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The Cell Biology of Antigen Processing

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ABSTRACT: T lymphocytes recognize antigen only after a series of intracellular events known as antigen processing. The result of antigen processing is the production of short segments of the primary peptide sequence bound to a polypeptide-binding groove on major histocompatibility complex (MHC) molecules. Antigen originates from one of two sites: intracellular or extracellular. There are two corresponding pathways for antigen processing and two corresponding classes of MHC molecule. Analysis of each pathway has demonstrated that their separation is not purely anatomical, but is maintained by molecular interactions with other molecules. Antigen processing has been shown to regulate the overall immune response, but the mechanisms involved remain obscure.

KEY WORDS: major histocompatibility complex (MHC), intracellular traffic, proteinase, T cell epitope, epitope hierarchy.

I. INTRODUCTION

The primary and unique property of the vertebrate immune system lies in its ability to distinguish between closely related, but different molecular structures. It is this recognition event that enables it to respond to the presence of any "foreign" matter that may penetrate the body, but at the same time ignore the multitude of "host" molecules with which it is constantly in contact. A striking feature of this recognition system is that it operates at two quite different levels (Figure 1). The adaptive (antigen-specific, memory forming) immune system, has two arms: humoral and cellular, consisting of B and T lymphocytes, respectively. The humoral immune system can recognize with extreme specificity the external three-dimensional configuration of almost any molecular species with which it is confronted. Antibody, derived from B lymphocytes, recognizes parts of the specific conformation of an antigen molecule, defined as a B cell epitope. The epitope is sensitive to small changes in the outer "accessible" surface of macromolecular antigens, even when the primary structure or chemical composition remains unchanged. In contrast, T cells, which make up the cellular immune system, have evolved a separate mechanism that recognizes the primary protein structure. Most T cell epitopes are not found in proteins in their natural state, and must be actively created, either during protein synthesis (i.e., before the molecule has folded into its correct final shape) or by a reductive process that involves some form of protein denaturation. T cell epitopes are therefore independent of the native conformation (secondary and tertiary structure), and this second form of recognition inevitably has less discriminatory power than B cell epitopes. In return, however, it exposes to the immune system a set of structures that are normally buried within the native conformation of macromolecules and, therefore, would otherwise be invisible to immune recognition. This double ability to recognize both internal structure and external configuration is integrated into an overall unitary system.

The second major difference between the two arms of the adaptive immune system is that T cell recognition does not occur with antigen alone. The two molecules, T cell receptor and epitope, only interact on the surface of a separate cell,

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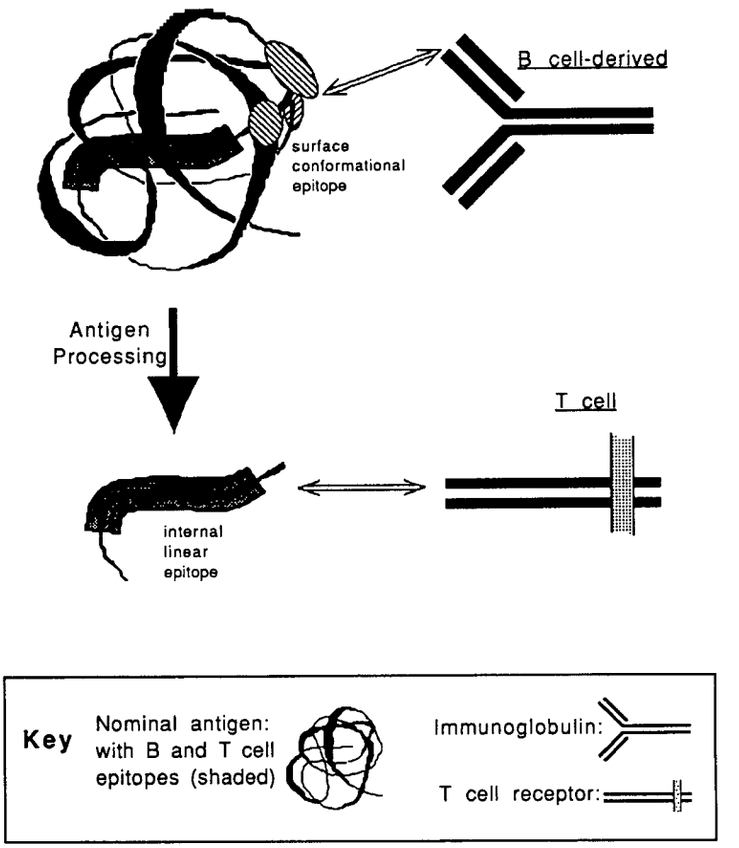

FIGURE 1. The two arms of the adaptive immune system recognize antigen at different levels. Antibody, produced by B cells, recognizes predominantly conformational epitopes on the surface of antigen molecules. T cells, via their antigen-specific receptor, recognize linear sequences of peptide, often buried within the antigen molecule.

the antigen presenting cell, and require this cell to express a third molecule, either class I or class II of the major histocompatibility complex (MHC). These cell-surface glycoproteins have been shown to directly bind T cell epitopes. This binding step is the essential screening process by which appropriately processed antigen is selected from the unprocessed majority (Figure 2). Although outside the scope of this review, overwhelming evidence exists that correct T cell recognition can only occur if the appropriate antigen epitope is positioned within a relatively small molecular groove at the distal end of a MHC molecule. The constraints of this trimolecular interaction ensure that intact, unprocessed molecules cannot normally be recognized by the T cell compartment of the immune system.

The molecular and cellular mechanisms by which T cell epitopes are created from complex macromolecules, and are then expressed by antigen presenting cells in a form that can be recognized together constitute antigen processing. In contrast, antigen presentation is defined as the subsequent interaction between antigen presenting cell and T cell in the presence of antigen. Therefore, antigen processing is a crucial step in the overall series of events leading to T cell stimulation, and hence to an effective immunological response. A highly simplified overview of the major phases of antigen processing is given by Figure 3. This discussion focuses on four separate stages in antigen processing: (1) entry into the processing pathway, (2) intracellular processing pathways, (3) enzymatic mechanisms in pro-

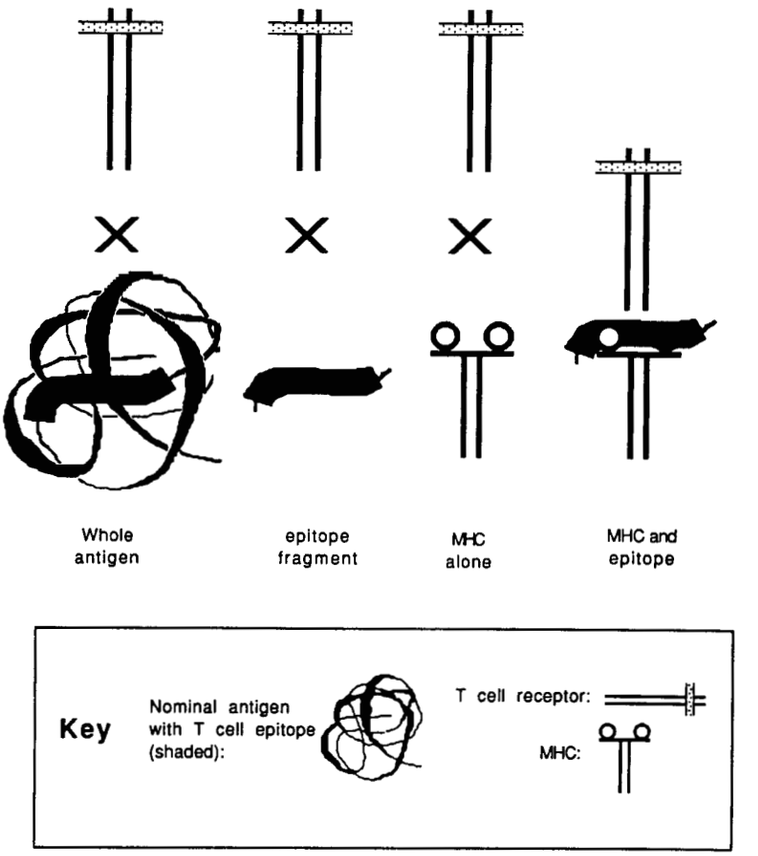

FIGURE 2. T cell recognition of protein antigen requires the antigen to be both in a processed form and presented by a MHC molecule. Thus, T cells do not usually recognize whole antigens, processed epitopes, or MHC molecules individually. Recognition requires fragmentation followed by binding to MHC molecules. Via this mechanism, T cells can limit recognition to processed antigen.

cessing, and (4) antigen/MHC binding. At many stages the discussion of processing is divided into halves corresponding to the two possible points at which antigen can enter the processing pathway (see below). Also discussed is the influence of the antigen processing mechanisms on functional aspects of the T cell response, in particular on the unresolved question of what determines which regions within a protein molecule elicit the major responses (hierarchy). Many of the issues are discussed in the contributions to the 1989 volumes of *Immunological Reviews* (Vol. 106) and *Cold Spring Harbor Symposia* (Vol. 54). In addition, the 1991 issue of *Annual Reviews of Immunology* contains a review of this issue.

A. Two Pathways of Antigen Processing

The presence of two separate classes of MHC molecule on the cell surface has long been known.[1,2] It is also well established that this class division is related to the separation of mature T lymphocytes into two distinct groups. The mutually exclusive T cell surface antigens CD8 and CD4 provide specificity for classes I and II, respectively.[3] In contrast, it has been shown that antigen-specific T cell receptors use the same pool of germline elements for recognition of antigen bound to both classes of MHC.[4]

Although immunologically distinct, the two classes of MHC were not assigned distinct pro-

I	II	III	IV
Entry into processing pathway	Processing Pathway	Processing events	MHC binding
Antigen uptake (extracellular)	Endocytosis	Proteolysis, other?	Low affinity, but peptide selective
Antigen synthesis (intracellular)	Export via Golgi		

FIGURE 3. The major stages of antigen processing. Note the existence of two pathways of processing, one for endogenous and one for exogenous antigens.

cessing pathways until quite recently. A series of studies, carried out predominantly on the recognition of viral antigens has demonstrated convincingly that two pathways of antigen processing exist, and that each pathway is associated with a separate class of MHC.[5,6] The pathways correspond to the two possible points of entry into the processing pathway shown in Figure 3. Thus, class I-associated antigens derive predominantly from "internal" antigens synthesized endogenously by the antigen presenting cell, while class II-associated antigens are "external", acquired exogenously by endocytosis from the extracellular environment. While the general principle of this dichotomy in processing is well established,[7,8] it is clear that a number of exceptions exist.[9-14] These are discussed below, in the context of the cellular pathways for class I and class II MHC processing, and the molecular mechanisms whereby each pathway is kept separate from the other.

The cellular distribution of the two MHC classes reflects the function of the relevant subset. MHC class I is found on all nucleated cells. Therefore, via the class I processing pathway, the immune system can "see" and react to the whole array of intracellular antigens that, in their native state, are never exposed to the extracellular environment. This is consistent with the function of CD8+ T cells, to scan universally for parasitized or neoplastic cells, and it has major implications in the fields of antiviral, tumor, and transplantation immunity.

MHC class II is found mainly on specialized cells within the immune system, in particular B cells, macrophages, and dendritic cells. In addition, it is expressed in certain epithelia and by abnormally activated cells of many types.[14a] This is in keeping with the helper activity of CD4+ cells that act as immune regulators, and so need to have restricted interactions, mainly with other immune cells. More importantly, all cell types expressing MHC class II, including experimentally transfected nonimmune cells,[15] show similar (though not always identical) antigen processing capabilities. The mechanisms of antigen processing for both class I and class II are therefore likely to be ubiquitous and to form part of the basic cellular machinery of nucleated cells.

