Rous-Whipple Award Lecture

Chemical Features of Peptide Selection by the Class II Histocompatibility Molecules

Emil R. Unanue

From the Department of Pathology and Center for Immunology,
Washington University School of Medicine, St. Louis, Missouri

I summarize here some of our studies on antigen processing and presentation that have taken place in the last several years. Our recent efforts attempt to explain the cellular and chemical basis for T cell recognition of peptide epitopes from protein antigens. I start with a brief introduction to the main features of antigen presentation and proceed to examine our experiments using the model protein hen egg-white lysozyme (HEL). In these experiments we have focused our attention on the class II major histocompatibility (MHC) molecules and their role in the selection and presentation of peptide epitopes to CD4 T cells. I will also include our recent studies on antigen presentation involving the diabetogenic histocompatibility molecules. My purpose in this paper is to review our own studies. Key literature citations are included but they are in no way comprehensive.

Protein antigens must be handled by antigen-presenting cells (APC) to be recognized by the T cell system. This interdependency between the T cell and the APC system of cells is the basis of what we have termed the symbiosis between the innate and adaptive immune systems: both cells depend on each other for full activation of the immune response.¹ In general terms, the first encounter with a foreign element is usually made by the cells of the innate system (ie, the macrophages, the dendritic cell system, and the granulocytes). This is best noted in the response to microbial infections. From this first encounter three positive results can emerge.

The first result is the release of cytokines from APC. This release affects the biology of the surrounding cells and tissues. This early response immediately poises the cellular adaptive system. Prominent are the vascular changes characteristic of inflammation as the endothelium becomes activated and receptive to the interaction with circulating leukocytes. Also prominent is the cellular migration that occurs, in part modulated by chemokines. Important among the cytokines released by APC are interleukin-12 (IL-12), the family of tumor necrosis factor (TNF) molecules, and IL-1 molecules. In some of our own studies, we described an interaction that took place rapidly after infection with the intracellular pathogen *Listeria*

monocytogenes.² This interaction involved IL-12 and TNF and resulted in the stimulation of natural killer (NK) cells to release interferon- γ (IFN- γ). Once IFN- γ was released various cells become activated, in particular the macrophage system.³ NK cells form part of the very early system of defense and participate in part through their properties of recognizing and lysing infected cells. In the *Listeria* infection, the prominent role of macrophage $\rightarrow$ NK interaction was noted best in mice with severe combined immunodeficiency. These mice do not have lymphocytes. They partially resist and control the infection through this interaction, described above, between macrophages and NK cells.²

The second result is that the APC are involved in antigen presentation and activation of the T cell system. T cells will not recognize protein antigens unless the antigens are taken by APC. This is done by the processing of protein antigens and the generation of peptides that become bound to MHC molecules, as will be described below.

A final result is the modulation of the lymphocyte once its receptor for antigen (the T cell antigen receptor, or TCR) has been engaged by the peptide MHC complex of the APC. The APC does this modulation by expressing molecules that promote cell-to-cell contact and the activation of the T cell (ie, costimulatory molecules)⁴ and by releasing cytokines that influence the differentiation of T cells to express different cytokine genes. As recorded by the work of Murphy and O'Garra, the release of IL-12 by the APC is a major reason for the differentiation of T cells to a Th1 pattern of differentiation.⁵

The reciprocal component of the symbiosis is activation of the innate cellular system by the products of the activated lymphocytes. Prominent among these cytokines is the role of IFN- γ molecules.^{3,6,7} Once IFN- γ is released, the macrophage system, for one, becomes activated. The activated macrophage is a highly cytotoxic cell that will restrict the growth of intracellular pathogens.⁸

Accepted for publication September 11, 1998.

Address reprint requests to Emil R. Unanue, M.D., Department of Pathology and Center for Immunology, Campus Box 8118, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110-1093. E-mail: unanue@pathology.wustl.edu.

Initial Studies on Antigen Processing

In our early studies we showed that generation of a T cell epitope required that the protein antigens be processed by APC. This was indicated in experiments that examined the interaction of T cells and APC involving the bacterium *L. monocytogenes*.^{9,10} Following a period of internalization by the macrophages, the T cells were able to recognize products of *Listeria* and strongly adhered to the macrophages. However, chemical neutralization of proteolytic activity abolished the expression of the T cell epitope. Thus, proteolytic processing was required.

