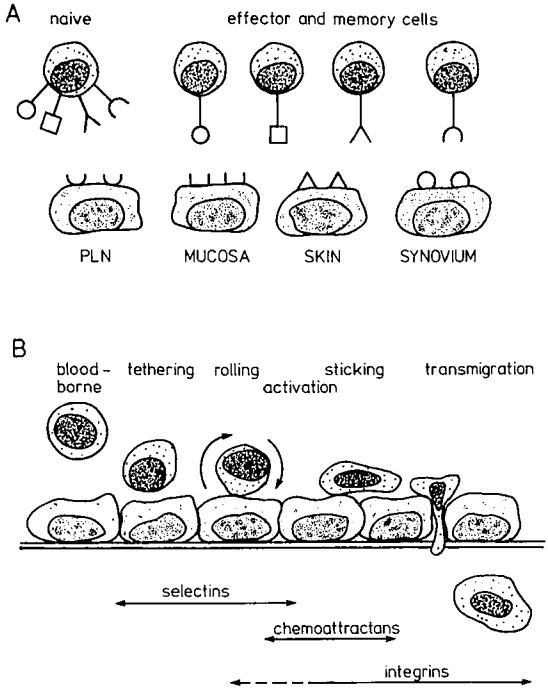


FIG. 3. Models of lymphocyte–endothelial cell interactions. (A) The key and lock model. Naive lymphocytes express distinct homing receptors for each endothelial specificity. In contrast, effector cells lose all but one homing receptor directing trafficking back to the site of activation. Endothelial cells in different organs also selectively synthesize only one type of addressin. (B) The multistep adhesion paradigm. Lymphocytes make multiple successive contacts with vascular lining during the emigration cascade. The specificity of interaction rises from unique combinations of different steps rather than from exclusive expression of unique adhesion molecules.

four types of interactions—tethering/rolling, activation, sticking, and transmigration—can be reconstituted in *ex vivo* systems and the three first ones even in cell-free models with recombinant proteins. When the role of individual adhesion molecules was studied, it soon became apparent that antibodies against selectins abolished both rolling and firm adherence under conditions of flow. On the other hand, blocking of $\beta 2$ integrin function had no effect on leukocyte rolling but it completely prevented stable arrest and transmigration (Lawrence *et al.*, 1995; Lawrence and Springer, 1991; Luscinskas *et al.*, 1994). These data indicate that selectins mediate the first reversible steps of interaction (i.e., transient tethering and rolling), integrins mediate the firm adhesion, and the distinct steps of

the adhesion cascade are sequential rather than parallel events (von Andrian *et al.*, 1991). Further refinement of the model has revealed that the four steps are overlapping rather than strictly sequential (Springer, 1994). For instance, $\alpha 4$ integrins are able to mediate both rolling and attachment, especially if $\alpha 4$ is in activated stage, and in inflammatory conditions, when the shear rates are reduced, CD18 may mediate rolling (Berlin *et al.*, 1995; Gaboury and Kubes, 1994). Although the adhesion cascade was initially depicted using granulocytes (Butcher, 1991), this model has been shown to be applicable to lymphocytes as well (Alon *et al.*, 1994, 1995b; Bargatze and Butcher, 1993; Luscinskas *et al.*, 1995).

The lectin-like nature of selectins is ideally suited for transient interactions because their association and dissociation kinetics with the oligosaccharide-based ligands are very rapid (the formation and disruption of a receptor–ligand interaction occurs within milliseconds) (Alon *et al.*, 1995a). Interestingly, shear over critical threshold is required for the function of L-selectin but not for that of P- and E-selectin (Finger *et al.*, 1996). Furthermore, selectins are constitutively active and the presentation of L-selectin on cell protrusions greatly facilitates initial contacts between leukocytes and endothelial cells (Picker *et al.*, 1991b). The sialomucin-like nature of physiological selectin ligands also predicts that they present multivalent binding sites on rigid rods that extend well above the cell glycocalyx to be maximally available for the blood-borne lymphocytes (Shimizu and Shaw, 1993). When the lymphocyte velocity diminishes due to transient contacts, cells become exposed to local activators. Activation signals can be provided by secreted molecules from endothelial cells or leukocytes or possibly from the engagement of selectins as such. After an activation-dependent switch in integrin conformation, lymphocytes arrest on the vascular lining, mainly via the ligation of Ig superfamily counterreceptors. This intercellular adhesion is very tight and can resist considerable shear forces (Butcher, 1991; Springer, 1994). Finally, additional molecules such as CD31 are brought into play during the transmigration phase (Muller *et al.*, 1993; Zocchi *et al.*, 1996). The exact mechanisms whereby the cells penetrate the interendothelial junctions and the basement membrane remain to be established (Oppenheimer-Marks and Lipsky, 1994), but the induction of proteolytic enzymes in lymphocytes by VLA-4–VCAM-1 ligation may provide some clues (Romanic and Madri, 1994). Other T cell gelatinases have been identified that mediate transmigration *in vitro* (Goetzl *et al.*, 1996; Leppert *et al.*, 1995). Interestingly, another extracellular matrix degrading enzyme heparanase has been proposed to have a dual function in lymphocyte migration. Depending on the local pH of the tissue, it can either act as a T cell adhesion molecule by anchoring them to

heparan sulfate proteoglycans or as an enzyme that degrades the matrix (Gilat *et al.*, 1995).

I. Do the Currently Available Technologies Allow Unraveling of Lymphocyte Recirculation?

Lymphocyte migration studies have heavily exploited mAbs to characterize the distribution and function of putative adhesion molecules in animal and human systems. Recently, studies with recombinant proteins, transfectants, and gene knockout animals have become possible. Ever-increasing complexity of subtle variations in the adhesion cascade is needed to explain the partially conflicting and even overtly contradictory results obtained by using these refined techniques. Although the results may truly represent the extremely specific and yet redundant process of lymphocyte migration, a legitimate concern remains regarding how well these techniques really mimic the *in vivo* situation.

When distribution of an adhesion molecule is assessed by staining with a mAb, a change in the apparent expression may truly represent a difference in the amount of the protein synthesized in a cell or, alternatively, merely reflect differences in the availability of the epitope recognized by the mAb. For instance, masking of an adhesion molecule with posttranslational modifications, typically glycans, or removal of sterically inhibiting modifications of the given molecule, or even in the adjacent molecules, may give an erroneous impression that the amount of the molecule has altered. Moreover, positive reaction in staining requires a certain threshold number of target antigens, and it may be that even a lower copy number of a given molecule renders it functionally active. Unfortunately, analyses of changes in mRNA levels do not provide more information and, in fact, they will not even tell whether the adhesion molecule is ever translated into a protein product. Moreover, the functional activity of molecules cannot be predicted by simply assessing the expression levels. This is particularly true for integrins and CD44, which functionally exist in inactive, partially active, and fully active forms (Diamond and Springer, 1994; Lesley *et al.*, 1993). Expression and function of endothelial molecules can also dissociate, as exemplified by nonglycosylated GlyCAM-1 and CD34, which are incapable of binding to L-selectin (Dowbenko *et al.*, 1993; Imai *et al.*, 1991).

In the functional studies of lymphocyte–endothelial interactions both *in vitro* and *in vivo* assays have been employed. The Stamper–Woodruff frozen section HEV-binding assay (Stamper and Woodruff, 1976) has been used to identify many of the known lymphocyte and endothelial adhesion molecules. The *in vitro* HEV-binding preference of cells has been shown to accurately reproduce the *in vivo* homing specificity of tissue-selective lymphoma cell lines and different lymphocyte subpopulations (Butcher *et*

al., 1980; Stevens *et al.*, 1982). This assay has the benefit of using HEVs, which represent the major extravasation route *in vivo*, rather than nonspecialized vessels as a target endothelium. HEVs also display endothelial cell ligands at a natural density and array and contain the rich glycocalyx with potential adhesion modulating effects. However, the HEV assay also faces several potential problems. The target endothelium is nonvital, therefore it cannot play as active a role in lymphocyte activation as live endothelial cells, and cryostat sectioning exposes cytoplasmic as well as luminal antigens available for interactions with lymphocytes. Moreover, the assay is performed at a temperature of 7°C and under conditions of mild (nonlaminar) shear. Thus, the HEV-binding assay may be biased toward measuring initial lymphocyte tethering. However, lymphocytes remain viable during the assay and can undergo activation. For instance, integrin function is clearly observable, even though it may not be maximal (Hamann *et al.*, 1988a; Holzmann *et al.*, 1989; Salmi *et al.*, 1995a; Yednock *et al.*, 1992). Moreover, the HEV assay only measures adhesion to endothelium and as such does not tell anything about the subsequent fate of the cell (transmigration vs detachment). Finally, a common pitfall in all systems using mAbs is that these large molecules can cause steric hindrance of physically adjacent molecules, which may functionally be more important in the adhesion than the actual mAb epitope-bearing molecule. However, blocking of highly abundant irrelevant endothelial molecules (such as HLA class I) with mAb does not affect HEV adhesion (Salmi *et al.*, 1995a).

Alternative adhesion assays commonly exploited also suffer from several limitations. HUVECs are widely used as an endothelial cell model because, although few encouraging results with animal HEVs have been obtained (Ager and Mistry, 1988; Ise *et al.*, 1988), human HEVs have resisted all attempts of prolonged culturing. However, HUVECs are derived from fetal macrovascular endothelium that normally does not support any lymphocyte trafficking. HUVECs also lack constitutive and inducible surface expression of several human endothelial molecules, such as PNAd, MAdCAM-1, and VAP-1, resulting in unavoidable overestimates on the importance of other molecules under physiological settings. Moreover, differences in the expression and regulation of adhesion molecules in anatomically different microvascular beds makes the generalization of HUVECs results somewhat difficult.

The use of transfectants has become increasingly fashionable in adhesion studies. However, apart from the requirement for a cloned gene, this assay system is inherently liable to variations caused by host-specific differences in gene expression and posttranslational modifications, notably in oligosaccharide synthesis. For example, Chinese hamster ovary cells lack α1-3-fucosyltransferase activity precluding the expression of sLe^x (Bierhuizen

et al., 1994; Li *et al.*, 1996). The function of the host homolog molecule is also sometimes ignored, especially if it does not cross-react with the mAb used to detect the transfection product. Moreover, the use of transfectants easily introduces abnormally high concentrations of adhesion molecules on a background of nonrelevant surface molecules (e.g., expression of endothelial adhesion molecules in fibroblasts or epitheloid cells).

Analyses with purified, surface-immobilized adhesion molecules also suffer from an unnaturally dense display of critical epitopes and in these systems all the physical constraints of the cell surface glycocalyx are ignored. Moreover, contaminants and copurifying molecules may render the interpretation of results difficult. On the other hand, it is virtually impossible to get negatively charged molecules [such as pure preparations of glycosaminoglycans (hyaluronate, heparin, etc.)] to bind to plain plastic (P. Heldin and M. Bernfield, personal communications). Nevertheless, use of purified molecules and transfectants in parallel plate flow chamber assays has for the first time allowed for the study of the effect of hydrodynamic stress on leukocyte adhesion in a controlled way.

Intravital microscopy bypasses many of the drawbacks of other models (Menger and Lehr, 1993). Most notably, it allows studies to be done under physiological shear in intact vessels. However, surgical manipulation invariably appears to activate the endothelial cells, fluorescent labeling of leukocytes may affect their binding properties, and this technique only allows the visualization of easily accessible vessels such as mesenterial and scrotal venules.[4]

Finally, knockout animals have become the golden standard to evaluate the significance of a given molecule in biological systems. This ingenious technique has repeatedly resulted in unexpected results in the adhesion (see Section VII,B). In most published examples, the phenotype of the knockout animal has been essentially normal under physiological conditions and only relatively mild aberrations in lymphocyte homing have been detected. Lymphocyte homing is also much less affected in these mutant animals than seen *in vivo* in antibody-treated animals. The reasons for this discrepancy remain unclear. Theoretically, in antibody-treated animals impurities of antibody preparations may cause nonspecific effects and the ligand engagement by the antibody may result in signaling with further modulation of cellular behavior. On the other hand, in gene knockouts there may be cryp-

[4] A technique to visualize HEV in mouse PLN by intravital microscopy has been worked out. These studies show that L-selectin and MECA-79 antigens mediate lymphocyte rolling under physiological shear stress in PLN. This technique also revealed that binding of activated platelets to lymphocytes provides a cellular bridge by which platelet P-selectin binding to PNAd in PLN is used for lymphocyte rolling (Diacovo *et al.*, 1996).

tic promoters or alternative splicing mechanisms that override the disruption of certain targeted exons. Also, embryonic development may give time to slow adaptation to the defect and redundant compensatory recirculation mechanisms can be brought into play more effeciently than in the case of an abrupt disruption by a mAb of an ligand–receptor pair in the fully developed normal animal. Finally, the effects of a gene knockout and its clinical correlate seem disparate: A reduction of CD18 expression by 90–95% results in a dramatic and even lethal illness in man (Anderson *et al.*, 1985), whereas CD18 mutant mice with similar LFA-1 levels are apparently healthy (Wilson *et al.*, 1993). In addition, the role of clearly crucial adhesion molecules such as the VLA-4–VCAM-1 pathway cannot be studied using knockout animals due to their requirement for the embryonic development (Gurtner *et al.*, 1995; Kwee *et al.*, 1995; Yang *et al.*, 1995). Of course, targeting of the defect to certain tissues or to certain developmental periods by inducible constructs will alleviate these problems in future.

The homing mechanisms (Wu *et al.*, 1988) and the primary structures of adhesion molecules are evolutionarily well conserved. Nevertheless, it remains to be seen whether the results from *in vivo* experiments obtained in laboratory animals can be directly generalized to humans because there are already a few examples of differences between mouse and human adhesion molecules. For example, there are distinct numbers of selectin CR domains in rodent and human selectins (see Section IV,A). These differences may not only affect the distance of extension of the sugar-binding lectin domain from the cell surface but also directly translate into differences in ligand recognition if the CR module proves to be an additional independent adhesion motif. Also, the membrane association and hence the cleavibility of molecules differ. For instance, unlike in mouse no GPI-linked form of VCAM-1 is predicted from a human gene (Cybulski *et al.*, 1991). Moreover, in MAdCAM-1 there appears to be marked differences in the length of the mucin-type domain and cytoplasmic tail and in the existence of the IgA-like domain between mouse and man (Shyjan *et al.*, 1996). Some adhesion molecules such as $\alpha D\beta 2$ even appear to be expressed on different subpopulations of leukocytes in man and animals (Danilenko *et al.*,1995; Van der Vieren *et al.*, 1995). There is also evidence for more subtle differences in the function of adhesion molecules because it is clear that human L-selectin can present sLe^x motifs to endothelial E-selectin (Picker *et al.*, 1991b), whereas mouse L-selectin does not bind to mouse E-selectin (D. Vestweber, personal communication).

VI. Lymphocyte Homing and Inflammation

Normal lymphocyte patrolling is vital because no gross defects in lymphocyte homing are known and they are likely to be incompatible with life.

On the other hand, exaggerated and prolonged responses to innocent antigenic stimulation exemplify the other extreme of how lymphocyte recirculation can go awry.

A. Lymphocyte Homing into Sites of Acute Inflammation

Lymphocyte homing under physiological conditions and during local acute inflammation evoked by physical or chemical agents, hypersensitivity reactions, microbial infections, or tissue necrosis follows the same basic lines. However, several hemodynamic changes take place during the inflammation that make it easier for a cell to extravasate (Cahill *et al.*, 1976; Herman *et al.*, 1972; Smith *et al.*, 1970). Initially, the blood vessels dilate and blood is shunted into the affected microvasculature, resulting in a phase of increased blood flow. Soon, however, the blood volume in the still dilated vessels diminishes leading to slowing of the flow and eventually even stasis (Gallin *et al.*, 1988). These changes result in a profound increase in the proportion of leukocytes in the marginal stream and more cells impinge on endothelial cells. Simultaneously, the local microvasculature becomes activated (Osborn, 1990; Pober and Cotran, 1990) and endothelial gaps emerge with a concomitant increase in vascular permeability. Granulocytes typically enter the scene of inflammation within minutes, whereas after 12 hr increasing numbers of mononuclear cells are encountered and these cell types constitute almost the sole infiltrating population after 20 hr. The induction kinetics of different endothelial adhesion molecules can explain, in part, the sequential appearance of leukocytes in the infiltrate. For example, rapidly induced P- and E-selectin mainly support granulocyte extravasation, whereas upregulation of ICAM-1 and VCAM-1, which are needed for the binding of mononuclear cells, takes longer (Osborn, 1990). Normally, granulocytes perform most phagocytic and degradative functions at the site of insult. At the same time, antigens are transported to local lymph nodes either free in the lymph or carried by the cells. Within a few hours a local inflammatory response develops in the node leading to augmented lymphocyte extravasation and a transient shutdown of cell output (Cahill *et al.*, 1976). Later, lymphocyte trafficking through the node increases dramatically but antigen-specific lymphocytes are selectively retained and they undergo proliferation and differentiation (Cahill *et al.*, 1976; Zatz and Lance, 1971). After 4 or 5 days, immunoblasts leave the node and specifically recirculate back to the same type of lymphatic tissue, or to its peripheral drainage area, marking the start of the effector stage of the inflammatory response. Still later, memory cells are released from the node. Should a rechallenge to the same antigen occur, memory cells can immediately extravasate into the peripheral site of insult and provide help for other leukocytes in eliminating the intruder.

In acute inflammation, the relative contribution of different adhesion pathways apparently changes because, for example, the VCAM-1–VLA-4 interaction has been shown to be relevant in determining the homing of elicited peritoneal T cells into inflamed PLNs (Issekutz, 1991; Picker and Butcher, 1992). The causative agent may also influence the usage of adhesion molecules by lymphocytes in the similar manner as has been shown in the lung infection model with neutrophils: Their emigration to *Escherichia coli*-induced infection is CD18 dependent, whereas in *Streptococcus pneumoniae* infection CD18-independent migration is significant (Doerschuk *et al.*, 1990). Microbes may exert their effects by regulating the appearance of inflammatory mediators, which in turn determines the expression status of homing-associated adhesion molecules. For instance, unlike gram-positive bacteria, gram-negative ones contain LPS, which is an extremely potent inflammation inducer.

Under normal conditions the insulting stimulus is successfully eliminated, mainly via granulocyte-dependent mechanisms, and the acute inflammation resolves. However, chronic inflammation can supervene on the acute one if the stimulus cannot be removed. Certain specific antigens (mycobacteria and silica) can also trigger chronic inflammation *ab initio*.

B. Lymphocyte Homing into Sites of Subacute and Chronic Inflammation

A lymphocyte-predominant infiltrate is a hallmark of chronic inflammation. During a prolonged ongoing inflammatory process, in fact, several characteristics typical to organized lymphoid tissues can be encountered in nonlymphatic tissues (Smith *et al.*, 1970). The morphology of venules changes so that vessels with high endothelial cells indistinguishable from HEVs are found. The phenotype of the vessels changes concomitantly and they start to express vascular addressins and inducible endothelial molecules in a sustained manner. These vessels are often surrounded by lymphocytes that can be organized into primary follicles and even germinal centers are not uncommon. Two well-known examples of functionally distinct lymphocyte recirculation pathways during chronic inflammation are the skin and synovium.

Lymphocytes in subacute and chronic skin infiltrates are brightly CLA positive (Barker, 1995; Picker *et al.*, 1991a). In contrast, CLA is present in only a small subpopulation of memory PBLs, which have a CLA-high, L-selectin-high, $\alpha E\beta 7$-negative, and $\alpha 4\beta 7$-low phenotype thought to represent skin-seeking cells (Picker *et al.*, 1994; Schweighoffer *et al.*, 1993). E-selectin has been proposed to function as a skin-selective addressin in lymphocyte homing because high levels of E-selectin expression associated with perivascular lymphocyte infiltrates are more common in inflamed skin than in other sites of the body (Picker *et al.*, 1991a). Involvement of E-

selectin in lymphocyte recruitment into the skin has been confirmed in *in vivo* studies using nonhuman primates (Silber *et al.*, 1994b). The direct interaction of E-selectin with CLA implies a special role for this receptor–ligand pair in determining skin tropism of lymphocytes (Berg *et al.*, 1991b), although the widespread expression of CLA in HEVs (Duijvestijn *et al.*, 1988) is not easily reconciled with these data. The induction of PNAds in HEV-like vessels during chronic skin inflammation also deserves emphasis (Michie *et al.*, 1993), particularly because most CLA-positive cells in skin coexpress L-selectin (Picker *et al.*, 1993a). As a matter of fact, recent experiments demonstrate the importance of PNAds in mediating lymphocyte binding to the vascular endothelium in inflamed skin. Moreover, these same studies suggest a central role for endothelial VAP-1 in the development of lymphocyte infiltrations in the inflamed skin (Arvilommi *et al.*, 1996). Collectively, these data indicate that at least three distinct, but most likely overlapping, adhesion mechanisms are involved in a relatively early phase in the multistep cascade mediating lymphocyte adhesion to vascular endothelium in inflamed skin. In addition, in animal models, lymphocyte homing into inflamed skin has been shown to be dependent on the function of LFA-1, $\alpha 4\beta 1$–VCAM-1, and CD44 (Camp *et al.*, 1993; Issekutz, 1992; Issekutz and Issekutz, 1991; Silber *et al.*, 1994b). According to the multistep paradigm LFA-1 and $\alpha 4/\beta 1$ may be required during the firm adhesion phase. The exact position of CD44 in the cascade is more speculative, but both the rolling phase and the signaling step could be possible in light of the published studies (DeGrendele *et al.*, 1996; Koopman *et al.*, 1990).

Lymphocyte migration into arthritic joints is a well-studied model of long-standing inflammation (Cronstein and Weissmann, 1993; Jalkanen and Salmi, 1996; Picker and Butcher, 1992). PBLs bind specifically to the HEV-like vessels in the inflamed synovium, whereas certain PLN- or mucosa-selective lymphomas do not (Jalkanen *et al.*, 1986a). Moreover, cell lines with nearly exclusive adherence to synovial endothelium only have been described indicating that in the synovial vasculature some sign is imprinted for a subpopulation of lymphocytes to adhere (Salmi *et al.*, 1992). In synovitis, almost all endothelial adhesion molecules are expressed at increased levels (Grober *et al.*, 1993; Koch *et al.*, 1991; Morales-Ducret *et al.*, 1992), although the amount of P-selectin, E-selectin, and VCAM-1 may not be sufficient to explain the marked lymphocytic influx. L-selectin and CLA obviously do not play any prominent role in PBL binding to the synovium (Fischer *et al.*, 1993; Pitzalis *et al.*, 1996), whereas *in vitro* binding assays and *in vivo* animal studies have shown that LFA-1–ICAM pathway and $\alpha 4\beta 1$ may be relevant for lymphocyte homing into the synovium (Iigo *et al.*, 1991; Jasin *et al.*, 1992; Salmi *et al.*, 1995a). Also, fibronectin may play a role in lymphocyte homing to synovium because synthetic

fibronectin peptides interrupt leukocyte recruitment into the synovium (Wahl *et al.*, 1994). However, the high amount of soluble fibronectin present in normal serum may effectively compete with the fibronectin attached to the lumen of the synovial vasculature. Therefore, the fibronectin may prove to play a more prominent role in lymphocyte migration within the synovial stroma than in initial endothelial adherence. Antibody treatment against CD44 has proved to be an especially effective way to prevent development of collagen-induced arthritis in mice, suggesting a central role for CD44 in the entrance of lymphocytes into the synovium and/or in intercellular or cell–matrix interactions within the synovial tissue (Mikecz *et al.*, 1994; Verdrengh *et al.*, 1995).

Intestinal inflammations are not uncommonly followed by joint symptoms. Inherent capacity of mucosal immunoblasts to bind efficiently to mucosal vessels and inflamed synovial vasculature but not to HEVs in inflamed or normal lymph nodes may help to explain the connection between these diseases (Salmi *et al.*, 1994; Salmi *et al.*, 1995a). Immunoblasts activated in the gut may not only home back to bowel, as expected, but aberrantly to joints as well. In joints, mucosa-derived, preactivated immunoblasts may initiate or accelerate the inflammatory cascade by reacting with the microbial or other triggering components or cross-reactive autoantigens. Interestingly, mucosal immunoblasts do not use the mucosal homing receptor $\alpha 4\beta 7$ to bind to synovial vasculature. CD44 and LFA-1 contribute to the immunoblast binding to both mucosal and synovial vascular endothelium, but no molecule demonstrating a major role in mucosal lymphocyte binding to synovial endothelium has been identified (Salmi *et al.*, 1995a). These findings show that mucosal immunoblasts have dual homing specificities and these specificities partly rely on the usage of different sets of receptors on a given cell.

Mucosa-derived effector cells may also be important in the development of diabetes. Evidence for that has been gathered from both animal studies using nonobese diabetic (NOD) mice and studies using human. Activated T cells propagated from a diabetic pancreas at the onset of juvenile diabetes display preferential binding to pancreatic and mucosal endothelium, whereas only poor binding to peripheral lymph nodes is observed (Hänninen *et al.*, 1993a). In a transfer model of diabetes in NOD mice, the first cells entering the pancreas bear the mucosal homing receptor $\alpha 4\beta 7$ and lack L-selectin. Compatible with this is that the vascular endothelium of pancreas brightly expresses MAdCAM-1 (Hänninen *et al.*, 1993b). Moreover, the NOD mice suffer from a more general homing abnormality, namely, the mucosa-seeking $\alpha 4\beta 7$ lymphocytes have an aberrant homing pattern in these mice as best demonstrated by the high frequency of these cells in peripheral lymph nodes (Hänninen *et al.*, 1996).

Other typical examples of lymphocyte-dependent pathological conditions are graft rejections and various autoimmune diseases (Bevilacqua *et al.*, 1994). For instance, host lymphocytes gain entrance into transplanted heart by binding to ICAM-1 and VCAM-1 of myocardial vessels, and in primate kidney allografts an ICAM-1-mediated pathway appears critical for rejection (Cosimi *et al.*, 1990; Isobe *et al.*, 1992, 1994). In the pathogenesis of experimental autoimmune encephalitis a role for the VLA-4 and in glomerulonephritis for the LFA-1–ICAM-1 pathway have been implicated (Kawasaki *et al.*, 1993; Nishikawa *et al.*, 1993; Yednock *et al.*, 1992). Currently, it thus appears that the specificity of lymphocyte infiltration into sites of inflammation is more likely dictated by the unique multidigit area code than by any individual inflammation-specific adhesion molecule.

VII. Adhesion Deficiencies

A. LAD I and II: Adhesion Deficiencies Created by Nature

In type I leukocyte adhesion deficiency (LAD), newborns manifest with recurring bacterial infections and the inability to form pus (Hayward *et al.*, 1979). In laboratory tests, neutrophilia and impaired leukocyte function are observed (Arnaout, 1990a). This rare syndrome is attributed to multiple different genetic defects that prevent synthesis, pairing, or expression of the common $\beta 2$ integrin chain in an autosomal recessive trait (Kishimoto *et al.*, 1987a). Complete and partial defects have been defined and their symptoms vary: Total lack of $\beta 2$ normally leads to death during early postnatal life (Anderson *et al.*, 1985). The leukocytes from the affected individuals show markedly reduced sticking to endothelium, although the initial rolling is apparently normal (von Andrian *et al.*, 1993). The defect leads to the complete inability of granulocytes to extravasate, except to the lungs (Arnaout, 1990a). Lymphocytes, however, can adhere to endothelium by relying mainly on the VLA-4–VCAM-1-dependent pathway (recall that neutrophils lack $\alpha 4\beta 1$) (Vennegoor *et al.*, 1992) and at sites of inflammation accumulating lymphocytes are indeed observed in these patients.

The two known cases of leukocyte adhesion deficiency type II are characterized by anomalies, mental retardation, neutrophilia, and increased susceptibility to infections (Etzioni *et al.*, 1992). These patients have normal CD18, but a general defect in fucose metabolism leads to inappropriate fucosylation of selectin ligands, the hallmark of which is lack of leukocyte sLe^x. Leukocytes from these patients display greatly impaired rolling, whereas under static conditions they function normally (von Andrian *et al.*, 1993). Thus, these two examples of experiments of nature recapitulate the aptness of multistep adhesion models.

B. Lessons to Be Learned from Gene Targeted Animals

The method to selectively ablate gene expression has also provided the adhesion field with a possibility to gain an insight into the fundamental importance of several homing-associated molecules.

Genes for all known selectins have been disrupted. The lymphocyte trafficking of L-selectin knockout mice is deficient both to PLNs and to Peyer's patches. Moreover, leukocyte rolling in venules and lymphocyte, neutrophil, and monocyte migration into inflamed peritoneum are severely compromised (Ley *et al.*, 1995; Tedder *et al.*, 1995b). These mice do not create normal delayed hypersensitivity reactions, and they show significant resistance to LPS-induced toxic shock (Tedder *et al.*, 1995b). E-selectin mice are apparently healthy and do not show any impairment in leukocyte trafficking to sites of inflammation (Labow *et al.*, 1994). Interestingly, however, anti-P-selectin mAb treatment of E-selectin-deficient mice prevents leukocyte accumulation into thioglycollate-induced peritoneal inflammation. The same blocking effect of the anti-P-selectin antibody cannot be observed in wild-type mice, indicating considerable redundancy in the function of E- and P-selectin (Labow *et al.*, 1994). Greatly diminished leukocyte rolling is observed in early stages of an inflammatory response in P-selectin-deficient mice, but a few hours after the injection of the inflammatory stimulus the leukocytes do roll without P-selectin (Mayadas *et al.*, 1993). Despite the low numbers of rolling cells in these mutant mice, the conversion of the rolling cells to adherent and extravasated cells is more efficient than in the wild-type mice (Johnson *et al.*, 1995). These studies have also shown that long-term macrophage accumulation in the inflamed peritoneum of P-selectin null animals does not reach the level observed in wild-type mice (Johnson *et al.*, 1995). This suggests a role for P-selectin in a prolonged tissue damage involving mononuclear cells (see footnote 3).

Genes of the L-selectin ligands, CD34 and GlyCAM-1, have also been targeted, but detailed descriptions of these mice have not yet been published. CD34 knockout mice have been shown to have normal lymphocyte recirculation system (see footnote 3), whereas the GlyCAM-1 knockouts show enlarged lymph nodes, suggesting a regulatory role for GlyCAM-1 (Krause *et al.*, 1996; Ley, 1995).

As can be suspected on the basis of central functions of many $\beta 1$ integrin heterodimers in several biological systems, homozygous embryos lacking $\beta 1$ integrins die during early postimplantation period (Stephens *et al.*, 1995). Other studies have shown that $\beta 1 -/-$ embryonic stem cells are able to differentiate even to mature B lymphocytes *in vitro*, but these cells cannot colonize the fetal liver (Hirsch *et al.*, 1996). Similarly, $\alpha 4$ knockout

mice die in early embryonic life precluding any observations concerning lymphocyte homing (Yang *et al.*, 1995). Partially β2-defective mice generated by gene targeting express 2 and 16% of normal granulocyte level of β2 in the resting and activated stage, respectively. Despite rather low β2 expression these mice are surprisingly healthy. The mutant mice show partially impaired neutrophil migration into inflamed peritoneum and delayed rejection of cardiac allografts (Wilson *et al.*, 1993) (see footnote 3).

Due to the essential role of VCAM-1 in the formation of umbilical cord and placenta, the VCAM-1-deficient mice die during embryonic life (Gurtner *et al.*, 1995; Kwee *et al.*, 1995). However, a small number (around 1%) of VCAM-1-deficient mice stay viable and develop to fertile, apparently healthy mice with an elevated number of circulating blood mononuclear leukocytes (Gurtner *et al.*, 1995). In ICAM-1 knockouts the rolling flux and velocity of neutrophils are normal but these mice show diminished response to contact hypersensitivity and impaired neutrophil migration into chemically induced peritonitis (Sligh *et al.*, 1993).

In general, the knockouts surviving beyond the embryonic life exhibit less drastic phenotype than would have been suspected based on the data obtained after blocking the function of a particular molecule with antibodies. Only the double knockouts have clearly demonstrated the importance of certain molecular systems as exemplified by mice in which the genes of both E-selectin and P-selectin have been mutated (Frenette *et al.*, 1996). Those mice display massive leukocytosis and elevated levels of IL-3 and granulocyte/macrophage colony-stimulating factor leading to an increase in hematopoietic activity. A nearly total lack of leukocyte rolling and a low number of cells adherent to the vascular wall make the animals extremely susceptible to bacterial infections (Frenette *et al.*, 1996). Altered hematopoiesis in these mice suggests that endothelial selectins may actually have more functions than previously anticipated. On the other hand, the mice lacking both P-selectin and ICAM-1 do not display the residual trauma-induced rolling detectable in P-selectin single deficient mice. This is a rather surprising finding in the light that leukocyte rolling in ICAM-1-deficient mice is intact and ICAM-1 is not thought to be a rolling mediator. However, E-selectin- and L-selectin-dependent rolling can be induced in these mice with TNF-α. Therefore, it is thought that lack of ICAM-1 somehow incapacitates L-selectin-dependent rolling and this can be reinduced with TNF-α (Kunkel *et al.*, 1996).

The studies with animals defective in one or more adhesion molecules have clearly reinforced some earlier findings obtained with other methods and demonstrated additional complexity and redundancy in mechanisms mediating leukocyte–endothelial cell interactions. For example, the role of L-selectin in mediating lymphocyte traffic to mucosal sites was confirmed

by L-selectin-deficient mice (Arbones *et al.*, 1994). Moreover, the knockout animals illustrated the sequential roles of P- and L-selectins in mediating early phases of leukocyte interaction with endothelium: P-selectin seems to be essential in initiating the leukocyte rolling following trauma, whereas at later time points L-selectin supervenes as the major rolling mediator (Ley *et al.*, 1995; Ley and Tedder, 1995).

VIII. Antiadhesion and Proadhesion Therapy

The idea of prevention of inflammation by modulating adhesion molecule function is probably as old as the concept of leukocyte recirculation itself. In fact, many long-known anti-inflammatory drugs, such as corticosteroids, gold, and salicylate, seem to partially exert their effects by modulating the expression and function of adhesion molecules (Cronstein and Weissmann, 1993). However, only during the past few years have more rational intervention strategies become technically feasible. The most dramatic effects have been seen in the inhibition of granulocyte extravasation, but examples of the applicability of these techniques to interfering with the lymphocyte–endothelial cell interactions have started to emerge as well.

The main strategies utilized in antiadhesion therapy are the use of mAbs or fragments thereof, chimeric molecules, soluble ligand analogs, and small molecular drugs (Dasgupta and Rao, 1994; Travis, 1993; Waldmann, 1991). Intravenous application of a mAb can temporarily block the function of the receptor almost completely and by repeated treatments cells can be effectively saturated with a mAb for prolonged periods. The use of only the antigen-recognizing part of the molecule (Fab and Fv), humanized mAbs, or synthetic human mAbs made by phage display techniques reduces the risk of untoward side effects during successive administrations. The possibilities of soluble ligand analogs have drawn increasing attention, especially within the selectin field. Both naturally occurring sugars and small synthetic water-soluble oligosaccharides appear to effectively compete with selectins for their ligands. For example, glycyrrihizin, a component of licorice, was found in a three-dimensional database search to have spatial similarity with sLe^x and it indeed shows inhibitory activity against selectins (Rao *et al.*, 1994). On the other hand, peptides and peptidomimetics can saturate the ligand binding sites of integrins (Dasgupta and Rao, 1994; Travis, 1993; Waldmann, 1991). Lately, the search for rationally designed small molecular drugs that inhibit the function of a single adhesion molecule has become a boom within the pharmaceutical industry.

Antiadhesion therapy has shown great promises in animal and phases I and II human studies (Bevilacqua *et al.*, 1994; Carlos and Harlan, 1994). Especially encouraging results have been obtained in acute neutrophil-mediated inflammations. Many selectin-mediated acute inflammatory reac-

tions such as reperfusion injuries can be blocked by a pretreatment with mAbs, chimeras, or oligosaccharide-based ligand analogs: some even after the onset of the disease (Mulligan *et al.*, 1993; Seekamp *et al.*, 1994). Targeting of firm adhesion and transmigration phases by blocking the functions of ICAM-1 and CD31, respectively, seems to also be feasible (Ma *et al.*, 1991; Murohara *et al*, 1996). Development and production of larger sugar complexes to replace simple sugar structures may greatly facilitate the therapeutic potential of oligosaccharide analogs (Turunen *et al.*, 1995).