B. Which Antigens Need Processing?

An essential function of antigen processing is to bring about the interaction between antigenic epitopes and a specific binding site on MHC molecules. Since the dimensions of this binding site are 2.5 nm long × 1.1 nm deep × 1 nm wide,[16] the majority of intact protein antigens are effectively excluded. However, no obvious correlation exists between antigen size and processing requirement, as illustrated in Figure 4, which lists some of the antigens whose processing requirements have been studied. Thus, fibrinogen (MW 340,000) does not require processing,[18] but can be presented intact, while some small proteins[19,20] and even some peptides apparently require processing.[22] These data can best be interpreted according to a model that requires the destruction of a sufficient amount of the secondary or tertiary

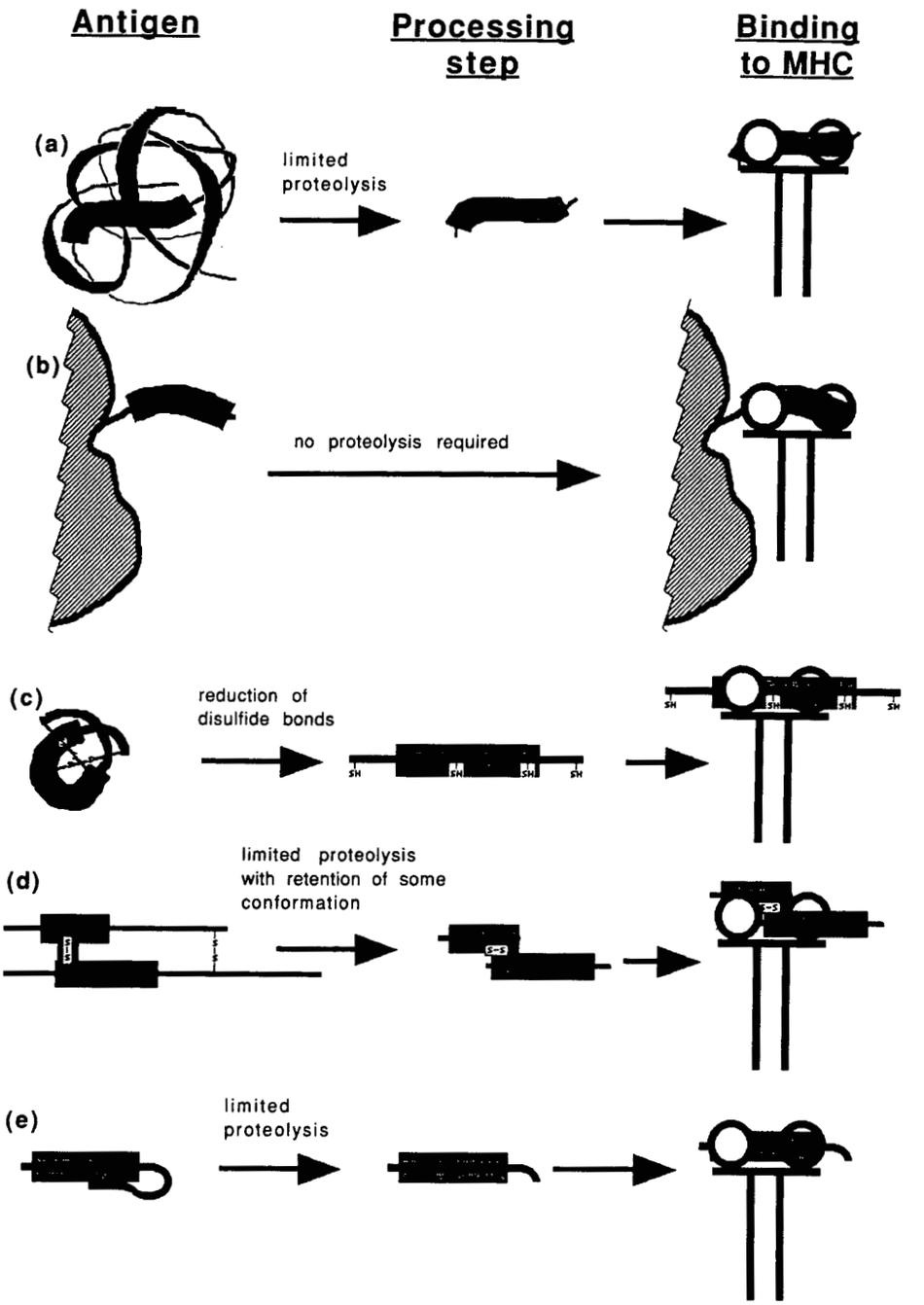

FIGURE 4. The need for antigen processing by different antigens is not dependent on size alone. (a) Large protein molecules that require processing (e.g., ovalbumin)[17]; (b) large protein molecules that do not require processing (e.g., fibrinogen);[18] (c) small protein molecules that require processing (e.g., apamin);[19] (d) protein antigens that retain some tertiary structure after processing (e.g., insulin,[20] hemagglutinin[21]); and (e) small peptide antigens that may nevertheless retain "hindering" structures that interfere with processing.[22]

structure of a protein to allow the particular sequence containing the epitope to adopt the conformation necessary to fit within the MHC binding site. In some proteins (e.g., fibrinogen) such sequences with little native structure already exist. In others, small proteins are tightly held in particular conformations by covalent crosslinks and require modification to allow MHC interaction. In some cases certain elements of native conformation are retained after processing and form part of the structure that interacts with the MHC binding site effectively.[21,23] In such antigens the total destruction of structure can result in the loss of antigenicity.[20,21,24] Such structure dependent (or conformational) epitopes are rare in T cell recognition, but evidence is increasing for their existence. In conclusion, the extent of, and requirement for, processing is determined not by an end point of size, but rather by an end point dependent on conformation. Consequently (discussed further below), processing neither requires nor involves the total fragmentation of an antigen.

II. THE PROCESSING PATHWAY FOR CLASS II MHC AND ITS ASSOCIATED ANTIGENS

A. Synthesis of Class II MHC

Class II MHC is expressed on the cell surface as a heterodimer of α- and β-glycoprotein chains, with molecular weights of 33 and 28 kDa, respectively. The class II genes are expressed together with a third nonpolymorphic protein called the invariant chain (Ii) or MHC class IIτ, which is encoded separately. As discussed below, the function of the invariant chain is the source of much speculation. All class II genes including invariant chain are coregulated, for example, showing the same pattern of upregulation by interferon-τ(IFNτ).[25]

B. Export

After synthesis, the α and β chains form a heterotrimer with Ii in the endoplasmic reticulum (ER). Ii is normally synthesized in greater numbers than the other chains.[26] The excess Ii.s forms homotrimers that are sequestered in the ER.[27] Within 30 min of formation the α/β/Ii heterotrimers are transported through the Golgi to the trans-Golgi network (TGN). Here, unlike class I, which continues rapidly to the cell surface, class II molecules reside for 2 to 3 h before they exit.[28,29] Immunoelectron microscopy has shown that class II is concentrated within the TGN in areas devoid of class I, but no mechanism for this subcompartmentalization has been found. Replacement of the short cytoplasmic tails by those of MHC class I does not affect function,[30] and complete absence of the α chain cytoplasmic domain makes little difference.[31] Therefore, routing to a peptide loading compartment appears to be controlled by the extracellular domains. It has been suggested that one role of the invariant chain may be to direct class II traffic into the correct compartment for peptide binding. Recent studies have shown that the cytoplasmic domain of the invariant chain contains a number of targeting signals that control the intracellular distribution of α and β chains.[31a,31b] In contrast, fibroblast transfectants have similar MHC II traffic with and without the cotransfected invariant chain.[32] Also, a mutant with defective antigen processing and folding of MHC II showed an apparently unaffected relationship between Ii and α/β.[33] Thus, a consensus view at present is that the invariant chain regulates MHC II traffic, but that its function is not an absolute requirement.

When class II is finally exported to the cell surface it is associated with only small amounts of Ii.[29] The degradation of Ii from α/β occurs stepwise, with dissociation of Ii occurring after the polypeptide has been considerably reduced in size. The breakdown and loss of Ii is postulated to be relevant to the binding of antigenic fragments to α/β (see the section following, "Invariant Chain and Peptide Loading"), and so occur after the intersection between endocytic and exocytic pathway, either in the class II-rich parts of the TGN or further in the exocytic pathway. Two pH-dependent phenomena have been described in the export of class II, both of which are thought to occur in the mildly acidic TGN: (1) the sialylation of Ii, which affects its function, is inhibited by monensin,[26] and (2) one of the steps in the degradation and dissociation of Ii is chloroquine sensitive.[29a]

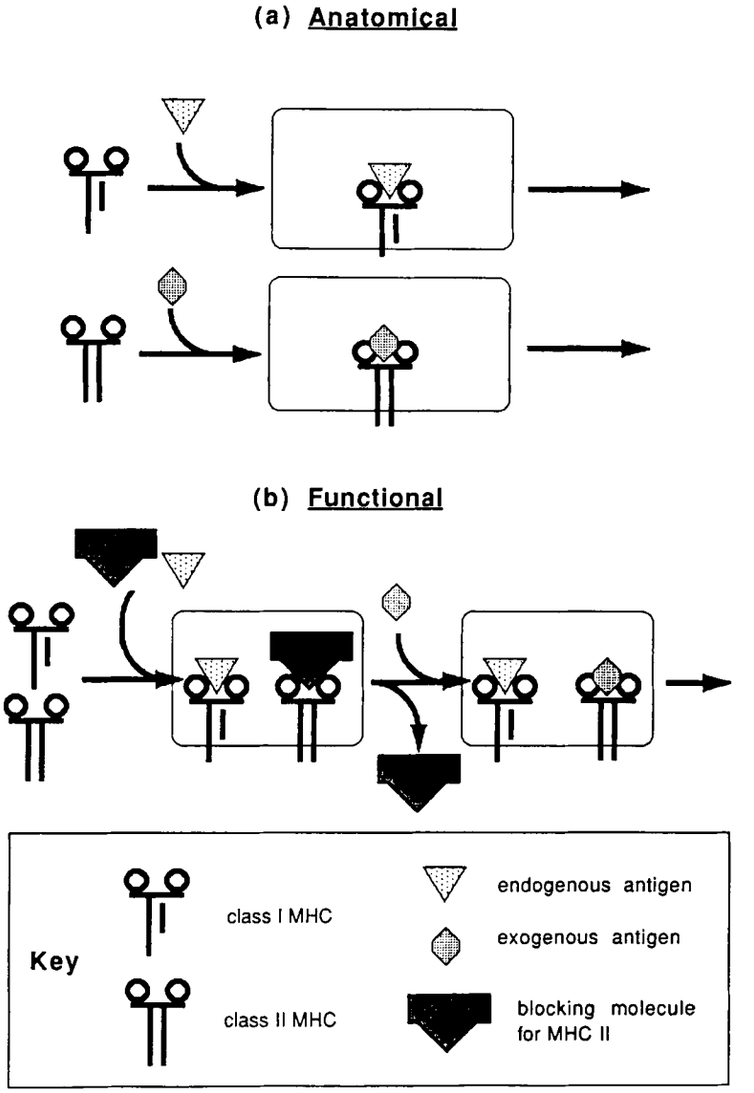

FIGURE 5. The separation of the antigen processing pathways for MHC class I and class II may be achieved either (a) anatomically or (b) functionally. (a) The two classes of MHC molecules traffic through different compartments, and thereby are exposed to different sets of processed antigen; (b) MHC molecules of both classes traffic through the same compartment, but specialized molecules block peptide loading of one class.