Insights into the events taking place were obtained using model proteins. We decided to examine the protein HEL and found, likewise, that processing was needed.¹¹ Similar results were obtained by Gray's lab using ovalbumin.¹² However, tryptic peptides derived from HEL did not require intracellular processing, ie, the peptides were presented by chloroquine-treated macrophages. Moreover, formaldehyde-fixed macrophages pretreated with peptides were effective in stimulating T cell hybridomas.^{11,13} Among the tryptic peptides of HEL, that from residues 46 to 61 stimulated many of our T cell clones. In brief, proteins like HEL required intracellular proteolysis, but a peptide derived from it did not.

At the time of these studies, a role for MHC molecules in physiological immune response had been identified. Discoveries about the MHC's role started with the experiments of McDevitt and Benacerraf in which they found MHC genes controlling responses to protein antigens^{14,15} and continued with the experiments of Rosenthal and Shevach,¹⁶ Katz and Benacerraf,¹⁷ and Zinkernagel and Doherty¹⁸ showing the involvement of the MHC proteins in cell-to-cell interaction (those of APC-T cell interaction, B cell-T cell collaboration, and cytolytic T cell-virally infected cell interaction, respectively). The issue that remained unknown was the nature of the relationship between the requirements for peptide and the involvement of the MHC molecules.

In our next series of studies Paul Allen and I, with Bruce Babbitt, showed that the class II MHC molecule I-A^k bound the 46–61 peptide to form a peptide-MHC complex that stimulated the T cell.^{19,20} This was done with purified MHC molecules in detergent solution in binding assays, first using equilibrium dialysis. The binding could also be found directly using radiolabeled peptides and APC.²¹ The binding was saturable, with affinities in the $\mu\text{mol/L}$ range. Thus, processing involved generation of peptides that upon binding to class II MHC molecules created the epitope recognized by T cells. Indeed, MHC and peptides directly triggered T cell hybridomas.^{20,22}

These results were confirmed by studies of others using a variety of proteins.^{23–26} They were extended to class I MHC molecules particularly by the efforts of A. Townsend, A. McMichael, and their associates²⁷ testing the response to influenza virus antigens. The X-ray crystal structure of a class I MHC peptide complex, initially the effort of Pam Bjorkman, Don Wiley, Jack Strominger, and their associates, explained the structural features required for the interaction.²⁸ The binding site for class I and II MHC molecules have been shown by X-ray crys-

tallography to have the same basic features, which are described later.²⁹

The MHC molecules are peptide-binding molecules that rescue peptides from lysosomal catabolism.³⁰ Each MHC molecule samples preferentially, but not exclusively, an intracellular compartment. The class I MHC molecule preferentially samples peptides that are derived from the cytosol, partially catabolized in the multicatalytic proteasome, and transported into the endoplasmic reticulum, where they assemble with nascent class I MHC molecules. The class II MHC system, the central focus of our study, samples proteins taken from the vesicular system, many of which represent internalized proteins.

Selection of Peptides Bearing a High-Affinity Binding Sequence

The relationship between the chemistry of the peptide and its presentation by a class II MHC molecule, and that between the display on the surface and the stimulation of T cells, are only partially known. We and others have indicated that T cells require engagement of very few peptide-MHC complexes, on the order of fewer than 100 per APC. Because of the uncertainties of T cell assays it becomes difficult to relate peptide display directly to T cell responses. For these reasons our major effort has turned recently to the direct isolation of peptide-MHC complexes from APC.

APC represented by B lymphoma lines were either incubated with HEL or transfected with the HEL gene bearing a transmembrane or cytosolic portion of the class I MHC molecule, L^d. The I-A^k molecules were subsequently isolated. The peptides contained in I-A^k molecules were released at acid pH and the peptides were analyzed and sequenced. All of the mass spectrometry analyses were made in the Division of Chemistry at Washington University by Ilan Vidavsky and Michael Gross.

The evaluation of peptides extracted from MHC molecules had been pioneered by Rammensee, who analyzed peptides extracted from either class I or II molecules and obtained partial purification and sequence analysis.³¹ Other groups were also able to isolate them from MHC molecules and to sequence those that were heavily represented.^{32–34} The breakthrough in the analysis of MHC peptides comes from the initiatives of D. Hunt, V. Engelhard, and their associates using tandem mass spectrometry to isolate and sequence peptides bound to either class I or II MHC molecules.^{35,36}

Initially, by examining CD4 T cells, clones, or hybridomas from mice immunized with HEL, Paul Allen and we had identified the tryptic peptide 46–61 as containing the epitope that stimulated many of the T cells. This peptide was therefore assumed to be a dominant stimulatory one. The 46–61 peptide contained a core segment responsible for the binding to I-A^k molecules.³⁷ Other laboratories, particularly that of Sercarz and his group, confirmed this result and extended it by analyzing the various segments of HEL that stimulated different T cells.³⁸ In our chemical studies, led by Chris Nelson, we characterized