Also, chronic lymphocyte-mediated inflammations have been successfully treated with antiadhesion therapy. An up to 50% increase in kidney graft acceptance and delay in rejection can be achieved with anti-ICAM-1 antibodies in primate models (Cosimi *et al.*, 1990). These promising results have led to clinical trials in human patients undergoing kidney transplantation (Haug *et al.*, 1993). Autoimmune diseases such as diabetes can be prevented in disease-prone NOD mice by anti-α4 and L-selectin mAbs (Burkly *et al.*, 1994; Yang *et al.*, 1993). Anti-α4 treatment has also been shown to be effective in preventing colitis in the cotton-top tamarin (Podolsky *et al.*, 1993). In chronic inflammatory disorders, e.g., arthritis, considerable amelioration has been seen in both animal and human studies with anti-ICAM-1 mAb treatment (Iigo *et al.*, 1991; Kavanaugh *et al.*, 1994). Also, synthetic fibronectin peptides have shown efficacy in preventing experimental arthritis (Wahl *et al.*, 1994). However, careful evaluation of the numerous ongoing animal and human studies is needed to understand the most suitable forms of applications, pharmacokinetics, timing in respect to the disease onset, long-term outcome, risks of general side effects, and compromised immune defense. Only when the most optimal conditions and formulas have been worked out can the best possible clinical benefits from the new forms of anti-inflammatory therapy targeted to inhibit leukocyte interaction with vascular endothelium be expected.

In certain special cases enhanced lymphocyte adhesion is desired. A typical example is immunotherapy of cancer with TILs. A prerequisite for successful therapeutic outcome is proper entrance of the *in vitro*-activated and expanded TILs into the malignant tissue when given back to the patients. Therefore, it would be of essential importance to identify those molecules mediating the extravasation of TILs preferentially into the tumor tissue and to learn to regulate the expression of these molecules in an appropriate way. Finally, gene therapy may provide a modern way to correct the leukocyte adhesion deficiencies (Wilson *et al.*, 1990).

IX. Concluding Remarks

The past 15 years have witnessed a rapid and successful dissection of the molecular architecture of interactions between lymphocytes and

endothelium. This field of investigation has combined in an effective way the power of new technologies, including recombinant DNA technology and methods of glycobiology, to analyze the multidimensional phenomenon of lymphocyte recirculation. A useful working model of lymphocyte extravasation cascade has been compiled on the basis of numerous elegant *in vitro* and *in vivo* studies. This paradigm envisions lymphocyte emigration as a multistep process with sequential but partially overlapping stages of interaction between the lymphocyte and the vascular lining.

Many aspects of lymphocyte recirculation, nevertheless, require further elucidation. The exact mode and stage of action of many known adhesion molecules in the extravasation cascade still requires refinement. For example, the elucidation of biophysics of selectin bonding to their carbohydrate ligands and the activation signals leading to integrin activation under natural conditions in the body will remain a formidable task for investigators in the future. The experimental analyses of these questions will probably require development of new techniques that will enable measurements of very rapid and transient interactions. Moreover, it is highly likely that completely new adhesion and signaling molecules guiding the lymphocyte traffic will be found.

The lymphocyte homing studies began from the observation of tissue-selective recirculation of immunoblasts. Although the molecular basis of this phenomenon was thought to be resolved rather early, recent findings have again rendered this issue more complicated. At the moment we are therefore in a position in which we cannot predict the homing behavior of a given lymphocyte on the basis of the adhesion molecule machinery it is carrying. It remains to be seen whether the multidigit area codes from the cascade model can be elucidated, which will explain the remarkably selective lymphocyte homing to secondary lymphoid organs. Furthermore, almost all lymphocyte homing experiments have been done with T cells. Therefore, analysis of the subsets of T cells and other lymphocytes, B cells in particular, will be rewarding in the future.

What happens to a lymphocyte when it has stably arrested to the endothelium? There are surprisingly few studies on the mechanisms of lymphocyte transmigration through the vessel wall, its chemotactic and haptotactic movements within the tissue stroma, its intraorgan segregation into discrete lymphoid areas, and its exit into the efferent lymphatic system. Reconstitution of these stages of lymphocyte recirculation will be a real methodological and intellectual challenge in the coming years.

Finally, the great premise of adhesion therapy has to be fulfilled in man. In animal models a huge variety of life-threatening acute and chronic conditions can be drastically alleviated by rationally modulating the leukocyte trafficking. Even if some of these results can be applied to humans, the studies on lymphocyte recirculation have demonstrated in a convincing

way how the basic molecular understanding of lymphocyte–endothelial interaction can be translated to a modern rational drug design to prevent inappropriate inflammation. These very same basic biological principles can also be used to efficiently direct the body's own defense mechanisms, e.g., in immunosuppressive and malignant diseases, to the sites of interest to combat a foreign insult or tumor growth.

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ADVANCES IN IMMUNOLOGY, VOL. 64

Plasma Cell Dyscrasias

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I. Introduction

Plasma cell dyscrasias are defined as hematological disorders with monoclonal proliferation of the cells from later stages of B lymphocyte lineage differentiation. These disorders include multiple myeloma, plasmacytoma, Waldenström's macroglobulinemia, heavy chain diseases, monoclonal gammopathies of undetermined significance (MGUS), and primary amyloidosis (Greaves, 1986) (Fig. 1). The common clinical manifestations of these diseases originate from (i) the expansion of malignant or nonmalignant plasma cells, (ii) the secretion of large amounts of monoclonal immunoglobulins and constituent subunits (M component), and (iii) the production of certain cytokines.

Multiple myeloma grows in the bone marrow, whereas plasmacytoma develops in extramedullar lymphadenotic and nonlymphadenotic organs. Myeloma is primarily a disease of the elderly and increases in incidence with age, whereas plasmacytoma tends to affect younger individuals. The bone marrow environment is considered to be important for the development of multiple myeloma. Patients with multiple myeloma suffer from bone pain or fracture, hypercalcemia, renal failure, hyperviscosity syndrome, anemia, and recurrent infection with immunodeficiency. Conventional chemotherapy and radiation therapy may induce remission, but problems include drug resistance, infrequent complete remission, and inevitable relapse (Alexanian and Dimopoulos, 1994). Early myeloablative therapy supported by autologous stem cells appears effective (Jagannath *et al.*, 1992; Alexanian *et al.*, 1994; Harousseau *et al.*, 1995), but there still remain problems such as residual myeloma cells detectable after autografting (Corradini *et al.*, 1995). Therefore, the need remains for a new therapeutic strategy.

Waldenström's macroglobulinemia is a neoplasm of lymphoplasmacytoid cells with IgM monoclonal gammopathy first described by Waldenström in 1944. The major clinical manifestation is hyperviscosity syndrome caused by an increase in IgMs, about 15% of which are cryoglobulins. Lymphadenopathy and hepatosplenomegaly are frequently observed in these cases. In contrast to myeloma, macroglobulinemia does not manifest osteolytic lesions or hypercalcemia. Patients with increased bone marrow plasma

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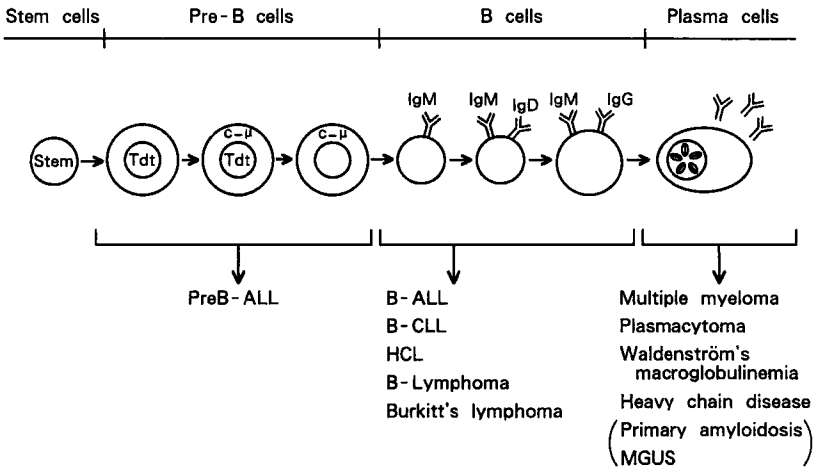


FIG. 1. Schematic representation of human B cell neoplasms categorized according to stage of B cell differentiation. Abbreviations used: ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; HCL, hairy cell leukemia; MGUS, monoclonal gammopathies of undetermined significance. Primary amyloidosis and MGUS are referred to as plasma cell dyscrasias but are not categorized as neoplasms.

cells, lytic bone lesions, and hypercalcemia are diagnosed as having IgM myeloma.

Heavy chain diseases are relatively rare lymphoplasmacytic neoplasms characterized by the secretion of defective heavy chains devoid of light chains (Franklin *et al.*, 1964). γ, α (Seligmann *et al.*, 1968), and μ heavy chain diseases (Forte *et al.*, 1970) have been described. Recent molecular analysis of these diseases has revealed the structural defects and generating mechanisms of the aberrant immunoglobulin heavy chains of these diseases (Cogne *et al.*, 1992).

MGUS denotes a condition characterized by the presence of M protein in the absence of clinical or laboratory evidence for multiple myeloma, macroglobulinemia, or other related diseases. The condition was initially referred to as benign monoclonal gammopathy. In 1978, Kyle proposed MGUS for this condition because 11% of the patients evolved into cases of malignant plasma cell dyscrasia such as multiple myeloma.

Primary amyloidosis is generally characterized by the deposition of amyloid of light chains of immunoglobulin (AL), whereas secondary amyloidosis is caused by the deposition of amyloid protein A (AA). Patients with AL amyloidosis have monoclonal immunoglobulins in the serum, Bence Jones proteins in the urine, and increased numbers of plasma cells in the bone

marrow (Husby and Sletten, 1986). Therefore, primary amyloidosis is included in plasma cell dyscrasias.

In this review, we first describe plasmacytomagenesis in mice, which has been thoroughly analyzed in experiments, to provide an understanding of human diseases through a comparative approach. Second, we report on new insights into the cellular and molecular biology of human plasma cell dyscrasias, particularly multiple myeloma.

II. Plasmacytoma in Mouse

A. Origins of Plasmacytoma Cells

1. Mouse B Cell Development

Plasma cells are terminally differentiated B cells secreting immunoglobulin. In this past decade, the study of mouse B cell development has remarkably progressed with the aid of molecular and developmental biology techniques (Chen and Alt, 1993; Kantor and Herzenberg, 1993; Rolink and Melchers, 1993; Löffert *et al.*, 1994). The differentiation of B lymphocytes from pluripotent stem cells goes through serial stages that are defined by successive immunoglobulin gene rearrangements and expression of stage-specific molecules. B cells differentiate into plasma cells as a result of antigen, B cell mitogen, and T cell–B cell interaction.

It has been demonstrated that aberrant chromosomal rearrangement during B cell development occurs in most mouse plasmacytomas, whereas most of the chromosomal aberration observed in plasmacytomas has been shown to result in deregulated c-*myc* gene expression. These events are strongly suspected of being involved in mouse plasmacytoma development (Fig. 2).

2. Possible Involvement of CD5$^+$ B Cells in Plasmacytomas

CD5$^+$ B cells (also called Ly-1 B cells or B-1a cells) constitute a unique B cell population with self-renewing potency (Kantor and Herzenberg, 1993; Kearney, 1993; Hardy and Hayakawa, 1994). In adult mice, bone marrow is considered to be the primary site for B cell development. However, a cell reconstitution experiment has demonstrated that CD5$^+$ B cells are generated only from fetal or newborn liver or from bone marrow from young mice, but not from adult bone marrow. In adult mice, CD5$^+$ B cells are predominantly detected in the peritoneal cavity and sparsely in the spleen. They are rare in lymph nodes, Peyer's patches, and peripheral blood.

Although the function of CD5$^+$ B cells remains to be elucidated, there is substantial evidence suggesting that CD5$^+$ B cells may be precursors of

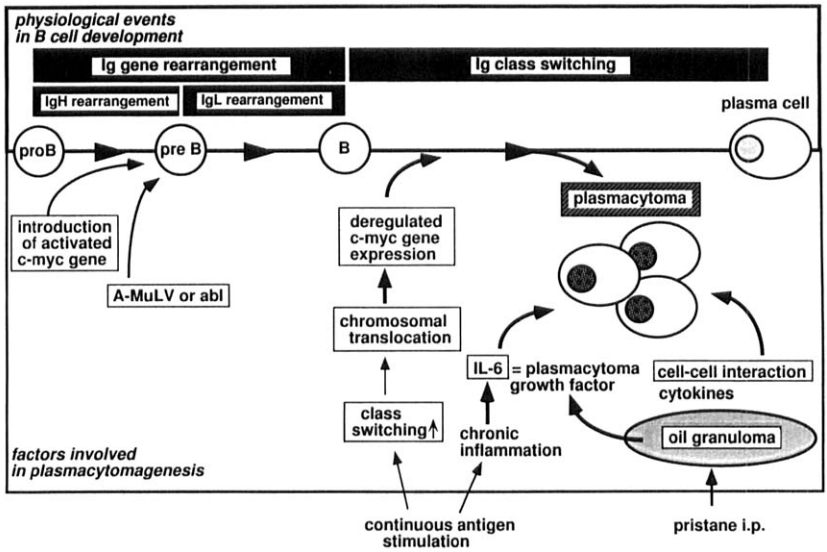


FIG. 2. Factors involved in mouse plasmacytomagenesis. Factors that have been suggested to be involved in plasmacytomagenesis are boxed. Cooperation of these factors is necessary to induce mouse plasmacytomas.

plasmacytomas. Histologically, plasmacytomas have been shown to exclusively develop in granulomatous tissues, which are induced in peritoneal cavities by chronic irritation. Furthermore, plasmacytomas can be induced with high frequency in conventionally raised BALB/c mice. On the other hand, the incidence is very low in BALB/c mice housed in a very clean environment (McIntire and Princler, 1969; Byrd *et al.*, 1991), indicating that the gut mucosal immune system may be important in plasmacytoma development. More than 60% of plasmacytomas in BALB/c mice are IgA producers (Potter, 1984). It has been suggested that IgA plasma cells in gut tissue are derived from $CD5^+$ B cells (Kroese *et al.*, 1989, 1993). To investigate the origin of IgA plasma cells in gut-associated lymphoid tissues, chimeric mice were generated with bone marrow-derived B cells and $CD5^+$ B cells of different immunoglobulin allotypes (Kroese *et al.*, 1989). Only plasma cells of the $CD5^+$ B cell allotype were shown to be IgA plasma cells in gut tissue, whereas IgM plasma cells were of either the bone marrow or $CD5^+$ B cell allotype.

B. Pristane-Induced Plasmacytomas

It is well known that mouse plasmacytomas are observed only in a few inbred strains of mice (Potter, 1984; Potter and Wiener, 1992). Some

spontaneous plasmacytomas have been reported in aged mice, although the incidence was very low. Therefore, studies for mouse plasmacytomas have focused on experimentally induced plasmacytomas (Table I).

Intraperitoneal administration of some specific nonmetabolizable materials induces chronic inflammation in mouse peritoneal cavities. These

TABLE I
CHARACTERISTICS OF EXPERIMENTALLY INDUCED PLASMACYTOMAS IN MICE

Intraperitoneal injection of pristane
Only BALB/c and NZB are susceptible (other strains are resistant)
Oil granuloma formation and IL-6 are important
Incidence is influenced by immune status of mice
Most of PCT[a] carry c-*myc*-associated chromosomal aberration; 90% of PCT have t(12;15), 10% t(6;15)
IgA PCT are dominant, some are IgG producers
Pristane + virus infection
Pristane + A-MuLV
Short latency in comparison with PCT by pristane alone
No PCT without pristane priming
Increase in PCT carrying t(6;15)
Pristane + c-*myc*/v-Ha-*ras* retrovirus
Short latency
PCT develop in both susceptible and resistant mouse strains
PCT lack rearrangement and expression of the endogenous c-*myc* gene
No PCT without pristane priming
No PCT by either c-*myc* virus of v-Ha-*ras* virus alone
Most of PCT are IgM producers
Pristane + ABL-MYC retrovirus
Most effectively induce PCT (mainly IgM producers)
PCT develop even without pristane treatment (mostly IgA PCT)
PCT develop in both susceptible and resistant strain
PCT lack rearrangement of the endogenous c-*myc* gene
Transgenic mice
Eμ-*myc* Tg
Develop B lymphomas, but no PCT without treatment
PCT induced by pristane + A-MuLV lack c-*myc* gene associated chromosomal translocation
Eμ-v-*abl* Tg
Develop PCT without treatment
PCT develop in both susceptible and resistant strain
PCT carry c-*myc* rearrangement by t(12;15)
Most of PCT are IgA or IgG producers
In Eμ-v-*abl* $\times$ Eμ-*myc* Tg, PCT develop with short latency
L^d-IL-6 Tg
Tg crossed to BALB/c develop IgA PCT without treatment
PCT carry typical t(12;15)

[a] PCT, plasmacytomas; Tg, transgenic mice.

chronic inflammatory changes then evoke malignant plasma cell transformation in a few selected strains such as BALB/c and NZB mice. The first plasmacytoma induction method was accidentally discovered in an experiment using BALB/cAn mice implanted with Millipore diffusion chambers in peritoneal cavities. Since then, a variety of materials, including plastics, paraffin oils (Potter and Boyce, 1962), 2,6,10,14-tetramethylpentadecane (pristane) (Anderson and Potter, 1969), and silicone gels (Potter *et al.*, 1995), have been introduced intraperitoneally to BALB/c mice to induce plasmacytomas (Potter and Wiener, 1992). Because pristane has been proved to be most effective for plasmacytoma induction, pristane-induced plasmacytomagenesis has been thoroughly studied as a mouse model system. Pristane is a nonmetabolizable pure alkane, and pristane itself does not attack DNA (Potter, 1984). Incidence of plasmacytomas depends on the pristane administration procedure and on the mouse strain. Three 0.5-ml intraperitoneal injections spaced 2 months apart have been shown to be most effective because this induces plasmacytomas with an incidence of 50–70% and with a mean latency of 190–220 days in BALB/cAn and BALB/cπ mice (Potter and Wax, 1983; Potter, 1984). The striking characteristic of BALB/c plasmacytomas is that most of them carry a chromosomal translocation involving one of the immunoglobulin loci and the c-*myc* locus.

1. Oil Granulomas

Intraperitoneally injected pristane induces influx of inflammatory cells, such as macrophages and neutrophils, in a few days (Cancro and Potter, 1976). Macrophages phagocytose oil droplets and adhere to the peritoneal surface, forming inflammatory granulomatous tissues called oil granulomas. The oil granulomas are immediately vascularized and numerous nucleated cells are found to exist in these granulomas (Potter and MacCardle, 1964). Histological examination shows that plasmacytomas occur exclusively in these oil granulomas. Furthermore, intraperitoneal transplantation of primary plasmacytoma cells into syngeneic mice without pristane conditioning has not been successful (Potter *et al.*, 1972; Cancro and Potter, 1976), suggesting that the microenvironment in oil granulomas is essential for plasmacytoma development.

It has been suggested that some cellular interactions are necessary for plasmacytomagenesis. *In vitro* long-term culture of plasmacytoma cells was accomplished by using stromal feeder cell layers derived from the initial site of plasmacytomagenesis (Degrassi *et al.*, 1993). Degrassi *et al.* prepared stromal cells from mesenteric tissue of pristane-induced plasmacytoma-bearing BALB/cAnPt mice. The basement membrane was used in the stromal cell and plasmacytoma primary cultures, and both

stromal cells and plasmacytomas could be expanded for at least 20 months. The stromal feeder cells were used to produce several plasmacytoma cell lines, all of which were shown to produce certain amounts of interleukin-6 (IL-6). The proliferation of plasmacytomas was inhibited by more than 85% with IL-6 antibody, and some of the plasmacytomas showed IL-6-dependent growth characteristics. However, none of the established plasmacytomas could be expanded in the presence of IL-6, basement membrane + IL-6, stromal cell-conditioned medium, or the physically separated culture from the stromal cells. These findings suggest that these stromal cells support at least expansion of transformed cells by producing growth factors (e.g., IL-6) or by providing unknown stimulatory signals induced during the physical interaction of the cells. After long-term culture, some plasmacytoma cell lines lost their stromal cell-dependent growth characteristics, indicating they had changed into a more malignant phenotype.

2. *Factors Influencing Pristane-Induced Plasmacytomagenesis*

a. Genetic Background. Pristane-induced plasmacytomagenesis exhibits remarkable strain dependency. Most inbred strains commonly used in laboratories, such as A, AL/N, AKR, C3H, CBA, C57BL, DBA/2, SWR, and STS/A, are resistant to plasmacytoma induction (Potter, 1992; Potter and Wiener, 1992) so that many of these strains in fact do not develop plasmacytomas. Although a few of these strains do develop plasmacytomas, the incidence is less than 5%. In contrast, in the most plasmacytoma-susceptible BALB/cAn strain, from 50 to 70% of the mice develop plasmacytomas as a result of three bimonthly intraperitoneal injections of pristane. The incidence varies, however, even between sublines of the BALB/c strain. BALB/cJ was reported to be relatively resistant to plasmacytomagenesis in comparison with BALB/cAn. The incidence for BALB/cJ was 11%, whereas it was 61% for BALB/cAn mice given pristane with the same regimens (Potter and Wax, 1981). NZB, which is well known to be an autoimmune disease-prone strain, is another strain susceptible to plasmacytomagenesis. Large numbers of NZB mice were analyzed for susceptibility to pristane-induced plasmacytomagenesis (Morse *et al.*, 1978). NZB mice developed plasmacytomas with almost the same frequency (77%) as did BALB/c mice under identical conditions. However, plasmacytomas of NZB mice showed a significantly longer latency than did those of BALB/c mice.

b. Immune Responses. The incidence of plasmacytomas is also influenced by the immune status of mice. It has been shown that mice should be housed conventionally, not in a pathogen-free environment, to obtain a high incidence of plasmacytomas. McIntire and Princler (1969) reported

that germ-free BALB/cAn mice were relatively resistant to plasmacytoma induction. Only 6% of germ-free BALB/cAn mice developed plasmacytomas with an average latency of 18 months, whereas a more than 10-fold (70–77%) raised in conventional conditions developed plasmacytomas with a latency period of only 11.3–11.5 months. Three kinds of antigen were intraperitoneally injected with mineral oil adjuvant to investigate the effect of external antigenic stimulation on plasmacytomagenesis in germ-free mice. There was no significant difference between the results with or without antigen stimulation in terms of plasmacytomagenesis (McIntire and Princler, 1969), suggesting that continuous antigen stimulation by microbial flora may be more important for plasmacytomagenesis than exogenous antigen stimulation. Byrd *et al.* (1991) studied plasmacytoma induction in further detail using specific pathogen-free (SPF) BALB/cAn mice. Similar low incidences and long latencies were observed as in the germ-free mice experiment. Five percent of the SPF mice developed plasmacytomas with a mean latency period of 300 days, whereas 55% of the conventionally raised mice developed plasmacytomas with a latency of 230 days. Another difference between the two sets of mice was that the influx of $CD4^+$ T cells into the peritoneal cavity of SPF mice was significantly reduced compared to that for conventional mice, although both groups of mice were shown to have similar gut flora. Analysis of serum antibodies against several kinds of viruses demonstrated that SPF mice had much less antigen to challenge these viruses, indicating that exogenous antigen stimulation is necessary to activate immune responses, which may result in a high incidence of plasmacytomas.

3. *Interleukin-6*

It has been suggested that IL-6 plays an important role in pristane-induced plasmacytomagenesis. It was demonstrated that a murine adherent phagocytic cell line produces growth factor(s) for a MOPC 104E plasmacytoma cell line (Namba and Hanaoka, 1972). Nordan and Potter (1986) reported identification of a plasmacytoma growth factor (PCT-GF) produced by the murine macrophage cell line P388D1, and determined its NH_2 terminal sequence (Nordan *et al.*, 1987). A hybridoma growth factor, designated interleukin hybridoma plasmacytoma 1 (IL-HP1), was purified from a murine helper T cell line (Van Snick *et al.*, 1986). All these plasmacytoma growth factors were shown to be identical and are now called IL-6 (for review, see Akira *et al.*, 1993).

As mentioned previously, the microenvironment of oil granulomas has been implicated in plasmacytoma development. Macrophages, which are one of the major cell components of oil granulomas, are known to produce large amounts of IL-6 following certain stimuli (Akira *et al.*, 1993).

Large amounts of IL-6 were also detected in the peritoneal cavities of plasmacytoma-bearing mice (Shacter *et al.*, 1992). The addition of indomethacin, a nonsteroidal anti-inflammatory drug, to drinking water dramatically inhibited pristane-induced plasmacytoma development (Potter *et al.*, 1985). It was demonstrated that intraperitoneal injection of pristane causes elevation of IL-6 in peritoneal cavities (Shacter *et al.*, 1992), whereas indomethacin administration was shown to decrease the peritoneal IL-6 level by 75–80% (Shacter *et al.*, 1992). The production of IL-6 from stimulated pristane-elicited peritoneal exudate cells *in vitro* was inhibited by 54–64%. An *in vitro* plasma cell culture system was established using stromal feeder cell layers (Degrassi *et al.*, 1993). The growth of plasmacytomas on the feeder cells was inhibited by antibody against IL-6.

C. Chromosomal Translocation and c-*MYC* Gene Rearrangement

Chromosomal translocations that lead to the juxtaposition of the c-*myc* locus and an immunoglobulin locus are regularly observed in three kinds of B cell tumors: human Burkitt's lymphoma, mouse plasmacytoma, and rat immunocytoma (for reviews, see Klein and Klein, 1985; Cory, 1986; Spencer and Groudine, 1991). Cytogenetic analysis has revealed that more than 95% of mouse plasmacytomas carry one of the chromosomal translocations involving chromosome 15, such as t(12;15), t(6;15), or t(15;16) (Potter and Wiener, 1992). In virtually all mouse plasmacytomas, chromosomal translocation juxtaposes the c-*myc* or *pvt-1* locus on chromosome 15 to one of the immunoglobulin loci on chromosomes 12 (IgH), 6 (Igκ), or 16 (Igλ) (Potter and Wiener, 1992). These chromosomal translocations cause the constitutive expression of the c-*myc* protooncogene (Adams *et al.*, 1983; Stanton *et al.*, 1983; Ohno *et al.*, 1984).

The most frequent chromosomal translocation observed in pristane-induced plasmacytomas is a t(12;15), which occurs in about 90% of plasmacytomas carrying translocations (Ohno *et al.*, 1979, 1984; Wiener *et al.*, 1990). Therefore, t(12;15) was designated "typical" and the others "variant" chromosomal translocations. The ratio of these chromosomal translocations changes according to the method of plasmacytoma induction (Sugiyama *et al.*, 1989; Wiener *et al.*, 1990; Potter and Wiener, 1992). About 90% of pristane-induced plasmacytomas have t(12;15) and 10% have t(6;15) (Ohno *et al.*, 1979; Webb *et al.*, 1984; Cory *et al.*, 1985). Following pristane + A-MuLV (Abelson murine leukemia virus) induction, t(6;15) is observed more frequently (Ohno *et al.*, 1984). Furthermore, t(15;16), which occurs rarely in response to pristane alone or pristane + A-MuLV, was found with equal frequency as that for t(6;15) as a result of new induction methods (Sugiyama *et al.*, 1989; Wiener *et al.*, 1990) in which A-MuLV-infected

spleen or bone marrow cells are transferred to pristane-treated BALB/c mice.

In the typical t(12;15) chromosomal translocation, the break point on chromosome 15 is in the 5′ flanking region of the c-*myc* gene, and the break on chromosome 12 occurs in immunoglobulin heavy chain (IgH) switch (S) regions, predominantly in Sα or Sμ (Harris *et al.*, 1983; Yang *et al.*, 1985; Cory, 1986). Furthermore, the immunoglobulin heavy chain S region in most cases faces the 5′ regulatory sequence in the c-*myc* locus in a head-to-head arrangement (Shen *et al.*, 1982; Erikson *et al.*, 1985; Wirschubsky *et al.*, 1985). On the other hand, t(6;15) is characterized by juxtaposition in head-to-tail fashion of the immunoglobulin κ chain C region on chromosome 6 with the *pvt-1* locus on chromosome 15, which is a 15-kb region located approximately 200–300 kb 3′ of the c-*myc* gene (Webb *et al.*, 1984; Banerjee *et al.*, 1985; Cory *et al.*, 1985; Axelson *et al.*, 1991). In another variant translocation, t(15;16), immunoglobulin λ chain on chromosome 16 breaks between the V1 and C3 regions and joins *pvt-1* locus in a head-to-tail manner (Axelson *et al.*, 1991). A novel t(12;15) has been reported in which the Sα region (on chromosome 12) juxtaposes with the *pvt-1* locus (on chromosome 15) in head-to-tail arrangement as in the case of t(6;15) (Shaughnessy *et al.*, 1994). All these chromosomal translocations replace the regulatory element of the c-*myc* and can mostly be detected as c-*myc* gene rearrangements, resulting in deregulated c-*myc* expression.

Other chromosomal aberrations involving chromosomal 15 have also been reported. Interstitial deletion in band D2 of chromosome 15, in which the c-*myc* gene is located, occurred in three plasmacytomas lacking chromosomal translocations (Wiener *et al.*, 1984). One of three plasmacytomas was shown to have an insertion of the immunoglobulin heavy chain enhancer (Eμ) in the 5′ flanking region of the c-*myc* gene (Fahrlander *et al.*, 1985).

Shaughnessy *et al.* (1993) reported another chromosomal aberration resulting in a c-*myc* gene rearrangement in chromosomal translocation-negative and in trisomy 11-containing plasmacytomas (Shaughnessy *et al.*, 1993). This c-*myc* rearrangement was caused by retroviral enhancer insertion in the 5′ flanking region of the c-*myc* gene, which also induced upregulation of the c-*myc* gene expression. The expression level of one of these plasmacytomas, ABPC22, was much lower than that of other plasmacytomas, suggesting even a low level of c-*myc* gene expression can result in plasmacytoma development. Trisomy 11 is the second most frequent chromosomal aberration occurring in plasmacytomas induced by pristane and A-MuLV (Ohno *et al.*, 1984). It was demonstrated that nearly 90% of plasmacytomas induced by pristane priming and v-*abl*/v-

myc-bearing retrovirus (ABL-MYC) contained trisomy 11 (Wiener *et al.*, 1995). The ABL-MYC retrovirus was shown to induce plasmacytomas with high frequency and with a shorter latency period than that of any other plasmacytomas (Largaespada *et al.*, 1992). Although the significance of the duplicated chromosome 11 remains unclear, it has been suggested that a gene dosage of unknown gene product(s) may be sufficient for plasmacytoma development in concert with v-*abl* and c-*myc* oncogenes.

Recently, a polymerase chain reaction (PCR) method was developed to detect the rearrangement between c-*myc* and immunoglobulin Sα, which frequently occurs in t(12;15). Surprisingly, the IgH Sα/c-*myc* rearrangement was detected in oil granuloma tissues from 40% of pristane-treated BALB/c mice as early as 30 days after pristane injection (Janz *et al.*, 1993). Considering the relatively long latency of plasmacytoma development, the rearrangement does not seem to be directly linked to plasmacytomagenesis so that certain additional genetic changes are thought to be necessary for plasmacytoma development.

D. Spontaneous Plasmacytomas

As described previously, mouse plasmacytomas are observed in only a few restricted inbred strains, even in mice treated with pristane (Potter, 1984; Potter and Wiener, 1992). However, some spontaneous plasmacytomas with a quite low incidence have also been reported.

C57BL/KaLwRij is an inbred strain of mice that frequently develops a benign B cell monoclonal proliferation referred to as "benign monoclonal gammopathy." It was reported that malignant plasma cell tumors arose in about 0.5% of aged, i.e., more than 2 years old, C57BL/KaLwRij mice (Radl *et al.*, 1988). Most of these were found to be IgG producers with the exception of a few IgD producers. Pathological analysis revealed that bone marrow had been replaced with typical myeloma cells, and bone lytic lesions were especially found in ribs, femora, and tibiae. A myeloma kidney-like change was occasionally observed. These findings were very similar to those for human multiple myelomas. The spleen was also shown to be an organ affected by myeloma cell infiltration. Bone marrow cells and spleen cells of tumor-bearing mice could be transplanted to syngeneic mice by either intravenous or intraperitoneal injection. Transplanted tumor cells were found to be predisposed to disseminate into bone marrow even if they had been transplanted into the peritoneal cavity. Radl *et al.* (1988) established myeloma cell lines after serial transplantation and demonstrated that most of the myeloma cell lines of earlier transplantation generations did not have c-*myc* gene rearrangement (Radl *et al.*, 1990), and there was no mention of c-*myc* gene expression of these cell lines. A comparison of

this result with those for both pristane-induced plasmacytomas and human multiple myelomas might well prove to be of interest.

E. Plasmacytomagenesis-Induced Virus Infection

To date, many experiments have suggested that a single oncogene is not sufficient for malignant transformation. For example, deregulated expression of the c-*myc* oncogene, which has been implicated as playing an important role especially in hematopoietic tumor development, can induce mono- or oligoclonal tumors *in vivo,* but not polyclonal tumors, suggesting that some other oncogenic genetic changes are needed for tumorigenesis (Schwartz and Witte, 1988; Klein, 1991). Avian retroviruses inducing hematopoietic tumors have been demonstrated to carry two oncogenes, indicating that the expression of two oncogenes is more effective than a single oncogene for tumor development in certain cell types (Schwartz and Witte, 1988). Several types of recombinant retroviruses carrying oncogene(s) have been introduced *in vitro* or *in vivo* to investigate the cooperative function of oncogenes in plasmacytomagenesis.

1. Abelson Murine Leukemia Virus

It has been demonstrated that A-MuLV causes mouse plasmacytomas under certain conditions. The type of lymphoid tumor induced by A-MuLV depends on the age of the recipient mice, the route of virus infection, and the existence of a helper virus (for review, see Rosenberg and Witte, 1988; Kerr, 1994; Rosenberg, 1994). Potter *et al.* (1973) demonstrated that pristane-induced plasmacytomagenesis was greatly accelerated by intraperitoneal injection of A-MuLV in adult BALB/c mice. However, A-MuLV alone exclusively induced pre-B cell lymphomas without pristane treatment. In this experiment, mice were primed with a single 0.5-ml intraperitoneal injection of pristane for 30–60 days before A-MuLV infection. With pristane-conditioning, A-MuLV induced plasmacytomas in 28% of the mice (26 of 92) within 77–149 days after the pristane injection, and it induced lymphosarcomas in 58% (54 of 92). When the mice were treated with pristane alone, less than 4% (2 of 57) developed plasmacytomas between Days 138 and 166 after a single injection of pristane. Without pristane priming, A-MuLV alone induced lymphosarcomas in 42% of the mice (16 of 38), but no plasmacytomas were found.

Ohno *et al.* (1984) demonstrated that A-MuLV-induced plasmacytomas carried a nonrandom chromosomal translocation, such as t(12;15), indicating that the cooperative action of v-*abl* and the activated c-*myc* gene may be necessary for plasmacytomagenesis.

2. c-myc/v-Ha-ras Retrovirus

Clynes *et al.* (1988) introduced a retrovirus harboring both Eμ-driven c-*myc* and Molony murine leukemia virus long terminal repeat (Mo-MuLV

LTR)-driven v-Ha-*ras* into BALB/cAnPt or plasmacytoma-resistant (BALB/cAnPt × DBA/2N)F1 (CDF1) mice after pristane treatment. In pristane-treated mice, the Eμ-c-*myc*/v-Ha-*ras* virus induced plasmacytomas in both BALB/cAn and CDF1 mice after a short latency period, whereas only a few plasmacytomas were induced by the v-Ha-*ras* virus alone. However, the Eμ-c-*myc*/v-Ha-*ras* virus did not induce plasmacytomas without pristane priming, whereas the Eμ-c-*myc* virus alone did not induce plasmacytomas even in pristane-treated mice. Induced plasmacytomas were mono- or oligoclonal and transplantable to syngeneic mice, either pristane treated or untreated. Interestingly, plasmacytomas induced by the Eμ-c-*myc*/v-Ha-*ras* virus + pristane lacked the rearrangement and the expression of endogenous c-*myc* gene. Furthermore, most of them were IgM producers. These phenomena can be explained by the hypothesis that the synergistic action of v-Ha-*ras* and activated exogenous c-*myc* gene can overcome the chromosomal translocation that is commonly observed in pristane-induced plasmacytomagenesis.