C. Invariant Chain and Peptide Loading

The picture described above of MHC class II in the exocytic pathway has led to the hypothesis that Ii prevents endogenous peptides binding in the ER and Golgi.[34] This would make Ii a major factor in maintaining the division between the two pathways of antigen processing for class I and II — a functional rather than anatomical separation (see Figure 5). The discussion centers on the molecular mechanisms that underlie the reaction:

$$\alpha/\beta/\text{Ii} + \text{peptide} \longrightarrow \alpha/\beta/\text{peptide} + \text{degraded-Ii}$$

The function of Ii has been studied in transfected cell lines containing α and β chains with or with-

out cotransfected Ii.[32,35] The authors overall interpretation is that invariant chain is required for normal processing. Without it, exogenous peptides and endogenously synthesized processed antigens can be presented, but the normal chloroquine sensitive processing of whole exogenous antigens is diminished. However, contradictory results have been obtained from different cells. One reason for this apparent heterogeneity is that there may be other unknown molecules that control peptide loading (see below), whose presence cannot be controlled for, especially in cells that do not express the usual transcription factors for class II expression.

Techniques that study the direct binding of peptide to MHC in suspension[36] have been applied to class II molecules containing Ii.[37] α/β/Ii trimers after a long incubation (15 h) with specific peptide showed an affinity about 30-fold less than α/β dimers in the absence of Ii. Interestingly, a subpopulation of the trimers spontaneously lost Ii and had almost 100% peptide occupancy. On- and off-rates of binding to the trimers were not estimated, so that the lower affinity of trimers for peptide may be due not to a reduced on-rate but to a much faster off-rate than for dimers where the half-time is >40 h (see Peptide/MHC Binding). This raises the possibility of peptide binding (and exchange) to α/β/Ii during transit from ER to TGN. The data go some way to supporting the claim that class II trimers do not bind peptide. This may occur by direct binding of a free loop of Ii in or near to the groove, described as a "fig leaf" protecting the binding site from occupation. However, there is no evidence that this occurs. An alternative idea is that Ii holds the α/β dimer in a nonbinding conformation.

Altered conformation of MHC molecules associated with changes in antigen processing ability have been documented both with respect to Ii and independently. In one study MHC molecules synthesized in the absence of Ii were not recognized by a number of monoclonal antibodies that recognize class II determinants on normal presenting cells.[38] Presentation of peptides in this case was in fact enhanced in the absence of Ii. Other experiments have detected a group of antigen processing defective mutants that have unaffected class II genes, and in which class II molecules are expressed at normal levels but with altered conformation.[33] This was associated with a decreased stability of the α/β dimer in the presence of mild detergent. In this case the kinetics of invariant chain association with α/β were unaffected. Both sets of studies point to a close correlation between peptide loading and MHC conformation (as in MHC class I, see below). Additionally, the processing mutants point to a role for another as yet unidentified molecule in peptide loading. A potential candidate is a heat shock or related protein, one of which, HSP-70, is coded for in the MHC region. These molecules are thought to assist in the acquisition of conformation by correct folding. Specifically, a HSP-70-like protein able to bind peptide has been found in antigen presenting cells, and which may be important for the processing of intact antigen.[39] A possible sequence of events is suggested by the data from *in vitro* binding of peptide to α/β/Ii (see above). Given the high percentage of trimers spontaneously losing Ii that were then found to be bound to peptide, it is possible that both Ii degradation and the control of conformation occur after, and may be driven by, the interaction of peptide with class II.

D. Class II MHC and Endogenous Antigens

Contrary to the straightforward two pathway model there are examples in which endogenous antigens are presented by class II MHC and active CD4+ T cells.[11,12,32] Endogenous plasma membrane and endosomal and lysosomal molecules are likely to be processed for both class I and class II. However, there are some endogenous cytosolic molecules that are presented by class II in a Brefeldin A sensitive, chloroquine-resistant fashion. Since Ii is not an absolute inhibitor of peptide binding, circumstances are envisaged under which an endogenous peptide can load enough class II in the early synthetic pathway to survive transport through the exocytic pathway and stay bound until Ii is lost. A second form of endogenous presentation by class II has been shown to be chloroquine sensitive and Brefeldin A insensitive. Endogenous molecules may also be presented on class II after autophagy, a well-

established route by which cytosolic proteins are included into autophagosomes that then fuse with lysosomes or endosomes.[41] This could explain the occasional discovery of chloroquine sensitive endogenous antigen processing.[11]

E. The Endocytic Pathway

Significant advances have been made over recent years in the understanding of the endocytic pathways relevant to the processing of exogenous antigen (reviewed in Reference 42). For this reason a brief outline is presented of the consensus view of the endocytic pathway, particularly in its relationship to antigen uptake. Exogenous antigen enters a cell via either endocytosis (pinocytosis) or phagocytosis. The intracellular pathway has been separated into the various distinct organelles that exogenous material enters. There is one major route of entry, after which incoming material proceeds to a number of destinations. This divergence is brought about by a series of sorting steps, which are discussed in turn.

1. Antigen Uptake

Initial uptake is functionally divided into receptor-mediated and fluid phases. Both phases gain access to the cell via clathrin-coated vesicles, which invaginate from the plasma membrane via clathrin-coated pits.[43] Receptors that require internalization to carry out their function, for example, transferrin receptor (TfR) and low-density lipoprotein (LDL) receptor, are endocytosed at the highest rates. This is achieved by direct interaction with endocytic adaptor proteins (adaptins), and hence indirectly with clathrin, thus concentrating the receptor into incoming vesicles.[43] A consensus sequence has been identified within the cytoplasmic tail of all such receptors, consisting of ten residues, including a tyrosine.[44] For antigen presenting cells there is specialized use of the receptor-mediated pathway. B cells express antigen-specific membrane immunoglobulin (mIg), which has implications for specific B-T interaction.[45] B cells share with macrophages the nonspecific Fc receptor that takes up immune complexes.[46] Macrophages also have complement receptors for which a role in endocytosis has been indicated.[47] Uptake of antigen can also be enhanced by targeting to any other selectively endocytosed receptor, including TfR.[45]

In antigen presenting cells, as in all cells, nonspecific uptake of exogenous molecules occurs as a mixture of membrane adsorption and fluid phase endocytosis, concepts representing extreme stickiness and nonstickiness, respectively. The rate of uptake, much less than receptor-mediated endocytosis at physiological concentrations, is enhanced by favoring membrane adsorption, for example, by increased hydrophobicity. There is evidence for a second route of entry[48] other than via clathrin-coated pits/vesicles, but its normal role remains unclear.

2. Early Endosome

The structures involved in the early part of the endocytic pathway are illustrated in Figure 6. Clathrin-coated vesicles rapidly uncoat and fuse in the peripheral cytosol with early endosomes. These structures, as identified by electron microscopy, are a mixture of tubulocisternal and vesicular elements.[49,50] The cisterna is characteristically folded into the shape of a cup, diameter 1 to 2 μm, open to the cytosol; the function of this semi-enclosed cytoplasmic space is unknown. Tubular extensions 30 to 50 nm in diameter have been seen to extend some distance from the main structure and are the proposed site for molecules recycling back to the cell surface.[51] The vesicular portion is 300 to 500 nm in diameter and contains small membrane vesicles. For this reason these structures have been named multivesicular bodies (MVBs). These may be the source of the carrier vesicles between early and late endosomes.[50] The total surface area of the early endosome is at least 20% of the plasma membrane.[49]

The early endosome has no specific markers, although a number of as yet undefined proteins are uniquely found there.[52] Some cell functions are carried out within the early endosome, such as the removal of iron from transferrin. The most important function of the early endosome appears to be the provision of an internal milieu for the rapid sorting of endocytosed material,[53] either for

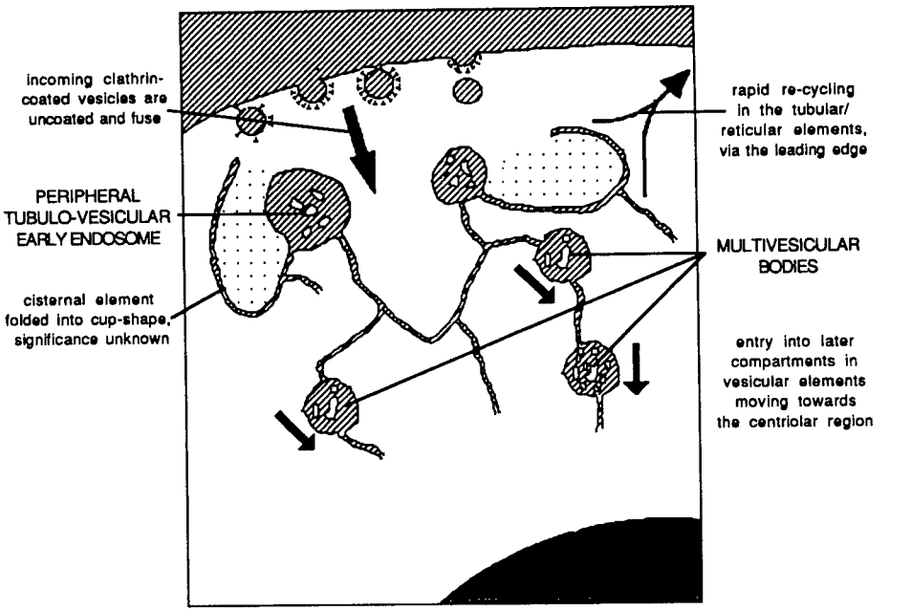

FIGURE 6. The early endosomal pathway, showing both the peripheral tubulovesicular elements and the endosomal reticulum, including multivesicular bodies.

recycling (exocytosis) to the cell surface with an average transit time of <3 to 10 min,[52,54] or for entry into the next endocytic compartment (the late endosome). Acidification is an important component of the endosomal environment.[55] At 6.0 to 6.2 the pH is higher than that of the late endosome and lysosome. This has been attributed to a partial inhibition of the endosomal/lysosomal proton-ATPase by an opposing charge gradient created by sodium-potassium ATPase.[56] Inhibition of acidification leads to failure of recycling with continued uptake. The sorting mechanism in the early endosome is highly efficient. For example, the fraction of molecules routed to the late endosome is 0.5% for TfR and many other membrane molecules,[57] compared with up to 100% for some aggregated molecules.[46,58] The fraction of fluid phase contents directed to the late endosome is 5 to 25%.[54,178] No specific signal sequence has been identified among those membrane molecules directed into the late endosome, but many are polymeric.[58]

Recent evidence on the nature of the early endosomal sorting mechanism has been provided by video microscopy.[59] A network of thin strands forms an "endosomal reticulum" labile to most fixation techniques and present throughout the cytoplasm. The reticulum contains molecules to be rapidly recycled, flowing at 0.5 to 1.0 μm/s in apparently random directions. Recycling of the bulk of TfR occurs from the leading edge of cells,[179] which is often close to the TGN and the bulk export pathway. Varicosities are seen in the network, which consists of MVBs. The extensive inner membranes of the MVBs contain an excess of molecules to be directed to the late endosome. They move slowly (approximately 0.05 μm/s) toward the pericentriolar area. Therefore, sorting within the early endosome appears to occur within, or close to, the MVBs.