Table 1. Naturally Processed I-A^k-Associated Peptides

Peptide	Position		−4	−3	−2	−1	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10		RIC ^{−1}	% SDS-Stable
HEL	48–61		D	G	S	T	D	Y	G	I	L	Q	I	N	S	R		1.1	92
			D	G	S	T	A	Y	G	I	L	Q	I	N	S	R		185	10
Hsp70	28–41		I	I	A	N	D	Q	G	N	R	T	T	P	S	Y		19	66
			I	I	A	N	A	Q	G	N	R	T	T	P	S	Y		32	9
Ryudocan	84–101	E P	L	V	P	L	D	N	H	I	P	E	N	A	Q	P	G Y	21	91
		E P	L	V	P	L	A	N	H	I	P	E	N	A	Q	P	G Y	257	7
mIL-1 Receptor	185–199		A	I	L	L	D	K	G	N	K	E	F	L	S	A	G Y	8	39
DNA-B	92–108		A	I	L	L	A	K	G	N	K	E	F	L	S	A	G Y	18	0
		D	P	F	K	G	D	D	F	N	E	E	N	P	T	E	P S Y	28	72
		D	P	F	K	G	D	A	F	N	E	E	N	P	T	E	P S Y	32	69
		D	P	F	K	G	A	D	F	N	E	E	N	P	T	E	P S Y	125	1
Aβ ^k	37–53		F	V	R	F	D	S	D	V	G	E	F	R	A	V	T E L Y	2	0
			F	V	R	F	A	S	D	V	G	E	F	R	A	V	T E L Y	65	0
			F	V	A	F	D	S	A	V	G	A	A	R	A	V	T E L Y	12	90
Transferrin Receptor	103–117		K	G	T	D	F	Q	L	N	Q	L	E	G	K	K	G Y	14	96
Cathepsin H	72–92		K	G	T	A	F	Q	L	N	Q	L	E	G	K	K	G Y	32	17
		Y	I	L	Y	N	K	G	I	M	G	E	D	S	Y	P Y		23	0
		Y	I	L	Y	A	K	G	I	M	G	E	D	S	Y	P Y		140	0
FcE R II	298–310		S	F	L	D	A	W	V	C	E	Q	L	A	T	Y		28	19
			S	F	L	A	A	W	V	C	E	Q	L	A	T	Y		<200	0
MB1	177–193		E	D	E	N	L	Y	E	G	L	N	L	D	D	C S M Y		28	ND
			E	D	E	A	L	Y	E	G	L	N	L	D	D	C S M Y		80	ND

Indicated above are a number of autologous peptides isolated from APC and their amino acid composition in the standard one-letter code. The dominant HEL peptide residue 48-62 is also indicated. With many of these peptides, the binding to I-A^k was tested directly on I-A^k molecules. RIC refers to a binding assay: the relative amounts of peptide competing for binding with radiolabeled standard was determined. Higher numbers indicate weaker binding peptides that require more peptide to inhibit the standard reference. Another parameter indicated is the capacity of the peptide to form a stable structure resistant to SDS denaturation. The role of anchor residue was tested on peptides mutated with alanines. Indicated in bold are the main sites that produced changes and which were taken to bind to the P1 site.

a family of peptides from HEL representing the major or dominant epitope as well as the major autologous peptides.^{39,40} The set of HEL epitopes contained the core sequence from residues 52-61, previously recognized in the early studies with tryptic HEL peptides. The peptide sequence is Asp-Tyr-Gly-Ileu-Leu-Gln-Ile-Asn-Ser-Arg. However, the core sequence was presented as a nested set with amino and carboxy terminal extensions, the most frequent peptides starting at residue 48 and terminating at residue 62 or 63 (both tryptophans), ie, 15 or 16 residues. The 52-61 set of peptides were high-affinity binders to I-A^k molecules (Table 1).

The pattern of MHC-extracted peptide follows the rule of a core segment responsible for MHC binding and irregular extensions on both ends. We have found this pattern with two HEL peptides and also with autologous peptides. A similar result was obtained in the analysis of peptides derived from membrane proteins, like invariant chain.^{32,34}

Thus, the isolation of the 52-61 set of peptides confirmed that it was the immunodominant set, but with the important addition of the identification of the precise sequence that was being selected and presented. Indeed, the naturally selected set was larger than the core sequence, extending to terminal residues that were previously missed because of the use of a tryptic digest. The identification of natural sequences has permitted us to understand important biological correlates.