3. *ABL-MYC Retrovirus*

There is little doubt that the v-*abl* oncogene plays an important role in plasmacytomagenesis, even though pre-B cell transformation is a preferential activity of this oncogene (Kerr, 1994; Rosenberg, 1994). Infection with A-MuLV of pristane-treated mice induces accelerated plasmacytomagenesis (Potter *et al.*, 1973). However, A-MuLV alone can induce pre-B cell lymphomas without pristane but not without plasmacytomas. Moreover, pre-B cell lymphomas occur more frequently than plasmacytomas even in pristane-treated mice. Eμ-v-*abl* transgenic mice were shown to develop B cell lymphomas and plasmacytomas (Rosenbaum *et al.*, 1990). In addition, Eμ-v-*abl* × Eμ-*myc* double-transgenic mice developed plasmacytomas very rapidly, even in the pristane-induced plasmacytomagenesis-resistant mouse strain (Rosenbaum *et al.*, 1990). All these results strongly suggest that v-*abl*-integrated B lineage cells require *myc* gene upregulation by either chromosomal translocation or introduction of the activated *myc* gene to be transformed into plasmacytomas. To study the synergistic function of the c-*myc* and the v-*abl* in further detail, a recombinant murine retrovirus encoding both oncogenes was created. Largaespada *et al.* (1992) constructed a recombinant retrovirus, designated ABL-MYC, by insertion of the c-*myc* coding sequence into A-MuLV under the control of the herpes simplex virus thymidine kinase promoter. This ABL-MYC retrovirus has been found to be the most effective so far for the induction of plasmacytomas. Plasmacytoma induction by ABL-MYC is influenced by several factors such as age and strain of mice, the route of virus infection, and the existence of a helper virus. Intravenously injected helper virus-free ABL-MYC (HF ABL-MYC) induced plasmacytomas in all adult BALB/c

mice and most adult C57BL/6 and SWR mice, both of which are plasmacytoma-resistant strains, after 75–91 days. No other tumor types were induced in these adult mice. Furthermore, HF ABL-MYC was shown to be able to induce plasmacytomas without pristane pretreatment. Strain- and pristane-independent plasmacytoma induction after a short latency period is a striking feature of this HF ABL-MYC retrovirus. A control virus containing either v-*abl* or v-*myc* alone did not induce plasmacytomas (v-*abl* virus induced pre-B cell lymphoma in 9 of 10 adult BALB/c, and v-*myc* virus induced two plasmacytomas, five monocytic tumors, and two thymomas in a total of 10 young BALB/c mice).

F. Plasmacytoma in Transgenic Mice

Many transgenic mice were designed to clarify the role of oncogenes and growth factors in the development of various tumors. These mice were often subjected to retrovirus infection experiments to investigate the synergistic action of these factors. Therefore, some parts of this section may overlap with parts of the preceding retrovirus section.

1. Eμ-c-myc Transgenic Mice and Eμ-N-myc Transgenic Mice

It has been demonstrated that deregulated c-*myc* protooncogene is involved particularly in lymphoid malignancies, such as mouse plasmacytoma and human Burkitt's lymphoma. Virtually all mouse plasmacytomas express high levels of c-*myc* transcripts. Most of them have been shown to carry a nonrandom chromosomal translocation between the c-*myc* locus and one of the immunoglobulin loci (Potter, 1992; Potter and Wiener, 1992). Transgenic mice were generated by introducing Eμ-c-*myc*, which was derived from pristane + A-MuLV-induced plasmacytomas (Adams *et al.*, 1985; Harris *et al.*, 1988). The transgene was expressed exclusively in the B-lymphoid cells. All Eμ-c-*myc* transgenic mice were highly predisposed to monoclonal pre-B or B lymphomas, with the tumor onset ranging from 3 to 80 weeks of age (median 9 weeks). However, Eμ-c-*myc* transgenic mice did not develop plasmacytomas. In young transgenic mice, polyclonal proliferation of immature B cells was observed, but these cells did not contain a malignant phenotype (Langdon *et al.*, 1986). These results suggest that a high-level expression of the c-*myc* gene from the pre-B cell stage causes mainly polyclonal pre-B or B cell proliferation so that certain as yet unknown genetic changes must be necessary for malignant transformation of B lineage cells. The Eμ-c-*myc* transgenics were crossed to plasmacytoma prone Eμ-v-*abl* transgenic mice, resulting in a dramatic acceleration of plasmacytomagenesis (Rosenbaum *et al.*, 1990).

The N-*myc* gene, which has been implicated in only a few types of tumors such as neuroblastoma, retinoblastoma, and small cell lung carcinoma, was

introduced into a mouse germ line under the control of Eμ (Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989). These Eμ-N-*myc* transgenic mice showed quite similar phenotypes to those of Eμ-c-*myc* transgenics; that is, they displayed a predisposition to develop B lymphomas, indicating that the deregulated c-*myc* and N-*myc* expressions play a similar role in B cell tumorigenesis. Interestingly, Eμ-N-*myc* B lymphoid tumors were found to lack endogenous expression of either c-*myc* or N-*myc*.

It was also demonstrated that A-MuLV retrovirus infection of Eμ-c-*myc* mice and Eμ-N-*myc* mice induced plasmacytomas in both of these transgenic mice (Sugiyama *et al.*, 1990; Wang *et al.*, 1992). Transgenic mice backcrossed to BALB/c mice for two or three generations were subjected to plasmacytoma induction experiments with pristane + A-MuLV. None of the plasmacytomas induced in either of these transgenic mice strains with this method carried c-*myc*-associated chromosomal translocations. As previously described, exogenous c-*myc* or N-*myc* expression suppresses endogenous c-*myc* gene expression. These results indicate that a high level of exogenous *myc* gene expression can substitute for chromosomal translocations causing deregulated c-*myc* expression, thus resulting in the development of plasmacytomas without chromosomal translocations.

2. *Eμ-v-abl Transgenic Mice*

Rosenbaum *et al.* (1990) introduced the v-*abl* oncogene fused with Eμ into mouse germlines. Although one line developed lymphomas, three of four established Eμ-v-*abl* transgenic mice lines developed only plasmacytomas. One year later, the plasmacytoma incidence was 60%. The mice showed lymph node enlargement especially in the peritoneal cavities, and tumor nodules were often detected on the peritoneal connective tissue. Plasmacytoma invasion was observed in the bone marrow and the spleen, whereas some of the mice developed hindlimb paralysis due to tumor masses in the vertebral canal. About 70% of the tumors were transplantable to recipient mice by intraperitoneal injection even if they had not been treated with pristane. Furthermore, pristane treatment did not shorten the latency period of transplanted tumor development. Plasmacytomas occurring in Eμ-v-*abl* transgenic mice were clonal, and most of them were IgA or IgG producers. More than 80% (27 of 32) of Eμ-v-*abl* transgenic plasmacytomas were shown to carry a c-*myc* gene rearrangement coupled with Cα, which is mostly caused by typical t(12;15) in pristane-induced BALB/c plasmacytomas. Eμ-v-*abl* × Eμ-*myc* double-transgenic mice were shown to develop tumors after a much shorter latency period. These facts—monoclonality of plasmacytomas, c-*myc* gene rearrangement, and acceleration of tumor onset by crossing with Eμ-*myc* transgenic mice—suggest that the synergistic action of the activated c-*myc* gene is necessary

for plasmacytoma development in Eμ-v-*abl* transgenic mice. The following similarities between plasmacytomagenesis in Eμ-v-*abl* mice and that in pristane-induced BALB/c mice have been identified: (i) A relatively long latency period is needed, suggesting that other oncogenic factor(s) is required; (ii) plasmacytomas arise mainly from the mesenteric lymph nodes and Peyer's patch of the gut; (iii) most plasmacytomas are IgA or IgG producers, but not IgM producers; and (iv) the majority of plasmacytomas carry a c-*myc* gene rearrangement. However, Eμ-v-*abl* transgenic plasmacytomas have more striking characteristics than do pristane-induced plasmacytomas. First, Eμ-v-*abl* plasmacytomas do not require pristane priming for the plasmacytomagenesis itself or for intraperitoneal transplantation to recipient mice. Second, Eμ-v-*abl* plasmacytomas develop in (C57BL/6 × SJL)F1 mice, a strain likely to be resistant to plasmacytoma induction by pristane. These differences can be explained by the assumption that a high level of v-*abl* oncogene expression has a higher plasma cell transformation activity and thus does not require the cofactor(s) needed for conventional plasmacytoma induction.

3. *Eμ-IL-6 Transgenic Mice and H-2L^d-IL-6 Transgenic Mice*

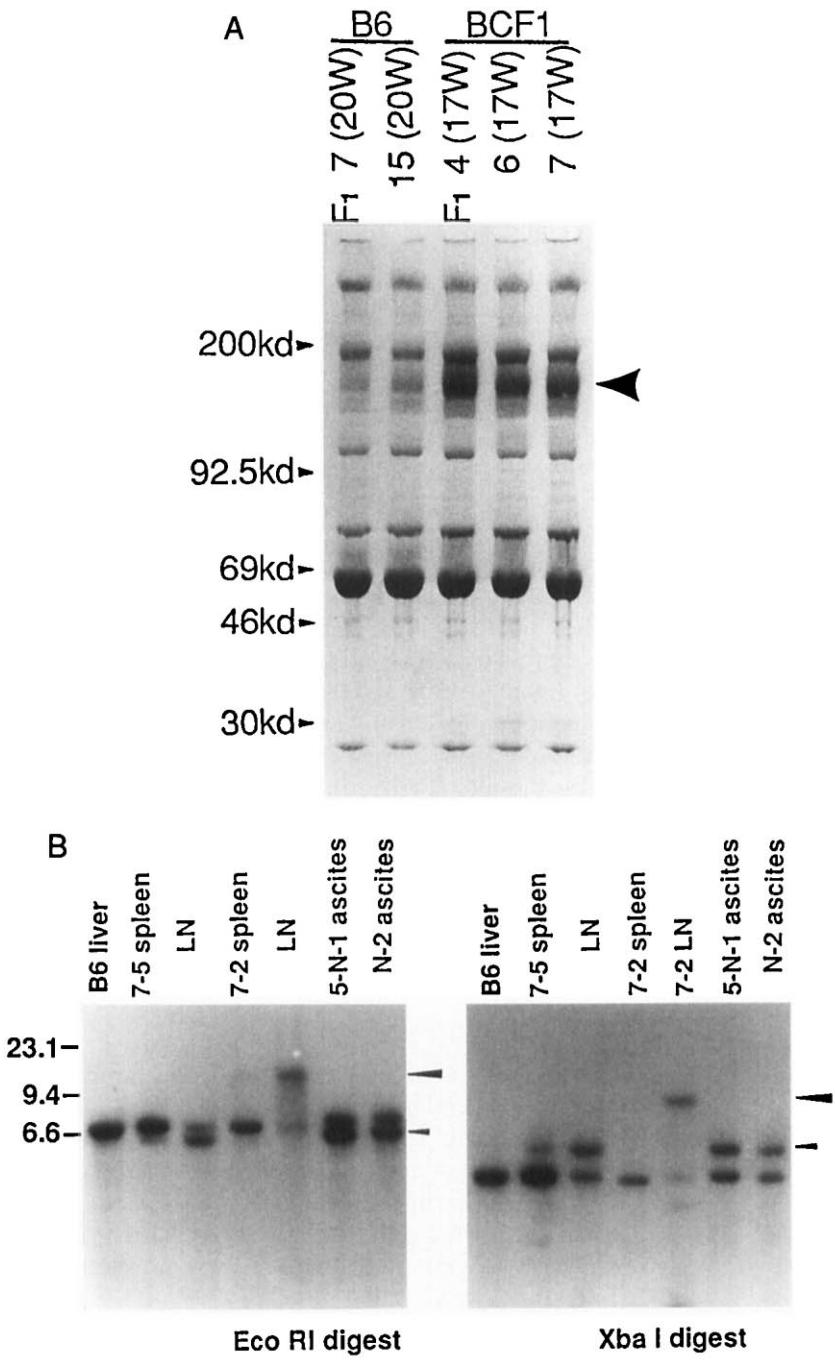
Interleukin-6 has been suggested to be involved in plasma cell dyscrasia in both humans and mice (Kawano *et al.*, 1988; Barlogie *et al.*, 1989; Akira *et al.*, 1993; Klein, 1995; Klein *et al.*, 1995). Transgenic mice were produced by introduction of human IL-6 gene under the transcriptional control of Eμ into a plasmacytoma-resistant strain, C57BL/6 (Suematsu *et al.*, 1989). Eμ-IL-6 transgenic mice developed a fatal proliferation of plasma cells and died within 4 or 5 months. Proliferation of plasma cells in Eμ-IL-6 transgenic mice was observed not only in hematopoietic tissues but also in many other organs, with histopathological features in distinguishable from those of plasmacytomas. Serum IgG1 levels dramatically increased 100- to 400-fold, whereas other types of immunoglobulins remained at less than 10-fold levels. Immunoglobulin gene rearrangement analysis indicated that the plasma cell proliferation was polyclonal. Furthermore, transgenic plasma cells were not transplantable to the peritoneal cavities of pristane-primed syngeneic C57BL/6 mice. These results demonstrated that Eμ-IL-6 transgenic mice developed fatal IgG1 plasmacytosis but not fully transformed malignant plasmacytomas. Another transgenic line, named L^d-IL-6, was produced by introduction of human IL-6 cDNA under the control of the murine histocompatibility class I (H2-L^d) promoter also into C57BL/6 mice (Suematsu *et al.*, 1992). Although the expression level of the L^d-IL-6 transgene was relatively low compared to that of the Eμ-IL-6 transgene, the pathological features of L^d-IL-6 transgenic mice were essentially the same as those of Eμ-IL-6 transgenic mice; that is, they

developed polyclonal IgG1 plasmacytosis. To observe the influence of genetic background, the L^d-IL-6 transgenic mice were backcrossed to plasmacytoma-susceptible BALB/c mice. Interestingly, (C57BL/6 × BALB/c)F1 IL-6 transgenic mice showed a faster increase in serum immunoglobulins, mainly in IgG1, than did C57BL/6 transgenic mice. In the next backcrossed generation (designated BCF2 IL-6 transgenic mice), about 30% of the transgenic mice developed ascites and monoclonal-transplantable IgA plasmacytomas at approximately 40 weeks of age (Fig. 3). This latency was comparable to that of pristane-induced plasmacytomagenesis in BALB/c mice. In plasmacytoma-bearing BCF2 IL-6 transgenic mice, enlargement of mesenteric lymph nodes was prominent in comparison with C57BL/6 IL-6 transgenics. The IgA plasmacytomas that developed in BCF2 IL-6 transgenic mice were shown to carry t(12;15) translocation, which is most frequently observed in pristane-induced plasmacytomas.

These two IL-6 transgenic experiments provide the first direct evidence that IL-6 plays an important role in plasmacytomagenesis. Oil granulomas, which are generated in the peritoneal cavity by pristane injection, are supposed to constitute an important microenvironment for plasmacytomagenesis (Potter *et al.*, 1972; Cancro and Potter, 1976; Degrassi *et al.*, 1990, 1993; Potter and Wiener 1992). Cell components of oil granulomas have been shown to produce growth factors including IL-6 and are suggested to express certain as yet unknown cell–cell interaction signals to plasmacytoma cells and their precursors. It is conceivable that IL-6 is a major and necessary growth factor for plasmacytoma development in many growth factors produced by oil granulomas. Furthermore, the BALB/c genetic background is suggested to be important in plasmacytomagenesis in IL-6 transgenic mice. If IL-6 transgenenic mice could be generated by using plasmacytoma-susceptible BALB/cAn mice, the incidence of plasmacytomas might be comparable to that of pristane-induced plasmacytomas in the same strain. In other words, the function of oil granulomas could be replaced by overexpression of IL-6.

4. Eμ-bcl-2 Transgenic Mice

The *bcl-2* gene was originally identified as an oncogene implicated in the t(14;18) chromosomal translocation, which is typical in human follicular B cell lymphomas (Tsujimoto *et al.*, 1984; Bakhshi *et al.*, 1985; Cleary and Sklar, 1985). The Bcl-2 protein is now known to inhibit apoptosis and prolong the survival of several normal cells and cell lines including the lymphoid lineage (Korsmeyer, 1992a,b; Cory, 1995). To clarify its role in lymphoid malignancy, the *bcl-2* gene fused with Eμ was introduced into a mouse germline (McDonnell *et al.*, 1989; Strasser *et al.*, 1990a). It was demonstrated that bcl-2 transgene expression induced accumulation of


A
B6
BCF1
F1
7 (20W)
15 (20W)
F1
4 (17W)
6 (17W)
7 (17W)
200kd
92.5kd
69kd
46kd
30kd
B
B6 liver
7-5 spleen
LN
7-2 spleen
LN
5-N-1 ascites
N-2 ascites
23.1
9.4
6.6
Eco RI digest
B6 liver
7-5 spleen
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5-N-1 ascites
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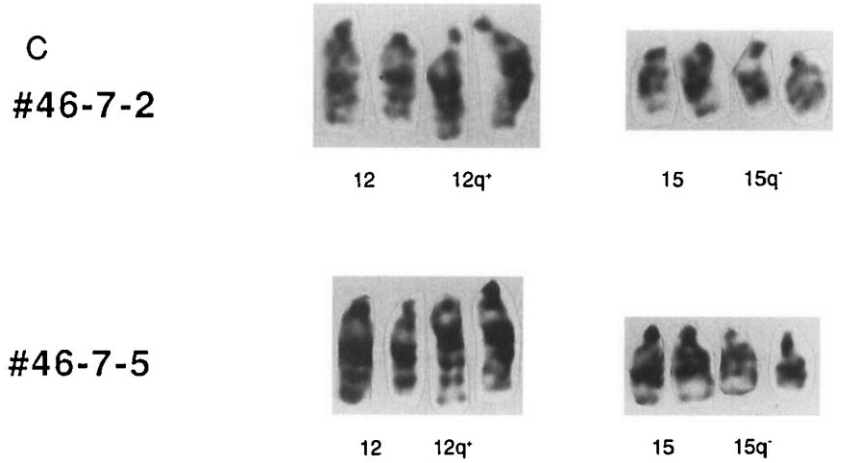

FIG. 3. Plasmacytoma development in IL-6 transgenic mice. (a) Acceleration of an increase in the level of serum immunoglobulins in L^d-IL-6 transgenic mouse with a BALB/c genetic background. The arrowhead indicates the band that contains the immunoglobulins. The first two lanes from left: sera from two C57BL/6 L^d-IL-6 transgenic mice (20 weeks old); other three lanes: sera from three C57BL/6 × BALB/c) F1 transgenic mice (17 weeks old). (b) Southern blot analysis of the immunoglobulin gene of L^d-IL-6 transgenic plasmacytomas. Immunoglobulin heavy chain region 4 (J_{H4}) was used as a probe. A single rearranged immunoglobulin heavy chain gene band was observed in each plasmacytoma (the arrowheads indicate rearranged monoclonal J_{H4}-bearing fragments). 7-5 Spleen (or LN), DNA from spleen (or lymph node) from BCF2 L^d-IL-6 transgenic mice (L^d46-7-5); 7-2 spleen (or LN) DNA from spleen (or lymph node) DNA from BCF2 L^d-IL-6 transgenic mice (L^d46-7-2); 5-N ascites, DNA from ascites cells of BALB/c nu/nu mouse transplanted with plasmacytomas from L^d46-7-5. (c) Chromosomal translocation in L^d-IL-6 transgenic plasmacytomas. G-banded karyotyping showed both L^d46-7-2 and L^d46-7-5 plasmacytomas were tetraploid and contained "typical" t(12;15) chromosomal translocation.

resting small B cells in lymphoid follicles and promotion of B cell survival in vitro (McDonnell *et al.*, 1989; Strasser *et al.*, 1990a). Some Eμ-*bcl-2* transgenic lines also expressed the transgene in T cells, which resulted in prolonged T cell survival (Strasser *et al.*, 1991b). Strasser *et al.* (1991a) demonstrated that transgene expression in B cells gave rise to amplified and prolonged antibody responses, which in turn caused autoimmune-like diseases. They also demonstrated that Eμ-*bcl-2/myc* double-transgenic mice developed transplantable lymphomas of novel but presumably lymphoid-committed precursors and became terminally ill in about 6 weeks (Strasser *et al.*, 1990a), much faster than in the case of Eμ-*myc* transgenic mice. Although accumulation of large pre-B and B cells was also observed in Eμ-*bcl-2/myc* double-transgenic mice, even in 2-day-old mice they were

not tumorigenic. Acceleration of lymphomagenesis in Eμ-*bcl-2/myc* mice is supposed to be caused by suppression of Myc-induced apoptosis by Bcl-2. After a long latency period, Eμ-*bcl-2* mice developed B cell lymphomas (McDonnell and Korsmeyer, 1991) or both B cell and T cell lymphomas, depending on the cell types in which the transgene expressed (Strasser *et al.*, 1993). The B cell lymphomas arose primarily from mesenteric lymph nodes (McDonnell and Korsmeyer, 1991; Strasser *et al.*, 1993). The latency of lymphoma development was more than 1 year with an incidence of less than 10% (McDonnell and Korsmeyer, 1991; Strasser *et al.*, 1993). Most of the B cell lymphomas were shown to have rearranged immunoglobulin heavy chain genes (McDonnell and Korsmeyer, 1991; Strasser *et al.*, 1993). Some of the Eμ-*bcl-2* transgenic mice developed plasmacytomas and almost all of them carried IgH and c-*myc* gene rearrangement (Strasser *et al.*, 1993). McDonnell and Korsmeyer (1991) mentioned that c-*myc* gene rearrangement was observed in half of the B cell lymphomas; therefore, they could be included in plasmacytomas.

G. Factors Determining Susceptibility and Resistance to Mouse Plasmacytomagenesis

Mouse plasmacytomas can be induced in genetically susceptible strains of mice, such as BALB/cAn and NZB, by intraperitoneal injection of mineral oil, plastics, or pristane. On the other hand, most other inbred strains are resistant to plasmacytoma induction (Potter, 1984; Potter and Wiener, 1992). For example, F1 hybrid mice between susceptible and resistant strains are resistant to plasmacytoma induction (Potter, 1984). Using BALB/c and DBA/2 mice as prototypes of susceptible and resistant strains, respectively, many experiments have been designed to clarify factors that define susceptibility and resistance to plasmacytoma development.

1. Cell Transfer Experiments

To investigate whether the donor BALB/c-derived B lineage cell itself has plasmacytoma-prone characteristics or whether the host BALB/c environment determines susceptibility, several cell transfer experiments have been performed. Chimeric mice were produced by intravenous transfer of cells from donor mice to recipients, which were either whole body-irradiated mice or newborn mice, and subjected to plasmacytoma induction by pristane + A-MuLV (Silva *et al.*, 1989, 1991). To distinguish the origin of the cells, BALB/c mice carrying a Robertsonian fusion chromosome (BALB/c6;15) were utilized for these experiments. Radiochimeras between BALB/c sublines were treated with pristane or pristane + A-MuLV (Silva *et al.*, 1989). As in a previous experiment (Potter *et al.*, 1973), latency of plasmacytoma development was shortened by A-MuLV infection, and

plasmacytomas were induced to carry chromosomal transactions. Most of the plasmacytomas were shown to be of donor origin. However, the incidence was lower than that for nonirradiated BALB/c mice (Silva *et al.*, 1989). Histopathological analysis of radiochimeras revealed that formation of oil granulomas was insufficient. The low incidence of plasmacytomas in radiochimeras may be explained by insufficiency of the growth factors supplied by oil granulomas.

Silva *et al.* (1991) generated reciprocal chimeras between BALB/c mice and DBA/2 mice by transferring bone marrow cells into newborn mice through the periorbital vein. The BALB/c bone marrow cell-transferred DBA/2 chimera was designated C > D and the reciprocal chimera D > C. Chimerism and tumor origin were determined by detection of Robertsonian fusion chromosomes in BALB/c cells as in a previous experiment (Silva *et al.*, 1989). Plasmacytomas were induced in both chimeras by pristane (incidence: 60% C >D, 47% D >C) or pristane + A-MuLV (32% C > D, 24% D > C) regardless of their chimerism (10–70% chimerism). All plasmacytomas occurring in chimeric mice were of BALB/c origin, regardless of whether BALB/c mice were donors or hosts, even in chimeras containing only 10% of BALB/c cells. This result implies that the environment of DBA/2 mice, for example, oil granulomas, can support plasmacytoma development. All the plasmacytomas carried Ig/*myc* chromosomal translocation and were predominantly IgAκ or IgGκ producers. Some chimeric mice developed other kinds of tumors, such as lymphosarcomas, monocytic tumors, and leukemia, which were of either BALB/c or DBA/2 origin. These cell-transfer experiments using BALB/c chimeras indicate that the difference in susceptibility of BALB/c mice and DBA/2 mice to plasmacytoma induction is determined by the precursor cell itself.

Hilbert *et al.* (1993) investigated the same question by using severely combined immunodeficient (SCID) mice reconstituted with BALB/c or DBA/2 cells. SCID mice reconstituted by intravenous transfer of cells from BALB/c or DBA/2 mice were subjected to plasmacytoma induction by means of pristane + v-*raf*/v-*myc* retrovirus (J3V1). Approximately 50–75% of BALB/c bone marrow cell-reconstituted SCID mice developed tumors; half of these developed plasmacytomas and the rest myeloid tumors. However, DBA/2 bone marrow-reconstituted SCID mice developed only myeloid tumors (incidence of 45–60%) and not plasmacytomas, with one exception. This finding supports the view that susceptibility or resistance to plasmacytomagenesis is determined by the precursor cell itself. To investigate whether the inability of the DBA/2 precursor cell to develop into plasmacytomas is caused by other cell components of DBA/2 mice, SCID mice were reconstituted with a combination of BALB/c peripheral lymphocytes from several sources and DBA/2 bone marrow cells. These

double-chimeric SCID mice developed plasmacytomas of BALB/c origin with a comparable incidence to that for BALB/c mice or BALB/c cell-reconstituted SCID mice. No plasmacytomas of DBA/2 origin developed even in the presence of BALB/c peripheral lymphocytes in these SCID mice.

T cell–B cell interaction is essential for cell growth and differentiation in an immune system. Therefore, it is conceivable that T cells play a certain role in plasmacytoma development. To clarify T cell function in plasmacytomagenesis, T cell-deficient BALB/c *nu/nu* (nude) mice were reconstituted with BALB/c-derived T cells (BALB-nude) and subjected to plasmacytoma induction by pristane and J3V1 (Hilbert *et al.*, 1995b). This induction method is known to induce plasmacytomas and myeloid tumors in BALB/c mice. With this induction method, both the control nude mice and reconstituted BALB-nude mice developed B-lineage tumors and myeloid tumors. The tumor phenotype was determined by cell surface markers specific for B cells, plasma cells, and myeloid cells. The tumor origin, whether BALB/c or nude mice, was identified by restriction fragment length polymorphism (RFLP) of the nude mice-specific locus. Interestingly, plasmacytomas were preferentially induced in BALB/c-nude reconstituted mice. Furthermore, the ratio of plasmacytomas/B lymphomas increased according to the number of transferred BALB/c T cells. On the other hand, most of the B-lineage tumors induced in nude mice were B cell lymphomas. All these findings suggest that the plasmacytoma phenotype is likely to be determined by interaction with T cells at a late stage in B cell development.

2. *DNA Repair at the Chromosomal Translocation Break Point*

Cell transfer experiments raise the question of whether the plasmacytoma precursor cells of BALB/c mice are more predisposed to Ig/*myc* translocation than are B cells of DBA/2 mice.

Beecham *et al.* (1991) demonstrated defective DNA repair in the c-*myc* locus in BALB/c mice. Lipopolysaccharide stimulated B cells were prepared from both BALB/c and DBA/2 mice and subjected to an ultraviolet (UV)-induced excision repair experiment. B cells from both strains retained almost the same viability after UV exposure. The extent of repair of UV-induced DNA damage was estimated by Southern blotting using probes (DNA probe or RNA probe) that detect the 5′ or 3′ flanking region of the c-*myc* gene. In the case of DBA/2 DNA, UV-induced DNA damages were removed from both the 5′ and 3′ flanking regions of the c-*myc* in both the transcribed and nontranscribed strand. On the other hand, in the case of BALB/c DNA, DNA damage was repaired only in the transcribed 3′ flanking region of the c-*myc*. None of the 5′ flanking regions in both

strands, nor the 3′ flanking region in the nontranscribed strand, were repaired in BALB/c DNA. Both the damaged BALB/c DNA and DBA/2 DNA were repaired to the same extent in the dihydrofolate reductase (DHFR) gene. Because the DHFR gene is actively transcribed and DNA damage is effectively repaired in this gene, the DHFR gene was used as the c-*myc* gene unrelated control gene. The results suggest the possibility that frequent rearrangements in the 5′ region of the c-*myc* gene in BALB/c plasmacytomas may occur as a result of lack of DNA repair activity in this region in BALB/c B cells. The difference in DNA repair efficiency in the 5′ flanking region of the c-*myc* between BALB/c and DBA/2 B cells may determine the extent of susceptibility or resistance to plasmacytoma induction. Beecham *et al.* (1994) analyzed DNA repair in other loci involved in chromosomal transactions in plasmacytomas using the same system as described previously. The *pvt-1,* IgH-Cα, and Igκ loci were analyzed. In this experiment, the c-*abl* protooncogene was used as control. The c-*abl* gene has been implicated in plasmacytomagenesis but not in plasmacytoma-related chromosomal translocations. Ultraviolet-induced DNA damages in all these loci were effectively repaired in DBA/2 B lymphoblasts. In contrast, damages in corresponding loci were not repaired in BALB/c B lymphoblasts. Furthermore, DNA damages in the BALB/c-derived loci of CDF1, which is a strain resistant to plasmacytoma induction, were shown to be repaired comparably to those in DBA/2. These results are consistent with the previously established concept that chromosomal translocation is one of the critical steps in plasmacytomagenesis. All these outcomes suggest that susceptibility and resistance to plasmacytoma induction may be explained by the correlation between DNA repair defect and chromosomal translocation in BALB/c mice. Efficient DNA repair in the BALB/c loci of CDF1 mice suggests that a certain dominant transacting factor derived from DBA/2 promotes DNA repair, resulting in the resistant phenotype of this strain.

3. *Genes Determine Susceptibility and Resistance to Plasmacytoma Induction*

A large-scale genetic analysis was performed in BALB/c, CDF1, and BALB/c × (BALB/c × DBA/2)F1 N2 backcross mice (Mock *et al.*, 1993). Intraperitoneal injection of pristane induced plasmacytomas in 47% of BALB/c mice, 2% of CDF1, and 11% of N2 backcross mice, indicating that susceptibility is likely to be subject to multigenic control. Using a panel of 83 susceptible backcross N2 progeny and 68 resistant backcross N2 progeny, RFLP and simple sequence length polymorphism of the simple sequence repeats for BALB/c and DBA/2 mice DNA were analyzed and compared. As a result of these analyses, a major genomic region

influencing susceptibility to plasmacytoma development was localized to the distal portion of chromosome 4 (Chr 4), within 11 cm of the marker gene trap insertion site 10. Another possible susceptibility gene was shown to be linked to distal Chr 1 near low-affinity FcIgG receptor 2.

Potter *et al.* (1994) identified two genes that determine resistance to plasmacytoma induction. To identify these resistant genes, BALB/c.DBA/2 (C.D2) congenic strains were generated. Plasmacytoma development was estimated by counting focal proliferations of atypical plasma cells (foci) to reduce the time needed for typing susceptible or resistant phenotypes. These foci correlated well to plasmacytoma development. Previously, C.D2-Fv-$1^{n/n}$ (Chr 4) congenics had been constructed by introgressive backcrossing Fv-1^{n} alleles from DBA/2 to BALB/c (Fv-1^{bb}) (Potter *et al.*, 1984). Congenics carrying DBA/2 alleles other than Chr 4 were shown to be susceptible to plasmacytoma induction. Furthermore, new C.D2-Chr 4 congenic strains, C.D2-MIA and C.D2-TF3, were generated. After serial backcrossing C.D2-MIA to BALB/cAnPt, the C.D2-Lgm-1 and C.D2-Pnd series of congenic strains were selected. These C.D2-Chr 4 congenics were mated to create homozygotes at certain loci and were subjected to plasmacytoma induction. At least two Chr 4 DBA/2 genes suspected of determining resistance, designated Pct^{r1} and Pct^{r2}, were located on the distal end. Further investigation into searching for resistant genes at DBA/2 loci on other chromosomes is now in progress.

III. Human Plasma Cell Dyscrasias

A. Immunophenotype of Human Multiple Myeloma

1. Identification of Multiple Myeloma Cells

Differentiation of hematopoietic cells is accompanied by the sequential acquisition and loss of multiple cell surface and internal protein markers. By using specific monoclonal antibodies against these markers, the lineages and maturational stages of the cells can be identified (Loken *et al.*, 1987). Normal and neoplastic plasma cells in the terminal stage of B cell differentiation do not express surface immunoglobulin, HLA-DR, Fc receptors, or C3 receptors (Stashenko *et al.*, 1981). B cell-associated antigens CD9, CD19, CD20, CD21, and CD24 are also lost in most cases (Anderson *et al.*, 1984a; Katagiri *et al.*, 1984).

Many laboratories have attempted to obtain monoclonal antibodies for the specific antigens expressed by multiple myeloma cells. Such monoclonal antibodies can be employed for (i) the indentification and isolation of myeloma cells from bone marrow that contains multilineages of hematopoietic cells and for the analysis of myeloma cell biology, (ii) the clinical

diagnosis of multiple myeloma, (iii) immunotherapy targeting myeloma cells or myeloablative therapy with monoclonal antibody-purged bone marrow cells, and (iv) an understanding of normal plasma cell development. Not a few monoclonal antibodies have been obtained using neoplastic plasma cells as immunogens (Table II). Although most of these monoclonal antibodies lack restrictions on lineages or stages of differentiation, PC-1 (Anderson *et al.*, 1984b), BU11 (Nathan *et al.*, 1986), R1-3 (Gonchoroff *et al.*, 1986), MM4 (Tong *et al.*, 1987), and HM1.24 (Goto *et al.*, 1994) monoclonal antibodies reacted more specifically with plasma cells. When the bone marrow cells of a myeloma patient were sorted into R1-3-positive and R1-3-negative cells, 90% of the R1-3-positive cells consisted of plasma cells with labeling index of 4%, whereas the R1-3-negative population contained 3% plasma cells with labeling index or 1.8% (Gonchoroff *et al.*, 1986), indicating that this reagent can be used for the isolation of myeloma cells and the analysis of myeloma cell biology. The *in vitro* effect of MM4 on myeloma cell proliferation was inhibitory, suggesting that this monoclonal antibody might be potentially useful in the regulation of myeloma cell growth (Tong *et al.*, 1987). To purge myeloma cells, a combination of PCA-1 (Anderson *et al.*, 1983) and BL-3 prepared by immunization with a Burkitt's lymphoma cell line, B35M (Wang *et al.*, 1984), was effective without a significant loss of normal hematopoietic progenitors as measured by CFU-GM, CFU-GEM, and BFU-E *in vitro* (Shimazaki *et al.*, 1988). In practice, autologous bone marrow transplantation therapy purged with a cocktail of PCA-1, anti-CALLA (CD10), and anti-B1 (CD20) monoclonal antibodies in combination with high-dose chemoradiotherapy could achieve a high response without unexpected toxicity in myeloma patients (Anderson *et al.*, 1991). The fact that HM1.24 was found to be specific for plasma cells and did not inhibit the growth of normal CFUs *in vitro* suggested that this reagent could be quite useful for purging malignant cells from patient bone marrow (Goto *et al.*, 1994).