3. Late Endosome

The pathway exogenous antigens take before processing and presentation involves routing via the late endosome. Various definitions of this compartment have been made, and between them these definitions demonstrate much of what is known. The late endosome remains obscure because it lacks a specific marker, making biochemical analysis difficult.[52] Morphological studies show an accumulation of markers in the late endosomes after 15 min or more.[49] They are

large, pericentriolar, reticular bodies containing internal membranes. Late can also be distinguished from early endosomes due to the delivery of lysosomal contents.[60] Both the specific transmembrane proteins (e.g., lgp120) and the hydrolases (acid phosphatase, cathepsin D, etc.), together with mannose-6-phosphate receptors (MPR), which deliver them, appear in the endocytic pathway simultaneously at the late endosome stage. Whereas lgp120 is routed to lysosomes, MPR recycles back to the TGN. Therefore, the only compartment on the endocytic pathway that is MPR positive is the late endosome. This fact has provided the best working definition of the late endosome to date. Recent immunoelectron microscopic findings showed that parts of the late endosome are located near the Golgi and TGN.

Other features of the late endosome include a pH estimated at 5.0 to 5.5, which is marginally less acidic than the lysosome at 4.8 to 5.0.[52,56] A distinguishing feature of its function is the complete block of traffic through the late endosome by temperatures below 18 to 20°C.[50,60] Entry into MVBs still occurs, so the early endosomal sorting mechanism is not the site of the block. This is one of many intracellular processes that has this temperature cut-off and so is not the most ideal experimental tool. Kinetic analysis of fluid phase transit through late endosomes of macrophages[54] and other antigen presenting cells (unpublished observations) has been estimated to be 4 h, assuming equivalence throughout the late endosome and first-order kinetics for exocytosis. In fibroblasts the turnover was slower — 10 to 15 h. Macrophages have been found to have a relatively large late endosome.[49] This is consistent with the major proportion of the uptake of these cells occurring via phagocytosis delivering exogenous molecules directly to the late endosome.

4. Lysosomes

The original considerations of antigen presenting cell function envisaged routing of antigen via lysosomes where processing would occur.[61] There are two reasons that this idea has fallen from favor in recent years: (1) many of the probable processing enzymes have been found in the prelysosomal portion of the endocytic pathway;[62,63] and (2) the limited degree to which antigens need to be modified prior to binding to their restriction element suggests that excessive proteolysis would occur in lysosomes, destroying T cell epitopes. However, with increasing understanding of the biogenesis of lysosomes it is becoming clear that they represent a range of structures. Except for the residual bodies, which form from lysosomes and contain nondegradable material, they are in apparent dynamic equilibrium with each other[64] and with the endosomes, inasmuch as exit from them and reentry into the endocytic pathway can occur.[65] The minimum time in which antigen processing and presentation occur (45 to 60 min) could therefore involve relatively rapid cycling through the lysosome with limited proteolysis, but would not require long-term lysosomal residence.

5. The Exocytic Pathway

It has been demonstrated biochemically and morphologically that certain plasma membrane proteins, for example, TfR, return to the TGN (i.e., the exocytic pathway) while recycling back to the membrane.[28,66,67] This pathway has been demonstrated as an intersection between endocytic and MHC class II positive compartments, and may be a novel pathway with particular relevance to antigen processing. However, the general significance of the TGN in endocytosis is not clear.[68] Many endocytosed molecules, including the fluid-phase endocytic marker horseradish peroxidase, do not enter the TGN even after a long period of incubation. The close proximity of late endosome and TGN and their exit pathways in the same pole of the cell may facilitate selective traffic in the absence of free exchange of contents.

F. Class II MHC in the Endocytic Pathway

1. Newly Synthesized MHC Class II

The presence of MHC class II together with the invariant chain in compartments accessible to

endocytic tracers has been known for some time.[66] The cross-sectional site between the endocytic and exocytic pathways was assumed to be the TGN. Recent studies in both macrophages and B cells have used immunoelectron microscopy to visualize compartments positive for the markers of processing: class II, Ii, low pH (using DAMP), acid cathepsins, endocytic tracers, and endocytic adaptins.[28,69,70] Reservations about these studies are inevitable given that T cell activation only requires a fraction of the class II of an antigen presenting cell to be occupied by a determinant, and that the exact site where these are processed could be easily overshadowed by irrelevant compartments. However, these data are considered the best guess until the important class II/peptide conjugates can be identified.

These studies have demonstrated Ii together with α/β in both early and late endosomes. The presence of Ii is particularly significant, as it marks a MHC molecule as newly synthesized, and suggests that class II still bound to Ii can pass from the TGN to the endocytic pathway. Certain acid cathepsins were also found to be present throughout the endosomal system. Thus, while these studies do not exclude a role for the TGN as an important site of antigen processing, they suggest that both processing and MHC peptide binding also could occur in earlier compartments of the endocytic pathway.

2. Recycling Class II and Reuse

In B cell lines the recycling of MHC class II from the cell surface into internal compartments has been demonstrated to occur within a small early endosomal pool that turns over very rapidly.[71] A large proportion of the surface MHC is available to enter this pathway. This recycling pathway has been demonstrated only recently with newer, more sensitive techniques[28,72] that have yet to be applied to class II recycling in macrophages.[73]

The more pressing question that needs to be addressed is whether the recycling class II molecules are reused by more than one peptide. This has been considered important in terms of the flexibility of antigen presenting cell function in the face of changing exogenous antigens. Class II-restricted presentation in the presence of the inhibitors cycloheximide and Brefeldin A has demonstrated heterogeneity in the ability of different antigen presenting cells to present new exogenous antigen without new synthesis or export, respectively.[73,74a] In general, processing by B cells appears to be more resistant to these reagents than macrophages, although there are discrepancies in data from different laboratories. The reuse of MHC is often given as an explanation of the continuing ability of B cells to present, but this interpretation fails to take into account the possible presence of a large intracellular pool of MHC trimers (i.e., including invariant chain) distal to the action of these two inhibitors.

Further support for MHC reuse has been deduced from the demonstration that functional peptide/MHC complexes are lost much more quickly from the surface of B cells than can be explained by the overall turnover of MHC class II.[74] These results are interpreted in terms of a model in which antigenic peptide is replaced by endogenous or serum-derived antigens. An alternative explanation of peptide/MHC complex loss is that selected complexes may be preferentially lost from the plasma membrane/early endosomal pool and even degraded.[76] This may be especially so for short-length peptides, including most of those used experimentally. In contrast, peptides derived for processing *in vivo* may be quite large when they initially bind to MHC, to be digested by exopeptidases while in the plasma membrane/early endosome recycling pool.

A third experimental system that purports to demonstrate MHC reuse is the inhibition of presentation of one peptide that occurs if a competing peptide is fed to live B cells even 6 to 12 h after the removal of the first peptide.[75] Both this and the previous experiment may alternatively be interpreted in terms of the presence of a large pool of intracellular peptide, which binds only slowly to MHC molecules. Thus, peptide competition may appear to occur after binding, but in fact it occurs during the formation of new peptide/MHC complexes.

Finally, more recent studies have provided evidence against a substantial degree of MHC reuse. Human B cells specific for a known antigen (tetanus toxoid) have been studied for reuse of MHC class II. The surface molecules were "labeled" by removing terminal sialic acids, making them detectable by changing lectin af-

finity.[180] Processed fragments retrieved from immunoprecipitated class II were all associated with the sialic acid positive fraction, i.e., with MHC internal to the cell at the beginning of the experiment. Assuming that the desialylation did not affect MHC function, this is good evidence against the reuse of class II by these cells.

The authors' overall conclusion is that MHC class II recycles into endosomes but that reuse has not yet been proven, and may not occur at all. The relevance of class II in the endocytic pathway, rather than reuse per se, is discussed further below.

3. Site of Peptide Loading

The above findings must be seen in light of one of the more difficult questions in antigen processing — what is the relevance of the requirement for 1 h or more after the introduction of exogenous intact antigen, even at very high concentrations, before presentation is detectable?[77] This could reflect a slow rate of antigen processing or, alternatively, the time taken to traffic through the cellular processing pathway. Careful quantitative studies of the kinetics of processing suggest that trafficking contributes substantially to this lag phase. If this interpretation is indeed correct, passage through relatively inaccessible compartments of the cell (e.g., the lysosome or TGN) may be an obligate step in antigen processing. In light of this conclusion, the role of each compartment of the endocytic pathway in peptide loading is considered in turn.

The **plasma membrane** and **early endosome** are, over a 1-h period, in effective continuity with each other because of the turnover time of 5 to 10 min on average, including surface MHC class I and II, as discussed above. They are not therefore involved in the much longer process of antigen processing, but may be the site of both class I and II binding to antigens that do not require processing, such as exogenous peptide, at high concentrations. However, rapid exposure to the peripheral endosomal milieu is insufficient in some cases of peptide loading where entry into the late endosome appears to be required.[76]

The **late endosome** is the most likely candidate, containing all the processing enzymes required as well as a low pH. The most promising factor in its favor is the recent identification of multivesicular bodies near the TGN with late endosomal characteristics, rich in MHC class II, containing some but not all acid hydrolases, and generally slightly more acidic than the late endosome.[70] Traffic to and from this subcompartment would be expected to take roughly 30 min in total. Processing occurring en route and then *in situ* may well be slow enough to account for the overall hour needed. How does this tie in with the concentration of newly synthesized class II in the TGN? There are two possibilities: either there is transport of α/β/Ii from the TGN into the late endosome, or alternatively, the class II-rich areas are in the TGN or an as yet undescribed intermediary, with antigen being transported into it.

Finally, it may be that rapid transit through the **lysosome,** however unfashionable, would be required for adequate processing of some antigens. This coincides with the current view that two-way traffic to and from lysosomes does occur, probably as an expression of lysosomal continuity with the late endosomes.

III. THE PROCESSING PATHWAY FOR CLASS I MHC AND ITS ASSOCIATED ANTIGENS

A. Synthesis and Export of Class I MHC

Class I MHC molecules are expressed at the cell surface of most nucleated cells, as an α chain of 45 kDa, in noncovalent association with the nonpolymorphic molecule β_2-microglobulin (12 kDa). The synthetic pathway for class I MHC molecules is that of a typical membrane glycoprotein. The polymorphic heavy chain and β_2-microglobulin are translocated into the rough ER during synthesis, pass through the Golgi where glycosylation occurs, and are exported to the plasma membrane within approximately 30 min to 1 h.[28] The two chains are believed to interact while still within the endoplasmic reticulum, and both chains are required in order to achieve their final stable conformation.[78] Class I MHC molecules, in common with a number of other molecules, may require the presence of "chaperon" molecules to facilitate correct assembly.[79] In the

absence of these molecules excess peptide, or in certain cases excess β_2-microglobulin,[80] may be able to substitute for their function.

Controversy surrounds the extent to which MHC class I molecules recycle once on the cell surface. In contrast to class II MHC, class I molecules were believed not to recycle extensively, except in certain specific cell types (particularly activated T cells).[81] More careful measurements have suggested that a small proportion of class I molecules are in fact in an endosomal recycling pool, with a very fast half-life (2 to 3 min).[71] The presence of a recycling pathway suggests that MHC molecules could be reused by exchanging one processed antigen for another. However, since the interaction between MHC and processed antigen, at least *in vitro*, is very stable (see below), the biological significance of recycling remains obscure. The issue of recycling has been studied in greater depth for class II molecules (see above).