The MHC molecules did not discriminate between self- and foreign peptides; the rules for selection applied equally to both. Indeed, many of the peptides identified derived from self-proteins. This point was brought forth in

our early experiments, in which we found that murine 46-61 or 52-61 bound equally well to I-A^k.²⁰ Later, Paul Allen reported finding autologous peptides bound to APC in normal tissues.⁴¹ Importantly, the first chemical analyses of MHC-extracted peptides found that the number of self-peptides was in the order of hundreds.^{32,34-36} Many of these are in the realm of a few molecules per APC, but others are heavily represented. Table 1 shows a list of the autologous peptides bound to I-A^k molecules identified by our laboratory and that of Marrack and Kappler.⁴²

Finally, the finding of self-peptides associated with MHC class II molecules must be placed in the perspective of autoimmunity. Many of these can potentially activate self-reactive T cells. In the future it will be important to identify those peptides from a tissue captured by the local APC, which could be involved in local antigen presentation. Autologous peptides are found in local APC, presumably, in part, from normal turnover of tissue proteins, but also from local tissue damage.

One final issue for comment is the source of the peptides. Three sets of peptides have been identified in our studies and others'. First, as expected, are the peptides derived from proteins taken in by the APC, which in our case consisted of serum proteins or HEL. Second are the transmembrane proteins in the vesicular system, including peptides derived from invariant chain and class I and II molecules. In our APC, a frequent peptide is one derived from the I-Aβ^k chain itself.^{40,43} In the third group are peptides derived from cytosolic molecules, a finding which indicates that the class II MHC can also sample the cytosol.⁴⁴ In our case we have identified peptides from heat shock proteins and RNA and DNA binding proteins.

Figure 1. The time of persistence of class II MHC molecules depends on their content of strong or weak binding peptides. The C3F6 line was pulsed with radioactive amino acid precursors, then chased. At each time point the radioactivity associated with total class II MHC molecules and with the two SDS subsets was determined. The T 1/2 calculated by linear regression analysis in cells cultured without HEL was 12.6 hours: 10.2 hours for the unstable SDS set and 20.3 hours for the stable set. In cells cultured with HEL, the T 1/2 was 23 hours, with 10.9 hours for the unstable and 43 hours for the stable SDS set. Reprinted with permission from *Nature* 371:250–252, copyright 1994 Macmillan Magazines Limited.

Selection and Persistence of Extended Dominant Peptides

Cellular and binding analysis established some of the biological features for these extended sets of 52–61-containing peptides. First, purified I-A^k molecules bound the 52–61 core of HEL peptide, but its binding strength increased about 10-fold when the peptide was extended, particularly at the amino terminus.^{40,43} Thus, there was a chemical basis for selection of longer peptides in that their binding affinity increased up to 10-fold.

Importantly, also, the time of persistence of the peptide-MHC complex in APC increased.⁴⁵ Indeed, the time of persistence of the entire class II MHC molecules in APC (its T 1/2) depended on its content of strong or weak binding peptides. This point was best seen when examining the T 1/2 of metabolically labeled I-A^k molecules in B lymphoma lines (Figure 1). Metabolically labeled class II MHC molecules could be separated into two sets when run in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under conditions where the immunoprecipitate was not boiled.^{46,47} Stable SDS molecules contained strong binding peptides and resisted denaturation migrating as an $\alpha\beta$ dimer-peptide complex (~50 kD),^{48,49} but unstable SDS complex having weak binding peptides denatured, and the α and β chains dissociated into single chains. The strong binding peptides were found in the stable $\alpha\beta$ dimer at the top of the gel, but the peptides that bound weakly fell off, to be found at the front of it (Figure 2). In our experiments, I-A^k molecules were labeled in C3F6 B lymphoma lines, then chased in unlabeled media, isolated at various times, and the amount of labeled determined as well as the ratio of SDS stable and unstable sets. Whereas the T 1/2 of the unstable set was ~10 hours, that of the stable set was ~20 hours. If cells were given HEL, which donated good

Figure 2. Two sets of class II MHC molecules can be distinguished by their migration in SDS-PAGE when the molecules are not boiled (NB). After 100°C (B), the molecule dissociates in the α and β chains. The I-A^k molecules contain about 50% stable, which contain the strong binding peptides. The SDS-unstable are made up of weakly binding peptides. The I-A^{g7} molecule bind peptides weakly and are mostly found in SDS-unstable molecules.

binding peptides, the percentage of stable molecule increased, and so did their T 1/2 in the APC.