Although the availability of monoclonal antibodies specific to plasma cells is limited, multiparameter flow cytometry analysis permitted the identification and extensive characterization of the antigens expressed on plasma cells. CD38, ADP-ribosil cyclase (Howard *et al.*, 1993), is one of the antigens expressed by plasma cells, although it is not specific for plasma cells and expressed on a large variety of other cells such as myeloid cells, monocytes, natural killer cells, pre-B cells, and a subset of T cells. Because of its consistent and strong reactivity, anti-CD38 mAb has been used in conjunction with other parameters to identify plasma cells in both normal and neoplasms (Terstappen *et al.*, 1990; Van Camp *et al.*, 1990; Leo *et al.*, 1992; Harada *et al.*, 1993; Hata *et al.*, 1993; Kawano *et al.*, 1993; Huang *et al.*, 1993). Terstappen *et al.* (1990) described how the plasma cells could

TABLE II

MONOCLONAL ANTIBODIES OBTAINED BY USING NEOPLASTIC PLASMA CELLS AS IMMUNOGENS

mAb	M_r (kDa)	Antigen distribution other than in plasma cells	Immunization	Reference
PCA-1	NA	PWM-stimulated B cells, HCL, granulocytes, monocytes	Plasma cells from PCL	Anderson *et al.* (1983)
PCA-2	NA	Granulocytes, monocytes	Plasma cells from PCL	Anderson *et al.* (1983)
PC-1	28	PWM-stimulated B cells	Plasmacytoma cells	Anderson *et al.* (1984b)
BU11	58	Some B cells, some B-CLL, HCL	MM cell line (RPMI8226)	Nathan *et al.* (1986)
R1-3	NA	Some B cells, some B-CLL, NHL, HCL	Plasmacytoma cell line (MCX-REN)	Gonchoroff *et al.* (1986)
MM4	39–40	PWM-stimulated B cells	MM cell lines (RPMI8226 and GM1312)	Tong *et al.* (1987)
8A	NA	Pre-B, B cells, B-CLL, NHL, HCL, PWM-stimulated B cells, some granulocytes	Plasma cells from MM	Tazzari *et al.* (1987)
8F6	NA	B cells, B-CLL, NHL, HCL, PWM-stimulated B cells	Plasma cells from MM	Tazzari *et al.* (1987)
62B1	NA	HCL, some B-CLL	Plasma cells from MM	Tazzari *et al.* (1987)
MPC-1	48	B cells, monocytes	MM cell line (KMS-5)	Huang *et al.* (1993)
D2	54	PBL	MM cell line (KMS12PE)	Hata *et al.* (1994a)
HM1.24	29–33	PWM-stimulated B cells	MM cell line (KPC32)	Goto *et al.* (1994)

Note. mAb, monoclonal antibody; M_r, molecular weight; NA, information not available; PWM, pokeweed mitogen; HCL, hairy cell leukemia; PCL, plasma cell leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; PBL, peripheral blood lymphocytes.

be uniquely identified by their location in a three-dimensional space created by forward light scattering, transformed orthogonal light scattering, and quantitative CD38 expression. More than 99% of this fraction consisted of plasma cells examined by cell sorting. Hata *et al.* (1993) obtained pure myeloma cells by sorting the cells into either $CD38^{2+}$ $CD45^{-}$ (mature plasma cells) or $CD38^{2+}$ $CD45^{+}$ (immature plasma cells) because end-stage plasma cells lose the CD45 expression (Jensen *et al.*, 1991).

Comparative studies of multiple myeloma, MGUS, and normal plasma cells clarified, with the aid of multiparameter flow cytometry, that myeloma cells expressed CD56 antigen, identical with the expression by the neural cell adhesion molecule N-CAM, whereas all the normal plasma cells did not (Van Camp *et al.*, 1990; Van Riet *et al.*, 1991). A subsequent study on multiple myeloma, Waldenström's macroglobulinemia (WM), MGUS, and normal individuals revealed that plasma cells of myeloma and MGUS had a phenotype of $CD38^{2+}$, $CD54^{+}$, $CD56^{+}$, cytoplasmic immunoglobulin^{+} (cIg^{+}), whereas those of WM and normal individuals had a phenotype of $CD38^{2+}$, $CD54^{+}$, $CD56^{-}$, cIg^{+} (Leo *et al.*, 1992). Thus, the only immunophenotypical difference between myeloma and normal plasma cells was the expression of CD56. Loss of CD56 on the plasma cells of WM suggested that WM was a B cell neoplasm of lower differentiation than that of multiple myeloma and MGUS. Harada *et al.* (1993) reported that the phenotype of normal plasma cells was $CD38^{2+}$, $CD10^{-}$, $CD19^{+}$, $CD20^{-}$, $CD21^{+}$, $CD24^{-}$, $CD44^{+}$, $CD54^{+}$, $CD56^{-}$, VLA-4^{+}. On the other hand, myeloma cell phenotypes all contained $CD19^{-}$ and most of them contained $CD56^{+}$. In MGUS, both normal plasma cells with $CD19^{+}$ $CD56^{-}$ and malignant cells with $CD19^{-}CD56^{+}$ or $CD19^{-}CD56^{-}$ were found (Harada *et al.*, 1993). Adhesion molecules such as N-CAM have been implicated in cell migration, embryonic development, and cellular adhesion. Thus, the expression of CD56 may be relevant to the pathology of multiple myeloma.

Markers of multiple hematopoietic cell lineages and of early B cells have been identified on a subpopulation of myeloma cells, although multiple myeloma is considered to be a neoplasm in the late stage of B cell differentiation. Common acute lymphoblastic leukemia antigen (CALLA, CD10), thought to be expressed in the very early stage of B cell differentiation and on granulocytes, was detected in bone marrow myeloma cells and some peripheral B cells of myeloma patients as well as in leukemic cells of plasma cell leukemia patients (Durie and Grogan, 1985; Caligaris-Cappio *et al.*, 1985; Epstein *et al.*, 1988, 1990). The occurrence of CALLA expression was identified as a clinically significant finding portending a poor prognosis (Durie and Grogan, 1985; Epstein *et al.*, 1988). When $CALLA^{+}Ig^{-}$ cells with lymphoid morphology were separated with a fluorescence-activated cell sorter and stimulated with phorbol ester *in*

vitro, the induced cells transformed into $CALLA^-$ plasma cells that synthesized the heavy and light chains of the same isotype as expressed by the patients' myeloma cells, suggesting that $CALLA^+Ig^-$ cells might be precursors of myeloma cells (Caligaris-Cappio *et al.*, 1985).

Expression of myelomonocytic antigens (CD11c, CD13, CD14, CD15, butyrate esterase, and/or chloroacetate) on myeloma cells has been reported (Grogan *et al.*, 1989; Durie *et al.*, 1989). In some cases, functional surface markers such as Fc receptors and a functional capacity such as phagocytosis were observed in addition to CD11c, CD14, CD15, and butyrate esterase expression. Megakaryocytic (CD41) and erythroid (glycophorin A) antigens as well as myelomonocytic antigens (CD11b and CD33) were also expressed in some myeloma cases (Epstein *et al.*, 1990). These reports indicated that myeloma cells appear to be malignant cells with aberrant disorganized expression of differentiation antigens due to loss of their gene regulation. Alternatively, malignant transformation in multiple myeloma occurs in an early hematopoietic progenitor capable of differentiation into various lineages. On the other hand, Terstappen *et al.* (1990) detected myeloid-specific antigens, such as CD33 and CD13, and the pre-B cell antigen CD10 expressed on a portion of normal plasma cells. Therefore, the phenomenon may not be associated with neoplasia but rather with enhancement of a nonpathologic process of normal differentiation.

2. *Adhesion Molecules on Myeloma Cells*

One of the distinctive features of multiple myeloma is that proliferation of malignant plasma cells is localized in the bone marrow until the terminal stage of the disease, suggesting that some homing receptors are involved in the pathology of this disease. The microenvironment of this site seems to be favorable for myeloma growth. This is probably because bone marrow stromal cells produce several cytokines including IL-6, which has been identified as the major growth factor of myeloma cells (Kawano *et al.*, 1988; Klein *et al.*, 1989). Intimate cellular contact between the myeloma cells and bone marrow stromal cells is important not only for raising the local concentration of cytokines to which the myeloma cells are exposed but also for stimulating the stromal cells to secrete cytokine (Carter *et al.*, 1990; Uchiyama *et al.*, 1993; Lokhorst *et al.*, 1994). Several adhesion molecules are thought to mediate the interaction between myeloma and stromal cells (Table III). Most of the myeloma cells strongly express the intercellular adhesion molecule (ICAM-1, CD54) (Kawano *et al.*, 1991; Hamilton *et al.*, 1991a; Leo *et al.*, 1992; Van Riet and Vam Camp, 1993), VLA-4(CD49d) (Uchiyama *et al.*, 1992; Moller *et al.*, 1992; Kawano *et al.*, 1993; Harada *et al.*, 1993; Lokhorst *et al.*, 1994), and human lymphocyte

TABLE III
ADHESION MOLECULES ON MYELOMA CELLS

Molecule	Family	Ligand
ICAM-1 (CD54)	Ig superfamily	LFA-1, Mac-1
N-CAM (CD56)	Ig superfamily	Heparan sulfate, N-CAM
LFA-3 (CD58)	Ig superfamily	CD2
VLA-4 (CD49d)	β_1 integrin	VCAM-1, fibronectin
VLA-5 (CD49e)	β_1 integrin	Fibronectin
VLA-6 (CD49f)	β_1 integrin	Laminin
LFA-1 (CD11a)	β_2 integrin	ICAM-1, -2, -3
H-CAM (CD44)	Cartilage-link protein	Hyaluronate

Note. ICAM, intercellular adhesion molecule; N-CAM, neural cell adhesion molecule; LFA, lymphocyte function-associated antigen; VLA, very late activation antigen; H-CAM, homotypic cell adhesion molecule.

homing receptors (H-CAM, CD44) (Hamilton *et al.*, 1991a, Uchiyama *et al.*, 1992; Kawano *et al.*, 1993; Harada *et al.*, 1993; Lokhorst *et al.*, 1994).

ICAM-1 is one of the ligands of lymphocyte function-associated antigen 1 (LFA-1) and the binding of this ligand facilitates the cell-to-cell interaction of T and B lymphocytes, neutrophils, macrophages, and endothelial cells (Wawryk *et al.*, 1989). Although myeloma cells express ICAM-1, it is unlikely that ICAM-1/LFA-1 interaction is involved in the adhesion of myeloma cells to bone marrow stromal cells or the proliferation of myeloma cells because anti-ICAM-1 antibody was found not to affect these functions (Kawano *et al.*, 1991). However, Kawano *et al.* (1991) found coexpression of LFA-1 and ICAM-1 on myeloma cells and this coexpression was related to the formation of homotypic cell aggregates *in vitro*. LFA-1 on myeloma cells was also reported to correlate with disease activity in multiple myeloma (Ahsmann *et al.*, 1992). Together with the evidence that bone marrow stromal cells from myeloma patients were ICAM-1$^+$ and able to support myeloma cell growth (Caligaris-Cappio *et al.*, 1991), these findings suggest that ICAM-1/LFA-1 interactions may be involved in the development of myeloma. However, Van Riet and Van Camp (1993) reported the absence of LFA-1 on myeloma cells. Thus, further analysis is required to explain these discrepancies in observations.

N-CAM (CD56) and LFA-3 (CD58) are also members of the Ig superfamily. As previously described, CD56 is a distinctive marker for malignant plasma cells, although its pathological function has not been determined. Expression of LFA-3 has been reported by Barker *et al.* (1992), but its function has not been identified.

The fibronectin receptor VLA-4 is also strongly expressed on malignant plasma cells from nearly all patients with myeloma and plasma cell leuke-

mia. This molecule was reported to mediate the adhesion of B cell precursors to the bone marrow microenvironment by means of interaction between VLA-4 and vascular cell adhesion molecule-1, which is known as the other counterreceptor for VLA-4 besides fibronectin (Ryan *et al.*, 1991). Furthermore, Roldan *et al.* (1992) showed that VLA-4/fibronectin interaction was essential for the induction of plasma cells in normal bone marrow. In multiple myeloma, malignant plasma cells adhered to fibronectin through VLA-4 as well as through RGD peptide-dependent mechanisms. This binding action could be downregulated by IL-6, suggesting that IL-6 may be involved in the loss of adhesive capacity upon release of malignant plasma cells into blood (Uchiyama *et al.*, 1992). Furthermore, VLA-4 and H-CAM permit cell-to-cell contact and facilitate stimulation of IL-6 secretion by bone marrow stromal cells in a paracrine growth mechanism (Uchiyama *et al.*, 1993; Lokhorst *et al.*, 1994).

VLA-5 and VLA-6 also belong to the $\beta 1$ integrin family and their respective ligands are fibronectin and laminin. The proportion of myeloma cells expressing these molecules differs from report to report (Moller *et al.*, 1992; Uchiyama *et al.*, 1992; Harada *et al.*, 1993; Kawano *et al.*, 1993; Van Riet and Van Camp, 1993). Kawano *et al.* (1993) and Huang *et al.* (1993) proposed a classification of myeloma cells at maturation based on the expression of adhesion molecules such as VLA-5 and MPC-1. Most VLA-5^- myeloma cells presented immature or plasmablastic morphology, whereas VLA-5^+ cells were classified as mature myeloma cells. VLA-5^- myeloma cells proliferated *in vitro* and responded to IL-6, a growth factor for myeloma cells, whereas VLA-5^+ cells did not proliferate in response to IL-6 but secreted higher amounts of M protein (Kawano *et al.*, 1993). MPC-1^- myeloma cells showed immature morphology, but MPC-1^+ cells showed mature morphology. Furthermore, MPC-1^-VLA-5^- cells were found to have lost their capability to adhere to bone marrow stromal cells (Huang *et al.*, 1993). Subpopulations identified by these molecules may reflect the developmental stages of normal plasma cells in bone marrow. Because Kawano *et al.* reported that the proportion of VLA5^+ mature myeloma cells and VLA5^- immature myeloma cells varies among patients, the discrepancies among previous reports may be due to differences in the proportion of patients with aggressive disease examined for each of these studies.

Sugahara *et al.* (1994) reported that VLA-5/fibronectin interactions induced the apoptosis of human hematopoietic cell lines. VLA-5^- cells might be able to avoid apoptosis, resulting in an aggressive form. Myeloma cells themselves produce fibronectin, and fibronectin is a crucial cofactor with IL-6 in the regulation of the terminal B cell differentiation, suggesting an autocrine process for this disease (Van Riet *et al.*, 1994). It is also interesting

that fibronectin and laminine induce not only the adherence and spread of myeloma cells but also their migration chemotaxis (Shibayama *et al.*, 1995).

B. Oncogenic Transformation of Multiple Myeloma

1. Precursors for Myeloma Cells

Although multiple myeloma cells morphologically represent plasma cells in the terminal differentiation stage of B cells, a number of reports have suggested that various populations of B cells are clonally related to myeloma. First, anti-idiotypic antibodies generated to the monoclonal immunoglobulin of myeloma have traced B cells with different isotypes or even pre-B cells expressing the same idiotype as myeloma protein in bone marrow and peripheral bood (Kubagawa *et al.*, 1979; Boccadoro *et al.*, 1981; Bast *et al.*, 1982a,b; Osterborg *et al.*, 1991). Second, coexpression of early B cell lineage markers and plasma cell markers has been observed on malignant plasma cells (Caligaris-Cappio *et al.*, 1985; Pilarski *et al.*, 1985; Grogan *et al.*, 1987; Epstein *et al.*, 1988, 1990; Grogan *et al.*, 1989). Third, studies on immunoglobulin gene rearrangement have detected monoclonal B cells related to myeloma cells in peripheral blood of patients (Berenson *et al.*, 1987; Berenson & Lichtenstein, 1989; Van Reit *et al.*, 1989; Cassel *et al.*, 1990). Furthermore, differentiation from such B cells into plasma cells *in vitro* with the aid of phorbol ester (Caligaris-Cappio *et al.*, 1985) or cytokines (Bergui *et al.*, 1989) has been reported. Recently, it has also been reported that $CD19^+CD34^+$ B cells in the blood of patients contain the cells expressing clonotypic immunoglobulin heavy chain sequences, suggesting that so-called myeloma precursor cells exist in $CD34^+$ pre-B cells (Belch *et al.*, 1994). These findings imply that oncogenic transformation in this disease occurs in the early stage of B cell differentiation. However, the existence of myeloma cell precursors is still a subject of controversy because anti-idiotypic antibodies have limited specificity and may cross-react with some normal B cells (Kiyotaki *et al.*, 1987). Pre-B cell antigens as well as myeloid antigens have been expressed as a proportion of normal bone marrow plasma cells (Terstappen *et al.*, 1990). In addition, some reports indicate that clonal immunoglobulin gene rearrangement has not been detected in peripheral blood (Clofent *et al.*, 1989a,b; Levy *et al.*, 1991a).

The immunoglobulin heavy chain variable region (VDJ region) genes can serve as markers of clonality during B cell differentiation; specifically, the complementarity determining region 3 (CDR3) of this VDJ region is a unique marker for the myeloma clone. The sequence analysis of this region showed that somatic hypermutation occurred without intraclonal

variation in VDJ genes (Bakkus *et al.*, 1992; Ralph *et al.*, 1993). Somatic mutation does not occur during the primary response to antigen but rather after antigenic stimulation, as a proportion of the stimulated B cells become memory B cells or plasmablasts, both of which result from rescue of germinal center cells from apoptosis (Liu *et al.*, 1992). This suggests that the clonogenic cell in myeloma is a postgerminal center cell, which can be a memory B cell or plasmablast, that has already gone through the stage of somatic hypermutation and antigen selection (Bakkus *et al.*, 1992; Ralph *et al.*, 1993). Further analyses of expressed immunoglobulin sequences by reverse transcription-polymerase chain reaction (RT-PCR) using patient-specific CDR primers and isotype specific primers (Cμ, Cγ, and Cα) have revealed the presence of preswitched B cells bearing plasma cell V regions still joined to the Cμ gene (Billadean *et al.*, 1993; Corradini *et al.*, 1993a; Bakkus *et al.*, 1994). Therefore, myeloma precursors can be situated in the preswitched B cell compartment and heavy chain switching can occur without further somatic mutation. Billadeau *et al.* (1993) further showed that Cμ, Cδ, and Cα transcripts were observed in $CD38^-CD45^+$ sorted populations, whereas the $CD38^+CD45^-$ fraction expressed only Cγ transcript, suggesting myeloma precursors represent a mature B cell phenotype. Bakkus *et al.* (1994) identified the VDJ–Cμ sequence in peripheral blood cells, whereas Corradini *et al.* (1993a) did not. This discrepancy could be due to the sensitivity of PCR. Bergsagel *et al.* (1995) reported that clonotypic B cells could be detected among $CD19^+CD38^+CD56^+$ cells in peripheral blood, thus supporting their presence. Previous observations, such as IL-3 and IL-6-dependent proliferation and differentiation of malignant precursors from blood of myeloma patients to plasma cells *in vitro* (Bergui *et al.*, 1989), also support the presence of myeloma precursors in blood.

Recently, myeloablative therapy has been performed and the peripheral blood is considered an alternative source of normal stem cells for autografting. Although it is unlikely that myeloma precursors exist in the population of $CD34^+$ cells (Vescio *et al.*, 1994), the presence of preswitched myeloma precursors in the peripheral blood should be taken into account when contemplating autografting (Bakkus *et al.*, 1994).

2. *Abnormality of Chromosome and Oncogenes in Multiple Myeloma*

Cytogenetic studies of multiple myeloma have been relatively rare and difficult because of the very low proliferation rate of myeloma cells. A relatively frequent, but not very high, chromosomal abnormality was found in chromosome 14 on which the immunoglobulin gene is located (Taniwaki *et al.*, 1994; Lai *et al.*, 1985). Recently, several laboratories have reported

anomalous chromosomes on which have been found certain oncogenes or a suppressor gene such as N-*ras*, c-*myc*, *bcl-2*, or *p53*.

Structural alterations of the c-*myc* oncogene in human Burkitt's lymphoma and mouse plasmacytoma suggest the possibility of c-*myc* alterations in human myeloma. Selvanayagam *et al.* (1988) described alterations in the c-*myc* locus with concomitant elevated expression of mRNA in 2 of 37 cases with multiple myeloma. Moreover, elevated mRNA expression without demonstrable gene alteration was observed in approximately one-fourth of the patients analyzed. Similar findings were reported by Nobuyoshi *et al.* (1991). Greil *et al.* (1991) used *in situ* hybridization to demonstrate c-*myc* expression by plasma cells in a majority of patients. However, they could not find a correlation between proliferation rate and the expression of c-*myc*.

Mutations of N- and/or K-*ras* gene have been demonstrated with considerable frequency (Neri *et al.*, 1989; Paquette *et al.*, 1990; Portier *et al.*, 1992; Corradini *et al.*, 1993b). The frequency of *ras* mutations found in treated patients is higher than that in primary cases, suggesting the accumulation of point mutations during the progression of disease.

The *bcl-2* gene is located on chromosome 18. This protooncogene was originally described in follicular lymphomas with chromosome translocation t(14;18). Hamilton *et al.* (1991b) demonstrated the presence of Bcl-2 protein in myeloma cell lines and freshly isolated plasma cells from both myeloma patients and normal individuals. According to the data from cell lines, *bcl-2* expression does not depend on the presence of t(14;18). A similar observation was reported by Pettersson *et al.* (1992). Whether or not *bcl-2* prevents apoptosis of myeloma cells is unknown at the present time.

The *p53* gene is a tumor suppressor gene often inactivated by deletion and/or point mutation in various types of human cancers (Harris and Hollstein, 1993). Point mutations of the *p53* gene in myeloma cells were detected in approximately 20% of myeloma patients (Portier *et al.*, 1992) and 80% of myeloma cell lines (Mazars *et al.*, 1992). The mutations were specifically associated with the more advanced and clinically aggressive leukemic phase of multiple myeloma (Neri *et al.*, 1993). Because *p53* mutations are also frequently observed in patients with drug-resistant hematologic malignancy (Wattel *et al.*, 1994), they may be involved in generating malignant characteristics.

These observations indicate that oncogenes and tumor suppressor genes may be involved in the development and progression of multiple myeloma, although the mechanisms have not been as clearly identified as have those in mouse plasmacytomagenesis.

C. Cytokines Involved in the Pathogenesis of Multiple Myeloma

1. IL-6

a. In Vitro IL-6 Is a Potent Growth Factor for Myeloma/Plasmacytoma Cells. Since IL-6 was originally identified as a B cell differentiation factor (BCDF/BSF-2) that induces final maturation of B cells into antibody-producing cells (Hirano *et al.*, 1986), it has been shown to be a pleiotropic cytokine with a wide range of biological activities such as support of hematopoiesis, regulation of acute phase reactions and immunoresponse, and neural differentiation (Akira *et al.*, 1993). One of the most interesting activities of IL-6 is the induction of the growth of myeloma/plasmacytoma cells. In mice, it is thought to be a growth factor of plasmacytomas arising in the granulomatous tissue formed by the intraperitoneal injection of pristane (Nordan and Potter, 1986; Van Snick *et al.*, 1987; Vink *et al.*, 1990; Degrassi *et al.*, 1993). IL-6-deficient mice are completely resistant to *myc/raf* plasma cell tumorigenesis, defining an essential role for IL-6 in the development of plasmacytoma *in vivo* (Hilbert *et al.*, 1995b). In human, Kawano *et al.* (1988) first reported that IL-6 is a possible autocrine growth factor for both myeloma cells freshly isolated from patients and myeloma cell line U266. The evidence supporting this conclusion is threefold. First, recombinant IL-6 was seen to induce *in vitro* growth of myeloma cells freshly isolated from patients. Second, myeloma cells spontaneously produced IL-6 and expressed the IL-6 receptor. Third, *in vitro* growth of myeloma cells was specifically inhibited by anti-IL-6 antibody. In support of the existence of IL-6-mediated autocrine growth, Schwab *et al.* (1991) have shown that the addition of a neutralizing anti-IL-6 monoclonal antibody or IL-6 antisense oligonucleotides inhibits proliferation of the U266 myeloma cell line, and this effect is reversed by adding IL-6. Similar findings supporting the autocrine hypothesis have been reported by several laboratories using myeloma cell lines (Levy *et al.*, 1991b; Jernberg *et al.*, 1991; Hitzler *et al.*, 1991; Goto *et al.*, 1994) or malignant plasma cells freshly isolated from patients with multiple myeloma (Hata *et al.*, 1993) or plasmacytoma (Nishimoto *et al.*, 1994). On the other hand, Klein *et al.* (1989) have proposed a paracrine instead of an autocrine growth mechanism. They also observed a spontaneous proliferation of myeloma cells freshly isolated, but not purified, from patients in short-term culture that was inhibited by neutralizing antibodies to IL-6 and reinduced by adding recombinant IL-6. However, they demonstrated significant *in vivo* IL-6 mRNA expression in bone marrow stromal cells but not in myeloma cells from most of their myeloma patients. Moreover, they described that myeloma cell line U266 and RPMI8226 did not secrete IL-6 or express mRNA

for IL-6, whereas anti-IL-6 antibodies did not inhibit their proliferation nor did recombinant IL-6 stimulate it. Several reports have also described paracrine growth of myeloma cells induced by IL-6 (Anderson *et al.*, 1989; Shimizu *et al.*, 1989; Sonneveld *et al.*, 1991; Okuno *et al.*, 1991; Barut *et al.*, 1992; Zhang *et al.*, 1994a). Specifically, the evidence that macrophages that produce a large amount of IL-6 or recombinant IL-6 can support the establishment of reproducible myeloma cell lines that used to be extremely difficult to establish, especially from bone marrow (Shimizu *et al.*, 1989; Zhang *et al.*, 1994a), supports the paracrine hypothesis. Bone marrow stromal cells from multiple myeloma have been found to actively produce IL-6, although normal bone marrow stromal cells have not been found to do so (Nemunaitis *et al.*, 1989; Portier *et al.*, 1991). Activated bone marrow stromal cells, after trigering with myeloma cells, may play a role in supporting the growth and final differentiation of malignant B cells of peripheral origin and in further promoting the recruitment of circulating osteoclast precursors and the growth of osteoclast, resulting in bone destruction (Caligaris-Cappio *et al.*, 1991; Uchiyama *et al.*, 1993; Lokhorst *et al.*, 1994). Because IL-6 production from myeloma cells is much less than that from stromal cells, and also because myeloma cell lines with autocrine growth mechanism mediated by IL-6 are rare, the paracrine hypothesis has been sustained. Myeloma cells may, however, require additional mutation to obtain autocrine characteristics. Barut *et al.* (1993) obtained evidence that IL-6 functions as an intracellular growth factor in hairy cell leukemia. In such a case, the cells do not increase DNA synthesis *in vitro* in response to exogenous IL-6 despite IL-6 receptor expression. However, the fact that the cells produce IL-6 and IL-6 antisense oligonucleotide, but not anti-IL-6 antibody, inhibits their proliferation. Similarly, IL-6 may be an intracellular growth factor in some cases of multiple myeloma. Taken together, the evidence indicates that IL-6 is a growth factor for multiple myeloma cells *in vitro*.

b. IL-6 Is Also Thought to Stimulate Myeloma Growth in Vivo. IL-6 is a likely *in vivo* myeloma cell growth factor. IL-6 serum levels reflect disease severity in multiple myeloma. In the sera of patients with advanced multiple myeloma, increased levels of IL-6 have been observed, whereas IL-6 is usually not elevated in early stages (Bataille *et al.*, 1989; Nachbaur *et al.*, 1991; Solary *et al.*, 1992; Tienhaara *et al.*, 1994; Pelliniemi *et al.*, 1995). Serum IL-6 levels correlate with bone marrow plasmacytosis, serum lactate dehydrogenase, and serum β_2 microglobulin, which are the known prognostic markers of this disease (Solary *et al.*, 1992), and inversely correlate with hemoglobin (Nachbaur *et al.*, 1991). Furthermore, when patients were divided into two groups according to normal or raised serum IL-6

levels, the patients with high IL-6 levels had more frequent osteolytic bone lesions (Pelliniemi *et al.*, 1995). Zhang *et al.* (1989) also demonstrated that IL-6 responsiveness of myeloma cells *in vitro* is directly related to their *in vivo* proliferative status. Such evidence strongly suggests that IL-6 is a major growth factor for myeloma cells *in vivo* as well as *in vitro,* although some reports with different conclusions have been published (Asaoku *et al.*, 1988; Bell *et al.*, 1991; Ballester *et al.*, 1994). On the basis of the positive findings, Klein *et al.* (1991) showed that the *in vivo* administration of murine anti-IL-6 monoclonal antibodies to a patient with plasma cell leukemia was therapeutically effective, thus confirming the *in vivo* function of IL-6 in this disease.

c. IL-6 as a Differentiation Factor for Myeloma Cells. Ballester *et al.* (1994) proposed that a primary role for IL-6 is as a differentiation rather than a growth factor, as it is in normal plasma cells, because they found a positive correlation between spontaneously secreted IL-6 levels and the rate of IgG secretion in IgG myeloma, but not with the disease severity determined by serum β_2 microglobulin, bone marrow myeloma cells, or a proportion of the proliferative compartment. However, not enough direct evidence of augmented production of M-protein by IL-6 could be provided to refute previous studies showing that IL-6 had no differentiation effect on myeloma cells (Tanabe *et al.*, 1989; Sonneveld *et al.*, 1991).

For patients with MGUS or Waldenström's macroglobulinemia, serum levels of IL-6 are lower than those for patients with multiple myeloma or plasma cell leukemia, although the actual IL-6 levels differ between studies (Bataille *et al.*, 1989; Nachbaur *et al.*, 1991; Solary *et al.*, 1992). In addition, IL-6 was found to affect neither the proliferation nor the differentiation of malignant plasma cells of Waldenström's macroglobulinemia *in vitro* (Levy *et al.*, 1990). Therefore, the function of IL-6 seems less significant in these diseases.

d. IL-6 Is Responsible for the Progressive Bone Resorption Characteristic of Myeloma. IL-6 is involved in the pathophysiology of osteolytic bone lesions and hypercalcemia of multiple myeloma. In normal individuals, there is a balance between the process of bone formation by osteoblasts and bone resorption by osteoclasts. Such bone remodeling is regulated by local factors, referred to as osteotropic cytokines, that are generated in the microenvironment of the remodeling unit. The osteotropic cytokines include IL-1, tumor necrosis factor (TNF), colony-stimulating factors, and IL-6. IL-6 is locally produced in bone by osteoblasts under the influence of the parathyroid hormone or other cytokines such as IL-1 and TNF (Lowik *et al.*, 1989; Ishimi *et al.*, 1990; Feyen *et al.*, 1989). IL-6 is also

produced by osteoclasts and stimulates early osteoclast precursor formation from cells present in CFU-GM colonies (Kurihara *et al.*, 1990). Although IL-6 alone did not induce osteoclast-like multinucleated cells from bone marrow cells *in vitro*, simultaneous treatment with IL-6 and soluble IL-6 receptors (sIL-6R) induced authentic osteoclasts characterized by the presence of tartrate-resistant acid phosphatase activity, calcitonin receptors, and pit formation in dentine slices (Tamura *et al.*, 1993). Black *et al.* (1991) demonstrated that a significant increase in blood calcium associated with increased levels of serum IL-6 was induced in nude mice inoculated with Chinese hamster ovary cells transfected with the human IL-6 gene, indicating that IL-6 stimulates bone resorption. Add to this evidence the finding that myeloma patients with high IL-6 levels had more frequent osteolytic bone lesions (Pelliniemi *et al.*, 1995), and IL-6 can be postulated as playing a major role in generating osteolytic bone lesion and osteoporosis in myeloma.

e. Other Pathophysiological Functions of IL-6. IL-6 is probably involved in anemia of patients with multiple myeloma because cancer patients receiving recombinant IL-6 for antitumor immunotherapy showed a rapid induction of anemia, which was reversed after the cessation of IL-6 treatment (Nieken *et al.*, 1995; Atkins *et al.*, 1995).

Another important function of IL-6 in multiple myeloma is to prevent dexamethasone-induced apoptosis of myeloma cells (Hardin *et al.*, 1994). Because dexamethasone provides an effective treatment for many patients with both primary and resistant myeloma (Alexanian *et al.*, 1992), this finding should be taken into account when planning therapeutic strategy.

f. Soluble IL-6 Receptors in Multiple Myeloma. The IL-6 receptor (IL-6R) complex comprises two functional different membrane proteins: a ligand-binding chain (80-kDa IL-6R, CD80) and a nonbinding but signal-transducing chain (gp130, CD130). IL-6R has a very short intracytoplasmic portion containing only 82 amino acid that is not essential for signal transduction. IL-6, when bound to either a membrane-anchored or soluble form of its receptor (IL-6R or sIL-6R), induces the formation of gp130/gp130 homodimer, resulting in a high-affinity functional receptor (Murakami *et al.*, 1993). Increased and high levels of sIL-6R are found in the serum of patients with multiple myeloma when compared with that of healthy individuals (Gaillard *et al.*, 1993). It has also been found that sIL-6R plays an agonistic role and can produce a 10-fold increase in IL-6 sensitivity during the growth of myeloma cell lines. sIL-6R may also play a role in inducing osteoclasts in bone marrow as described previously. To date the origin of sIL-6R observed in patient sera has not been determined.

2. *IL-6-Related Cytokines That Use gp130 as a Signal Transducer*

Functional pleiotropy and redundancy, common characteristics of cytokines, can be explained by interactions between multiple ligand-binding receptors and a common signal transducer that lacks direct ligand-binding activity (Kishimoto *et al.*, 1994, 1995). IL-6, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OM), and IL-11 have been proven to use the common signal transducer, gp130. Recently, cardiotrophin-1 (CT-1) has been identified as a factor that induces hypertrophy of cardiac myocytes, and it has been suggested that CT-1 uses gp130 as a component of its receptor complex (Pennica *et al.*, 1995). Thus, it seems likely that these cytokines as well as IL-6 may be growth factors of human myeloma cells. In fact, LIF and OM as well as IL-6 have been reported to stimulate the *in vitro* growth of malignant plasma cells freshly explanted from a patient with plasmacytoma (Nishimoto *et al.*, 1994). These growth stimulation activities were inhibited by the addition of anti-gp130 monoclonal antibody, thus confirming that these cytokines transduce the growth signal through the common signal transducer, gp130 (Fig. 4). IL-11 failed to stimulate the growth of the explanted cells. This observation is consistent with a previous report that no growth-stimulation activity of IL-11 was shown in either myeloma cell lines or freshly isolated myeloma cells (Paul *et al.*, 1992). On the other hand, Zhang *et al.* (1994b) showed that IL-11 and CNTF, as well as LIF, OM, and IL-6, are growth factors of human myeloma cell lines. The responsiveness of the myeloma cells to these cytokines seems to depend on whether the cells express the ligand-binding receptors. To date, fresh myeloma samples whose growth depends on multiple cytokines as described previously have seldom been identified. However, because these cytokines exist *in vivo*, they may be involved in the pathogenesis of myeloma.

3. *Other Cytokines Involved in the Pathophysiology of Multiple Myeloma*

IL-3 has been reported to be a proliferation and differentiation factor of myeloma cells, acting synergistically with IL-6 (Bergui *et al.*, 1989). A large number of malignant plasma cells could be obtained from peripheral blood cells cultured *in vitro* in the presence of IL-3 and IL-6, suggesting the presence of myeloma precursors in blood. However, this observation could not be confirmed by others (Sonneveld *et al.*, 1991; Tokumine *et al.*, 1993). Granulocyte macrophage colony-stimulating factor (GM-CSF) can accelerate growth of myeloma cells synergistically with IL-6 (Zhang *et al.*, 1990). Because this stimulatory effect of GM-CSF was abrogated by anti-IL-6 monoclonal antibodies but GM-CSF did not increase the

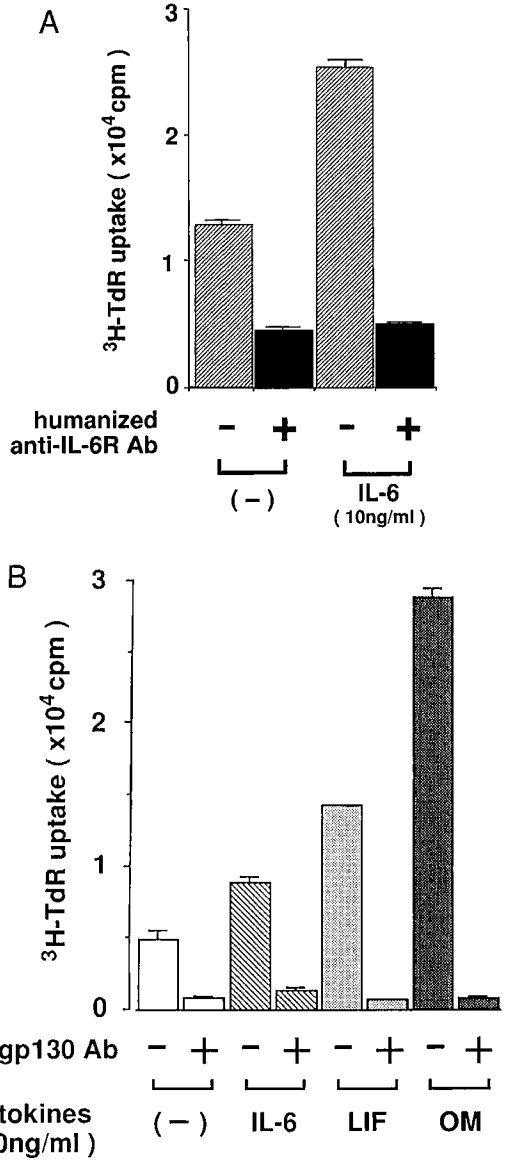

FIG. 4. Autocrine and/or paracrine growth of myeloma cells. (A) IL-6 promotes growth of human myeloma cells and anti-IL-6 monoclonal antibody inhibits their growth. (B) IL-6 family of cytokines promote growth of myeloma cells and anti-gp130 monoclonal antibody inhibits their growth.

endogenous IL-6 production, GM-CSF must enhance the IL-6 responsiveness of myeloma cells. IL-5 has been reported to stimulate myeloma cell growth independently of IL-6 (Anderson *et al.*, 1989). The functions of IL-3, GM-CSF, and IL-5 overlap in various hematopoietic cells and this functional redundancy is also reflected in the existence of a common β signal transducer (Miyajima *et al.*, 1992). Therefore, IL-3, GM-CSF, and IL-5 may act similary on myeloma cells.