B. Peptide Loading of Class I MHC

In contrast to the MHC molecules, which are translocated into the ER during synthesis, and which are therefore never present free in the soluble cytosolic compartment, the pathway for antigen that becomes associated with class I molecules starts in the cytoplasm. Antigens that are synthesized in the cytoplasm itself,[6] or soluble antigens that are artificially loaded into the cytoplasmic compartment across the plasma membrane,[82,83] can all be processed and associate with class I MHC molecules. However, in contrast to MHC molecules, antigen does not require conventional leader sequences to mediate transport into the ER.[5] The major hypothesis is that antigen is processed within the cytoplasm and then transported into the ER via a peptide transporter. A candidate gene encoding such a transporter recently was identified (see below).[83a-c] Alternatively, whole antigen may enter the ER via an unknown separate pathway and be processed subsequently by proteinases present within this compartment. Such a mechanism would explain the efficient processing of antigens that are translocated into the ER during synthesis, and are therefore never found in the cytoplasm. The nature of the enzymes involved in processing endogenous antigens remains totally obscure. Although it has been hypothesized that antigen processing may involve ubiquitin-dependent proteolysis,[84] this is unlikely to be the only pathway; cytoplasmic ovalbumin, for example, is processed and presented effectively in association with class I MHC,[82] but is not a substrate of ubiquitin-dependent proteolysis.[85]

Likewise, little direct data exist on the site of peptide/MHC interaction, although this is generally believed to occur within the ER.[86,87] A number of mutants with defects in antigen processing for class I MHC molecules have been described.[78,88,89] It is interesting in this respect that antigen presenting cells with mutant processing pathways for class II MHC have also been identified,[33] and that both types of mutant demonstrate aberrant assembly, rather than alterations in the MHC structural genes. In the class I processing mutants, MHC molecules fail to fold into their normal stable three-dimensional configuration, and are present at only low levels on the cell surface. MHC can be induced to adopt correct stable conformations in the presence of sufficient levels of certain peptides that bind tightly within the antigen binding groove on the molecule.[78,80] On the basis of functional and binding experiments, these peptides are thought to occupy the binding site on the MHC molecule. These data have led to the unexpected conclusion that peptides within the class I MHC binding site play an essential role in inducing the correct folding of the MHC, and/or in stabilizing the final conformation. This model has striking parallels to the older "instructionalist" models of antigen/antibody interaction.[90] In support of this hypothesis, those MHC molecules that are expressed on the cell surface in the mutant cell lines are less temperature stable, and are predominantly empty of peptide.[91,92] The molecular nature of the mutation in one of these cell lines has been determined recently.[83a-c] The gene implicated codes for half a member of the superfamily of ATP-driven transporters. It is found near a second, related gene that may make up the other half of the functional transporter. The sequence resembles those of the multidrug-resistance transporter family, which can translocate peptides, and could therefore deliver antigenic peptides from the cytoplasm into the ER.

Recent studies have begun to characterize the structure of the peptides bound within class I

MHC molecules.[92a-d] Peptides eluted from class I MHC are rather homogeneous in size and quite short (ranging from eight to ten amino acids).[92a] These peptides are selectively bound when present as minor contaminants of longer peptides,[92b] and only the short peptides allow the class I chains to adopt their mature conformation in long-lived complexes.[92c] The peptides appear to share certain structural features that are haplotype specific and believed to be essential for binding to MHC.[92d] These peptides cannot be detected in the absence of MHC expression, suggesting that they represent short-lived degradative intermediates which are stabilized by their interaction with MHC molecules.

C. Separate Processing Pathways for Class I and Class II MHC

The mechanisms by which the processing pathways for MHC class I and II are kept separate are still the central enigma in the field of antigen processing. Studies on the final binding interaction between peptide and MHC molecule have so far not revealed any major distinctions between the binding characteristics of the two classes that would explain this phenomenon (see below). Indeed, it seems unlikely that the distinction depends on intrinsic features of antigen structure, since in a number of cases, the same peptide can interact with both MHC classes.[93-95] Two basic models have been proposed to explain the separation (Figure 5). According to the first (anatomical) model, loading of peptides to each class of MHC occurs in separate locations, from which the other class of MHC is excluded. Thus, loading of class II MHC molecules is likely to occur at an intersection between the paths for endocytosis and synthesis, perhaps a specialized region of the TGN (see section on class II). In contrast, evidence exists that class I MHC molecules are transported to the cell surface via a different route without ever meeting endocytosed material.[28] The different pharmacological characteristics of the two processing pathways likewise argue for physically distinct cellular pathways. Thus, processing for class II,[61] but not class I, MHC[6] molecules is sensitive to the lysosomotrophic bases (chloroquine, primaquine, etc.). In contrast, processing for class I is more rapidly and easily inhibited by Brefeldin A,[11,86,87] an inhibitor that blocks passage through the Golgi network. According to this hypothesis, plasma membrane glycoproteins, some of which recycle extensively via an endocytic route, provide exceptions to the association between endogenous cellular proteins and class I binding.

Although distinct anatomical locations for peptide MHC interaction and separate processing pathways between the cell may explain why class I MHC molecules usually fail to interact with exogenous antigens, this hypothesis cannot explain the complementary exclusion of endogenously synthesized antigens from class II binding. In fact, strong evidence exists that both class II and class I MHC molecules passage through the rough ER and the Golgi, the two most likely sites of class I/antigen interaction. An alternative model postulating the existence of specific regulatory molecules that favor class I, and prevent class II MHC binding in these locations is summarized in Figure 5, and was discussed earlier in the article in the context of class II.

In conclusion, it seems increasingly likely that multiple mechanisms exist to maintain the separation between the two processing pathways, but that nonetheless the division may be less absolute than was first believed.

IV. ENZYMES IN ANTIGEN PROCESSING

The key step in antigen processing is the modification of native antigens into processed forms, which must involve the action of one or more enzymes. So far, no information is available on enzymes in the class I pathway, so this section is restricted to a discussion of processing of exogenous antigens prior to interaction with class II MHC molecules.

A. Proteinases in the Class II MHC Pathway

A number of studies have used a pharmacological approach to identify the nature of these enzymes. Two major classes of compounds interfere with antigen processing, those that disturb the pH gradients within the cell and proteolytic inhibitors (see Table 1). Certain classes of en-

TABLE 1
Selective Inhibition of Antigen Processing by Specific Proteinase Inhibitors

Antigens	Inhibitor	Class of proteinase inhibited	Ref.
Myoglobin	Leupeptin	Cysteine or serine	96
		Cathepsin B?	97
DNP-PLL	ZPADK	Cysteine	98
PPD	pepstatin	Aspartic	
Lysozyme	Leupeptin	Cysteine or serine	99
KLH	Leupeptin	Cysteine or serine	100
	TPCK	Serine	
Ovalbumin	TPCK	Serine	
Ovalbumin	Pepstatin	Aspartic	101, 102
		Cathepsin D?	
		Cathepsin E?	
	E64	Cysteine	
Various	Various	Various	103
Mycobacteria	Leupeptin	Cysteine or serine	104
Hemagglutinin	Leupeptin	Cysteine or serine	105

dopeptidase inhibitors have been shown to block processing of a number of protein antigens without interfering with the presentation of previously formed peptide/MHC complexes to T cells. Conversely, prior proteolytic fragmentation of certain antigens bypasses the requirement for processing.[17] Such antigen fragments can be presented by fixed antigen presenting cells, or by viable presenting cells in the presence of agents that block processing of the intact molecule. These studies suggest that processing consists of the proteolytic fragmentation of antigen. However, direct evidence of this hypothesis, namely, the recovery and identification of processed antigen from class II molecules, has so far been possible in few cases.[106,107] One such study[106] demonstrated that a proteolytic fragment of lysozyme (about 2 to 3 kDa) could indeed be recovered bound to class II MHC molecules from cells that were allowed to process lysozyme over an extended period. More detailed analysis of this fragment to determine possible cleavage sites within the molecule has thus far not been carried out. These studies cannot easily rule out the degradation of antigen occurring during its extraction from cell lysates. Therefore, the size of antigenic fragments at the time they initially bind to MHC remains unknown.

Examination of the data in Table 1 indicates that processing is blocked by two major classes of proteolytic inhibitors, those specific for cysteine and for aspartic proteinases (for a review of the major proteinase classes see Reference 108). Interpretation is sometimes complicated by the use of inhibitors that do not discriminate well between different classes of proteinase (e.g., leupeptin, TPCK). In general, inhibitors are added in high concentrations to the tissue culture medium and are assumed to enter the processing compartments of the cell by endocytosis. However, the effectiveness of the inhibitors in terms of inhibition of enzyme activity or antigen degradation is almost never assessed. Many of the inhibitors used are poorly soluble in aqueous solution, and must be added in small quantities of organic solvent. True concentrations over extended periods of culture are therefore difficult to estimate. Despite these methodological problems, it seems clear that both cysteine and aspartic proteinases may be involved in antigen processing, with the dominant enzyme perhaps being determined by the susceptibility of a particular antigen or epitope[105] to one or another enzyme. An alternative interpretation of some of the heterogeneity in the data has been suggested by more detailed studies on the processing of ovalbumin.[101,102] The presentation of ovalbumin is inhibited by inhibitors of *both* cysteine and

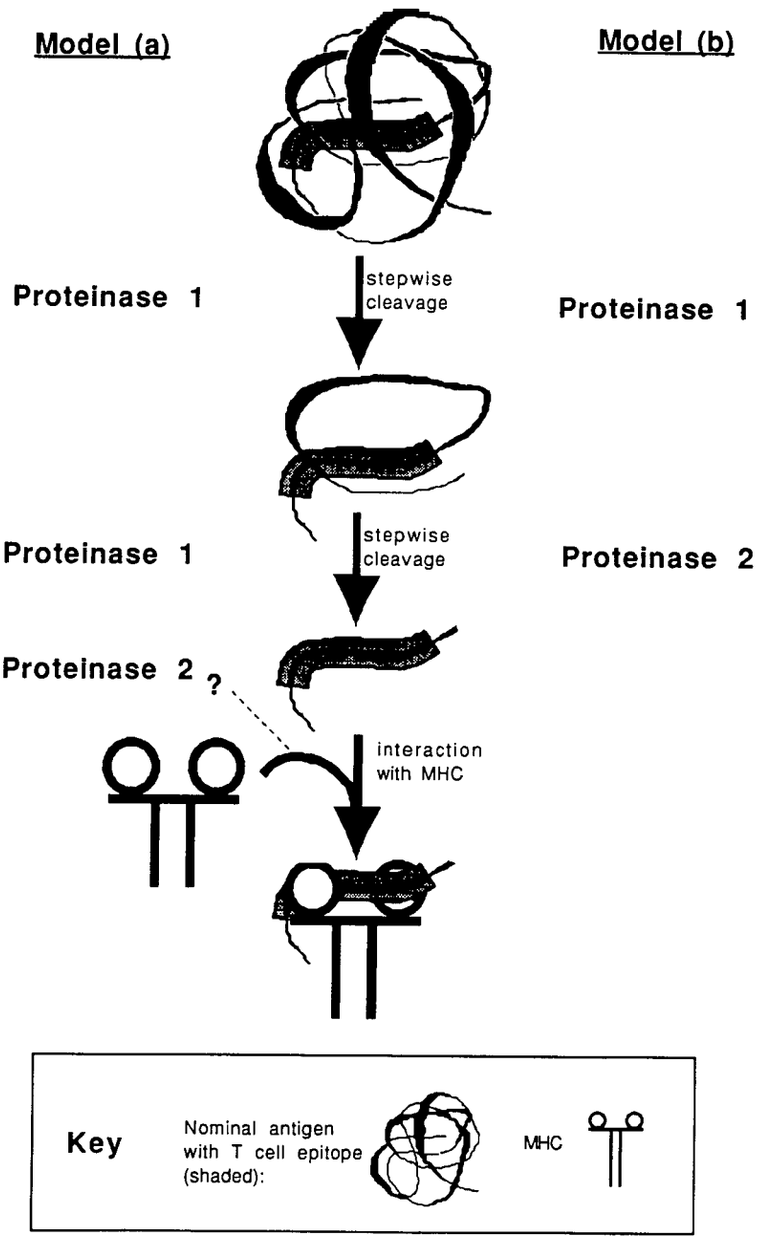

FIGURE 7. Two models for the requirement of two proteinases in the processing of some protein antigens. (a) One proteinase is sufficient to cleave the antigen for presentation, while the other carries out a separate function, such as a stage in MHC maturation. (b) Both proteinases are required sequentially at different steps in antigen cleavage.

aspartic proteinases. As illustrated in Figure 7, this could be explained either by the requirement for two successive steps of proteolysis by two distinct enzymes or by the involvement of one type of proteinase in the fragmentation of antigen and of another class of proteinase in some independent step in the processing pathway. The latter hypothesis is supported by several findings:

1. Cleavage by an aspartic proteinase alone is sufficient to abrogate the processing requirement

2. Processing by a cell-free system is inhibited only by aspartic proteinase inhibitors
3. Data suggest that cysteine proteinase inhibitors may, under certain conditions, also inhibit the presentation of peptide fragments

Although the second step in the processing pathway, which might be sensitive to proteinase inhibitors, has not been definitively identified, one possible target could be the dissociation of the invariant chain (Ii) from α/β class II, which occurs during processing and transport of class II to the cell surface. This step has been shown to involve proteolysis,[29] and may be an essential step in allowing processed antigen to interact with MHC molecules (see above).