A second approach was to culture the APC with peptides labeled with I¹²⁵ and then to estimate the amounts bound to I-A^k after periods of time. The results indicated that the 15- to 16-residue peptide, ie, 48–62/63 with its amino and carboxy extensions, had a prolonged T 1/2 of ~40 hours. In contrast, the shorter peptide (bearing only the ten-mer case sequence 52–61) had much shorter persistence.

How the peptide-MHC complex governs its lifetime in the B cell is not clear. We showed that the persistence was a feature of the entire complex not explained simply by dissociation of the peptide. We interpret our results to indicate that the vesicular system can sense and select a stable complex, favoring less those unstable ones which are catabolized.

The persistence of the peptide-MHC complex correlated with its immunogenicity, a point best established by *ex vivo* culture with APC briefly pulsed with peptides. For example, T cells added to culture pulsed 24 hours previously with 48–62 responded strongly, but did not when pulsed with the short 52–61 sequence, which had been significantly lost from the APC.

A final important lesson from studying the naturally processed peptide was the identification of terminal tryptophan residues in the C-terminus. These, Trp 62 and Trp 63, actually serve as contact residues for T cells, as was first shown in studies from Vignali's laboratory.⁵⁰ In our laboratory, Dan Peterson and Tom Cirrito found that about half of the T cells elicited against the core 52–61 actually recognized the terminal tryptophans.

Figure 3. A monoclonal antibody can be made that specifically recognizes the I-A^k-48-62 complex found in APC fed HEL. The panels to the left represent cells reacted with a monoclonal antibody (40F) that recognizes all I-A^k molecules. On the right is the specific monoclonal antibody Aw3.18. M12-A^k refers to the C3.F6 line bearing I-A^k, not exposed to HEL; the 40F-specific (bold line) can be seen, but there is no reactivity with Aw3.18. M12-A^k-48-61 is a line engineered to express Aβ^k chains with covalently linked 48-62 peptide sequence; these APC react with Aw3.18. M12-A^k-34-45 is the line containing an unrelated complex, indicating no reactivity with Aw3.18. Lastly, M12-A^k-mHEL is a line bearing HEL molecules, known to donate 48-62 peptide to their I-A^k molecules; these peptides are identified with Aw3.18.

Dominant Peptides Can Be Detected with a Monoclonal Antibody

The 52-61 series of peptides were heavily represented on the plasma membrane, occupying as much as 6-10% of the class II MHC molecules. These amounts were estimated not only by direct examination using mass spectrometry, but also by using a specific monoclonal antibody that recognized I-A^k with the 48-62 sequence.⁵¹

The specific antibody to the complex was produced in our laboratory by Brian Deck and Gilles Dadaglio and used to characterize this complex on APC or in solution. The antibody selectively bound to the complex, and was used to quantitate its display in APC (Figure 3). Indeed,

antibody binds to APC fed with HEL or cultured with the 48-62 peptide. The antibody also precipitated purified complexes: I-A^k molecules containing I¹²⁵-48-62 were bound to the antibody, but this did not take place with complexes formed with unrelated peptides. The majority of the I-A^k 48-62 complex was recognized by the antibody, an indication that most of the peptide was bound in a single register⁵¹ (unpublished studies with Robert Latek).

Cellular Studies of the Peptide Selection

There are three different ways in which a peptide-MHC complex can be formed in an APC, and these depend on the form in which the binding segment is presented (Figures 4 and 5). First, using purified peptides, eg, 48-62, the binding takes place in I-A^k molecules that are on plasma membrane or in recycling endosomes. This binding can even take place in APC after fixation, an indication that there is no need for internalization. This same phenomenon may take place with any unfolded proteins that have sufficient conformational flexibility.⁵²

Second, native proteins need to be taken to a processing vesicle where the protein is denatured and associates with class II MHC molecules recently arrived from Golgi vesicles. By feeding HEL to APC we quantitated the efficiency of peptide selection with the monoclonal antibody mentioned above.⁵¹ About 1 for every 500-1000 molecules of HEL internalized by fluid phase endocytosis was processed to yield the 52-61 core peptides. We envision HEL being taken to late-acidic compartments and going through a process of reduction before catabolism.^{53,54} This reduction gives the HEL molecule the flexibility to bind to MHC molecules. In early studies we examined the fate of the dominant peptide when the HEL molecule was funneled directly to a lysosome-rich compartment instead of to an endosomal vesicle.⁵³ We did this by encapsulating HEL in either of two chemically different liposomes. One liposome was made up of lipids that rapidly disassembled at the light acidic pH of the endosomes. The other, pH-resistant lipid coat was disassembled only by enzymatic digestion in a deep lysosomal vesicle. The results indicated that the HEL in pH-resistant liposomes were in fact the most efficient in yielding the I-A^k-bound peptides.