The growth-stimulating function of IL-1α or IL-1β is controversial. Kawano *et al.* (1989a) and Carter *et al.* (1990) reported that IL-1α or IL-1β enhanced [^{3}H]TdR uptake in myeloma cells through the enhancement of IL-6 production, but this activity could not be confirmed by Anderson *et al.* (1989). Because IL-1 can induce IL-6 production from various cells, IL-1 may stimulate the growth of myeloma cells through IL-6 *in vivo*. IL-1 is also responsible for the activation of osteoclasts. Initially, Garrett *et al.* (1987) found bone-resorbing activity in the culture supernatant of a tumor cell line derived from a myeloma patient with osteolytic bone lesion and hypercalcemia. They determined this activity originated from lymphotoxin (TNF-β) because it was suppressed by anti-lymphotoxin antibody. However, in the culture supernatant of freshly isolated myeloma compartments, a high amount of IL-1β and a lower amount of IL-1α were detected and found responsible for most of the osteoclast activating factor activity (Kawano *et al.*, 1989b; Cozzolino *et al.*, 1989).

IL-10 is another growth-stimulating factor for myeloma cells. Because anti-IL-6 and anti-IL-6R antibodies did not affect the growth activity induced by IL-10, IL-10 is thought to be an IL-6-unrelated growth factor for myeloma cells (Lu *et al.*, 1995).

TNF has also been identified as a growth factor for myeloma cells through a pathway that is independent of IL-6 (Hata *et al.*, 1990; Borset *et al.*, 1994).

Interferon-α (IFN-α) has been used as a therapeutic agent in multiple myeloma (Mellstedt *et al.*, 1979; Salmon *et al.*, 1983; Ahre *et al.*, 1984; Quesada *et al.*, 1986; Mandelli *et al.*, 1990; Attal *et al.*, 1992; Cooper *et al.*, 1993; Osterborg *et al.*, 1993). Most studies, but not all, have proved the benefits of IFN-α for improvement in overall response or prolonging the plateau phase of remission. However, the mechanisms for its therapeutic effect on myeloma remain unclear, whereas the *in vitro* effect of IFN-α on the growth of myeloma cells is still controversial (Brenning *et al.*, 1985; Tanaka *et al.*, 1989; Shimizu *et al.*, 1989; Jourdan *et al.*, 1991) IFN-α was found to augment myeloma cell line growth, which was dependent on IL-6 (Shimizu *et al.*, 1989; Jourdan *et al.*, 1991). IFN-α was seen to promote myeloma cell lines, thus assuming a characteristic of the IL-6-mediated autocrine mechanism (Jourdan *et al.*, 1991). On the other hand,

disruption by IFN-α of an autocrine IL-6 growth loop was reported in the IL-6-dependent U266 myeloma cell line as a result of homologous and heterologous downregulation of the IL-6R and gp130 signal transducer (Schwabe *et al.*, 1994). In the former case, treatment with IFN-α might result in producing an aggressive myeloma. In fact, development of aggressive plasma cell leukemia under IFN-α therapy has been reported (Blade *et al.*, 1991). Therefore, it may be helpful to check the efficacy of IFN-α before clinical application.

IFN-γ was reported to inhibit the growth of IL-6-dependent myeloma cell lines by downregulation of IL-6R *in vitro* (Portier *et al.*, 1993). Jernberg-Wiklund *et al.* (1991) also reported the growth inhibition of myeloma cells by IFN-γ, but this activity was not mediated by the downregulation of IL-6R. Our data support the former finding. However, the therapeutic application of IFN-γ must be performed carefully because IFN-γ was found to stimulate the IL-6 production from myeloma cells *in vitro* (Ogata *et al.*, 1994). Quesada *et al.* (1988) reported that *in vivo* administration of recombinant IFN-γ did not improve the disease activity.

IL-4 was also reported to block endogenous IL-6 synthesis in a dose-dependent fashion, resulting in the reduction of myeloma cell growth *in vitro* (Herrmann *et al.*, 1991).

All the cytokines described previously may well be involved in the pathophysiology of multiple myeloma. Therefore, further studies are needed to study the possible application of these cytokines to therapeutic strategy.

4. Expression and Function of Fas/APO-1 (CD95) in Myeloma Cells

When cytokine receptor expression was analyzed in freshly isolated myeloma cells and myeloma cell lines, most of the cells expressed Fas antigen/APO-1 (CD95) in addition to IL-6R, gp130, and IFN-γ receptors. The expression of IL-6R and gp130 is not surprising because IL-6 is a potent growth factor for myeloma cells, whereas IFN-γ inhibits myeloma cell growth by reducing IL-6R expression *in vitro*. However, the effect of Fas antigen on myeloma cells could not be clarified. Recently, similar observations have been reported by three laboratories using both freshly isolated myeloma cells and cell lines (Shima *et al.*, 1995; Westendorf *et al.*, 1995; Hata *et al.*, 1995). Fas antigen can mediate the signal for apoptosis of freshly isolated myeloma cells, although this effect varied from patient to patient. In some cell lines, apoptosis was not induced by cross-linking of Fas antigen with the aid of anti-Fas monoclonal antibody of IgM isotype. Whether the cells undergo apoptosis in response to the signal mediated by the Fas antigen could not be related to *bcl-2* expression because no correlation was observed between the inducibility of apoptosis and *bcl-2* expression. Further studies are required to define the pathophysiological

role of Fas antigen because the prevention of apoptosis seems to be very important for the development of very slowly proliferating tumors such as multiple myeloma.

D. New Therapeutic Approaches Utilizing Interference with IL-6 Signal Transduction

1. Biological Effects of Anti-IL-6 Murine Monoclonal Antibody on Advanced Myeloma

On the basis of the evidence that IL-6 is a potent growth factor for myeloma cells, Klein *et al.* (1991) treated a patient with plasma cell leukemia with murine anti-human IL-6 monoclonal antibodies. After *in vivo* administration of antibodies, improvement of fever, serum M-component, serum calcium, and C-reactive protein was observed in association with a decrease in the number of malignant plasma cells in the S-phase of the DNA cell cycle. However, such therapeutic effects were transient. The loss of efficacy was thought to be due to either the emergence of human antibodies against murine anti-IL-6 monoclonal antibodies or the insufficient concentration of anti-IL-6 monoclonal antibodies to neutralize IL-6 that had increased after the treatment. In fact, myeloma cells explanted from a patient after recurrence showed the same IL-6-dependent growth *in vitro* as before treatment. Furthermore, the same group of investigators reported the clinical data for 10 patients with advanced myeloma who were treated with murine anti-IL-6 monoclonal antibody (Bataille *et al.*, 1995). Two patients with data suitable for evaluation exhibited marked inhibition of plasmablastic proliferation, and 3 showed an objective antiproliferative effect marked by a significant reduction of the myeloma cell labeling index within the bone marrow. However, none of the patients studied achieved remission or improved outcome as judged by standard clinical criteria. The loss of the effect of anti-IL-6 monoclonal antibody is likely due to a higher production of IL-6 and inability of the monoclonal antibody to neutralize IL-6 in four patients. The generation of human antibodies against murine anti-IL-6 monoclonal antibody appeared to be another reason for the resistance, especially in the patients who were treated for longer than 1 month. Because all the patients had advanced myeloma, the results do not seem to be discouraging.

2. Inhibition of Myeloma Cell Growth by Humanized Anti-IL-6R Antibody

Murine antibodies are highly immunogenic in human, especially when they are repeatedly administered. Thus, their therapeutic value for human patients is limited. In order to be effective as therapeutic agents administered to patients in repeated doses, mouse antibodies must therefore be

engineered to look like human antibodies. Sato *et al.* (1993) succeeded in reshaping a mouse monoclonal anti-human IL-6R antibody, PM-1, into a human antibody. Reshaped human PM-1 (rhPM-1) is constructed by grafting the CDRs from the mouse PM-1 into human IgG to re-create a well-functioning antigen-binding site in a reshaped human antibody. It is equivalent to both mouse and chimeric PM-1 in terms of antigen binding and growth inhibition of myeloma cells *in vitro*. rhPM-1 also inhibited both autocrine and paracrine growth of freshly isolated myeloma cells as well as that of goat anti-IL-6 polyclonal antibodies *in vitro* (Nishimoto *et al.*, 1994). Moreover, rhPM-1 looks very much like a human antibody and can therefore be expected to be a poor immunogen in human patients. The *in vivo* inhibitory effect of rhPM-1 on the growth of myeloma cells was also examined in IL-6 transgenic SCID mice inoculated with human myeloma cells. rhPM-1 inhibited both tumor mass formation and an increase in serum M-protein in this mouse model of human myeloma (N. Nishimoto, S. Suematsu, and T. Kishimoto, unpublished results). Clinical trials of rhPM-1 are now in progress.

3. *All-trans Retinoic Acid as a Modulator of IL-6 Signal Transduction*

A factor that modulates IL-6 signal transduction can be a potential therapeutic agent for multiple myeloma. All-*trans* retinoic acid (ATRA) derived from retinol (vitamin A) induced the differentiation of an acute promyelocytic leukemia (APL) cell line (HL-60) into mature granulocytes (Breitman *et al.*, 1980). On the basis of this finding, Huang *et al.* (1988) applied ATRA to the treatment of APL, with the result that 23 of 24 patients who received ATRA were induced into complete remission. Sidell *et al.* (1991) identified the antiproliferative action of ATRA on a myeloma cell line, resulting in down regulation of IL-6R and subsequent inhibition of IL-6-mediated autocrine growth. The growth of freshly isolated myeloma cells was also inhibited by ATRA *in vitro*, although the extent of the inhibitory effects varied among cases (Ogata *et al.*, 1994). This growth-inhibitory activity is partly due to a heterologous or homologous downregulation of IL-6R and gp130 signal transducers. Furthermore, ATRA inhibited the *in vitro* IL-6 production from both myeloma cells themselves and bone marrow stromal cells. Thus, ATRA can interfere with the autocrine and paracrine loop of IL-6-dependent myeloma cell growth. Recently, a phase II clinical trial using ATRA for patients with advanced refractory multiple myeloma was initiated (Niesvizky *et al.*, 1995). However, patients developed severe hypercalcemia following administration of ATRA, in association with an increase in serum IL-6 levels that had previously been observed in ATRA treatment for APL (Akiyama *et al.*, 1992). Further

investigation will be required to determine whether this treatment is beneficial.

4. Other Therapeutic Approaches

Schematic models of therapeutic approaches for multiple myeloma by means of interference with IL-6 signal transduction are shown in Fig. 5: (i) Inhibition of IL-6 production or neutralization of IL-6, (ii) blockade of IL-6 binding to IL-6R, (iii) blockade of IL-6/IL-6R complex binding to gp130, (iv) suppression of IL-6R and/or gp130 expression, and (v) blockade of intracytoplasmic signal from gp130 are possible approaches that have been described previously. If IL-6 is an intracellular autocrine growth factor, antisense oligonucleotides for transcription factors such as acute-phase response factor/signal transducers and activators of transcription 3

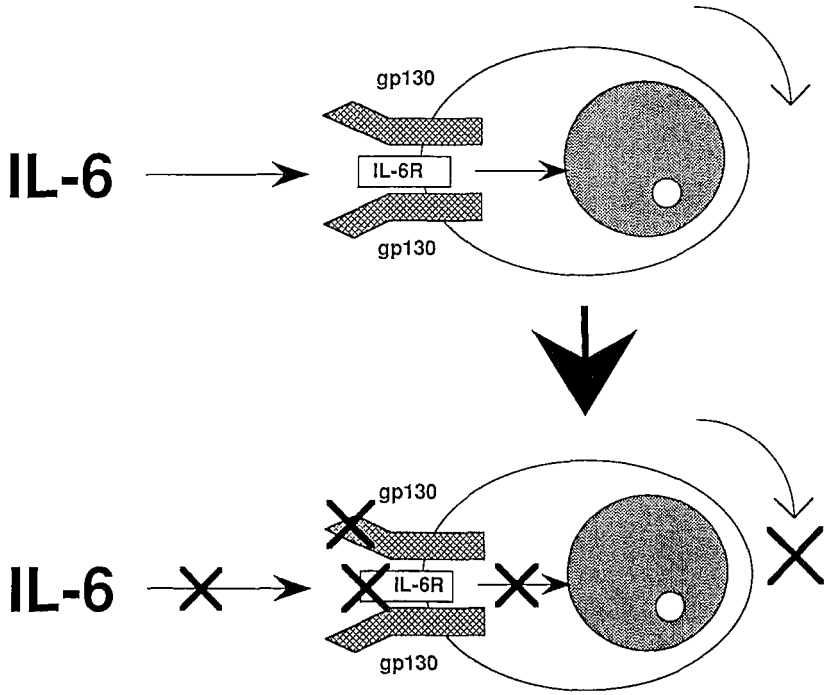

FIG. 5. Schematic models of therapeutic approaches for multiple myeloma by means of interference with IL-6 signal transduction. Curved arrows indicate proliferation of myeloma cells. X, potential targets for blockade of IL-6 signaling, including (i) inhibition of IL-6 production or neutralization of IL-6, (ii) blockade of IL-6 binding to IL-6R, (ii) blockade of IL-6/IL-6R complex binding to gp130, (iv) suppression of IL-6R and/or gp130 expression, and (v) blockade of intracytoplasmic signal from gp130.

(STAT3) or nuclear factor-IL6 might be effective in blocking intracellular signals.

Monoclonal antibodies specific to myeloma cells possess clinical utility not only for characterization of myeloma cells but also for *in vivo* or *in vitro* purging of human myeloma cells. Goldmacher *et al.* (1994) reported the development of a potent anti-CD38 immunotoxin capable of killing human myeloma cells. A chimeric anti-CD38 antibody consisting of the Fab portion of the mouse monoclonal antibody linked to an Fc molecule derived from human IgG1 was found to mediate antibody-dependent cellular cytotoxicity (ADCC). Antibody attack on tumor cells by ADCC has been predicted (Stevenson *et al.*, 1991).

IL-6-pseudomonas exotoxin derivative is cytotoxic for myeloma cells, suggesting that it can be effective in *ex vivo* marrow purging for myeloma patients (Kreitman *et al.*, 1992).

VI. Conclusion

Advances in molecular biology have made it possible to elucidate the mechanisms of proliferation and differentiation of plasma cells both from patients with plasma cell dyscrasias and from normal individuals. Especially in mouse, plasmacytomagenesis is well defined through experiments with the hyperexpression of cytokines and oncogenes and with viral infection. The experimentally developed tumors in mouse models are extremely useful for understanding the mechanisms of oncogenic transformation of human counterparts. Although the role of oncogenes in human multiple myeloma is not yet well understood, remarkable progress has been made in cytokine study. IL-6 has been identified as a major growth factor for myeloma/plasmacytoma, and it is now clear that blockade of the IL-6 function can inhibit myeloma cell growth and improve the clinical status of patients. However, further studies will be required to elucidate the intracellular events, such as signal transduction and gene regulation, in myeloma cells. An approach based on these findings should provide a new therapeutic strategy for myeloma.

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ADVANCES IN IMMUNOLOGY, VOL. 64

Anti-Tumor Necrosis Factor-α Therapy of Rheumatoid Arthritis

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I. Introduction

A. Etiology and Pathogenesis of Rheumatoid Arthritis

The etiology and pathogenesis of rheumatoid arthritis (RA), like that of many other chronic immune-inflammatory diseases, is poorly understood. The clearest genetic predisposition is with HLA, and this association was first described with Dw4 (Stasny, 1978). In many, but not all, populations there is a high association (>80% of patients) with DR4 or DR1. This is not true in some populations (e.g., Greeks). Within DR4, there are subtypes. Dw4, Dw14, and Dw15 are disease associated, but Dw10 and Dw13 are not. Based on sequence analysis of the variable DRβ chain of susceptible and nonsusceptible alleles, the concept of the "shared epitope," a sequence between amino acid positions 70 and 74 of the DRβ chain that is shared by the DR4 susceptible haplotypes and DR1, has been proposed (Gregersen *et al.*, 1987). This concept is illustrated in Fig. 1 (Maini *et al.*, 1995a; Woulfe *et al.*, 1995).

Because the only known function of HLA-DR molecules is to bind and present peptides to the T cell receptor (TCR), the shared epitope hypothesis is the strongest argument that T cells are involved in the pathogenesis of RA. However, it is not clear whether the DR and T cell involvement is in the thymic development of the T cell antigen receptor repertoire, in responsiveness (or lack of it) to extrinsic antigen, or, as widely favored but not established, in the autoantigen response (Todd *et al.*, 1988). The importance of the immune response in the late stages of arthritis has been challenged, and there are differences in opinion (Firestein and Zvaifler, 1990; Panayi *et al.*, 1992).

Twin studies have shed some interesting light on aspects of etiology. Although a previous study of hospital-treated RA patients revealed a 35% concordance in identical twins (Laurence, 1970), a more recent, wider community-based survey of RA in identical twins revealed a lower concordance of ~15% (Silman *et al.*, 1993). Identical twins have, by definition, all the genetic risk factors, and therefore the low concordance rates in identical twins (15–35%) places a limit on the genetic contribution to this disease. Because there is an increased likelihood of the unaffected twin developing RA in subsequent years, the genetic contribution is greater

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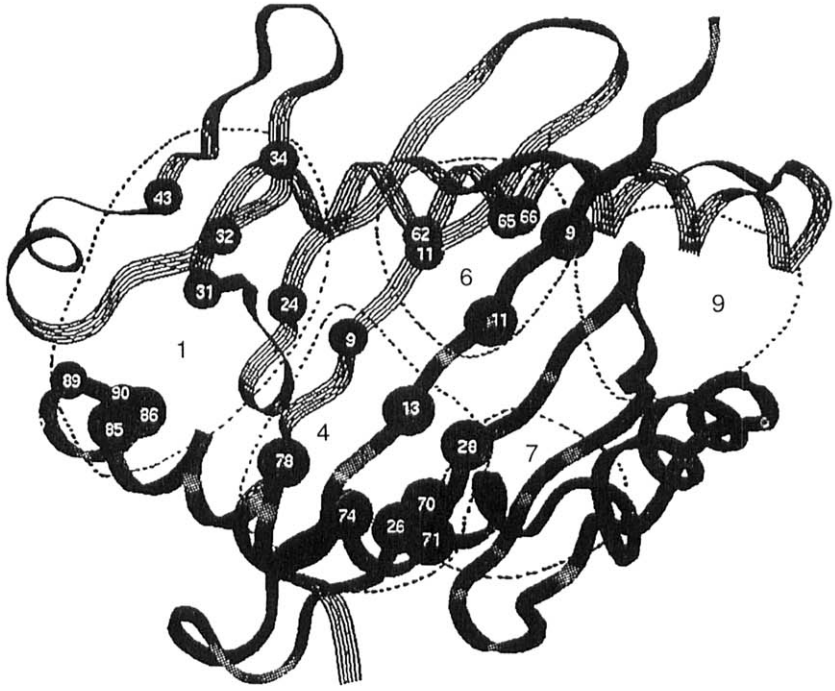
POLYMORPHIC SEQUENCES ASSOCIATED WITH RA				
HLA-DR type	**70**	**71**	**74**	**ASSOCIATION**
DR4 Dw4	Glutamine	Lysine	Alanine	+
DR4 Dw14	"	Arginine	"	+
DR4 Dw15	"	"	"	+
DR1	"	"	"	+
DR6 Dw16	"	"	"	+
DR4 Dw10	Aspartic	Glutamic Acid	"	-
DR4 Dw13	Glutamine	Arginine	Glutamic Acid	-



than the concordance suggests (approximately 50%) (Macgregor and Silman, 1994; Silman and Hochberg, 1993).

There is evidence that individuals homozygous for the shared epitope have a greater risk of developing RA and are more likely to have systemic disease (Weyand and Goronzy, 1995; Weyand *et al.*, 1992, 1995; Wordsworth and Bell, 1992). This raises the question as to whether DR4/DR1 is a risk factor or a severity factor. Comparisons of DR4 and DR1 patients have suggested that DR4 individuals have more severe disease than DR1 individuals, although RA patients with both DR4 and DR1 appear to have the same severity as DR4 homozygotes (Evans *et al.*, 1995; Wordsworth and Bell, 1992). The reasons for this are not yet known. There are data suggesting that DR4 and DR3 individuals produce more tumor necrosis factor-α (TNFα) on stimulation with lipopolysaccharide (LPS) compared to those expressing DR1 or DR2 (Bendtzen *et al.*, 1988; Jacob *et al.*, 1990); therefore, a possible contribution to differences between DR4 and DR1 may be linked to genes such as TNFα, lymphotoxin-α (LTα), or LTβ. However, studies of genetic polymorphisms of the TNF genes have yet to reveal any consistent differences between RA patients and normal individuals, in contrast to results in SLE (Wilson *et al.*, 1993, 1994).

Although the HLA genes are the dominant genetic influence, other genes contribute based on differences in concordance between HLA-matched individuals, HLA-matched sibs, and twins (Silman *et al.*, 1993). The nature of these genes is unclear but is under investigation. RA is three times more common in women than in men, which suggests an important role for sex hormones in its pathogenesis (Masi, 1994). A defect in the hypothalamic response has been proposed by Chikanza *et al.* (1992).

B. Clinical Features and Pathology of RA

Although the most obvious clinical features in RA are in the synovial joints of the hands, feet, wrists, knees, hips, etc., there is also clear evidence of a systemic disease. Active RA patients have an elevated erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) and other acute-phase protein levels, often a mild fever, a mild anemia, granulocytosis, and thrombocytosis (Gordon and Hastings, 1994). In more severe and prolonged cases, there is evidence of extraarticular disease. The most

FIG. 1. The shared epitope hypothesis. (Top) polymorphic amino acids: table modified from Maini *et al.* (1995a); data from Gregersen *et al.* (1987). (Bottom) amino acids 70, 71, and 74 are in the p4 pocket of HLA-DR. Reproduced with permission from Woulfe *et al.* (1995).

common feature is skin nodules, which occur in regions (e.g., elbows) prone to mild injury; other features include fibrosis in lung and less commonly in liver and kidney, vasculitis, and in ~1% of cases there is "Felty's syndrome" with hypersplenism and evidence of increased leucocyte and platelet turnover.

Only in recent years has it become appreciated that the survival of RA patients can be markedly impaired. Studies in the United Kingdom and the United States have revealed that although even mild RA has a detectable reduction in survival, severe RA, defined as having disease involving multiple joints and/or extraarticular manifestations, has increased mortality, with only 40% of such patients surviving 10 years (Erhardt *et al.*, 1989; Pincus and Callahan, 1993), as illustrated in Fig. 2.

The reasons for the increased mortality have been analyzed. There is a marked augmentation in the incidence of severe infections. There is also an increased incidence of lymphoma (Wolfe *et al.*, 1994). However, the majority of patients, like the overall population, die of cardiovascular disease and of cancer but at an earlier age. The reasons for this are not understood, and it is not evident if reduced mobility, therapy, or the disordered cytokine network in the RA are involved. Because some risk factors for cardiovascular disease are upregulated by cytokines, there is experimental evidence for the latter hypothesis (see Section IV,G2).

In the joints, there are two major features: synovitis characterized by hyperplasia and inflammation of the synovium, with an inflammatory exudate into the joint cavity; and erosions of cartilage and bone. The synovitis

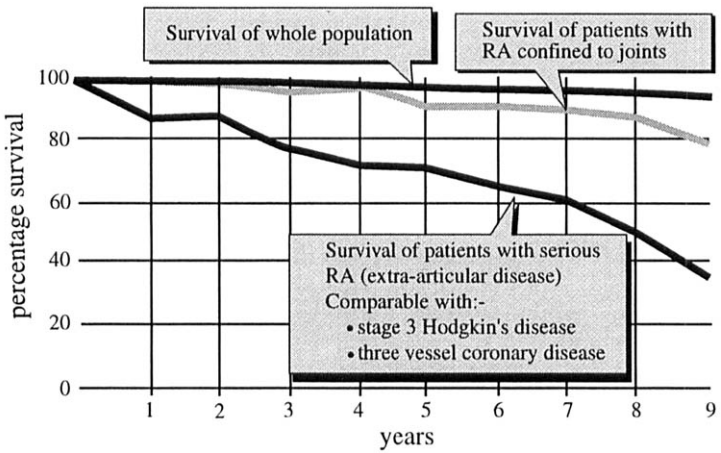


FIG. 2. The life expectancy of patients with severe rheumatoid arthritis is reduced. Reproduced with permission from Erhardt *et al.* (1989) and Pincus and Callaghan (1993).

is manifest symptomatically as joint pain and morning stiffness, with swelling and tenderness of joints, and sometimes marked increases in synovial fluid.

A normal joint has a lining layer, 1 or 2 cells thick consisting of fibroblastic or macrophage-type cells without a basement membrane, lying on an interstitutium of connective tissue with a few blood vessels and fibroblasts in deeper layers. In contrast, the RA synovium has a much thicker lining layer, and a markedly hypercellular deeper layer. The lining layer can be 6–10 or more cells thick, there are perivascular accumulations of cells, there is a marked increase in the number of small vessels and there are cells infiltrating in between the vessels (Gardner, 1994). The most abundant cells are T lymphocytes and macrophages, with plasma cells, dendritic cells, "activated" fibroblasts, and endothelium contributing to the rest of the synovial cell mass. Thus, the majority of the cells are blood borne, indicating the importance of cell trafficking and recruitment in the pathogenesis of the disease. The augmented number of capillaries supporting the increased cellularity of the tissue suggests that neovascularization and angiogenesis are important contributors to the disease process (Schrieber, 1994). Figure 3 illustrates the histology of RA synovium.

The erosion of cartilage and bone takes place mostly at the site where the fibrous capsule of the joint lined by synovium abuts with the cartilage and bone. This region of the synovium is sometimes referred to as the "pannus." The pannus overlies and locally erodes the cartilage and also

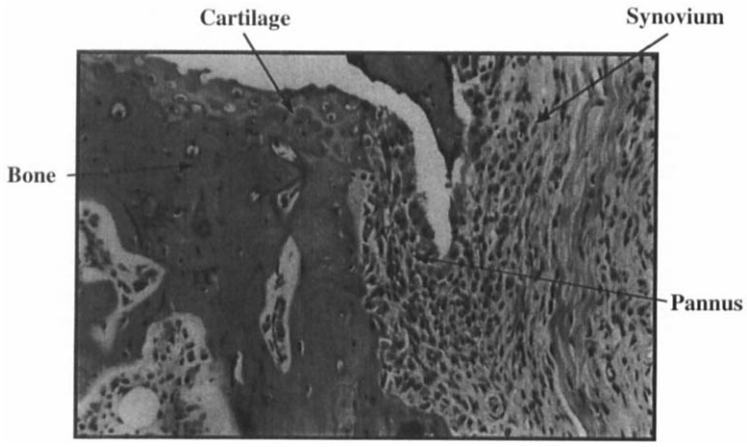


FIG. 3. The rheumatoid synovium. Erosion (invasion) of bone and cartilage by pannus tissue arising from adjacent synovium in inflammatory arthritis in collagen II-induced arthritis. Similar change is observed in rheumatoid arthritis.

invades bone. The major cell type here is the macrophage, with abundant vascular endothelium and fibroblasts (sometimes referred to as "synoviocytes"). T lymphocytes are less common here than in the rest of the synovium (Allard *et al.*, 1991; Kobayashi and Ziff, 1975). There is also a region of loss of bone around the joint at some distance (periarticular osteoporosis and osteopenia), visible on X ray.

C. Autoimmune Response in RA

Autoantibodies directed against a variety of antigens occur in the sera of patients with RA. IgM-rheumatoid factor (RF) was the first to be described by Waaler and Rose and occurs in 70% of patients with RA and has gained recognition as a diagnostic test. However, IgM-RF also occurs in other diseases, for example, Sjogren's syndrome, sarcoidosis, and chronic bacterial and protozoal infections. In the context of RA, it correlates with a poor functional outcome, systemic complications such as vasculitis and fibrosing alveolitis, and premature death. RF is more prevalent in $DR4^+$ than in $DR4^-$ patients with RA. Other diagnostically specific autoantibodies in RA include antiperinuclear and antikeratin antibodies, shown to be directed against profilaggrin and filaggrin, and antibodies to RA-33, a nuclear antigen (Hassfeld *et al.*, 1989; Nienhuis and Mandema, 1964; Sebbag *et al.*, 1995; Young *et al.*, 1979).

In recent years attention has focused on the T cell autoimmune response in RA, but there has been little success in defining autoantigens or their epitopes. Responses to hsp70 were proposed as important until it became apparent that they were also found in other diseases and could not explain the joint specificity of the disease process (Cohen, 1991). T cell responses to collagen type II (CII), in analogy to animal models, have been sought but are found in only a minority of patients (Londei *et al.*, 1989). The reasons for this are not clear but may include the endogenous immunosuppression found in RA patients with low T cell proliferation, low IL-2 production, and reduced skin test responses (Lotz *et al.*, 1986; Silverman *et al.*, 1976), especially in joints. Current hypotheses include that upregulated IL-10 and TGFβ are responsible for this endogenous immune suppression (Brennan *et al.*, 1990a; Katsikis *et al.*, 1994; Lotz *et al.*, 1990). Others have proposed that there may be anergy in RA tissues. Elispot assay for anti-CII IgG, an antibody class that needs T cell help, was found in >50% of patients, suggesting that T cell help was present (Ronnelid *et al.*, 1994). It is likely that other joint-specific or cartilage-specific autoantigens will be found to be important.

D. What Can Be Learned from Animal Models?

An erosive arthritis with synovitis, resembling RA to various extents, can be induced in a variety of animals. Mice of the $H\text{-}2^q$ and $H\text{-}2^r$ haplotypes

(e.g., DBA/1 and B10.RIII) develop arthritis after injection of chick, bovine, or human collagen type II in complete Freund's adjuvant (CFA). Other haplotypes do not (Holmdahl *et al.*, 1988), echoing the HLA DR4/DR1 preponderance of RA. Injection of other components of cartilage, such as collagens type IX or XI, in adjuvant also induces arthritis (Williams, 1995). Repeated cartilage proteoglycan injection induces a chronic erosive arthritis in BALB/c mice (Mikecz *et al.*, 1987). Thus, many cartilage-specific antigens are potentially arthritogenic. The same may be true in humans because in other autoimmune diseases, such as Grave's disease of the thyroid, multiple autoantigens are recognized (Dayan *et al.*, 1991). Collagen type II also induces arthritis in genetically susceptible strains of rats and monkeys (Bakker *et al.*, 1991; Klareskog and Olsson, 1990).

Arthritis can also be induced in other ways. For example, the injection of adjuvant and irritant pristane, which is necessary for inducing or transplanting myelomas in mice, leads to a late onset of chronic arthritis in genetically susceptible strains, e.g., CBA (Wooley *et al.*, 1989). Repeated injection of antigen into rabbit knees induces an arthritis, termed "antigen-induced arthritis" (Dumonde and Glynn, 1962). Bacterial antigens, such as streptococcal cell walls, induce arthritis in Lewis rats (Kaklamanis, 1992). Mycobacteria in adjuvant induces an arthritis, adjuvant arthritis, in Fisher rats (Kaklamanis, 1992). Despite the widely different causes of arthritis, it has been found that anti-TNFα therapy in animal models of arthritis is effective at ameliorating the disease if given after onset in all these models (Issekutz *et al.*, 1994; Lewthwaite *et al.*, 1995; Russell *et al.*, 1994; Thorbecke *et al.*, 1992; Williams *et al.*, 1992).

A question of interest is whether the processes of synovitis and of joint destruction have the same etiology and pathogenesis. There have been proposals that these may be distinctly regulated, although both may have the same unknown "cause" (Bresnihan *et al.*, 1995; van den Berg, 1995). In this context anti-TNFα therapy is of relevance. In animal models such as collagen-induced arthritis (CIA) and TNFα transgenics (Keffer *et al.*, 1991), anti-TNFα prevents or ameliorates both the synovitis and erosions (Williams *et al.*, 1992), indicating that TNFα is essential in both these processes and arguing against a discrete pathogenesis for synovitis and tissue damage.

E. Is There a Role for Infection?

The low rate of concordance in identical twins, which must by definition have all the genetic risk factors necessary for disease development, has raised obvious questions concerning other etiological factors. Certain non-heritable factors may differ between identical twins, such as different placental nutrition and influences at birth (Barker, 1995). There may be

differences in generation or selection of the T and B cell repertoires, but in view of the enormous numbers of cells generated and selected via identical genetic influences, including HLA, it is unlikely to account for the vast discordance (Gulwani-Akolkar *et al.*, 1991). The hypothesis that is most widely favored is that infectious agents may have a role in concert with a susceptible genotype. A number of observations lend credence to this possibility. First is the evidence that RA is a disease of recent (few hundred years?) onset, whose incidence appears to be diminishing postwar (Buchanan, 1994). Second is the evidence that in certain parts of Africa, rural Xhosa populations have less RA than the nearby towns (Soloman *et al.*, 1975).

However, most of the evidence does not support an infectious origin. Epidemiologically, there is no evidence of case clustering or of epidemics (Silman and Hochberg, 1993). Worldwide, the prevalence is always close to 1%, with lower levels in populations with a low prevalence of DR4 (e.g., Japan). Most important, attempts during the past 100 years to identify organisms have led to claims of microbial infection with organisms ranging from mycobacteria, diphtheroids, bacteria (e.g., proteus mirabilis), mycoplasma, viruses (e.g., parvovirus), and retroviruses, but none have been reproducibly found in the joints of patients. With the advent of polymerase chain reaction (PCR) it is possible to use conserved sequences, for example, in lentivirus polymerases, or eukaryotic mitochondrial DNA to look for the presence of minute quantities of infectious agents, although so far without success.

Nevertheless, there are lentiviruses that infect goats and sheep (maedi/visna) and cause an arthritis that closely resembles RA (Kennedy-Stoskopf, 1989). This resemblance, and the fact that "lack of evidence for infection," is different from "evidence of lack of infection," means that this issue is far from settled. The possibility that certain infections may protect from RA has not been investigated. In the nonobese diabetic mouse, streptococcal extracts and other immunostimulants such as CFA can prevent the onset of disease (Bach and Chatenoud, 1995).

II. Rationale for Anti-TNFα Antibody Therapy

A. Role of Cytokines in Rheumatoid Arthritis

1. Proinflammatory Cytokine Expression in RA Joints

In a chronic autoimmune disease with inflammatory features such as rheumatoid arthritis, it can be safely predicted that cytokines, major mediators of both immunity and inflammation, would be highly expressed. With the development of suitable sensitive assays for cytokine production, subse-

quent to the cloning of cytokine cDNAs from the early 1980s (Gray *et al.*, 1984; Taniguchi *et al.*, 1980), it became possible to verify that prediction. We initially used assays for detection of mRNA in RA synovium as an index of local production (Buchan *et al.*, 1988a,b; Hirano *et al.*, 1988), and subsequently extended this to verify that the cytokine proteins were indeed produced in short-term cultures with cells obtained from dissociated RA synovium. Others have used assays of cytokines in synovial fluid or *in situ* hybridization to evaluate cytokine expression (Di Giovine *et al.*, 1988; Firestein *et al.*, 1988; Fontana *et al.*, 1982; Hopkins *et al.*, 1988; Saxne *et al.*, 1988).