Although inhibitors can be used to identify the major classes of proteolytic enzyme involved in antigen processing, they have not given detailed information on the specific enzymes involved. One important factor in predicting whether an enzyme will have a significant role in processing is the intracellular localization of the various enzymes, in terms of the processing pathway. The major degradative enzymes of the cell (the cysteine proteinases cathepsins B, H, and L and the aspartic proteinase cathepsin D) were classically believed to localize to the lysosome, which, as discussed elsewhere, is unlikely to be a major processing site.[109] However, nonlysosomal forms of cathepsin D and some cysteine proteinases have now been identified both by enzymatic function[62,110,111] and by electron microscopy.[69] These forms are associated with plasma membrane or early endosomal compartments of the cell, and are therefore good potential candidates for processing enzymes. Very recently, it was demonstrated that processing of at least one antigen may involve the action of cathepsin E,[181] a non-lysosomal aspartic proteinase localized in tubular/vesicular structures in the cell, whose function was previously unknown.[112-115] At least in the mouse this enzyme is the major aspartic proteinase in lymphocytes, suggesting a role in immunological function. It is, of course, possible that different antigen presenting cell types use different sets of processing enzymes, and examples of functional heterogeneity in processing have been reported.[116,117]

In addition to its intracellular localization, a second feature that will determine the effectiveness of the processing function of an enzyme will be its substrate specificity. In general terms, enzymes with too broad a specificity run the risk of destroying the necessary antigenic epitopes, and such enzymes, if present, may in fact play a regulatory role in limiting the amount of immunogenic material produced during processing (antiprocessing enzymes).[118,119] It has proven very difficult to predict cleavage sites of cellular cathepsins on the sole basis of primary substrate sequence. Furthermore, the vast majority of substrate specificity studies on cellular proteinases have been carried out using short synthetic peptides without secondary/tertiary structural features, and are therefore of relatively little relevance to antigen processing. One study that looked in detail at the nature of cathepsin D cleavage sites within a number of protein antigens[120] found that this enzyme appears to show some preference for cleaving at sites that form amphipathic α-helices (as predicted by primary structure). Such structures are also relatively good predictive features for antigen/MHC binding sites (see below).

Further studies on cleavage specificities of putative processing enzymes, as well as characterization of the cleavage sites of "naturally" processed antigen will be required in order to interpret antigen processing in terms of the enzymology of the processing enzymes involved, and thus arrive at the rules that govern antigen processing at a molecular level.

B. Other Enzymes

If knowledge of the proteinases involved in processing is incomplete, information on other potential processing enzymes is only rudimentary. Although protein unfolding, induced artificially by reduction of disulfide bonds, is sufficient to remove the requirement for processing in some cases,[19,96,121] no evidence exists that such processes occur under physiological conditions, and, indeed, reduction of such bonds can under certain cases also lead to the destruction of T cell epitopes.[20,24] However, certain antigens that are highly resistant to proteolytic degradation may require partial denaturation or modification prior to entering the processing pathway. For example, we have shown that in the case of ovalbumin, processing and degradation can be greatly enhanced by protein chlorination, a form of protein

denaturation that occurs within granulocytes.[182] The inability of lymphoid dendritic presenting cells to process some, but not other, antigens[122] may likewise reflect an absence of some "preprocessing" denaturing pathway (e.g., oxygen radical formation). In such cases, processing can often be restored by the addition of cells with more potent degradative machinery, such as macrophages.[123,124]

While very large or resistant antigens may require additional processing steps, it has also been suggested that quite small antigenic fragments may acquire enhanced immunogenicity by further limited degradation, perhaps through the action of exopeptidase.[22] Such modifications may even occur after portions of the antigen are bound to the MHC binding site, and hence remain protected from further degradation. However, evidence for such "secondary" processing events is indirect, and there is no information on which enzymes might be involved. Similarly, there is no convincing experimental evidence for the occurrence of other forms of posttranslational modification (e.g., removal of sugars, side-chain modification) during processing.

V. INTERACTIONS BETWEEN PEPTIDES AND MHC

The interaction of processed antigen with MHC molecules to form a stable complex is the last step in the antigen processing pathway. Although such interactions had long been postulated and formed an essential element of the "determinant selection" model of antigen presentation,[125] direct evidence proved difficult to obtain. However, the development of an assay for the binding of synthetic peptide fragments to affinity purified MHC class II molecules in a cell free system,[126] and the description of the crystallographic structure of a MHC molecule showing a well-defined "binding site" containing non-MHC material,[16] provided convincing evidence for a physical binding of antigen to MHC. Aspects of this interaction, particularly in relation to cellular localization and intracellular trafficking, were discussed above (class I and class II MHC processing). In this section the concentration is on the biophysical factors that regulate MHC/antigen binding.

A variety of assays that measure "binding" of peptide to MHC molecules (both class I and class II) have now been reported (Table 2). Some, which measure binding on intact cells,[94,133] are of value in terms of screening large numbers of peptides and in terms of understanding the cell biology of binding, yet yield limited information on the more quantitative aspects of binding. Conversely, assays using purified MHC molecules suffer from problems of experimental artifacts (for example, MHC molecules can only be kept in solution in the presence of detergent, and the detergent may affect binding parameters[134]). Not all studies have correlated binding data to functional responses, and the interpretation of binding in such cases is unclear. A more fundamental problem is that in both types of assay, binding has been measured virtually exclusively for short synthetic peptides, rather than antigen processed by physiological routes. Processed antigen may in some cases consist of quite large antigenic fragments whose binding characteristics may be substantially different than those of small peptides. In the case of class I MHC, antigen may interact with and play an active role in the initial folding of MHC molecules, determining their correct configuration (see above). Assays that measure binding of peptides to preformed MHC sites (whether on empty molecules or by exchange with bound peptide) may therefore be of little physiological significance.

A. Peptide Binding and Immunogenicity

Despite these reservations, a number of general features of the interaction have become apparent. Perhaps the most important observation, and usually the only evidence that the observed binding is physiologically relevant, is that binding is antigen selective in the sense that some peptides bind with much higher affinity than others. In practice, binding of most random peptides is below the limits of sensitivity of the assays used, and such peptides are said not to bind the relevant MHC molecule. Peptide binding generally shows good correlation to immunogenicity for T cells of the appropriate MHC restriction, and there are no well-documented cases of peptides that do not bind a particular MHC molecule and yet stimulate a T cell response. The ability

TABLE 2
Physical Measurements of Peptide/MHC Binding

Species	MHC class	Type of assay	Affinity K_D (M^{-1} s^{-1})	Specificity*	Ref.
Mouse	II	Equilibrium dialysis	2×10^{-6}	+ +	126
	II	Gel filtration and equilibrium dialysis	2×10^{-6}	+ +	127
	II	Gel filtration	–	+ –	128
	II	Planar membrane	–	N.T.	129
Human	I	Gel filtration	–	+ –	130
	II	Gel filtration	2×10^{-8}	+ –	36
	I, II	Solid phase	–	—	95
	I	Solid phase	–	—	131
	I	Solid phase	6×10^{-4}	—	132
	II	Live cells	10^{-7}(estimate)	+ –	133
	II, I	Live cells	–	—	94

* + + allotype specific; + – isotype, but not allotype specific; — demonstrate both iso- and allotype crossreactivity.

to bind appropriate MHC molecules is therefore of fundamental importance in determining which epitopes within a protein are immunogenic. However, there are now many examples of peptides that do show good binding to a particular MHC molecule and yet fail to stimulate immunological responses. These examples can be divided into two classes: (1) derived from "self" proteins, which bind to self-MHC molecules, but do not elicit an immune response, presumably due to tolerance at a T cell level;[135,136] (2) a more complex group that derives from the apparent discrepancy between the lack of selectivity shown in the binding of many peptides to different MHC molecules, and the tight control that MHC exerts on immunogenicity of specific epitopes (Ir gene phenomena). This issue is discussed in more detail in the following section.

B. Determinant Selection and Degeneracy

Much controversy surrounds the question of the extent to which peptide binding is MHC selective, and thus what role peptide binding plays in determining immune response gene phenomena. Following immunization with intact protein antigens, responses to particular epitopes are usually tightly linked to presentation by a particular MHC molecule. If the MHC molecule is changed, the immunogenic epitopes within the antigen molecule will change (reviewed in Reference 137). It was clearly attractive to interpret this regulation in terms of different binding affinities of individual peptides for different MHC molecules (determinant selection), particularly since the majority of polymorphic residues within the MHC were found to map to the antigen binding site. However, experiments to test this hypothesis have provided very conflicting results. Thus, although initial reports did suggest that peptide binding was highly MHC specific,[126,127] many later studies found a great deal of cross-reactivity (degeneracy) between different MHCs for peptides involved in both class I and class II restricted responses (see Table 2). In the following discussion of the different factors that may explain this diversity, a distinction will be made between different structural forms of MHC molecule (isotypes, e.g., the A, B, and C class I molecules in the human) and the many allelic forms of each type that are found within the population because of the extensive polymorphism of this family of molecules (allotypes).

1. At least some discrepancies may well be attributable to experimental methods. Par-

ticularly in the case of class I binding, assays that have measured binding to MHC molecules in solution have shown a far greater degree of specificity than those in which peptides have been immobilized on plastic. The reasons for these differences are not yet fully understood.

2. In general, binding to MHC isotypes shows more specificity than to different allotypes.[138] In the binding of peptides to more than one MHC isotype, careful analysis has shown that at least in some cases these peptides in fact contain two quite distinct MHC binding sites.[139] In such cases one peptide probably binds to the two MHC types in quite different ways.
3. Immunological responses to short peptides are less tightly regulated by MHC type than when these same peptides form part of the larger protein structures (see the section on heirarchy). In some cases responses to the same peptide can be obtained not only in many different allotypes, but they also associated with the homologous MHC molecule of a different species.[140] This degeneracy in peptide responses may be due to the high concentrations of peptide used, compared with the very low levels of any one epitope produced during physiological processing.
4. In one case where peptides showed both differential immunogenicity and different total binding to different MHC haplotypes, kinetic and thermodynamic analysis showed that affinity constants and rates of reaction were in fact very similar.[36] This intriguing result suggests that actual binding may be largely controlled by the concentration and affinities of intrinsic cellular peptides, or competing antigens, which are already bound to the MHC molecules, and which will presumably vary from one MHC allele to another (see section on competition).
5. T cell recognition depends on the exact conformation the processed antigen adopts within the MHC binding site. Thus, while total peptide binding to different allotypes may be quite comparable, the small changes in binding site structure resulting from the polymorphisms in MHC sequence may be sufficient to alter the conformation of bound peptide sufficiently to alter T cell recognition (this idea is discussed in detail in Reference 141).