Recent studies carried out by Carlos Parra-Lopez in the laboratory are also informative.⁵⁵ He made a series of fusion proteins of cellular enzymes and HEL, transfected B cell lines with them, and then examined them for their localization and presentation of the 52-61 and other HEL peptides. Cathepsin-D-HEL was processed like a lysosomal enzyme and taken to prelysosomal compartments, yielding the 52-61 epitope. The same results were obtained by using a Cathepsin-D construct lacking its glycosylation sites; this fusion protein was taken to a lysosomal compartment and also displayed the 52-61 epitope bound to I-A^k molecules. To our surprise, a construct of pepsinogen-HEL likewise generated 52-61 peptides, albeit to a lesser extent. Pepsinogen-HEL was rapidly released from the cell after its synthesis, yet could

Figure 4. HEL is unfolded and binds to I-A^k through its 52–61 core segment. After binding, the unfolded polypeptide is trimmed up to the edge of the combining site.

Figure 5. A scenario for the processing of HEL in APC. The description is in the text.

generate the dominant epitope. The conclusions from these series of experiments were, first, that proteins taken to a lysosomal compartment effectively yielded peptides to the class II MHC molecules, but, second, that a dominant epitope could be generated from different sites in the APC.

The site of coupling of peptides derived from processing with class II MHC molecules has been the subject of analysis and much debate. Whether there is a single vesicular entity or more than one has not been resolved.⁵⁶ By subcellular fractionation and by electron microscopy class II MHC could be identified in a heavy density vesicle, together with Cathepsins, but other vesicles have been shown to contain class II molecules. It has now been well established that nascent class II MHC molecules leave Golgi vesicles associated with the invariant chain (Ii). Ii chain functions to transport the complex to vesicles while at the same time covering the combining site, blocking its binding to peptide molecules.⁵⁷ In vesicles, Ii is proteolyzed, leaving a peptide in the combining site (CLIP). An auxiliary molecule, HLA-DM or H-2M, now comes into operation, favoring the release of the CLIP peptide and the binding of any available peptide.⁵⁶

The scenario that we envision is a first step in which HEL is unfolded, after which the flexible HEL is sampled by nascent class II-MHC molecules⁵⁸ and bound to the segment containing the high affinity site ie, that having the core 52–61 sequence (Figure 4). Once bound, the 52–61 segment is protected from catabolism by the I-A^K molecule, and is then transported to plasma membrane.

This scenario is supported by recent experiments in which the amino acid residues that flank the 52–61 sequence were replaced by prolines.⁵⁹ The mutant HEL were transfected into APC and the peptides selected were then analyzed. Prolines are known to stop the action of amino- and carboxypeptidases. The results of these experiments, carried out mainly by C. Nelson, indicated that the selected peptides were extended in length if proline residues were placed near the N-terminus, ie, residue 48. These results agree with the finding of extended naturally processed autologous peptides, many of which contain proline residues (Table 1). Thus, we concluded that aminopeptidases are involved in the processing by trimming the segments of the peptide that extend beyond the binding groove.

Finally, I note a third pathway of processing recently identified by Robert Lindner.⁵⁸ He showed that a partially unfolded HEL molecule would bind I-A^K to form an SDS-unstable complex. Internalization of the partially unfolded HEL resulted in binding, in light endosomes, to I-A^K molecules, forming an SDS-stable complex. The polypeptide was then transported to plasma membranes without reaching deep endosomal compartments.

As shown in Figure 5, HEL or HEL peptides can interact at different sites in the APC with I-A^K molecules, depending on the form in which the protein or peptide is displayed. The two central squares denote the two functional cellular compartments: on the right the endosomal, early vesicular compartment, where endocytosed proteins pass on their way to lysosomes, and which includes recycling vesicles. The plasma membrane and endo-

somes contains I-A^K molecules that bind to 48–62 peptide directly. On the left is the deep endosomal or lysosomal compartment that receives class II MHC molecules from endoplasmic reticulum/Golgi vesicles. It also receives HEL which is first denatured before binding to I-A^K. I-A^K becomes competent to bind I-A^K after removal of the CLIP peptide of Ii, in an interaction with H-2M/HLA-DM. In the third pathway,⁵⁸ a partially unfolded HEL binds in the light endosomal compartment and is transported as a large bound polypeptide to plasma membrane.