From these studies it rapidly became apparent that the rheumatoid synovium was a site of extensive cytokine expression. A wide variety of cytokines were produced, as listed in Table I, many of which have proinflammatory properties, some have anti-inflammatory properties, and many have a mixture of both. As more cytokine cDNAs were cloned, the list of detectable cytokines grew. Thus, "cataloging" of cytokine expression was not yielding many clues as to which cytokines were the most important in the pathogenesis of RA because a great number were detectable. This aspect has been reviewed by Feldmann *et al.* (1996) and Maini *et al.* (1995b).

The results obtained had certain patterns. One was that in terms of abundance, cytokines characteristically produced by macrophages, such as IL-6, IL-1, TNFα, and IL-8, were most abundant. Those typically produced by T cells, such as IL-2 and IFNγ, although detectable at the mRNA level (Buchan *et al.*, 1988b), were less abundant at the protein level evaluated by antibody-based sandwich enzyme-linked immunoassay (ELISA), bioassay, or immunohistology (Firestein *et al.*, 1988). These observations, together with a lack of IL-4 (Miossec *et al.*, 1990; Simon *et al.*, 1994), could be explained in a number of ways. Because cloned T cells from joints did produce abundant IFNγ and IL-2 (Cohen *et al.*, 1995), the results are most compatible with immunoregulation occurring within the synovium. Another important pattern noted was that all RA synovial samples, ranging from joint replacement operations late in the disease to early arthroscopic biopsy, had similar patterns of cytokine expression, even if treated with drugs reputed to downregulate cytokine production such as corticosteroids (Feldmann *et al.*, 1996).

This last observation led us to hypothesize that in a chronic disease such as RA cytokine expression is prolonged, unlike the typically very brief cytokine production following immune or bacterial stimuli. To test this hypothesis, we cultured dissociated unpurified rheumatoid joint cell suspensions in the absence of extrinsic stimulation. It was found that the cells rapidly reaggregated, probably because synovial cells have upregulated

TABLE I
CYTOKINE EXPRESSION IN RA SYNOVIAL TISSUE

Cytokine		mRNA	Protein
Proinflammatory cytokine			
ENA-78	Epithelial neutrophil activating peptide 78	Yes	Yes
bFGF	Basic fibroblast growth factor	Yes	Yes
GM-CSF	Granulocyte macrophage-colony stimulating factor	Yes	Yes
Groα	Melanoma growth stimulating activity	Yes	Yes
IFNα	Interferon-α	Yes	Yes
IFNβ	Interferon-β	?	?
IFNγ	Interferon-γ	Yes	$\pm$
IL-1α, β	Interleukin-1	Yes	Yes
IL-2	Interleukin-2	Yes	$\pm$
IL-3	Interleukin-3	No	No
IL-6	Interleukin-6	Yes	Yes
IL-7	Interleukin-7	?	?
IL-8	Interleukin-8	Yes	Yes
IL-9	Interleukin-9	?	?
IL-12	Interleukin-12	Yes	Yes
IL-15	Interleukin-15	Yes	Yes
LIF	Leucocyte inhibitory factor	?	?
LT	Lymphotoxin	Yes	$\pm$
MCP-1	Monocyte chemoattractant protein-1	Yes	Yes
M-CSF	Macrophage-colony stimulating factor	Yes	Yes
MIF	Macrophage inhibitory factor	Yes	Yes
MIP-1α	Macrophage inflammatory protein-1α	Yes	Yes
MIP-1β	Macrophage inflammatory protein-1β	Yes	Yes
Onco M	Oncostatin M	?	?
PDGF	Platelet-derived growth factor	Yes	Yes
RANTES	Regulated upon activation T cell expressed and secreted	Yes	Yes
TNFα	Tumor necrosis factor-α	Yes	Yes
VEGF/VPF	Vascular endothelial cell growth factor	Yes	Yes
Immunoregulatory cytokines			
IL-4	Interleukin-4	?	No
IL-10	Interleukin-10	Yes	Yes
IL-11	Interleukin-11	?	?
IL-13	Interleukin-13	Yes	Yes
TGFβ	Transforming growth factor-β	Yes	Yes

adhesion molecules such as LFA-1, ICAM-1, VLA-4, VCAM-1, etc. (Koch *et al.*, 1991). Cytokine mRNA levels *in vitro* remained elevated, for example, for IL-1α over the 5- or 6-day period of culture, confirming that cells from disease sites have a different, more prolonged pattern of cytokine expression than extrinsically (e.g., mitogen) activated cells (Buchan *et al.*, 1988a). Most important, the prolonged and elevated cytokine levels indi-

cated that the signals regulating cytokine expression were present within the culture system and hence could be analyzed. Because IL-1 was believed to be important in the induction of cartilage and bone destruction (Fell and Jubb, 1977; Gowen *et al.*, 1983; Saklatvala *et al.*, 1985), and IL-1 was reported to be very arthritogenic, with injections into rabbit knee joints being capable of inducing marked arthritis (Pettipher *et al.*, 1986), we initiated our studies of cytokine regulation in synovial cultures with an analysis of IL-1 regulation.

Whereas the common *in vitro* mitogenic inducer of proinflammatory cytokines, bacterial LPS, is a candidate stimulus in some conditions (perhaps reactive arthritis, which is associated with infections), this is clearly not the case in RA. In view of reports that TNFα was a strong regulator of IL-1 (Dinarello *et al.*, 1986), we investigated the effects of blocking TNFα or LT with neutralizing antisera in RA joint cell cultures. The results were striking, and within 3 days of culture with anti-TNFα, production of bioactive IL-1 from RA synovial cultures virtually ceased. This was the case in numerous RA joint samples and was our first evidence for the dependence of proinflammatory cytokine expression in RA synovium on the presence of TNFα (Brennan *et al.*, 1989). Having found that IL-1 production was TNFα dependent, the effects of anti-TNFα on other proinflammatory cytokines were evaluated. GM-CSF production was also markedly reduced (Alvaro-Garcia *et al.*, 1991; Haworth *et al.*, 1991). Recent studies have extended the list of TNFα-dependent cytokines to include IL-6, IL-8 (Butler *et al.*, 1995), and IL-10 (Katsikis *et al.*, 1994), indicating that there is extensive interlinkage of these cytokines. Although these results were obtained with a pathological tissue, it is likely that they reflect normal cytokine physiology. The response to infectious agents is the normal function of the immune system, and in mice injected with LPS or gram-negative organisms anti-TNF antibody prevents a subsequent rise in serum IL-1 or IL-6 (Fong *et al.*, 1989).

In vitro, IL-1 is an inducer of TNFα (Dinarello *et al.*, 1986) and in order to establish whether IL-1 and TNFα interactions were bidirectional or unidirectional we have investigated the effects of IL-1 blockade in RA synovial cultures. Because there are two isoforms of IL-1 (IL-1α and -β) both highly expressed in RA joints, the IL-1 receptor antagonist was used, which at high concentration inhibits IL-1 signaling with both IL-1α and -β (Arend, 1993). It was found that although IL-6 and IL-8 were reduced by culturing RA joint cells with IL-1ra, there was no effect of IL-1ra on the production of IL-1 or TNFα. This indicates that IL-6 and IL-8 are "downstream" of IL-1 and TNFα and that there is little autocrine stimulation of IL-1. Because IL-1ra is not a signal blocking TNFα production,

IL-1 is not a significant stimulus for TNFα production in the RA joints (Butler *et al.*, 1995). This concept is summarized in Fig. 4.

This interlinkage of proinflammatory cytokines is a very important concept because it predicted that blocking TNFα alone would be effective in RA. In contrast, *in vitro* studies chiefly with cell lines had suggested that there is considerable "redundancy" in the properties of proinflammatory cytokines, with strong overlaps. Taken at face value, these results suggested that proinflammatory cytokines may not be good therapeutic targets because blocking one could still leave the others capable of driving the inflammatory process as discussed previously in Elliott *et al.* (1993). It is noteworthy that *in vivo* studies of anti-TNFα and other antibodies have confirmed the existence of the "hierarchy" of proinflammatory cytokines (see Section IV,C) in RA patients and in arthritic mice (see Section II,C).

Another family of important proinflammatory cytokines is the *chemokine* (chemotactic cytokine) family. This family is involved in the recruitment of polymorphs, lymphocytes, and monocytes, the major cell types in the RA synovial fluid and synovial membrane. There are significant amounts of chemokines produced in the joints. The first to be documented was IL-8, but since then other members, of both C–C and C–X–C chemokines, have been detected such as ENA78, GROα, MCP-1, MIP-1α, MIP-1β, and RANTES. These were detected by ELISA and bioassay of joint cell cultures and also by immunohistology, in which they are detected in the lining layer, lymphoid aggregates, and perivascular areas presumably derived from macrophages, fibroblasts, and also from endothelium (Brennan *et al.*, 1990b; Hosaka *et al.*, 1994; Koch *et al.*, 1992, 1994a, 1995; Rathanaswami *et al.*, 1993).

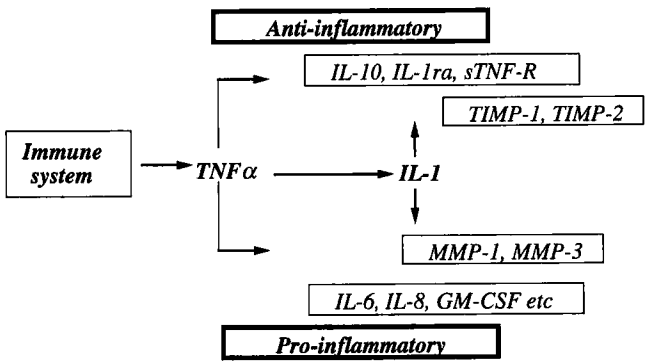


FIG. 4. Representation of the cytokine cascade in rheumatoid arthritis (based on data cited in text).

Because IL-8 production is dependent on TNFα *in vitro* (Butler *et al.*, 1995), we also investigated whether its production is also dependent on TNFα *in vivo,* and preliminary results suggest that this is indeed the case (P. Charles, A. Hogg, *et al.,* unpublished results). Whether other chemokines are also TNFα dependent is currently under investigation, and results so far indicate that MCP-1 is TNFα dependent (E. Paleolog, M. Hunt, *et al.,* unpublished data). In view of the extensive infiltration of blood-derived cells in RA joints, it is likely that chemokines that activate integrins, increasing their affinity, and generate chemotactic gradients are important in the pathogenesis of RA.

Angiogenesis is prominent in rheumatoid arthritis (Colville-Nash and Scott, 1992; Schrieber, 1994) and is likely to be an essential step in the maintenance of the recruited cell mass in the synovium. The importance of angiogenesis in experimental models of arthritis has been established because inhibitors of angiogenesis suppress collagen-induced arthritis (Oliver *et al.,* 1994; Peacock *et al.,* 1992). There are many cytokines that are reported to be involved in the process of angiogenesis. These include VEGF/VPF, basic FGF, TGFβ, TNFα, and IL-8. Most of these cytokines are present in rheumatoid joints; in particular, the presence of VEGF is well documented (Fava *et al.,* 1994; Koch *et al.,* 1994b). Which is the most important angiogenic factor in RA remains to be established.

2. *Expression of Anti-inflammatory Cytokines and Cytokine Inhibitors*

A chronic inflammatory disease could be chiefly due to the presence of excess inflammatory cytokines or a shortage of cytokine inhibitors or inhibitory cytokines or a combination of both processes. When the presence of cytokine inhibitors was measured, and it was found that the soluble TNF receptors derived from cleavage of both p55 and p75 membrane TNF-R were augmented in RA patients, with levels in serum two- or threefold higher than normal, and those in synovial fluid two- or threefold higher than in RA serum (Cope *et al.,* 1992; Roux-Lombard *et al.,* 1993). It was shown that the levels of sTNF-R tended to parallel the disease activity, with higher levels when patients were worse (Cope *et al.,* 1992). There did not appear to be a defect in production of sTNF-R, but there was an incapacity to totally neutralize TNFα presumably due to the greater rate of synthesis of TNFα than of its inhibitors (Brennan *et al.,* 1995).

Analogous data have been produced for the IL-1 receptor antagonist (IL-1ra), which was found to be upregulated in active RA patients (Deleuran *et al.,* 1992; Firestein *et al.,* 1992; Koch *et al.,* 1992; Malyak *et al.,* 1993; Roux-Lombard *et al.,* 1992). This has been assessed in various ways—by *in situ* hybridization, by immunohistology, and by assays of joint cell supernatants. Levels of IL-1ra in synovial fluid and in serum are augmented.

However, the rate of IL-1ra production also does not attain levels sufficient to neutralize IL-1 because IL-1 bioactivity is detectable in RA synovial cultures.

Cytokines with anti-inflammatory activity are also detectable in RA tissues. There is an abundance of TGFβ, both in the latent and in the bioactive form (Brennan *et al.*, 1990a; Fava *et al.*, 1991; Lafyatis *et al.*, 1989; Lotz *et al.*, 1990; Wahl, 1994). There is also abundant IL-10 (Cush *et al.*, 1995; Katsikis *et al.*, 1994). It is likely that the downregulated status of the T cell pool in the joints is due to the presence of these inhibitors as judged by the augmented IFNγ production of RA joint cultures in the presence of anti-IL-10 (Katsikis *et al.*, 1994). There is also evidence for some IL-13 (L. Klareskog, personal communication), but little IL-4 has been detected in RA joints (Miossec *et al.*, 1990; Simon *et al.*, 1994). This lack of IL-4 could be a fundamental part of the reason the disease is chronic: An excess of Th1 over Th2 cells is likely to favor a chronic inflammation (Liblau *et al.*, 1995). The balance of cytokines in the RA joint, as probably in all other chronic inflammatory diseases, is proinflammatory, but it is not a static equilibrium, with the balance of pro- and anti-inflammatory forces varying and probably accounting for the variations in disease activity with flares and remissions (Fig. 5).

B. Action of TNFα in Other Aspects of the Rheumatoid Arthritis Disease Process

Although most of the previous discussion has focused on the effects of TNFα on the cytokine network because TNFα influences most cell types, it might be anticipated that TNFα is involved in many of the pathogenic steps occurring in the rheumatoid process.

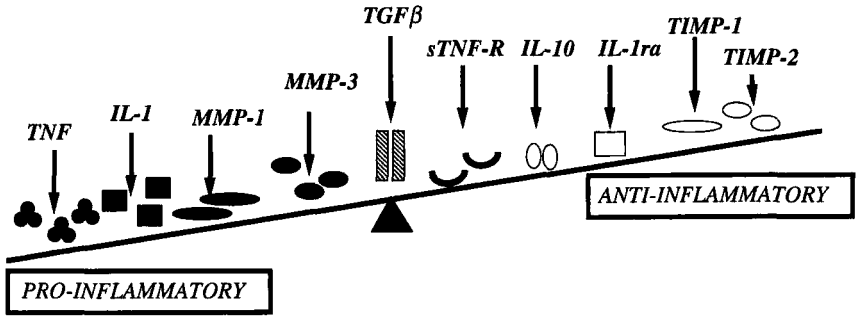


Fig. 5. Disequilibrium of cytokines and their inhibitors in active rheumatoid arthritis. This concept explains why both pro- and anti-inflammatory aspects are upregulated in RA.

It is known that TNFα markedly activates endothelium, upregulating E-selectin and other adhesion molecules such as ICAM-1, VCAM-1, as well as inducing endothelial cytokine production (Carlos and Harlan, 1994; Paleolog *et al.*, 1996). It could be presumed that control of TNFα would downregulate endothelial activation. This possibility is discussed under Section IV,D. The augmented synovial cell mass in RA contains many endothelial cells, and there is evidence for angiogenesis. TNFα has been described to be an angiogenic factor, and it is thus possible that blocking TNFα may reduce angiogenesis either directly or indirectly via TNFα-regulated angiogenic factors (see Section IV,E). TNFα is involved in both antigen-specific and nonspecific aspects of immunity. It activates neutrophils and macrophages; therefore, the activity of the cells in antimicrobial immunity may be diminished by anti-TNFα. TNFα is also involved in T cell and other immune responses, which paradoxically are diminished in active RA patients (Lotz *et al.*, 1986; Silverman *et al.*, 1976). *In vitro* studies revealed that exposure to excess TNFα for 10 days or more diminishes T cell responses, which can be restored by blocking TNFα (Cope *et al.*, 1994). The effect of anti-TNFα on immune responses, inasmuch as we know, will be discussed under Section IV,D. B cells are also stimulated by LT and TNFα (Kehrl *et al.*, 1987a,b); therefore TNFα could contribute to autoantibody production.

TNFα is a potent activator of antigen-presenting cell function, especially of Langerhans cells and dendritic cells (Sallusto and Lanzavecchia, 1994). Diminution of TNFα could thus reduce APC function, leading to long-term benefit by reducing the postulated T cell-dependent activation of the inflammatory processes.

The destruction of cartilage and bone in both osteoarthritis and rheumatoid arthritis is currently believed to be mostly due to the action of matrix metalloproteinase enzymes, which are serine proteinases. This is an expanding family that includes several collagenases that can cleave triple helical collagen, several gelatinases that can cleave "denatured" nontriple helical collagen, as well as other proteins and several "stromelysins." These latter enzymes are capable of degrading most of the proteoglycans of cartilage and bone and are considered to be of major importance in the destruction of joints (Birkedal-Hansen *et al.*, 1993; Buttle, 1994; Hill *et al.*, 1994). These serine proteases are secreted as proenzymes under the control of cytokines, such as IL-1 and TNFα, and need to be proteolytically or autocatalytically activated. There are a number of inhibitors of the matrix metalloproteinases (MMPs), including α_2 macroglobulin, which binds both active and latent enzymes. More specific inhibitors include the tissue inhibitors of metalloproteinases (TIMPs), which only bind active enzyme; only three of these have been identified. TIMP-1 is inducible by a spectrum

of cytokines—chiefly those with inhibitory function such as TGFβ, IL-10, IL-6, IL-11, and leukemia inhibitory factor (LIF) (Wright *et al.*, 1991).

Hence, physiologic tissue remodeling or tissue destruction can also be envisaged as an equilibrium between the processes of enzyme synthesis and activation, on the one hand, and the production of inhibitors on the other, with both processes being regulated by cytokines.

C. Immunotherapy with Anti-TNF in Animal Models of Arthritis

The previous cytokine analysis had led us to the conclusion that in RA there is an excess of proinflammatory over anti-inflammatory cytokines and an excess of TNFα over its inhibitors. This hypothesis predicts that blockade of TNF should be beneficial, and as a prelude to clinical trials this concept was tested in the DBA/1 mouse collagen type II model of arthritis using TN3, a hamster anti-mouse TNF antibody, generously provided by Dr. R. Schreiber (Sheehan *et al.*, 1989). A key aspect that was different from most prior studies was the treatment of animals *after* onset of disease. With an appropriate concentration and frequency of administration of TN3, a marked amelioration of disease activity was noted in all assessable parameters: footpad swelling, a marker of inflammation; clinical score, which measures degree of inflammation and number of affected limbs; as well as joint destruction, assessed by histology (Williams *et al.*, 1992). Analogous results were produced by others (Piguet *et al.*, 1992; Thorbecke *et al.*, 1992; Wooley *et al.*, 1993) using antibodies or Ig-TNF-R fusion proteins. Subsequently, it was found that other models of arthritis are improved by anti-TNF antibody (Issekutz *et al.*, 1994; Russell *et al.*, 1994).

The importance of TNFα in arthritis was further demonstrated in a transgenic model of arthritis. Mice that carry a human TNFα transgene with the 3′ untranslated region replaced by that of β globin develop an erosive inflammatory arthritis (Keffer *et al.*, 1991). Not surprisingly, this arthritis can be prevented by treatment with anti-human TNFα antibody. The proinflammatory cytokine cascade in RA joints discussed previously is recapitulated in these mice because blocking IL-1 action with an anti-IL-1 receptor (type 1) monoclonal antibody also leads to effective prevention of arthritis (Probert *et al.*, 1995). DBA/1 collagen arthritic mice can also be treated effectively after disease onset with a similar anti-IL-1R (type 1) mAb. As would be expected from the cytokine cascade concept, there is no additional benefit from combining anti-TNF and anti-IL-1R (type 1) mAb in these mice (Williams 1995; R. Williams *et al.*, unpublished results).

III. Clinical Studies with Anti-TNFα

The first aim of our clinical research program was to use anti-TNFα antibody to investigate disease mechanisms in RA. Specifically, we wanted to test the hypothesis that TNFα was of central importance in disease pathogenesis (Brennan *et al.*, 1989; Feldmann *et al.*, 1990, 1996) and that blockade of TNFα *in vivo* would result in downregulation of other inflammatory mediators. To a large extent, this aim has been accomplished, and the new emphasis is on the development of anti-TNFα antibody as a therapy for RA.

A. Chimeric Anti-TNFα Antibody cA2

The key to these studies is cA2, a chimerized mAb to human TNFα. cA2 was constructed by linking the variable regions of a mouse anti-human TNFα mAb (A2) to human IgG1 with κ light chains. Initial estimates of the binding affinity of cA2 to immobilized trimeric (3 × 17 kDa) TNFα (K_d, 550 p*M*) (Knight *et al.*, 1993) have been refined using an improved assay system in which [^{125}I]TNFα is bound to cA2 captured by anti-Fc (K_d, 100 p*M;* B Scallon, personal communication). cA2 has been shown to very efficiently inhibit the actions of soluble TNFα in many *in vitro* systems, including tumor cytotoxicity, procoagulant activity, and endothelial E-selectin expression (Knight *et al.*, 1993; D. Knight *et al.*, unpublished data). In cultures of rheumatoid joint cells, concentrations of 1 μg/ml are very effective at neutralizing endogenous TNFα (Butler *et al.*, 1995).

1. Dose and Pharmacokinetics of cA2

The initial dose and scheduling of cA2 administration in RA was arrived at by extrapolation from studies using analogous anti-TNF antibody reagents in murine CIA in which multiple doses of 10–12 mg/kg were needed to be optimally effective (Williams *et al.*, 1992). In the first uncontrolled open label trial in long-standing rheumatoid patients (Elliott *et al.*, 1993), a total cA2 dose of 20 mg/kg was administered during a 2-week period. Five patients received 4 × 5 mg/kg infusions at 4-day intervals and the remaining 15 received 2 × 10 mg/kg on Days 0 and 14. Each infusion was administered over 2 hr as a day procedure. The doses of antibody were considerably higher than those administered by investigators testing leukocyte-directed mAb such as anti CD4 in RA, but the high affinity of TNF receptors (Loetscher *et al.*, 1990; Schall *et al.*, 1990), being greater than that of anti-TNFα, has to be kept in mind (Choy *et al.*, 1992; Horneff *et al.*, 1991; Moreland *et al.*, 1995; Van de Lubbe *et al.*, 1995).

The subsequent multicenter-randomized, placebo-controlled, double-blind trial employed a single-infusion schedule and a dose of either 1 or

10 mg/kg cA2, with an option for re-treatment at an intermediate dose level on disease relapse (Elliott *et al.*, 1994a).

The initial assay for measurement of the level of cA2 in patient sera was on a ELISA that used a rabbit polyclonal anti-idiotype antibody that reacted primarily with the murine variable domains of the chimeric cA2. Recently, a monoclonal antibody-based assay using two noncompeting monoclonals has been developed. The solid-state capture antibody binds in the variable region and a second biotinylated monoclonal, specific for a framework epitope, is used for detection. Both assays provide similar results and have been validated for sensitivity, specificity, precision, accuracy, and reproducibility.

Results from blood samples collected at times ranging from 5 min to 4 months following infusion of cA2 have been analyzed and demonstrate that the serum concentration of cA2 at any given sampling time increases proportionately with the dose of cA2 administered. In both RA and Crohn's patient populations, the maximum levels of cA2 observed agree with the maximum concentrations predicted, based on the distribution of the total dose within the vascular space. The disappearance of cA2 from serum has likewise been shown to be equivalent. Detectable concentrations of cA2 were present in the serum at 8 weeks in patients receiving 10 and 20 mg/kg but were not present in patients receiving 1, 3, or 5 mg/kg.

General results for concentration (C_{max}) and area under the curve (AUC) were similar for patients with rheumatoid arthritis and Crohn's disease. At doses of 1, 5, 10, and 20 mg/kg, the C_{max} (μg/ml) was 23, 77, 277, and 603 and the AUC (μg/ml $\times$ hr) was 3672, 11,065, 54,775, and 82,409, respectively. Median volume of distribution (ml) at steady state ranged from 4094 to 5027 for 10 mg/kg doses in various studies, whereas median terminal half-life (hr) ranged from 219 to 240 for 10 mg/kg doses in various studies. Within each study the pharmacokinetic parameters show the expected trends as the dose was changed. The terminal half-life of 9 or 10 days for 10 mg/kg doses of the chimeric antibody is much longer than the 1- or 2-day elimination half-life observed for the majority of intact murine immunoglobulin (Trang, 1992; Winter and Harris, 1993); however, the cA2 half-life is somewhat less than the 23-day half-life reported for native human IgG1 γ-globulin (Waldmann, 1970).

2. *Clinical Benefit of cA2*

The patients selected for the cA2 trials had varying disease phenotype but on average were at the more severe end of the disease spectrum. In the placebo-controlled trial, for example, the mean duration of disease for the three treatment groups ranged from 7.3 to 9 years, the mean number of failed Disease Modifying AntiRheumatic Drugs (DMARDs) ranged

from 2.8 to 3.7, over 70% of patients showed significant titers of rheumatoid factor, and all had X-ray evidence of joint erosions (Elliott *et al.*, 1994a). All patients had active RA according to predefined clinical and laboratory criteria. For example, in the placebo-controlled trial the mean number of swollen joints in the three treatment groups ranged from 21.4 to 23.4 (58 joint count) and the mean CRP from 62 to 67 mg/liter (normal range, <10 mg/liter) (Elliott *et al.*, 1994a). In both trials, patients taking DMARDs were withdrawn from their therapy at least 1 month prior to trial entry, but patients were permitted to continue other therapies at stable dosage, including low-dose corticosteroids and nonsteroidal anti-inflammatory drugs. Analgesics were allowed *ad libitum.*

The primary aim of the open-label trial was to assess the safety and tolerability of cA2 in RA, but we also monitored the clinical response. We saw rapid, substantial, and statistically significant improvements in multiple clinical disease activity assessments, including the tender joint count (TJC), the swollen joint count (SJC), the pain score, and the duration of morning stiffness. In addition, we saw large and statistically significant falls in acute-phase measures such as the CRP and serum amyloid A (SAA), with many patients achieving normalization of values. Trends to improvement in hematological parameters and rheumatoid factor titers were also seen (Elliott *et al.*, 1993). Although we lacked a control group in this study, the consistency of the findings and the magnitude of the changes strongly suggested a true therapeutic effect. Without exception, these findings were later confirmed in the placebo-controlled trial (Elliott *et al.*, 1994a).

The aim of the placebo-controlled trial was to confirm the clinical benefit of cA2 in the short-term treatment of RA. The additional design strengths of this trial included its implementation at four separate sites, the randomization of patients to one of three treatment groups including placebo, the blinding of clinical observers and patients both to treatment group and to the results of laboratory tests of disease activity, and the use of standardized techniques in clinical assessments. Patients received 1 mg/kg cA2 ("low-dose" cA2; $n = 25$), 10 mg/kg cA2 ("high-dose" cA2; $n = 24$), or placebo (0.1% human serum albumin; $n = 24$) (Fig. 6). The three groups were well matched in terms of baseline demographic characteristics and disease activity.

The primary outcome measure was the achievement at Week 4 of a 20% "Paulus" response, a composite index incorporating swollen and tender joint scores, morning stiffness, assessments of global disease severity, and the ESR (Paulus *et al.*, 1990). Using this measure, 79% of patients treated with high-dose cA2 responded compared with only 8% of those treated with placebo. Patients treated with low-dose cA2 showed an intermediate response rate of 44%. Using the more stringent Paulus 50%

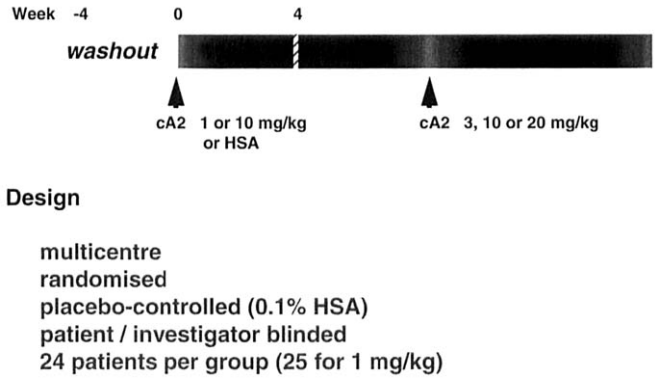


FIG. 6. Design for the placebo-controlled trial of cA2 in RA. Reproduced from Elliott *et al.* (1994) with permission.

measure, 28% of low-dose patients and 58% of high-dose patients responded at Week 4, demonstrating that the quality of the clinical responses was mostly good. The differences between the cA2 and placebo groups were highly significant ($p < 0.0001$) (Fig. 7). Secondary outcome measures in this trial included multiple individual clinical disease assessments, such as the TJC and SJC, the pain and fatigue scores, the duration of morning stiffness, the patient and observer assessments of global disease severity, and the grip strength. Each of these showed highly significant improvements in both low- and high-dose cA2 groups compared with no change in those receiving placebo. On average, the duration of morning stiffness showed the largest response to treatment (94% improvement from the high-dose group at Week 4), but major changes were seen in more objective clinical assessments such as the TJC and SJC, which showed 60 and 61% improvement, respectively, in the high-dose cA2 group at Week 4 (Elliott *et al.*, 1994a). The changes in a patient showing a good response to high-dose cA2 are shown in Fig. 8.

As in the open-label study, the clinical improvements were matched by changes in the acute-phase response and other laboratory measures. The CRP fell from a mean of 64 mg/liter at baseline in the high-dose group to 28 mg/liter at Week 2 and 35 mg/liter at Week 4, compared with no significant change in the placebo group ($p < 0.001$). Other acute-phase proteins showing significant improvement included SAA, fibrinogen, and haptoglobin (Charles *et al.*, 1996). Statistically significant restoration toward the normal were also seen for the ESR, hemoglobin (Hb), and platelet counts (Elliott *et al.*, 1994a).

Patients who received placebo treatment were eligible to participate in a follow-up phase of the trial during which they received an open-label

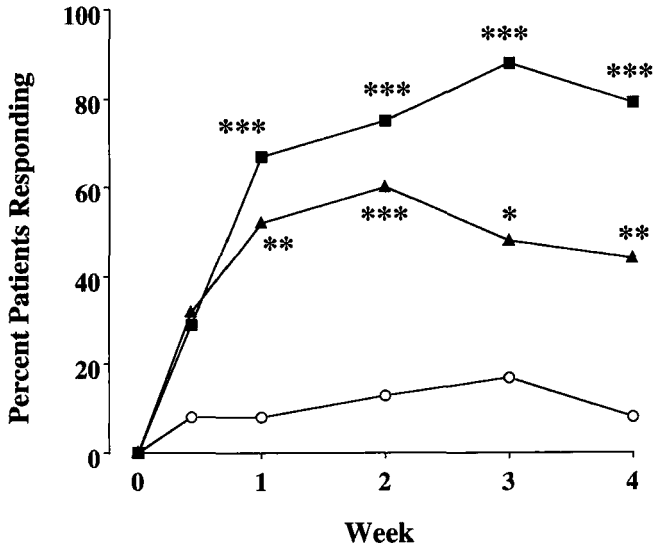


FIG. 7. Response rates in the placebo-controlled trial of cA2 in RA. Response was defined according to the criteria of Paulus *et al.* (1990). *p* Values represent significance versus the placebo group by ANOVA. (—o—) Placebo; (—▲—) 1 mg/kg cA2; (—■—) 10 mg/kg cA2; $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$. Data from Elliott *et al.* (1994a) reproduced with permission.

administration of one of several cA2 doses. Analysis of data from patients receiving 3 mg/kg cA2 in this phase, together with data from the blinded phase of the study, allowed the construction of a dose–response profile for cA2. Overall, the maximum proportion of patients responding to treatment increased modestly from 50–60% to 70–80% with increasing doses of cA2. The major effect of increased dose, however, was on response duration, with a median duration of Paulus 20% response in the 1, 3, and 10 mg/kg groups of 3, 6, and 8 weeks, respectively. The duration of response could be directly related to the persistence of circulating cA2, declining rapidly following a fall in the mean serum levels below 1 μg/ml (Maini *et al.*, 1995a).

Overall, the placebo-controlled trial provides the first convincing evidence that blockade of a specific cytokine could be effective treatment in human autoimmune or inflammatory disease. Indeed, this experience is so far unique in the history of immunotherapy of RA, in which early promising results with anti-CD4 mAb have not been confirmed in blinded, controlled trials (Moreland *et al.*, 1995; Van de Lubbe *et al.*, 1995). However, it is clear that the response to even high-dose single-

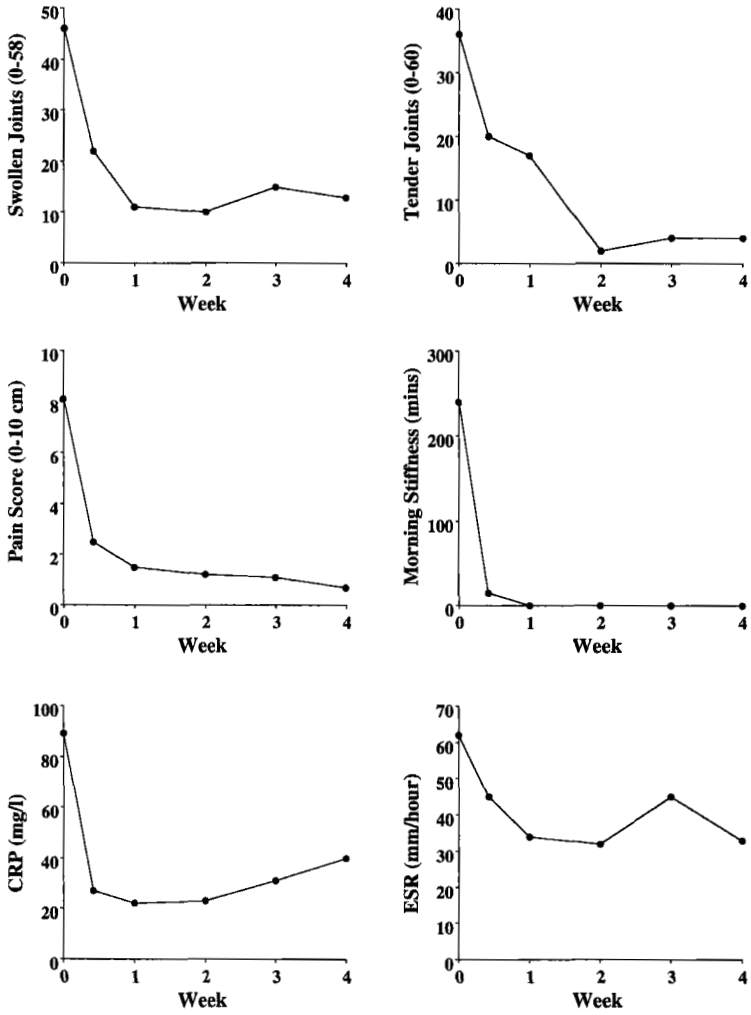


FIG. 8. A good response to cA2: Improvements in individual clinical and laboratory measures in a patient treated with 10 mg/kg cA2 at Week 0 in placebo-controlled trial. Data from Elliott *et al.* (1994a) reproduced with permission.

shot cA2 is temporary and that long-term disease control in this patient group would require multiple dosing. We have assessed the feasibility of repeated dosing with cA2 in a pilot study in which patients received up to four cycles of treatment. The timing of each treatment was dictated by disease relapse, defined as loss of Paulus 20% response to

the previous therapeutic cycle. Although the dose of cA2 was halved in cycles 2–4 from a total of 20 mg/kg to a single infusion of 10 mg/kg, patients showed continuing good-quality responses, with median improvements in individual disease activity assessments such as the SJC and CRP exceeding 80%. Although there was considerable variability in response duration between patients, overall we saw a trend (not statistically significant) to shorter response times with successive treatment cycles (median of 12 weeks in cycle 1, 20 mg/kg cA2; median of 7.7 weeks in cycle 4, 10 mg/kg cA2) (Elliott *et al.*, 1994b). The latter result is not significantly different from the median duration (8 weeks) of benefit in the 10 mg/kg group in the placebo-controlled trial (Maini *et al.*, 1995a). Although the reduction in response duration may have been a result of the dosage reduction in cycles 2–4, other factors, including the development of antiglobulin responses, may have played a role (see Section V,A). In order to further assess the potential for prolonged disease control with cA2 in RA, we have devised a new multicenter, placebo-controlled, multiple-dose level trial in which patients receive regular antibody administration. The results will be obtained in mid-1996.