In summary, there are as yet insufficient data and the available data has too many potential sources of error to assess quantitatively the degree of specificity that exists in terms of peptides binding to different allelic forms of MHC. The only general conclusion can be that the peptide/MHC binding affinity is one essential factor in determining the efficiency of the overall processing pathway.

C. Characteristics of Peptides that Bind MHC

A considerable effort has gone into identifying the features of a particular peptide sequence that allow it to bind to a MHC molecule, and hence to define the intermolecular interactions between peptide and MHC molecule. In the absence of crystallographic data on the structure of any peptide/MHC complex these features must be deduced indirectly from binding or functional studies. Analysis of the constantly expanding database of peptides whose binding has been measured has revealed elements common to many, though not all binding peptides. These elements include intrinsic preferences for the formation of secondary structural elements (especially amphipathic α-helices[142]), and particular "motifs" of peptide sequence.[143,144] Both approaches have yielded operational procedures with some predictive ability to identify likely immunogenic peptides within particular protein sequences. Data comparing the binding and immunogenicity of a large variety of analogs of specific peptides also suggest that some peptides bind within the MHC binding site in a helical configuration,[145] while others may adopt a more extended conformation.[146] The final stable binding conformation will be determined both by the intrinsic structural characteristics of the peptide itself and its interactions with the MHC molecule. In some cases the binding site may be able to accommodate the same peptide bound in two different configuratons.[146a]

The interpretation of these data is further complicated by the fact that binding strength can

be greatly influenced (both positively and negatively) by small modifications to the terminal amino acids of peptides and by the addition of extra sequences of amino acids to the minimal immunogenic epitope region.[147,148] Such changes could occur by stabilization of particular secondary conformational structures, or by secondary interactions occurring in a number of different ways, which will depend on the individual structure of MHC molecule and antigen involved.

It was shown recently that the self-peptides that associate with a particular class I molecule have some degree of common structure that is allotype specific.[92d] One or two positions in the sequences of these peptides (eight to ten residues in length) are highly specific for a single amino acid, and several other positions show lesser degrees of specificity. These findings indicate that only some zones in the peptide-binding groove have precise requirements. These may correspond to the location of the pockets in the groove into which specific amino acid side chains must fit.

D. Quantitative Aspects of Peptide/MHC Interaction

A number of studies have tried to obtain quantitative data on the kinetic and thermodynamic aspects of MHC/peptide interaction, particularly for class II MHC molecules. Measured equilibrium constants for this interaction (see Table 2) are on the order of 10^{-4} to 10^{-8}, two to three orders of magnitude lower than typical values for antibody/antigen binding. This is in agreement with the theoretical prediction and the experimental demonstration that the MHC/antigen interaction must be less specific than that involving antibody. However, a surprising finding was that low affinity derived largely from a decrease in the on-rate of the reaction (complex formation), while the off-rate (complex dissociation) was relatively low (with half-times on the order of hours or days).[36,126,127,133] In qualitative terms this implies that complexes take a long time to form, but once formed are rather stable. Kinetic intermediates in the "on" reaction have been observed.[149] In molecular terms these results have been interpreted as suggesting that MHC molecules form stable interactions only with peptide in highly specific configurations and the slow rate of association reflects the small proportion of antigen molecules in correct conformation in solution at any one time. This interpretation is equivalent to saying that peptide antigens need to form multiple interactions with the MHC binding site *simultaneously* in order to form stable interactions.

Irrespective of their molecular explanation, these findings pose a number of physiological problems in terms of processing and presentation. In particular these data seem to suggest that antigen and MHC molecules must be present together at relatively high concentrations for a considerable amount of time in order to produce a significant number of complexes. Furthermore, they raise the question of how the cell maintains a sufficient rate of formation of free ("empty") binding sites to bind new antigens as these are processed. Although the solutions to these problems are not fully resolved, they have raised a number of issues that are currently the focus of much processing research. The sites of MHC/peptide interaction have been discussed above in the section on class I and class II processing pathways, and there is at least some evidence that class II molecules do pass through compartments within which they are retained for at least a few hours.[28] This, in conjunction with the fact that possibly as few as 200 molecules of antigen need to be bound to MHC in order to trigger a T cell response,[150] may be sufficient to achieve a sufficient rate of processing. Alternatively, it has been suggested that processed antigen may also be retained by secondary interactions, either with lipid components of the cell membrane,[151] general purpose peptide binding proteins,[39] or in B cells by antigen-specific immunoglobulin.[152] Such secondary interactions may enhance the degree of antigen/MHC binding by increasing localized concentrations of processed antigen and allowing increased time for antigen/MHC binding interaction to occur.

The question of the availability of "free" MHC binding sites, in view of the slow complex dissociation rates, has been largely focused on the extent to which antigens bind newly synthesized MHC molecules, or alternatively exchange with bound antigen on previously occupied MHC molecules. As discussed above (see Section on MHC class I and class II processing), the data

suggest that new antigens bind predominantly to new MHC molecules for both classes of MHC. In such a model the off-rate of peptides bound to MHC is of little significance, since such MHC molecules are not reused; however, mechanisms for MHC reuse have been proposed. In some cases, peptide binding has been shown to be pH sensitive, favoring dissociation of complexes under acidic conditions.[127] This has led to the suggestion that MHC molecules are "unloaded" by passage through endosomal acidic microenvironments, and hence become free to bind new antigen.[127,153] However, the pH dependence of binding seems to be peptide specific, and the overall significance of the phenomenon has been questioned.[154]

VI. ANTIGEN PROCESSING AS A REGULATOR OF THE IMMUNE RESPONSE

A. Competition for Antigen Processing/ Presentation

Many mixtures of antigens restricted to a single class II molecule show competitive inhibition at the level of antigen processing/presentation (reviewed in Reference 155). It is thus apparent that processing, particularly at the level of peptide/MHC binding, acts as a limiting step in the ability of new antigens to stimulate an immune response.[156] Although the majority of such competition studies have been carried out *in vitro*,[75] there are reports of *in vivo* antigenic competition thought to occur at the processing/presenting cell step (for example, see References 157 and 157a), and this is the basis for an intensive search for a new class of specific immunosuppressants that would work by blocking available sites for processed antigen on MHC molecules.

Competition between soluble protein antigens (self or foreign) has not been documented to affect steps in antigen processing other than the peptide/MHC interaction. Very little evidence exists that competition could limit the availability of processing enzymes, even with large amounts of antigenic material. Ingestion of intracellular bacteria has been shown to affect the endocytic pathway, and in some cases to depress antigen processing, but the mechanism is less likely to be competition within the degradation pathway and more likely to be a specific effect of the microorganism adapting to its own survival.[158]

A major source of potential competitors for antigen processing are the set of self-proteins present within the antigen presenting cell and in serum. Because tolerance to self is achieved purely at the lymphocyte level by thymic and extrathymic tolerance induction, self-derived immunogenic epitopes are not excluded from antigen presentation. Evidence for the processing and presentation of self has been found in various systems.[135,136] As for foreign antigens, it is presumed that presentation of self occurs via two pathways. The direct evidence for self-binding to class I is lacking, although there is indirect evidence from the class I-restricted response to minor histocompatibility antigens.[107] In the class II-restricted response, self-products synthesized both exogenously and endogenously have been shown to bind to MHC. In addition, proteins in the medium, including normal serum proteins, can compete for processing with intact antigen, the competition acting at the level of binding to MHC.[159]

The above data point to the importance of processing of a number of self-peptides producing a background with which foreign epitopes must compete. A resulting prediction is that the self-molecule, which can be processed into a high-affinity, broad MHC-specificity peptide and which is able to reach MHC loading compartments in high enough concentration to bind all the available MHC, would raise the threshold for foreign peptide to be expressed. The authors presume that evolution has matched the existence of self-peptides, which have some of these properties, with an increased rate of synthesis of MHC molecules. In addition, competition by high-affinity binding of self-peptides may be the main driving force in the maintenance of MHC polymorphism, rather than the converse possibility that antigens from potential pathogens would be able to avoid MHC binding.

B. B Cells as Antigen Presenting Cells

B cells are the only antigen presenting cells

to have a specific receptor for native antigen — the membrane immunoglobulin (mIg). It has been known for some time that B cells have poor overall endocytic activity, particularly compared with macrophages.[160] This correlates with the much lower abiliby of B cells to present most antigens; however, B cells function well as antigen presenting cells under certain circumstances.[161] This activity was first seen using antigens targeted to the mIg, such as antiglobulin (antisera to Ig raised in other species); B cells presented antiglobulin to T cells as well as macrophages. It has been found that this enhanced processing/presentation function is provided only to antigens targeted for surface receptors, which are then internalized via receptor-mediated endocytosis. These include mIg of all isotypes: IgM, IgD, IgG, and even IgE.[45,162] B cells expressing the specific mIg can present antigen at an ambient concentration as low as 10^{-11} *M* rather than the 10^{-8} *M* or more required for nonspecific uptake.[163] This shows that internalization of receptor and ligand delivers the antigen efficiently to the endocytic pathway. In addition, internalized mIg has been shown to colocalize with MHC class II.[164] Targeting of antigen to many other membrane proteins such as transferrin receptor and MHC itself can also enhance antigen processing/presentation, but not to the same extent as mIg. Not all surface proteins facilitate uptake when used as targets in this way, for example, CD45. These findings correlate closely with the extent of endocytic recycling of the target receptors involved.

B cells express 10^4 to 10^5 antigen receptors per cell that, in the absence of ligand, are in internal and external pools of roughly equal sizes, constantly and rapidly interchanging through the early endosomal compartments with a cycling time of 15 min. After antigen binding the rate of internalization is unaltered but re-expression is diminished.[165] The intracellular routing of the mIg/antigen complex is the late endosome and then the lysosome.[166] Signals may exist that target antigen to subcompartments where processing is more effective. More importantly, monovalent antigen is routed in this fashion as well as polyvalent immune complexes and artificial ligands such as the antisera described above. The signals that control the routing of mIg are unknown, but are unlikely to reside in the short cytoplasmic domain (three residues) of mIg itself. Rather, the family of accessory molecules associated with mIg are the likely source of sequences that control intracellular traffic.[166a]

It has been estimated that at the lowest antigen concentrations sufficient to present antigen only 0.05% of the surface receptors of B cells are occupied by antigen.[152] During the time that B cells usually need to process and present antigen to T cells this would lead to the routing into the late endosome of over 1% of mIg complexed with antigen. Interestingly, this suggests that the number of molecules internalized by B cells is not much in excess of the estimated 200 minimum required to activate T cells during antigen presentation.[150] Thus, processing is very efficient for these molecules, particularly in view of negative factors such as competition with other peptides for MHC binding.

Some years ago Mitchison[167] proposed that an antigen molecule acts as a bridge between specific B and T cells, binding receptors on each simultaneously. Now that antigen internalization and processing is known to be required prior to T cell recognition this model has had to be modified to one in which binding is sequential. Thus, B cells bind first to a native epitope, and this is followed by the binding of a T cell to a processed epitope on the B cell surface (see Figure 8). However, this model retains all the inherent advantages of "linked recognition" between the B and T cell compartments, as originally postulated.