Binding Properties of the Dominant I-A^K-Bound Peptides

The interactions between peptides and MHC molecules have been examined by direct binding analysis as well as by the X-ray crystal structure of the complexes. Peptides bind as a result of interaction between critically placed amino acid side chains with sites or pockets found in the combining site of the MHC molecule. Important also are the sequence-independent interactions between the peptide backbone and conserved residues of the MHC molecule. In our case, we have been able to establish some important sequence motifs for I-A^K-bound peptides, derived in part from the evaluation of the extracted HEL and the autologous family of peptides. The X-ray crystal structure of a covalent complex^{60,61} of 48–62 with I-A^K was just solved by David Fremont in Wayne Hendrickson's laboratory⁶² (Figure 6).

The binding features of the 52–61 peptide for I-A^K were first examined using the 48–61 sequence and purified I-A^K molecules. The 48–61 peptide contains a major residue responsible for the allele-specific binding to I-A^K, and that is the aspartic acid at residue 52.^{37,40,43} This contribution of Asp 52 was ascertained by making synthetic peptides and mutagenizing each residue, substituting each for alanine. Each mutagen analogue was then examined by Shirley Petzold and Chris Nelson for its binding to the I-A^K molecule and for its stimulation of T cells. We found that only Ala substitution of the Asp 52 residue had an impact: about a 150 less binding than the wild-type peptide. The complex was highly SDS-unstable⁴⁸ and was not reactive with the 48–61 I-A^K-specific monoclonal antibody⁵¹ (Table 2).

The contribution of the Asp 52 side chain was made very evident when examining peptides made only of alanine residues.^{40,43} Polyalanine peptides did not bind to I-A^K molecules. However, the insertion of an Asp residue allowed for the binding, as shown in Table 2. The polyalanine peptide with Asp at the fifth position indicated the important contribution of side chain interaction of aspartic acid as well as the interaction of the backbone main chain interaction brought about by the length of the polyalanine chain. Indeed, the extent of the backbone interaction contributed to the binding affinity. Precedent for this result are the findings of Jardetsky with Wiley, who likewise found that a polyalanine peptide bound to HLA-DR2, provided that a tyrosine residue was included as the main anchor residue.⁶³ We have examined a number

Figure 6. Ribbon diagram of the I-A^k-48-62 complex; taken from the X-ray crystal structure by D. Fremont. The important P1 pocket is filled by the side chain of the aspartic acid (Asp 52). The TcR contacts are Tyr 53, Leu 56, Asn 59, and Trp 62.

of autologous peptides that are heavily represented on the I-A^k molecule and carried out Ala mutagenesis of them. Most of them indeed showed the critical presence of an aspartic acid (or Asparagine) residue, usually at the fourth or fifth position from the amino-terminus (Table 1).

The X-ray crystallographic analysis by Fremont⁶² showed the peptide in an extended conformation and indicated the three critical elements of the interaction. First is the sequence-independent hydrogen bonding between the peptide backbone and the MHC residues, many of which are the nonpolymorphic residues; indeed, hydrogen bonds were identified throughout the peptide sequence. The second critical element is the contribution of side chains, in particular that of Asp 52 residue. The Asp residue makes a tight fit at the P1 pocket, forming an ion pair with an Arg residue ($\alpha 52$). Mutation of this α -helical residue decreased the binding of 52-61 to I-A^k.⁶⁴ There are other side chains that interact with sites in I-A^k, but they contribute much less in the context of the 48-62 sequence. The third element is the contribution of exposed side chains that form the TCR contact residues. In 48-62 these include Tyr 53, Leu 55, Asn 58 and Trp 62, which are at least 50% solvent-exposed (Figure 6). Mu-

tagenesis of these side chains resulted in impairment of T cell responses.

Minor or Subdominant Peptides

It is important to define chemically what a minor/subdominant determinant is and what its main features are. The problem with the identification of minor peptides is one of sensitivity of both the T cell assay and the peptide isolation method. Because MHC-bound peptides are in the hundreds, it is impossible to sequence and identify each. The usual procedure is first to scan the peptide extract fractionated by reverse phase high-pressure liquid chromatography by performing a T cell assay. This step introduces two problems that are especially serious with regard to minor/subdominant peptides. One is peptide loss, which could be heavy, particularly noticeable for those peptides represented in small amounts. The other is that the T cell assay skews against those peptides with lower affinity for MHC molecules. To circumvent these issues, Raffi Gugasyan and Ilan Vidavsky introduced the use of a peptide capture step using monoclonal antibody-

Table 2.