The use of cA2 in rheumatoid arthritis has also been investigated by Drs. Saeki and Kishimoto in Osaka (Saeki *et al.*, 1996). Six patients with refractory RA were treated in an open-label trial receiving 1, 5, or 10 mg/kg. In this study all patients had a significant improvement in all clinical and laboratory parameters and there was a trend to longer responses with the higher doses. This group has also investigated the mechanism of action of cA2 in these patients and has made the intriguing observation that serum IL-10 levels were increased after cA2 in four of six patients (Ohshima *et al.*, 1996). Because IL-10 is an active anti-inflammatory agent (Moore *et al.*, 1993), this may be an important contribution to the mechanism of action of cA2. Currently, trials of cA2 are under way in the United States led by Dr. P. Lipsky. The results will be decoded in 1996.

B. Other Trials Targeting TNFα

The work of other groups targeting TNFα in RA with other agents has been reported (Table II). The results are consistent and confirm that TNFα is a good therapeutic target in rheumatoid arthritis. Administration of an humanized anti-TNFα mAb (CDP571) in a randomized, controlled trial led to significant improvements in some clinical parameters (Rankin *et al.*, 1995), although the responses were less marked than those seen with cA2. These quantitative differences in antibody efficacy may provide

TABLE II
CLINICAL APPLICATION OF SPECIFIC TNF ANTAGONISTS IN RA

Reference	TNF antagonist	Type	Study design	Efficacy
Full papers				
Elliott *et al.* (1993)	cA2	Chimeric mAb IgG1	Open	Good responses
Elliott *et al.* (1994a)	cA2	Chimeric mAb IgG1	RCT[a]	Proven
Elliott *et al.* (1994b)	cA2	Chimeric mAb IgG1	Open, repeated administration	Repeated good responses
Rankin *et al.* (1995)	CDP571	Humanized mAb IgG4	RCT	Proven
Abstracts				
Moreland *et al.* (1994)	sTNF:Fc	hIgG1 + p75 TNFR	RCT	Trends to response
Baumgartner *et al.* (1996)	sTNF:Fc	TNFR	RCT	Good response
Sander *et al.* (1995)	sTNF:Fc	hIgG + p55 TNFR	No details	Late follow-up data only
Hassler *et al.* (1996)	sTNF:Fc	TNFR	Open label	Moderate response
Eutolo *et al.* (1996)	sTNF:Fc	TNFR	RCT	Moderate response
Furst *et al.* (1996)	sTNF:Fc	TNFR	RCT	Minimal response

[a] RCT, randomized, placebo-controlled trial.

insight into the mechanism of action of such agents and are discussed in detail under Section IV,B.

In another study, a fusion protein consisting of two p75 soluble sTNFR (the extracellular binding domain) joined to the Fc portion of human IgG1 (sTNFR:Fc) was employed to block TNFα function (Moreland *et al.*, 1994). Sixteen patients with similar demographic features to those enrolled in the cA2 trials were administered an intravenous loading dose of sTNFR:Fc or placebo at doses ranging from 4 to 32 mg/m^2, followed by twice weekly subcutaneous maintenance dosing at 2–16 mg/m^2 for a period of 4 weeks. Compared to baseline, improvements were noted in the sTNFR:Fc group in several assessments, including the ESR, TJC, SJC, patient and physician global assessments, and duration of morning stiffness, but without a dose–response relationship or a statistically significant improvement compared to placebo. Adverse events were mild and included injection site reactions, which did not require discontinuation of the drug. A multicenter, double blind trial of 180 patients with active rheumatoid

arthritis, injected subcutaneously twice per week at up to 16 mg/m^2 for 3 months, yielded a dose-dependent improvement with up to 75% responding at 20% (ACR criteria), a 58% reduction in swollen joint count, and a 49% reduction in CRP compared to baseline (Baumgartner *et al.*, 1996).

The divalent TNF-R p55 IgG Fc fusion protein, also known as "Lenercept," has been tested in over 500 rheumatoid patients in a variety of trials lasting up to 3 months in blind controlled trials at doses of up to 100 mg monthly or up to 1 year in extensions of blinded trials. With the three doses of 100 mg, there was a 36% reduction in swollen joints (compared to 20% in placebo) and a 28% ACR response compared to 14% with placebo. Lenercept appeared to be safe at these doses in these trials. Twenty-nine to thirty-six percent of patients developed antibodies to Lenercept, with a reciprocal antibody titer >500. The results disclosed in abstracts (Hassler *et al.*, 1996; Cutolo *et al.*, 1996; Furst *et al.*, 1996) indicate that there is a degree of benefit, but with differences in protocols and patient populations and because of a lack of detailed information it is difficult to accurately compare these results with those with previously discussed inhibitors. However, as used and reported, a lesser degree of benefit is reported, which may be considered paradoxical in view of the high neutralizing capacity *in vitro* and in animal models (Lesslauer *et al.*, 1991).

C. Other Diseases Treated with Anti-TNFα Antibodies

1. Inflammatory Bowel Disease

a. Crohn's Disease. Crohn's disease is an inflammatory disease that affects any portion of the alimentary tract. The granulomatous inflammatory lesions extend through the bowel wall, resulting in mucosal inflammation, ulceration, stricturing, fistulous development, and abscess formation (Podolsky, 1991). TNFα is found in substantial amounts in the mucosa and stools of subjects with inflammatory bowel disease and is thought to play an important role as a mediator of inflammation in Crohn's (Braegger *et al.*, 1992; Lukacs *et al.*, 1994). Moreover, in patients with Crohn's disease, a high number of mucosal cells as well as cells in the lamina propria have been shown to express TNFα (Breese *et al.*, 1994; MacDonald *et al.*, 1990). The first clinical study examining the hypothesis that TNFα constitutes a major factor in the pathophysiology of Crohn's disease was conducted by van Deventer and colleagues (Derkx *et al.*, 1993). In this single patient, there was a dramatic response that persisted for a period of 3 months following the two 10 mg/kg doses of cA2. In addition to the substantial clinical benefit, hematologic markers of inflammation including ESR reverted to normal. To investigate the potential therapeutic effects of cA2

in a larger group of patients, van Deventer *et al.* (1995) conducted a 10-patient open-label single-dose (10 mg/kg) trial in patients with severe Crohn's disease. Eligible patients were required to have a Crohn's disease activity index (CDAI) > 150 and to have failed treatment with prednisone at doses of >20 mg/day for more than 2 weeks. The primary efficacy endpoint "clinical response" was defined as a 70-point reduction in CDAI score. Eight of the 10 patients showed improvement within 1 week after treatment, with a mean reduction in CDAI from 257 before treatment to 114 at 2 weeks, 79 at 4 weeks, 61 at 6 weeks, and 69 at 8 weeks ($p = <0.0001$). This was accompanied by a reduction in CRP and ESR to essentially normal levels. The duration of clinical response for these patients was 4 months. Of particular interest was the ability to totally withdraw patients from steroid therapy in 3/3 patients where it was attempted. An endoscopy score was assessed for each colonoscopy using the system of Modigliani (Mary and Modigliani, 1989). Endoscopic results were consistent with clinical responses in this study, with a mean reduction of the Modigliani score (0–30) from 18 pretreatment to 8 at 4 weeks and 8 at 8 weeks.

In a subsequent multicenter open-label dose-escalation study, five patients with steroid-resistant Crohn's disease per group received 1, 5, 10, or 20 mg/kg of cA2 as a single infusion (McCabe *et al.*, 1996). Clinical response (CDAI decrease by >70 points) was achieved in 70% of patients at Week 4 and clinical remission (CDAI < 150 with >70-point decrease) was seen in 20–60% without a clear dose effect. The duration of response was somewhat dose related in that only 20% of the 1 mg/kg group maintained a clinical response at 12 weeks, whereas 50–80% of the patients receiving higher doses were still responding. The same relationship was true for clinical remission in which none of the members of 1 mg/kg group was in remission at 12 weeks, whereas 25–60% of the higher dose groups continued in remission. These two open-label studies of cA2 showed a remarkably consistent rapid and sustained response in patients with steroid-dependent Crohn's disease. Furthermore, out of five patients with enterocutaneous fistulae, partial or complete closure was observed in four, a rarely observed event in patients with fistulous Crohn's.

The clinical utility, tolerance, and safety of cA2 was further investigated in a multicenter international randomized double-blind placebo-controlled trial in 108 patients with severe Crohn's disease whose CDAI scores ranged between 220 and 400 (Targan *et al.*, 1997). Patients were randomized in equal proportion to receive placebo, 5, 10, or 20 mg/kg of cA2 as a single 2-hr infusion. Subjects with severe disease who were receiving stable concomitant therapy with corticosteroids, 6-mercaptopurine, or amino salicylates continued their prescribed regimen throughout the 12-week trial,

although there was an option to taper steroids following Week 8. The primary efficacy outcome was measured by the CDAI score with a "clinical response" defined as a decrease in CDA of 70 points at Week 4. Clinical responses were observed in 16.7, 81, 50, and 64% of placebo, 5, 10, and 20 mg/kg dose–response groups, respectively. More than 80% of responses persisted until 8 weeks, and 63% persisted beyond 12 weeks.

Following the publication of van Deventer *et al.*'s (1995) initial study, other anti-TNFα monoclonal antibodies were evaluated in patients with Crohn's disease, and similar findings resulted. In a recently reported study of 31 patients with Crohn's disease, 10 patients received placebo and 21 received 5 mg/kg CDP571, a humanized anti-TNF monoclonal antibody. Nine of the treated patients showed "remission" of disease at 2 weeks (43%) as judged by reduction in CDAI score and colonoscopy results, whereas only 1 placebo patient (10%) responded. No duration of response was mentioned (Stack *et al.*, 1996).

b. Ulcerative Colitis. Ulcerative colitis (UC) is an inflammatory disease of the mucosal surface of the large bowel. Inflammation of the mucosal surface results in diffuse friability, erosions, bleeding, and bloody diarrhea (Podolsky, 1991). TNFα is postulated to be a central cytokine in the pathophysiology of inflammatory bowel disease and may represent a novel target for therapy in UC. To study this, a double-blind, placebo-controlled trial was undertaken to examine safety, tolerance, and efficacy of a single infusion of cA2 to treat severe, steroid refractory UC (Sands, *et al.*, 1996). Eligible patients were required to be hospitalized for UC and had to have a Trulove and Witts (T&W) score >10 despite at least 5 days of intravenous high-dose corticosteroid therapy. All patients were required to have moderate to severe sigmoidoscopic changes of UC. Subjects were randomly assigned to receive placebo or cA2 at 5, 10, or 20 mg/kg while continuing stable doses of other medications. In this study a clinical response was defined as a T&W score <10 plus a 5-point decrease from baseline. Efficacy was evaluated as the proportion of treatment failures defined as lack of clinical response, colectomy, or introduction of new immunosuppressive agent (cyclosporine, etc.). Eleven patients entered the study: 3 each receiving placebo, 5, or 10 mg/kg cA2 and 2 receiving 20 mg/kg cA2. Enrolled patients exhibited severe UC with a mean T&W score of 13. Seven patients went on to colectomy within a month. All patients (3/3) receiving placebo failed, whereas only 4/8 (50%) receiving cA2 were treatment failures. One patient receiving 20 mg/kg achieved a remission with quiescent disease by sigmoidoscopy at Week 2. Although the cA2 was well tolerated and the results were encouraging, the study was discontinued due to slow enrollment (<1 patient/month) because these severely ill patients are quite rare.

Other groups have also studied anti-TNFα monoclonal antibodies in patients with UC. CDP571, a humanized anti-TNFα monoclonal antibody (Celltech) was administered to 15 patients with mild/moderate UC at 5 mg/kg in an open-label trial. Six of the 15 patients were considered steroid unresponsive. Patients showed a 27% reduction in disease activity score after 1 week and a 48% reduction in disease severity score as accessed by sigmoidoscopy after 2 weeks. It was suggested that patients with steroid-unresponsive disease responded somewhat better to CDP571 therapy. No duration of response was mentioned (Celltech, unpublished observations).

2. *Sepsis*

Sepsis is a major cause of death for hospitalized patients, despite the availability of potent antibiotics, supportive care, and advanced medical and surgical therapies. It is known that serum levels of TNFα increase when animals or humans are given endotoxin (Beutler *et al.*, 1985; Michie *et al.*, 1988; van Deventer *et al.*, 1990) and further that elevated levels of TNFα are often observed in patients with sepsis (Waage *et al.*, 1987). It is thought that TNFα may be the mediator of the hypotension, disseminated intravascular coagulation, and organ failure observed in such patients (Beutler and Cerami, 1989). The hypothesis that neutralization of TNFα in such patients might prevent or reverse some of the detrimental findings has been tested using monoclonal antibodies that block TNF.

During the past decade a number of clinical trials have been conducted using anti-TNFα monoclonal antibodies or TNF receptor–IgG fusion protein constructs or "immunoadhesins" to treat patients with a diagnosis of "presumed sepsis" (van der Poll and Lowry, 1995; Wherry *et al.*, 1993). To date, there has only been limited or no clinical benefit, whether using a murine anti-TNFα antibody (CB0006; Celltech) (Fisher *et al.*, 1993) murine 1351 (Bayer), an antibody from Miles (Wherry *et al.*, 1993), or IgG-TNF-R immunoadhesin (Agosti *et al.*, 1994).

In a series of phase I and II trials, cA2 (Centocor) was administered to 141 patients with a diagnosis of presumed sepsis in various trials. There were no major differences in adverse events between the placebo and treatment groups, and the difference in mortality between those receiving placebo (10/16, 62%) versus those receiving cA2 (24/65, 37%) was not statistically significant, in part due to the small sample size (Centocor, unpublished observations). The role of TNFα as a major mediator of organ damage and tissue injury in sepsis is thus not formally proven by these clinical studies, and it is possible that, unlike RA, simultaneous blockade of other mediators may be necessary to reveal the clinical benefit of blocking TNFα.

3. *HIV and AIDS*

In vitro, TNFα has been shown to upregulate the transcription of HIV in various cells (Poli *et al.,* 1990). In the clinical setting, serum levels of TNFα have been elevated in some patients with AIDS, and levels have been observed to increase with advancing disease (Lahdevirta *et al.,* 1988; Reddy *et al.,* 1988). Furthermore, in patients with AIDS-related Kaposi's sarcoma, elevations in p24 HIV antigen were noted following administration of recombinant TNFα (Aboulafia *et al.,* 1989). These studies suggested that blockade of TNFα might be able to reduce HIV replication and benefit patients with AIDS. To examine this hypothesis, six patients with HIV infection and CD4 counts less than 200 cells/mm^3 were given two 10 mg/kg infusions of cA2 14 days apart. Serum TNF levels fell after each infusion and two patients exhibited a transient increase in CD4 cell counts. Plasma viremia, as measured by branched DNA assay, was unchanged and stable over the 42-day follow-up. During the study, one patient developed esophageal candidiasis, which resolved with therapy, one had recurrence of cutaneous herpes simplex virus, and one developed a sulfa-related skin rash. The patients had no other significant adverse events. None of the patients developed fulminant infections, and those listed previously responded to routine therapy.

IV. Mechanisms of Action of Anti-TNFα

A. *In Vitro* Biology

The construction of cA2 and the characterization of its binding to the soluble trimeric form of TNFα and neutralization of TNFα bioactivity was described previously (see Section III; Knight *et al.,* 1993). Recently, it was shown that cA2 also binds the 26-kDa monomeric transmembrane form of TNFα with an affinity at least as high as that for soluble TNFα (K_d 46 p*M*). Binding of cA2 to transmembrane TNFα induced both antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity in a tumor cell line (Scallon *et al.,* 1995). Interestingly, an IgG4 version of cA2 showed no cell-killing activity. These findings are in accord with the general notion that human antibodies of IgG1 isotype, once complexed with antigen, can efficiently bind and activate Fc receptor-bearing cells and also activate complement cytotoxicity.

B. Does Inhibition of TNFα Involve Neutralization of Soluble TNFα or Lysis of TNFα-Bearing Cells?

Based on these *in vitro* properties, two basic mechanisms of action, not mutually exclusive, can be proposed for cA2. In the first, complexing of

soluble TNFα with cA2 leads to loss of TNF bioactivity and downregulation of the downstream inflammatory events, which were detailed previously (see Section II,A). In the second, binding of cA2 to transmembrane TNFα results in destruction of TNFα-producing cells by Fc-mediated mechanisms, leading to a reduction in inflammatory and immune cell numbers.

Direct *in vivo* evidence supporting the first process in RA is difficult to obtain in part because even in active disease, there is little circulating immunoreactive and no bioactive TNFα. Paradoxically, treatment with cA2 in RA patients leads to a rapid and sustained rise in circulating immunoreactive TNFα, with peak levels exceeding 100 pg/ml (M. Feldmann, M. Elliott, J. Woody, and R. Maini, unpublished data). Because cA2 markedly interferes with TNFα detection in ELISA assay and is present in μg/ml quantities in the patient samples, the recorded values are likely to be an underestimation of the true TNFα levels. Interestingly, the same serum samples show little or no bioactive TNFα (M. Feldmann, M. Elliott, J. Woody, and R. Maini, unpublished data). Although our understanding of these events is still incomplete, it is likely that cA2 binds to TNFα in transit in the circulation, possibly in combination with sTNFR, leading to the formation of high-molecular-weight complexes with reduced clearance rates. The kinetics of rise in the immunoreactive TNFα, in which there is a characteristic delay of up to 24 hr before the cytokine becomes detectable in serum, may also indicate that clearance of TNFα from the joint is an important mechanism.

Treatment with cA2 also leads to a dose-dependent fall in circulating sTNFR (Charles *et al.*, 1995; unpublished data). Because TNFα is a major regulator of sTNFR release (Bemelmans *et al.*, 1993), this finding supports the notion that cA2 acts through neutralization of soluble TNFα. However, the best direct evidence is likely to come from measurement of bioactive TNF levels in serial SF samples or synovial membrane eluates.

Direct evidence supporting a cytolytic role for cA2 in RA *in vivo* is also lacking. Experience with the administration of mAb directed to CD4 and other leukocyte antigens in RA indicates the clinical consequences of substantial *in vivo* cell lysis: rapid falls in circulating cell counts and a clinical syndrome of chills, fever, headache, and myalgia resulting from the release of multiple cytokines from targeted leukocytes (Elliott and Maini, 1994). Circulating lymphocyte counts in cA2-treated patients show the opposite trend, with a significant rise within 24 hr of antibody administration, but the granulocyte and monocyte counts do fall. However, measurement of serum elastase as a marker of granulocyte lysis showed no increase post-cA2 (M. Feldmann, M. Elliott, J. Woody, and R. Maini, unpublished data). The most likely explanations for the reduced myeloid cell counts include a change in their distribution between circulating and

marginating or tissue pools or a switch off in myelopoietic signals (see Section IV,G,1). Similarly, with the exception of a few patients receiving multiple doses of antibody over prolonged periods, infusion reactions of the type described previously have not been seen with cA2. The significance of this observation is uncertain, however, given that neutralizing soluble TNFα by cA2 might mask the clinical features of the syndrome. Overall, although there is no evidence for massive lysis of transmembrane TNFα-bearing cells with cA2 therapy, smaller scale cell lysis, perhaps within the synovium itself where TNFα expression is expected to be highest, may still be of importance.

A direct comparison of the therapeutic efficacy of IgG1 isotype cA2 (the isotype used to date) with the IgG4 version (Scallon *et al.*, 1995) would help answer this question but is unlikely to be performed for practical reasons. However, some insight may be gained by comparing the clinical effects of cA2 with those reported for another engineered anti-TNFα antibody, CDP571. CDP571 consists of the hypervariable regions from a mouse anti-TNFα mAb grafted onto a human IgG4, with κ light chains. Like cA2, CDP571 is divalent and shows a similar affinity of binding to soluble TNFα (K_d ~100 p*M*), but details of its interaction with transmembrane TNFα have not been published. Also lacking are details about other physicochemical properties such as on and off rates, which may not be reflected in the affinity constant, and its precise specificity, all of which may influence neutralizing capacity.

The clinical effects of administration of CDP571 in RA have been described (Rankin *et al.*, 1995). The patient selection criteria employed in this study were similar to those used in the placebo-controlled trial of cA2 in RA and demographic features and baseline disease activity were also broadly comparable between the two studies. In the cA2 study, median baseline CRP in the three treatment groups was 56, 58, and 65 mg/liter (placebo and 1 and 10 mg/kg groups, respectively); in the CDP571 study, the equivalent values were 80, 37, and 51 mg/liter. Baseline SJC, recalculated in the case of the cA2 study as EULAR 28 joint counts to be directly comparable with the published CDP571 data, were also similar: 15.5, 14, and 11.5 for the cA2 groups and 17.5, 15.0, and 16.5 for CDP571. Both antibodies were administered by iv infusion as daily procedures at identical dosage.

Graphs comparing the efficacy of cA2 and CDP571 during the first 4 weeks after treatment are shown in Fig. 9. Low-dose treatment with either antibody was effective in suppressing CRP during the first 2 weeks, but there was loss of response by Week 4. At the higher mAb dose, the responses were again identical, with good suppression by both mAbs and prolongation of the therapeutic effect through Week 8 (Fig. 9, top). Al-

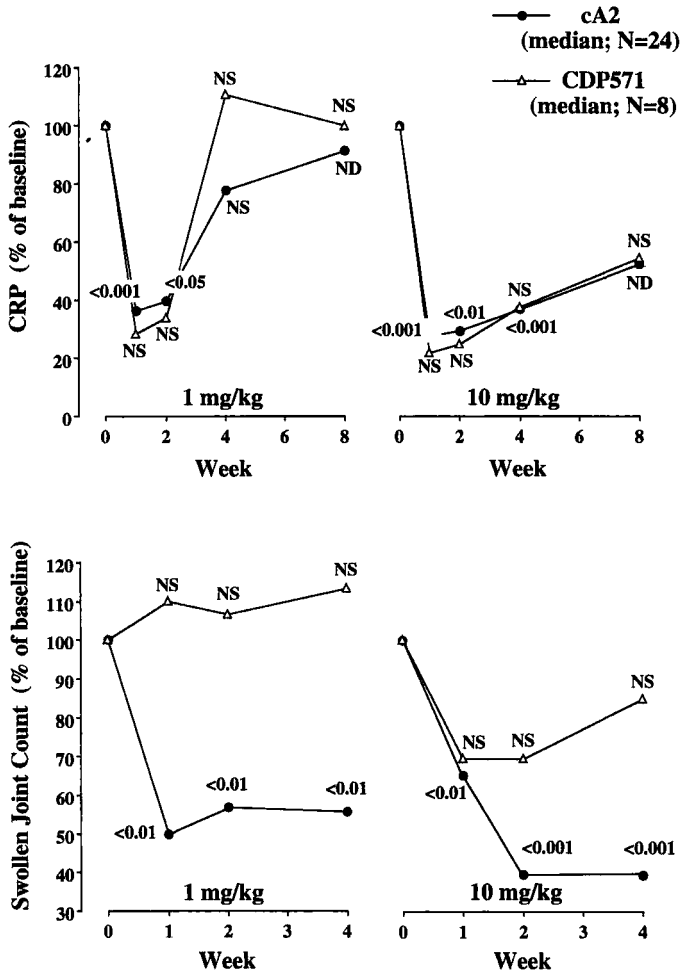


FIG. 9. Comparison of the effects of low- (1 mg/kg) and high (10 mg/kg)-dose cA2 and CDP571 in the treatment of patients with active RA. Data for CRP (top) and swollen joint count (28 joint count; bottom) are expressed as a percentage of baseline. *p* Values represent significance of change from baseline for that antibody versus the relevant placebo group (placebo data not shown); ns, not significant; nd, not done. Data from Elliott *et al.* (1994a) and Rankin *et al.* (1995).

though none of the changes in CRP reached statistical significance in the CDP571 study, an explanation may lie in the small sample size (8 patients per group compared with 24 in the cA2 trial) and the regression to the mean in the placebo group (Rankin *et al.*, 1995). The pattern of change

seen in the clinical assessments was quite different, with no effect of low-dose CDP571 on the SJC, whereas low-dose cA2 produced over 40% improvement in the SJC by Week 2 with maintenance through Week 4. Although high-dose CDP571 induced some improvement in the SJC, this was without statistical significance and clearly of smaller magnitude than that induced by cA2 (Fig. 9, bottom). Of the other clinical assessments reported by Rankin *et al.*, none showed more than a transient 25% improvement over baseline with low-dose mAb, compared with consistent and highly significant improvements of 60–80% for many clinical assessments in the low-dose cA2 group.

This analysis shows that CDP571 and cA2 are equally effective in controlling the early acute-phase response in RA, but that cA2 is up to 10-fold more effective in reducing joint counts and other clinical disease assessments. Because the only reported difference between the two mAbs that might influence single-shot efficacy is the antibody subclass, these observations suggest that isotype-dependent cell lysis could be an important element in the mechanism of action of cA2, at least in the control of articular disease. Consistent with this conclusion are the early results from synovial biopsy studies that show reduced synovial cellularity post-cA2, although this could also be due to reductions in cell migration (see Section IV,D) or changes in apoptosis. If confirmed, the significance of this conclusion is that in the future anticytokine reagents for use in RA should include lytic capability. The equal efficiency with which the two mAbs control CRP is consistent with the equivalence of IgG1 and IgG4 isotypes in neutralizing soluble antigen, resulting in this case in an equivalent interference in the systemic biology of TNF.

C. Downregulation of Cytokine Expression

The RA synovial tissue culture studies described previously (Sections II,A,1 and II,A,2) showed regulation by TNFα of many other cytokines *in vitro*. The clearest *in vivo* evidence supporting this concept has been provided by measurement of circulating cytokines in patients treated with cA2. Circulating IL-6 was detectable in more than 90% of patients enrolled in the placebo-controlled trial, and levels fell markedly following the administration of the antibody but not following treatment with placebo (Fig. 10). The changes were maximal within 24 hr of cA2 administration and were maintained for at least 4 weeks in patients treated with high-dose antibody. Of the high-dose cA2 patients with elevated circulating IL-6 at study entry, 70% achieved normalization of their levels posttreatment (Charles *et al.*, 1997).

Although the *in vitro* studies also predicted regulation of IL-1 production by TNFα (Brennan *et al.*, 1989), we were unable to directly test this

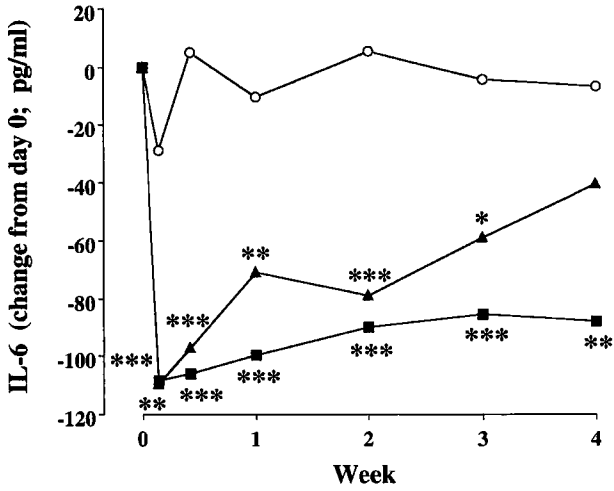


FIG. 10. Changes in serum immunoreactive IL-6 in the placebo-controlled trial of cA2 in RA. p Values represent significance versus the placebo group by ANOVA. (—o—) placebo; (—▲—) 1 mg/kg cA2; (—■—) 10 mg/kg cA2; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Data from Charles *et al.* 1996).

regulatory pathway *in vivo* because the majority of patients had very low or absent circulating IL-1 at trial entry. In contrast, we could detect the competitive receptor antagonist to IL-1, the IL-1ra, in all patients pretrial, with most showing values above the normal range. Treatment with cA2 induced marked downregulation of this mediator, with a similar temporal profile to the reduction in IL-6 (Charles *et al.*, 1996). Seventy-one percent of high-dose cA2 patients with elevated IL-1ra values at entry achieved normal values posttreatment.

These data confirm the important role of TNFα in regulating production of both inflammatory and anti-inflammatory mediators in RA. Together with the findings of reduced circulating soluble p55 and p75 TNFR discussed previously, the results also exclude the possibility that cA2 achieves a therapeutic effect by upregulating endogenous cytokine inhibitors. Downregulation of IL-6 production may help explain several important systemic phenomena seen after administration of cA2, including control of acute-phase proteins, and may possibly contribute to the reduction in thrombocytosis (Charles *et al.*, 1996; Elliott *et al.*, 1993, 1994a,b). IL-6 also plays a role in the control of maturation and terminal differentiation in the B cell lineage (Lipsky, 1989) and is known to promote autoantibody production in patients with cardiac myxoma (Hirano *et al.*, 1987; Jourdan *et al.*, 1990). Control of IL-6 might therefore explain the downward trend in rheumatoid

factor titers seen post-cA2 (M. Feldmann, M. Elliott, J. Woody, and R. Maini, unpublished data).

Other work aims to assess the role of TNFα in chemokine production in RA. Baseline levels of IL-8 and MCP-1 have been raised in most patients and have shown a dose-dependent reduction after cA2 (E. Paleolog *et al.*, unpublished data). These findings are consistent with data showing induction of MCP-1 by TNFα *in vivo* in mice and induction of IL-8 production by TNFα in cultured human umbilical vein endothelial cells and RA synovial cells *in vitro* (Butler *et al.*, 1995; Ohmori *et al.*, 1993; Paleolog *et al.*, 1994).

Although certain of the cytokines found in the circulation in RA have clear systemic roles (e.g., IL-6), others, such as the chemokines, have no known systemic biological function and may represent no more than spillover from the site of production. The degree to which circulating levels reflect synovial production rates is unknown. Other cytokines such as IL-1 are thought to have very important roles within the synovium but are difficult to detect in the blood. For these reasons, we have designed a study to test directly the effect of cA2 on synovial cytokine expression using both immunohistochemistry and semiquantitative PCR on tissue samples obtained at arthroscopy. The clearest early results are of a reduction in chemokine expression post-cA2 (M. Feldmann, M. Elliott, J. Woody, and R. Maini, unpublished data). Control of TNFα bioactivity within the synovial tissue and a consequent downregulation of expression of IL-1, IL-6, the chemokines, and other proinflammatory cytokines would provide an explanation for the impressive clinical effects consequent on the blockade of a single cytokine, TNFα.

D. Alterations in Cell Trafficking

Early indications in an effect of cA2 on cell trafficking in RA were provided by the rapid changes noted in peripheral blood leukocyte counts. Mean baseline circulating lymphocyte counts in both the open-label and placebo-controlled trials were in the low-normal range, as is common in severe RA, and showed a reproducible rise posttreatment with cA2, maximal by Day 1 (Paleolog *et al.*, 1996). These findings were consistent with the development of an alteration or block in lymphocyte margination and/or recirculation and prompted a search for other data to support this concept.

Because TNFα is a major endothelial activator, leading to increased expression of adhesion molecules required for leukocyte adhesion and diapedesis (Carlos and Harlan, 1994), and the soluble forms of the adhesion molecules are released and are at equilibrium" with the surface forms (Leeuwenberg *et al.*, 1992), we measured soluble forms of E-selectin, ICAM-1, and VCAM-1 in sera collected in the placebo-controlled trial.

Median baseline values for all three adhesins were markedly elevated and cA2 induced a dose-dependent statistically significant suppression in circulating levels of sE-selectin and sICAM-1, with a maximal fall in each of about 25% by Week 4 (Paleolog *et al.*, 1996). The best responses saw levels falling to the normal range and were typically seen in patients who experienced a good clinical response (Fig. 11). In contrast, levels of sVCAM were unchanged.

Soluble forms of E-selectin, ICAM-1, and VCAM-1 are released from cytokine-activated cultured endothelial cells (Pigott *et al.*, 1992) and it is likely that *in vivo* levels reflect changes in whole body endothelial activation. In agreement with this notion, we saw a significant reduction in synovial E-selectin expression post-cA2 in a small cohort of trial patients, and although the changes in synovial ICAM-1 expression were not statistically significant, there was a downward trend in the expression of both lining and sublining layer ICAM-1 (Tak *et al.*, 1996). Histological studies also showed a significant reduction in synovial $CD3^+$ cells posttreatment with cA2 and trends to reduced monocyte/macrophage infiltration (Tak *et al.*, 1996). Because the second of the serial biopsies on each patient was taken only 4 weeks after treatment, the effect of cA2 on the numbers of these cells, which are relatively long lived, may have been underestimated. Overall, however, the evidence supports the notion that cA2 acts to downregulate blood cell migration to the joint. This is likely to be a major mechanism of action for cA2 and may help explain the relatively prolonged clinical response observed (Elliott *et al.*, 1993; Maini *et al.*, 1995c). This concept is consistent with the results of a clinical trial of a murine anti-ICAM-1 antibody (BIRR-1) by Peter Lipsky's group (Kavanaugh *et al.*, 1994), which revealed clinical improvement in 9/13 rheumatoid patients lasting up to 3 months.

cA2-induced downregulation of endothelial adhesiveness would be expected to reduce trafficking by all leukocyte classes and result in abrupt increases in circulating counts, as seen for lymphocytes. In fact, circulating granulocyte counts were generally in the high-normal range at baseline and fell post-cA2, with maximal changes seen by Day 1 (Paleolog *et al.*, 1996). Similar but less marked changes were seen for monocytes (M. Feldmann, M. Elliott, J. Woody, and R. Maini, unpublished data). The high baseline granulocyte counts, which are commonly seen in patients with active RA, may result from the production of hemopoietic growth factors such as GM-CSF, which is highly expressed in RA synovium (Xu *et al.*, 1989). Studies with cultured RA synovial cells indicate that TNFα induces GM-CSF production in RA (Alvaro-Garcia *et al.*, 1991; Haworth *et al.*, 1991) and it is possible that cA2 acts to inhibit hemopoietic growth factor expression *in vivo*. A consequent drop in myeloid cell production

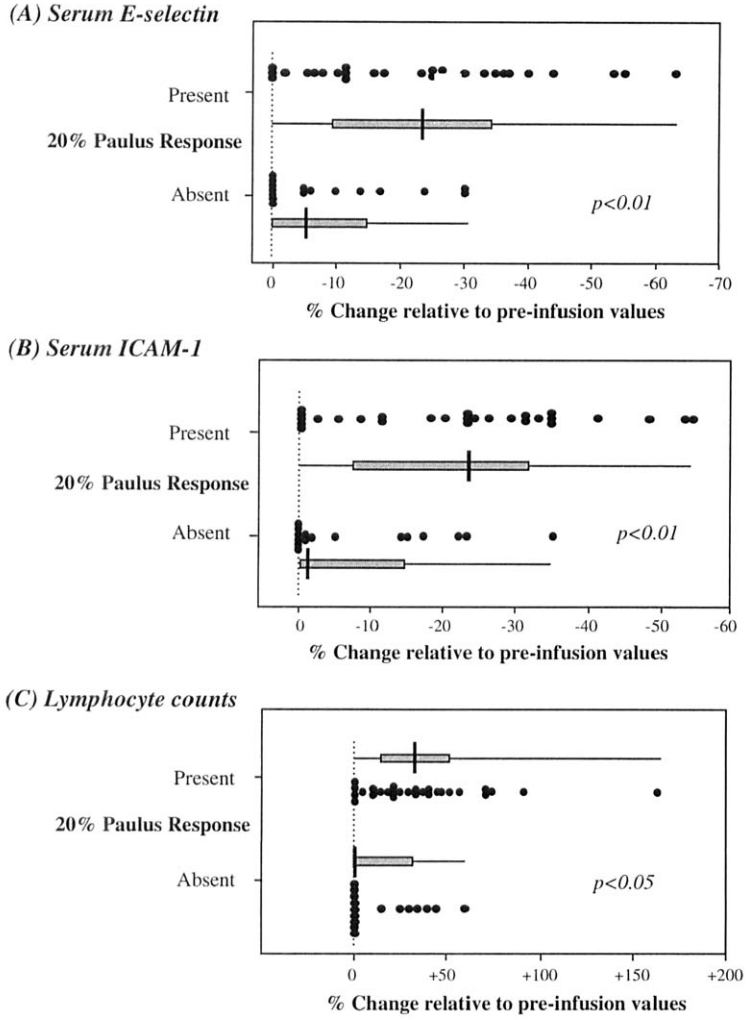


FIG. 11. Changes in serum E-selectin and ICAM-1 and circulating lymphocyte counts after addition of anti-TNFα antibody cA2 correlate with clinical parameters of RA. Correlation between the presence and absence of 20% Paulus response, a composite index of disease activate measurement, 4 weeks after infusion of cA2 (1 or 10 mg/kg) and percentage increase in peripheral blood lymphocyte counts. Solid bars represent median values, and hatched boxes enclose 25th and 75th percentiles. Statistical analyses were performed by Mann–Whitney U test; $n = 16$ for the absence of 20% Paulus; $n = 28$ for the presence of 20% Paulus. From Paleolog *et al.* (1996).

might mask any tendency for higher circulating cell counts induced by a migration block and could explain our findings. Indeed, the relatively short half-life of circulating granulocytes would ensure that a fall in production would be the dominant effect. The alternative of direct cA2-induced destruction of circulating granulocytes was discussed previously; another possibility is that cA2 may induce apoptosis. Nuclear medicine studies designed to directly measure the effect of cA2 on granulocyte distribution and migration *in vivo* should help resolve this question and have been planned.