C. Hierarchy

The T cell response *in vivo* to many whole proteins has been found to be focused on a limited part of the primary sequence. In some cases a single major epitope, which is described as immunodominant, may account for 90% of the total response.[168-170] Of the other epitopes that may be present in the antigen, or can be predicted by sequence, some will be immunogenic to a lesser extent, thus making up a hierarchy of immunogenicity below the principle epitope(s). The remaining epitopes do not form any part of the response. This variation is induced by factors that remain largely unclear. Many aspects of the immune response have been implicated, antigen processing among them.

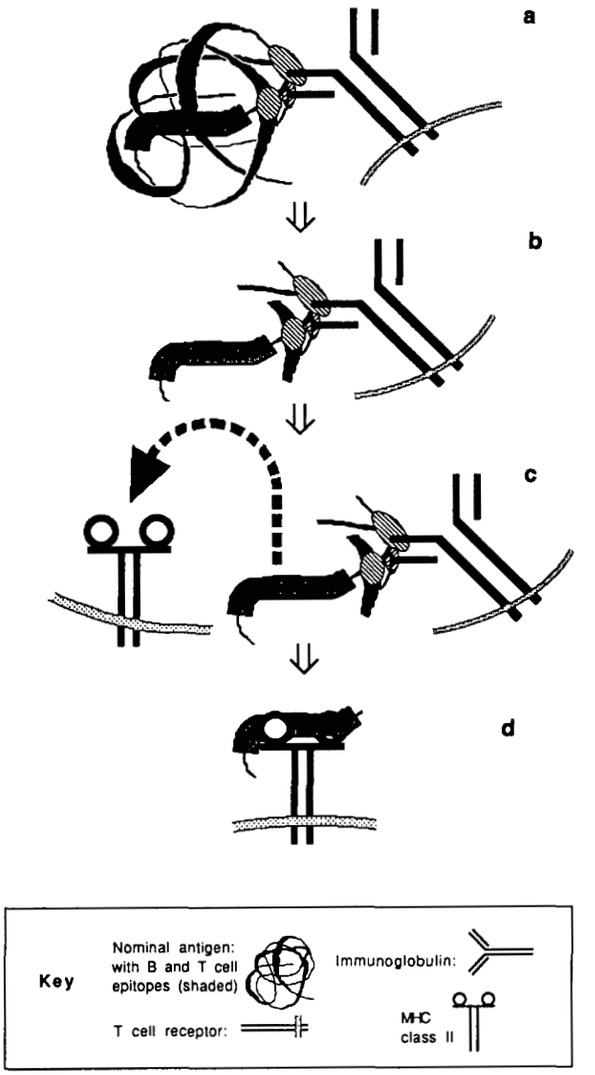

FIGURE 8. *B cells process and present their specific* antigen in a highly efficient manner. (a) Surface membrane immunoglobulin binds antigen. (b) Antigen processing of internalized antigen is affected by continued binding to immunoglobulin. (c) Processed epitopes bound to the endosomal membrane enter a class II *MHC positive compartment and bind. (d) Peptide/MHC* complexes are exocytosed for presentation to specific T cells.

Experiments to demonstrate an epitope hierarchy scan the response to whole protein with peptides derived from it which are immunogenic, as evidenced by their ability to prime T cell memory for themselves. Either the protein is used as primary immunogen and peptides used to generate a secondary response, or conversely the peptides prime for a challenge by intact antigen. Immunodominant peptide epitopes will prime for and be primed by the native protein. Nondominant epitopes can be immunogenic, priming response to themselves, but only when in the form of a peptide; they are not recognized in the context of the larger protein structure. Problems with

these techniques include the use of supraphysiological concentrations of peptide. This may overcome physicochemical barriers to processing. For example, antiprocessing enzymes that would destroy the epitope *in vivo* may be saturated at the concentrations used — up to 20 μM. In addition, the technique of cloning T cells from a primary response has been found to exaggerate dominance by selective outgrowth of cells responding to one epitope.[169]

1. Factors Other Than Processing

One possible factor in immunodominance is the affinity of an epitope for its binding site on MHC. Although this must play a part, there is not always a close correlation between affinity of an epitope for the binding site and antigenicity (see the above section on interaction between peptides and MHC — determinant selection and degeneracy).

The importance of the range of T cell receptors in establishing hierarchy (for example, by over- or underexpression of some families of variable regions with particular antigen specificities) has proven difficult to determine. In the case of epitopes that are immunogenic in the form of peptide but not when buried in the primary structure of protein, hierarchy cannot be explained by gaps or holes in the T cell receptor repertoire. T cell memory to cross-reacting epitopes, self or environmental, may influence hierarchy, leading to tolerance or enhancement, respectively, of a specific response. For example, if tolerance to the dominant epitopes is induced, it has been shown the response to whole antigen will be directed at the epitopes lower in the hierarchy.[171] Similarly, some peptides containing minor epitopes can induce T cell memory to whole protein.[170] Together these findings show that dominance in some cases is closely related to T cell repertoire; some nondominant epitopes are able to induce an immune response only when the T cell response is biased in their favor. One mechanism that may exclude these epitopes from the normal immune response is that the T cells specific for the dominant epitope(s), once stimulated, express high-affinity receptors for interleukin-2 (IL-2), the major T cell growth factor, which is then depleted from the local microenvironment. A further role for T cell memory has been suggested as the cause for the appearance of variable hierarchies in genetically identical animals responding to the same antigen.[169]

2. Differential Processing

The concept that processing of an antigen generates *all* of its potential epitopes, which are then free to bind MHC, does not explain the hierarchy of responses seen. In addition to the minor epitopes that can elicit T cell responses only under special circumstances, there remain some nonimmunogenic epitopes that are not expressed at all. The failure of these epitopes to be expressed is related to antigen processing. The degree of processing that different epitopes need before they are exposed from their native molecule was discussed earlier in the section on which antigens need processing. A further major factor is the stability of epitopes once exposed before binding to MHC can occur, in particular, resistance to exopeptidases.[172] Although it seems that the dominant epitopes are likely to be those that are both easily exposed and stable, little data exist on the subject. Differential processing of epitopes is thought to be controlled by neighboring sequences that fold back onto the epitope, thus producing "hindering structures".[168,170] Processing probably occurs via intermediates much larger than the 10 to 20 residue peptides used experimentally, therefore, it has been found in many cases that peptides cannot replicate the subtle structural context within which epitopes are sited.[173] To investigate the extent to which processing influences hierarchy *in vivo,* antigens must be made with unchanged epitopes but altered antigen processing sites. Before this can be done the enzyme specificity and major cleavage sites must be determined. A different approach to this problem would be to use *in vitro* techniques to replicate the differential processing seen *in vivo.*

Competition with the large number of self-derived peptides (see section above) may provide a high background of epitopes binding to MHC, but not seen by T cells due to self-tolerance. This background would act as a threshold for the expression of foreign epitopes, excluding those that are processed at low levels. Under conditions of reduced competition (e.g., in low serum), these

epitopes may become relevant. In contrast, epitopes that are not generated at all, or are destroyed during processing, would not appear even in the absence of competition. This hypothesis has yet to be tested fully.

3. The Role of Antibody in Hierarchy

The interaction between B and T cells that are specific for the same antigen (see discussion above) may affect epitope hierarchy through control of antigen processing. The pattern of fragmentation of exogenous antigen internalized within a B cell is dependent on the specificity of its mIg.[166,174] Thus, binding to mIg directs processing, possibly by inhibiting the action of (anti-) processing enzymes cleaving at or near the B cell epitope. The protected fragments still bound to mIg may, in view of the surprisingly high efficiency of antigen processing via this route, take a different intracellular pathway from the unbound fragments. In addition, the T cell epitopes that are included in the degradative intermediate bound to mIg will have a high concentration in the plane of the membrane, thus enhancing interaction with MHC class II. The rate at which this intermediate is degraded is indicated by the finding that Fab is cleaved from Fc 30 to 60 min after internalization.[165]

Macrophages can endocytose antigen complexed to antibody via Fc receptors, leading to enhanced processing and presentation. However, responses to a single antigen obtained by testing a panel of monoclonal antibodies in a checkerboard fashion against a panel of T cell clones specific for different T cell epitopes[175] showed that rare combinations of antibody and clone failed to enhance presentation. This is further evidence that specific interaction occurs between the B cell epitope bound to antibody and subsequent processing for T cell presentation, and may explain the close proximity of B and T cell epitopes on some antigens.[176]

4. MHC Directed Processing

How may the relationship between different processing steps affect immunological hierarchy? Given that many of the individual steps remain obscure, two complementary, extreme models may lead to some understanding of the overall mechanism. In one model there is complete microanatomical separation of crucial (anti-)processing step(s) from a later compartment where MHC binding occurs. Immunodominance would be achieved by a series of selective pressures as epitopes follow the pathway from initial uptake to MHC binding. The model predicts that antigen processing can be dissected ultrastructurally and biochemically.

The converse model is that processing and MHC binding occurs in the same compartment, and may be cooperative. The compartment in which this occurs for class II could be related to the late endosome, TGN, or lysosome,[70] whereas for class I the likely site is the ER.[86,87] Since MHC would be present, epitopes that are processed first would be able to bind immediately to MHC before other epitopes are exposed, thereby creating a hierarchy and directing the subsequent processing of the bound molecule.[168] In addition, antiprocessing enzymes would be inhibited by the protective effect of MHC. The model predicts that the pattern of antigen processing is dependent on the MHC molecules expressed by the cell. In the absence of MHC some nondominant epitopes will be produced that would otherwise not be functionally important, and others will be destroyed. The model predicts that experiments that perform the cleavage of antigen separately from MHC binding may fail to reproduce the true physiology of processing. Evidence for this model is quite strong for class I presenting endogenous antigen, where the peptides eluted from the class I are not found in cells that do not express that molecule.[92d]

VII. CONCLUSIONS

The authors would like to conclude this review of antigen processing by considering two related questions: why is antigen processing important either to us as immunologists, and, more significantly perhaps, of what importance is it to the overall function of the immune system? The first question has been dealt with, at least in part, within the review itself. Thus, the authors believe that antigen processing is the key step in the activation of most immune responses, and a ma-

jor regulatory influence on the antigen specificity of that response. Furthermore, although this question has been largely ignored, defects in antigen processing may play an important role in the pathogenesis of the increasing number of diseases in which defective or aberrant antigen presentation has been demonstrated. This list includes major diseases such as leprosy, AIDS, and rheumatoid arthritis. Thus, on both "pure" and "applied" grounds, there is ample justification for the enormous effort being put into the study of antigen processing by immunologists, cell biologists, biochemists, and molecular biologists.

The answer to the second question, which can be paraphrased as "why did antigen processing evolve?", must by its nature remain hypothetical. However, as alluded to in the introduction, the existence of antigen processing does mean that the immune system is able to "see" a whole second universe of antigens from which it would remain totally excluded were it to rely simply on antibody, and the recognition of molecular surface conformations. On a purely quantitative level this, of course, decreases the chances of any potential pathogen escaping detection. However, at a more subtle level, antigen processing can be seen as the evolutionary response to the problems of molecular camouflage and mimicry. It is now well established that many potential pathogens seek to escape detection by the immune system either by frequent changing of their antigenic surface or by mimicking the antigenic surface of their hosts ("the wolf in sheep's clothing"). In all these cases the changes tend to occur predominantly at those sites most exposed to the external environment, both at the molecular and supramolecular level. While the immune system is sometimes transiently fooled, the action of antigen processing is usually able to reveal inner features of an antigen which reveal its true identity and therefore make immunological recognition possible. Antigen processing is thus surely the embodiment of the ancient proverb "never judge a wine by its barrel".[177] Is this the lesson of antigen processing?

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