E. Does TNFα Induce a Reduction in Neovascularization?

A striking feature of the synovial membrane in active RA is its vascularity, which is seen well at arthroscopy and in tissue sections stained with an endothelial marker such as Von Willebrand factor. The augmented synovial cell mass requires, like a tumor, neovascularization to sustain it. Among the known regulators of neovascularization, vascular endothelial growth factor (VEGF) is of particular importance. VEGF is a selective mitogen for endothelial cells and also acts to increase vascular permeability, being identical to vascular permeability factor (Ferrara *et al.*, 1992; Leung *et al.*, 1989). VEGF also promotes endothelial chemotaxis, both directly and by promoting vascular leak and the consequent tissue deposition of fibrin (Koch *et al.*, 1994). VEGF and its receptors are expressed in RA synovial membrane endothelial cells and the cytokine is present in biologically significant concentrations in synovial fluid (Fava *et al.*, 1994; Koch *et al.*, 1994).

To investigate whether cA2 therapy influences the cytokines involved in neovascularization, we measured circulating VEGF by ELISA in patients and normal controls and showed significantly elevated levels in RA and some other forms of inflammatory arthritis. Treatment with cA2 resulted in a reduction in median circulating VEGF of up to 45% (Paleolog *et al.*, 1997). Interestingly, the changes in circulating VEGF were delayed relative to those of other cytokines, such as IL-6, which are also controlled by cA2. This may reflect a difference in the circulating half times of the respective cytokines or indicate that cA2 regulates VEGF production indirectly. Because hypoxia is a recognized inducer of VEGF expression (Plate *et al.*, 1993; Shweiki *et al.*, 1995) and joints are hypoxic (Blake *et al.*, 1989), one explanation for delayed control of VEGF may lie in reduced joint hypoxia, consequent on improving overall disease activity. Experimental analysis is necessary to discriminate between these possibilities.

Soluble adhesion molecules, such as sE-selectin and sVCAM-1, also possess angiogenic activity and may contribute to neovascularization in RA (Koch *et al.*, 1995). The combined reduction of sE-selectin and VEGF by cA2 in RA may result in reduced synovial vascularity, but histological

confirmation of this is still awaited. Reduced vascular permeability could contribute to the rapid improvements in clinical measures post-cA2, particularly reduced morning joint stiffness, swelling, and effusion, and the rapid kinetics of change is compatible with this notion.

F. TNFα and Mechanisms of Joint Destruction

TNFα is an important factor promoting cartilage and bone damage in RA (Bertolini *et al.*, 1986; Saklatvala, 1986). TNFα acts together with IL-1 to suppress synthesis of matrix components by connective tissue cells and to stimulate MMP production by fibroblasts and other synoviocytes. Preliminary data from the placebo-controlled cA2 trial show a dose-related reduction in serum MMP-1 and MMP-3 (collagenase and stromelysin-1) following treatment with the antibody but not following placebo (Brennan *et al.*, 1997). Thus, MMP-3 levels that were raised in the RA patients era fell to <50% of preinfusion values in the 10 mg/kg group 1 week after infusion and remained at significantly lower levels for up to 4 weeks. Similarly, the MMP-1 levels were also reduced but to a lesser extent (<85%) in the 10 mg/kg group, and throughout the 4-week period they remained at lower levels than those of the placebo-treated group. Although these results are encouraging, the relationship between circulating MMP levels and those in the joint has not yet been determined, but it is reasonable to assume that reduced serum levels posttreatment suggests reduced production and activation in the joint. Understanding the effects of TNFα blockade on traditional radiological measures of joint damage and on potential surrogate markers such as the MMPs and cartilage and bone markers is of prime interest but will require long-term clinical trials in early rheumatoid arthritis patients to establish whether TNF blockade in humans is as effective in joint protection as it is in mice with collagen-induced arthritis (Williams *et al.*, 1992).

G. Other Systemic Effects of cA2

1. Hemopoiesis

Anemia of chronic disease is common in active RA and was a feature of the long-standing active patient groups enrolled in the cA2 trials. Administration of cA2 in the placebo-controlled trial led to a small but significant rise in Hb by Week 4, contrasting with a fall seen in the placebo group (Elliott *et al.*, 1994a). Because the patients were subject to repeated venesection (~400 ml) during the trial, and in view of the slow response times of these patients in recovery from anemia, the 4-week rise in Hb is likely to be a significant underestimation of the benefit of cA2 therapy.

Although the normal erythropoietin (EPO) response to anemia is blunted in RA (Boyd *et al.*, 1991; Vreugdenhil *et al.*, 1990) and Hb levels respond

to EPO administration (Means *et al.*, 1989; Pincus *et al.*, 1990), it is likely that TNFα also contributes to anemia of chronic disease. TNFα acts directly to suppress erythroid development *in vitro* (Akahane *et al.*, 1987; Roodman *et al.*, 1987) and its unregulated expression in nude mice leads to reticulocytopenia and profound anemia (Johnson *et al.*, 1989). Our investigations to date show that the rise in Hb seen after cA2 treatment is not caused by an increase in the serum EPO response (Davis *et al.*, 1997, unpublished results), raising the possibility that cA2 acts directly in the marrow by blocking the TNFα-induced suppression of erythroid development.

The classic roles for IL-6 in hemopoiesis are those of a stem cell comitogen, acting in concert with IL-3 to promote the transition from G_0 to the G_1 phase of the cell cycle, and of a thrombopoietic factor inducing the maturation and release of platelets (Akira *et al.*, 1993). Interestingly, however, administration of IL-6 to experimental animals also led to the development of anemia (Burstein *et al.*, 1992; Herodin *et al.*, 1992). This led to the therapeutic use of IL-6 in patients with advanced malignancies (Weber *et al.*, 1993). Administration of IL-6 to cancer patients led to a profound anemia, resembling the anemia of chronic disease, at serum IL-6 levels that were comparable to those commonly measured in active RA (van Gameren *et al.*, 1994). Because levels of other erythroid-suppressive cytokines, such as IL-1 and TNFα, were unaffected by IL-6 use, the findings suggest a direct effect of IL-6 on erythropoiesis. cA2 may therefore have an additional, indirect mechanism of action in the control of anemia of chronic disease by downregulating IL-6 expression. Downregulation of IL-6 may also be responsible for control of platelet counts post-cA2 (Elliott *et al.*, 1994a), although other thrombopoietic cytokines, such as IL-11, thrombopoietin, and LIF, are likely to also be involved.

2. *Cardiovascular Risk Factors*

Patients with RA tend to die prematurely but largely of the same diseases that affect the general population. Overall, the most common causes of death in RA are cardiovascular and cerebrovascular disease (Wolfe *et al.*, 1994). TNFα may contribute to this excess risk through induction of IL-6 and, as a consequence, increased plasma fibrinogen levels (Meade, 1995). The promotion of SAA production through the same intermediary may also contribute to the development of atherosclerosis by altering HDL metabolism (Van Lenten *et al.*, 1995). Similarly, the thrombocytosis found in RA may contribute to cardiovascular risk because platelets play a central role in both early and late atherosclerotic lesions (Brown and Martin, 1994). It was found that treatment with cA2 normalizes all three of these risk factors, at least in the short term (Charles *et al.*, 1997; Elliott *et al.*, 1993, 1994a). The question of whether TNFα blockade will reduce vascular

deaths in RA is of great interest but could only be determined in large, long-term trials.

3. *Immune Response*

TNFα has well-recognized effect on the afferent limb of the immune response, acting as a comitogen with IL-2 on T cells, promoting the production of cytotoxic T cells in MLR, and augmenting the priming phase of DTH responses in mice (Vassalli, 1992). TNFα is also important in the activation of nonspecific effector cells, such as neutrophils and monocyte/macrophages, and plays a role in lysis of tumors and virus-infected cells. Studies in various animal models of acute infection have confirmed a marked protective effect of TNFα (Beutler and Cerami, 1989; Vassalli, 1992).

These generally beneficial effects of TNFα in short-term immune function experiments may be contrasted with the results of chronic TNFα exposure. Pretreatment of antigen-specific T cell clones with TNFα for up to 16 days impaired rechallenge responses, and chronic exposure to this cytokine impaired mitogen-stimulated lymphokine production (Cope *et al.*, 1994). Similarly, TNFα blocked cell cycle progression in 3-day cultures of PHA-stimulated RA synovial fluid T cells (Lai *et al.*, 1995). Measurement of PBMC proliferative responses to mitogens and recall antigens in patients treated with cA2 showed suppressed baseline responses and an improvement posttreatment, supporting the data from the long-term culture studies (Cope *et al.*, 1994). In the same trials, the skin test response has been assessed in a proportion of patients. The results so far indicate that the delayed-type hypersensitivity response is not diminished and there is a suggestion that it is increased (M. Elliott *et al.*, unpublished results). These findings provide some reassurance about the likely effects of long-term TNFα blockade on immune function. However, anti-TNF may still be disadvantageous when a strong effector response is required, such as during an acute bacterial infection.

V. Potential Problems

A. Anti-Immunoglobulin Antibodies

The formation of anti-idiotype antibodies following administration of murine monoclonal antibodies is a well-known phenomena (LoBuglio *et al.*, 1989; Schroff *et al.*, 1985). The use of chimeric antibodies is less well studied, although LoBuglio *et al.* (1989) were able to show a substantial decrease in antiglobulin response when chimeric 17-1A was administered to patients and compared to murine 17-1A. Recently, we were able to show a substantial reduction in the frequency of such antibodies in patients

when administration of murine Fab 7E3, with an 18% human anti-mouse antibody (HAMA) response, was replaced with chimeric Fab 7E3, yielding a 1% human antichimeric antibody (HACA) response (Jourdan *et al.*, 1990).

Recent reports with the use of totally humanized antibodies such as CAMPATH-1H show that even in these circumstances 3/4 (75%) patients formed human anti-human antibodies directed against both the variable regions and the allotype structures following the second dose (Isaacs *et al.*, 1992). In a subsequent, longer study, anti-idiotype antibodies were observed in $\frac{13}{24}$ (54%) patients after the first few weeks of therapy with humanized CAMPATH-1H (Matteson *et al.*, 1995; Weinblatt *et al.*, 1995). Therefore, the development of anti-idiotype or antivariable region antibodies may be expected in some patients, despite chimerization or humanization. The response may be dependent on dose, formulation, and variable region determinants of the monoclonal antibody as well as the immune status of the patients and the sensitivity of the assays used. The hypothesis that antibodies that are targeted to immune and antigen presenting cells are particularly immunogenic, made by Waldmann's group, is intriguing.

The assay used for detecting HACA to cA2 is a double-antigen system. Briefly, microtiter plates are coated with streptavidin, biotinylated cA2 is added, and unbound excess is washed off. Test sera are diluted twofold following an initial $\frac{1}{10}$ dilution. The assay format has the advantage that it can detect all types of HACA, including IgM and/or IgG, but has the disadvantage that free circulating cA2 in the serum interferes with the analysis. A second problem identified in sera from patients with rheumatoid arthritis was the presence of RF, i.e., antibodies against the Fc region of IgG molecules, which also interfered with the HACA analysis. Currently, when RFs are expected, the samples are absorbed with aggregated human Fc fragments prior to analysis. Although this step might theoretically remove antibodies to IgG1 heavy chain allotypes, from prior studies in sepsis patients we know that these are extremely rare because almost all HACAs are directed at the cA2 variable region. Once the presence of a HACA response is detected (OD > 0.25), precise HACA specificity is determined by solution phase neutralization with intact cA2 (human IgG1 constant domains and murine A2 variable region), murine A2 (murine Ig constant regions and murine A2 variable region), or cMT412 (human IgG1 constant domains and different murine variable region).

HACA responses in patients receiving single doses of cA2 are generally of low titer ($<1:100$) when compared to HAMA responses observed with murine antibody administration ($>1:1000$) when tested in similar assays. The frequency of HACA to cA2 varies from study to study. In some studies of RA and Crohn's patients the frequency is <5% even with long-term follow-up after single dose administration. Other studies show a 25–30%

frequency. Thus far, there is no relationship between dose and formation of HACA, although the data suggest that higher doses of cA2 result in a somewhat lower frequency of positives. Because the cA2 is deaggregated, this may suggest that high doses may be approaching the tolerogenic dose for deaggregated human IgG (Chiller *et al.*, 1970; Loblay *et al.*, 1980; Weigle, 1973). The HACA responses observed are IgM, IgG, or both, but no IgE HACAs have thus far been identified.

Studies with repeat dosing are still in progress, although one study, the first open-label RA study, showed a frequency of 50% after multiple infusions during a 2-year period. Preliminary data from other studies suggest that certain dose combinations may result in higher HACA rates. Patients with high levels of HACAs who receive re-treatments with cA2 generally experience the same magnitude of clinical response but of shorter duration, although there are patients with HACAs who have a longer duration of response. Current studies with multiple infusions will clarify this situation.

In primate studies, cA2 has been given in doses similar to those used in clinical studies without the formation of antichimeric antibodies, even when cA2 was intentionally heat aggregated. The reasons for this species difference are not understood.

B. Anti-Double-Stranded cDNA Antibodies

Seven of 69 cA2-treated patients in the first two RA trials (Elliott *et al.*, 1993, 1994a) developed anti-double-stranded (ds) DNA antibodies following cA2 treatment. Three of these patients received another infusion of cA2. In 1 patient the anti-double-stranded DNA antibodies decreased during follow-up after the first infusion and were not measurable after the second infusion. In a second patient the titers remained at approximately the same level, and in a third the titers rose after the repeat cA2 infusion. None of these patients developed signs or symptoms of autoimmune disease other than those considered to be part of their underlying RA (Maini *et al.*, 1994). However, in an ongoing cA2 multidose study in RA, 1 patient developed dyspnea, pleuritic chest pain, and a rebound of arthritis activity at study Week 14 (4 weeks after the fourth infusion of 3 mg/kg of cA2). The symptoms resolved, and she received her fifth dose of cA2. More severe and persistent symptoms recurred 3 weeks later. Examination of the serial blood samples revealed that the test for antinuclear antibodies and anti-DNA antibodies was negative prior to treatment but became positive at Week 6 of the study. The patient's symptoms responded to oral prednisolone at a dose of 20–30 mg daily. It is possible that cA2 precipitated a lupus-like syndrome in this patient. The generation of anti-ds DNA antibodies after cA2 is reminiscent of studies in murine NZB/W lupus in which blockade of TNF worsened disease (Ishida *et al.*, 1994; Jacob and

McDevitt, 1988). Anti-ds DNA antibodies have also been reported in the RA trials of the other anti-TNF biologic agents, such as CDP571 and TNF-R IgG/immunoadhesins (D. Isenberg, personal communication; P. van Auwera, personal communication), and thus cannot be an unusual anti-idiotypic reaction.

C. Safety Parameters and Adverse Events Attributed to cA2 Administration

1. Is There an Increased Susceptibility to Infection?

cA2 has been administered to more than 400 persons, including patients with rheumatoid arthritis, Crohn's disease, sepsis, and AIDs. We have access to all this information, which is discussed here. However, we do not have access to analogous information concerning the other anti-TNF agents. It would be likely, however, that exactly the same problems will occur, as is already known with anti-double-stranded DNA antibody. During the past two decades it has become apparent that TNF plays a role in protection against infectious immunity (Vassalli, 1992). There was thus concern that blockade of TNF might increase susceptibility to infection or accelerate minor infections to a more fulminant form.

Studies in patients with sepsis represent the worst case for risk and thus allow investigators to gain insight as to any additional risk engendered by the use of TNF blockers such as cA2. From the theoretical point of view, it is possible that the results obtained with the IgG TNF-R fusion proteins and anti-TNFα antibodies could be different. The fusion proteins will block both TNFα and the related LT, also known as TNFβ. Because LT is involved in immune function (De Togni *et al.*, 1994; Kehrl *et al.*, 1987a), it is conceivable that the IgG TNF-R fusion proteins may be more immunosuppressive.

As noted previously, cA2 was administered to 141 patients with a diagnosis of presumed sepsis. Fifty percent of these patients were in shock, 65% were septicemic, and 20% had both gram-positive and gram-negative organisms cultured from their blood. Administration of cA2 showed a trend toward mortality benefit at 28 days, adverse events were not different between cA2- and placebo-treated groups, and opportunistic infections did not occur. These data suggest that TNF blockade by cA2 does not predispose individuals to fulminant infections or increase the risk for opportunistic infections or lead to worsening of existing infections.

The adverse experiences that have occurred in the clinical trials in rheumatoid arthritis and Crohn's disease may reflect the complications of the underlying diseases, concurrent illnesses, and concomitant medications, as well as effects of the trial medication with anti-TNF. The patients

enrolled in the rheumatoid trials have severe, long-standing RA. From the data of Wolfe *et al.* (1994) it is known that patients with RA who develop infections have worse than expected outcomes. He noted a five or six times increased risk of death with pneumonia or other infections. It is also well known that patients with RA have increased susceptibility to infection, as do patients receiving long-term corticosteroids or immunosuppressive agents including methotrexate (Boerbooms *et al.*, 1995; Conaghan *et al.*, 1995; Dale and Petersdorf, 1973; Panillo and Fauci, 1979). Therefore, one might expect to note some infectious episodes over long-term follow-up of patients in these trials.

To understand the relationship of the adverse experiences to cA2 treatments in the nonsepsis trials, it would be helpful to compare the adverse experiences observed in cA2-treated patients to those occurring in matched placebo-treated patients in the randomized trials. At this time, however, there is no *long-term* placebo group comparable to the cA2-treated group. There were no placebo-treated patients in the two Crohn's disease trials. In the latter, randomized RA trial, placebo-treated patients (n = 24) received cA2 shortly after the 4-week evaluation point so that the active drug was eventually received by all trial patients. This is helpful for patient recruitment in these trials. The group of patients on placebo that would consitute a good control group for the incidence of infections unrelated to cA2 therapy is therefore small and followed up for only a short period. In contrast to those who had received placebo, patients treated with cA2 were followed as long as they responded—up to 6 months. Most were retreated and followed throughout the second response period—again up to 6 months. Adverse events were collected throughout these follow-up periods.

It is reasonable to categorize adverse events from three perspectives. First, those that are serious, defined as life threatening or requiring hospitalization are considered. Second, those that the clinicians have indicated as definitely, possibly, or probably related to the drug are likewise considered. Lastly, those events occurring while antibody might still be present in the serum (3 months after the last infusion) are included. Adverse events that were considered serious (resulted in death, were life threatening, or resulted in hospitalization) and were attributed (definitely–possible–probably related) to cA2 therapy and occurred within 3 months after the last infusion are included in Table III. Events outside this definition will be discussed later.

The infections are similar to what might be expected in this population of patients. Thes case of pleuropericarditis was discussed under the section on anti-double-stranded DNA antibody. The infusion reactions observed with cA2 are similar to reactions observed when intravenous γ-globulin is

TABLE III
SERIOUS ADVERSE EVENTS ATTRIBUTED TO cA2 THERAPY[a]

Serious adverse event	Number	cA2 dose	Time from infusion to event
Infectious	1	1 mg/kg	7 Weeks
Bronchitis			
Pneumonia	3	Various	2–6 Weeks
Purulent bursitis	1	na[b]	6 Weeks
Noninfectious	1	3 Weeks	4 Weeks
Pleuropericarditis			
Infusion reaction	6	Various	<12 hr

[a] Total patients treated = 336.
[b] na, not available, data blinded at present time.

administered too quickly (Ochs *et al.*, 1980). The symptom complex includes headache, nausea, myalgia, fever, vomiting, facial flushing, erythema, chest tightness, dyspnea, pleuritic pain, and occasionally hypotension or any combinations of these. For the six patients listed previously, the signs and symptoms were of sufficient severity to warrant continued hospital observation, although all fully recovered. Minor infusion reactions have been observed in 6 of 52 (12%) of re-treated patients in early studies. In recent studies, which included multiple re-treatments, the frequency to date has decreased to <2% of patients. Symptoms of allergy, including urticaria, pruritus, and skin erythema, have occurred in only a few patients (<2%).

One patient developed a serious postoperative infection following cataract surgery 9 weeks after receiving cA2. The infection spread leading to eventual removal of the eye. Of nine other patients undergoing surgery (bowel resection, etc), only one had a postoperative infection and the patient responded well to treatment.

2. *Nonserious Adverse Events: 3-Month Follow-Up*

a. Infectious Origins. Respiratory infections of various types, including upper respiratory infections (common cold), rhinitis, sinusitis, pharyngitis, etc., were most common. They have been observed in 21 patients of 226 (9.2%) analyzed thus far. None have been caused by opportunistic organisms and all have resolved with routine care. In one study in which a placebo group was compared to the cA2 treatment group the incidence per patient month of respiratory system infections was the same, expressed in patient months in the placebo- and cA2-treated groups, indicating a lack of increased risk during the 4-week follow-up period during which there were circulating blood levels of cA2.

Other infections include urinary tract infection in three patients, skin infection in two patients, and pneumonia in two patients. Furthermore, single cases of infections include severe cellulitis, herpes labialis, herpes zoster, scar abscess, moniliasis, fungal infection, and gastrointestinal infection.

b. Noninfectious Origins. One patient with Crohn's disease developed hemolytic anemia of unknown etiology 3 or 4 weeks after 5 mg/kg cA2. The hemolytic anemia resolved after transfusion.

3. Adverse Events Observed in Long-Term Follow-Up (3–24 Months)

Following therapy with cA2 in the clinical trial setting, these severely affected patients returned to conventional drugs. Severe infection has been observed in two patients at a time long after the antibody has cleared, infections similar to those observed in severe RA. From the data of Wolfe *et al.* (1994) it is known that patients with RA who develop infections have a worse than expected outcome. They noted a five- or sixfold increased risk of death with pneumonia or other infections.

a. Infectious Origins. One RA patient with a history of pulmonary emphysema and recurring pleural effusions related to RA developed empyema approximately 5 months after the second infusion of 3 mg/kg of cA2. This resolved with drainage and antibiotics. Another patient became ill 15 weeks after the last cA2 therapy. Six days later she was admitted to the hospital with untreated staphylococcal pneumonia and septicemia. She subsequently died. The event was judged to be unrelated to cA2.

b. Noninfectious Origins. One patient became pregnant 8 months after cA2 infusion and 2.5 months later lost the pregnancy for unknown reasons.

In summary, there is currently little evidence of impairment in handling infectious diseases in patients receiving cA2. Furthermore, the infections observed are typical of infections commonly seen in such patients. Although no additional risk to patients has been identified, we continue to carefully observe these patients and maintain a long-term follow-up database.

D. Malignancies in RA Trials

It has long been postulated that there is an increased risk of development of leukemia and lymphoma in patients with RA (Bannwarth *et al.*, 1995; LeGoff *et al.*, 1994). Wolfe and co-workers (1994) have reported an observed/expected ratio for leukemia/lymphoma of 8.016 in rheumatoid arthritis patients from the ARAMIS database. Patients with long-standing RA that have failed numerous drugs might be expected to be at even

higher risk than those identified in the Wolfe study. Of more than 400 treated patients, 4 have developed a malignancy. One patient had preexisting breast cancer that was discovered shortly after administration of cA2. Two patients with long-standing (16 years) severe RA developed lymphoma after receiving cA2. One patient received a single dose of 1 mg/kg and 6 months later, after resuming other medications, developed axillary lymphadenopathy diagnosed as Hodgkin's lymphoma. A second patient entered the study with lymphadenopathy that had been biopsied 14 years earlier to rule out lymphoma. The diagnosis was "reactive hyperplasia." This patient received 20 mg/kg and, following relapse, resumed other medications. Eighteen months later he developed a lymphoma. A fourth patient with HIV received 20 mg/kg cA2 and 8 months later developed lymphoma. We believe that the cancers developing in these patients are related to their underlying disease. We have initiated a long-term registry for patients receiving cA2 so as to clearly identify any risk potential.

Overall, the safety profile for cA2 administration at this point suggests that it is well tolerated and that suppression of this cytokine does not, as previously predicted, lead to a large increase in serious infections.

VI. Prospects for the Future

A. Other "Anti-TNF" Drug Modalities

The degree of effectiveness, reproducibility, and widespread benefit of anti-TNFα antibody therapy, extending to many, conceivably even all aspects of rheumatoid arthritis, implies that other approaches to inhibiting the excess of TNFα signaling that takes place during this disease are likely to be effective. Because anti-TNFα antibody has limitations in terms of convenience (injectable), the development of anti-globulin (HACA) antibodies, and potentially of cost, a number of other approaches to blocking TNFα actions are being investigated. These include drugs that reduce TNFα synthesis, including the phosphodiesterase type IV inhibitors (Semmler *et al.*, 1993) and corticosteroids (Han *et al.*, 1990), drugs that interfere with the stability of TNFα mRNA, such as thalidomide (Moreira *et al.*, 1993); drugs that interfere with the signaling that induces TNFα production, such as the pyridinyl imidazoles of Smith Beecham (Lee *et al.*, 1994); and those metalloproteinase inhibitors that interfere with the enzymatic cleavage of TNF from the membrane (Gearing *et al.*, 1994; McGeehan *et al.*, 1994; Mohler *et al.*, 1994).

There are probably other anti-TNFα drugs in various stages of development, and it is to be expected that some of these will prove to be both safe and effective and their effects in rheumatoid patients should mimic those of anti-TNFα antibodies. However, because drugs of this type do

not necessarily have only a single mode of action, it is conceivable that, in practice, in nontoxic doses they may not be as effective. An example of this problem is illustrated by the current generation of metalloproteinase inhibitors that can block the TNFα cleavage enzyme. It has been predicted that these drugs, by diminishing the cleavage of membrane TNFα and hence its bioavailability in the fluid phase, as well as reducing its synthesis by an unknown feedback mechanism, may lead to an effective anti-inflammatory action (Gearing *et al.*, 1994). However, these drugs also inhibit the cleavage of both forms of the TNF receptor, the end result of which is proinflammatory because higher TNF receptor expression on the membrane facilitates signal transduction. Using cultures of dissociated RA synovial cells as a model system to test these concepts, it was found that despite a marked reduction in both TNF and TNF-R release, these drugs had no effect on downstream cytokine production, with no change in the production of IL-1, IL-6, and IL-8 (Williams *et al.*, 1996). Hence, a relatively nonspecific metalloproteinase inhibitor did not possess the desired anti-inflammatory properties and was not an effective anti-TNF agent. The specificity of anti-TNFα biological agents (antibodies and Ig TNF-R fusion proteins) may thus confer advantages that will allow them to be used in the clinic for a good few years before being supplanted by cheaper organic chemicals that can be taken orally.

B. Synergy or Combinations of Anti-TNF Approaches

It is virtually axiomatic that all drug therapy has its problems, and although those of anti-TNFα biologicals are becoming known, those of anti-TNFα drugs are not yet known because these drugs are chiefly still on the drawing board. It is very likely that some problems are *intrinsic* to any form of effective TNFα inhibition, such as the increased susceptibility to develop anti-double-stranded DNA antibodies and SLE (Maini *et al.*, 1994). Other problems are likely to be drug specific, and these could be reduced by using combinations of agents that block TNF production or its action.

The phosphodiesterase (PDE) type IV inhibitors raise cAMP levels intracellularly, which influences a plethora of signaling pathways in cells in which PDE type IV is expressed, such as lymphocytes, macrophages, and the nervous system. Rolipram is a potent PDE type IV inhibitor that markedly reduces TNFα production *in vitro* (Semmler *et al.*, 1993) and is active in arthritis in the collagen-induced model (Williams *et al.*, unpublished results). It is also effective in animal models of experimental allergic encephalomyelitis (Genain *et al.*, 1995; Sommer *et al.*, 1995). In the monkey model of encephalomyelitis, the antiemetic ondansetron had to be administered concurrently to reduce side effects (Genain *et al.*, 1995).

It is a reasonable hypothesis that if an anti-TNFα drug such as rolipram is used after TNF is "cleared," and significant clinical improvement has been established using anti-TNFα antibody, then lower and thus potentially less toxic doses may be necessary in order to maintain clinical benefit compared to the doses needed to induce and maintain benefit de novo. Preliminary data in animal models of arthritis supports the possibility that anti-TNF antibody and anti-TNF drugs have additive effects (Williams *et al.*, unpublished results).

C. Synergy of Anti-TNF and Anti-T Cell Therapy

Our initial concept of the pathogenesis of autoimmune disease envisaged a cytokine (IFNγ)-dependent activation of local autoantigen presentation to the preexisting autoantigen reactive T cells (Bottazzo *et al.*, 1983). From that immune interaction the effector pathways of cytokines, etc. are activated, leading to inflammation and tissue damage. This concept suggests that interfering with the immune response, in addition to blocking TNFα, should have additive or synergistic benefit. This hypothesis was evaluated in the collagen type II model of arthritis in DBA/1 mice after disease onset. Either an optimal or a suboptimal (in itself ineffective dose) of anti-TNF antibody was used in combination with lytic anti-CD4 antibody that had been shown to be protolerogenic (Benjamin and Waldmann, 1986; Qin *et al.*, 1987). The anti-CD4 by itself was not effective in ameliorating collagen-induced arthritis, as previously reported (Ranges *et al.*, 1985). In both instances there was a marked augmentation of benefit as judged by inflammation (footpad swelling), number of affected limbs, and, most important, in joint histology, which revealed a diminished degree of joint damage, with less bone erosion and cartilage damage (Williams *et al.*, 1994) (Fig. 12).

The synergy of anti-TNF and anti-CD4 suggests a general principle: Anti-inflammatory cytokine and anti-immune therapies, targeting two distinct steps in the autoimmune process, may be synergistic. We have investigated this principle further and shown that cyclosporin A, a drug that blocks T cell activation via the T cell receptor, also synergizes with anti-TNF antibody (Williams *et al.*, unpublished results). However, this synergy is less dramatic at higher doses of cyclosporin because at higher concentrations cyclosporin is also able to reduce TNF synthesis and hence is directly anti-inflammatory.

The mechanism of synergy was of interest and has been investigated. The protolerogic effect of anti-CD4, well documented by Waldmann and colleagues (Benjamin and Waldmann, 1986; Waldmann, 1989), may account for some of the synergistic benefit detected. In experiments in which a strongly immunogenic human IgG1–human p55 TNF receptor fusion

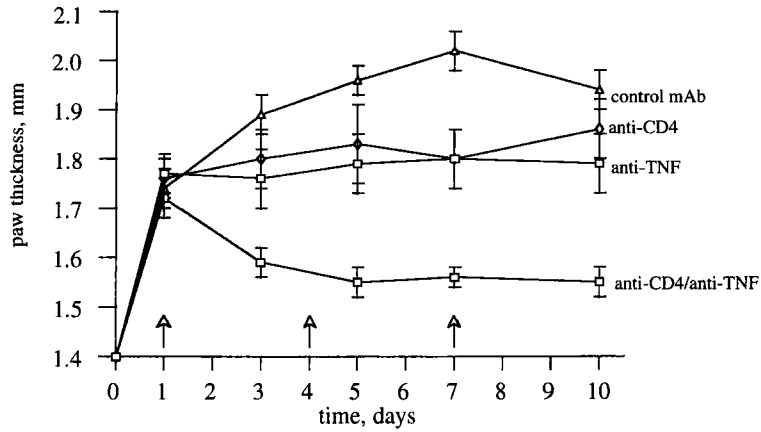


FIG. 12. Combination therapy. comparison of a suboptimal dose of anti-tnf (50 μg) and anti-CD4 (200 μg), given at times shown by arrows, with combination therapy with both agents. The footpad swelling at Days 3, 5, 7, and 10 was significantly reduced ($p < 0.05$). Reproduced with permission from Williams *et al.* (1994).

protein was used in CIA, there was synergy with anti-CD4 (Williams *et al.*, 1995). Blood levels of the IgG-TNF-R were much higher in the animals concomitantly treated with anti-CD4 antibody and the titers of anti-human IgG1 antibodies generated by the fusion protein were reduced in these mice. However, in experiments using a hamster anti-TNF antibody, which is weakly immunogenic in DBA/1 mice, there was no increase in the blood levels of anti-TNF in the presence of anti-CD4. Because the clinical benefit with low-dose anti-TNF in the presence of anti-CD4 was greater than that with high-dose anti-TNF alone, despite much lower serum anti-TNF levels, it suggests that the anti-CD4 is also downregulating proinflammatory pathways and not acting merely to increase anti-TNF levels, consequent on reducing its immunogenicity. The capacity of anti-CD4 to reduce the immunogenicity of a foreign protein may be a very useful property and in itself may help prolong the clinical benefit of anti-TNF antibody in humans.

The opportunity has not yet presented itself to perform clinical trials of anti-TNF antibody in combination with anti-T cell therapy in rheumatoid patients, but the outcome of such trials is eagerly awaited because it is likely that the duration and degree of benefit from a two-pronged attack on the disease process may be greater than with anti-TNFα alone. It is conceivable that long-term remission may be induced in a proportion of patients with combination therapy. Because cyclosporin A is moderately useful in itself (Tugwell *et al.*, 1990) and is licensed for use in RA in a

number of countries, synergistic therapy of anti-TNFα antibody and a nontoxic, low dose of cyclosporin A is possible in the near future.

VII. Concluding Remarks

In this review, we have attempted to summarize the rationale for anti-TNFα antibody therapy and its clinical effects, which have been remarkably reproducible, albeit temporary. With this resource of clinical samples and observations on rheumatoid arthritis patients who transiently but markedly improve, we have had a novel opportunity to unravel the pathogenesis of RA. Increasing our understanding of the "mechanisms of action" of this intervention will aid the next step—defining refinements that permit long-term control of disease.

Like all new therapeutic ventures, much remains to be learned about the optimal use of anti-TNFα therapy and also about its limitations and possible pitfalls. To date, the results in RA are highly encouraging, and the benefits of anti-TNFα therapy have rapidly been extended to Crohn's disease. It is likely that a number of other diseases, such as ulcerative colitis and multiple sclerosis, will also be helped by anti-TNFα therapy, although benefit has not yet been seen in the clinical trials of anti-TNFα in sepsis.

The validation of our proposal that TNFα is a good therapeutic target in RA is in one way merely the start of a long road of discovery and clinical development. Although there is a marked degree of benefit in patients with long-standing disease, and a very high frequency of responders to adequate doses, these responses are all transient. Clinical benefit is repeatedly observed during subsequent infusions leading to the conclusion that, unlike cancer, the molecular mechanism of RA, i.e., its TNFα dependence, does not alter. A number of approaches can be envisaged to attempt to obtain longer term effects, remissions, and, even in due course, some cures. For example, we think that combination therapy may be a useful approach, and our current favorite hypothesis, based on its efficacy in animal models, is to block TNFα and T cell function concomitantly.

The effectiveness of anti-TNFα therapy in RA will also be helpful in refining the design of clinical trials, in defining and validating criteria of benefit, and in developing surrogate markers for clinical efficacy. The trials of anti-TNFα have clearly shown that even complex multifactorial diseases, refractory to conventional therapy, may succumb to new approaches. This encouragement has stimulated the reevaluation of mechanisms of action of existing drugs and the search for new drugs that mimic the specificity and efficacy of anti-TNF. It is clear that the anticytokine field offers an enormous opportunity for developing research programs in which basic,

strategic, and clinical research interact effectively to yield improved clinical benefits for many crippling diseases.

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