Peptide	−4 48	−3 49	−2 50	−1 51	P1 52	P2 53	P3 54	P4 55	P5 56	P6 57	P7 58	P8 59	P9 60	P10 61		RIC ^{−1}	% SDS-Stable
A. Alanine Substitution of the HEL 48–61 Peptide																	
48–61	D	G	S	T	D	Y	G	I	L	Q	I	N	S	R		1.1	96
A48	A	G	S	T	D	Y	G	I	L	Q	I	N	S	R		0.6	79
A49	D	A	S	T	D	Y	G	I	L	Q	I	N	S	R		0.3	91
A50	D	G	A	T	D	Y	G	I	L	Q	I	N	S	R		0.3	94
A51	D	G	S	A	D	Y	G	I	L	Q	I	N	S	R		0.2	82
A52	D	G	S	T	A	Y	G	I	L	Q	I	N	S	R		150.0	9
A53	D	G	S	T	D	A	G	I	L	Q	I	N	S	R		1.6	ND
A54	D	G	S	T	D	Y	A	I	L	Q	I	N	S	R		1.4	82
A55	D	G	S	T	D	Y	G	A	L	Q	I	N	S	R		0.6	79
A56	D	G	S	T	D	Y	G	I	A	Q	I	N	S	R		0.9	86
A57	D	G	S	T	D	Y	G	I	L	A	I	N	S	R		0.6	57
A58	D	G	S	T	D	Y	G	I	L	Q	A	N	S	R		4.0	95
A59	D	G	S	T	D	Y	G	I	L	Q	I	A	S	R		0.1	94
A60	D	G	S	T	D	Y	G	I	L	Q	I	N	A	R		0.4	83
A61	D	G	S	T	D	Y	G	I	L	Q	I	N	S	A		0.1	83
Peptide	−4 48	−3 49	−2 50	−1 51	P1 52	P2 53	P3 54	P4 55	P5 56	P6 57	P7 58	P8 59	P9 60	P10 61	62	RIC ^{−1}	% SDS-Stable
B. Polyalanine Peptides Containing an Aspartic Acid Anchor																	
A ₄ DA ₁₀	A	A	A	A	D	A	A	A	A	A	A	A	A	A	A	21	ND
A ₄ DYA ₄	A	A	A	A	D	Y	A	A	A	A						>1000	ND
A ₄ DYA ₅	A	A	A	A	D	Y	A	A	A	A	A					16	1
A ₄ DYA ₆	A	A	A	A	D	Y	A	A	A	A	A	A				12	8
A ₄ DYA ₇	A	A	A	A	D	Y	A	A	A	A	A	A	A			12	6
A ₄ DYA ₈	A	A	A	A	D	Y	A	A	A	A	A	A	A	A		10	77
A ₄ DYA ₉	A	A	A	A	D	Y	A	A	A	A	A	A	A	A	A	6	77
A ₃ DYA ₉		A	A	A	D	Y	A	A	A	A	A	A	A	A	A	40	53
A ₂ DYA ₉			A	A	D	Y	A	A	A	A	A	A	A	A	A	34	49
A ₁ DYA ₉				A	D	Y	A	A	A	A	A	A	A	A	A	125	32
DYA ₉					D	Y	A	A	A	A	A	A	A	A	A	443	ND
A ₅ YA ₉	A	A	A	A	A	Y	A	A	A	A	A	A	A	A	A	>1000	ND
A ₁₅	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	>1000	ND

In A, the sequence of the 48–61 peptide is shown, with the results of the alanine scan. Amino acids are indicated in the standard one-letter code. RIC refers to the assay for binding strength shown in Figure 8. Binding was done with purified I-A^k binding to a standard labeled peptide. The amounts of unlabeled peptide that competes for the binding is indicated. SDS-stable means the capacity of the purified complex to resist denaturation (Figure 8). In B, polyalanine peptides were produced. Note that the binding depends on the presence of aspartic acid. Tyrosine was added in order to label the peptide. ND, not determined. Data taken from Nelson et al, Ref. 43.

ies to the peptide, ie, the APC-fed HEL were isolated and lysed, their MHC molecules were purified, and the peptides bound to them were released and then bound to an antibody affinity column.⁶⁵ The bound peptides were then released directly into the on-line tandem mass spectrometer (Figure 7).

Using this approach, we identified peptides represented by just a few molecules in the APC. The family that we have just reported consists of the 31–47 set of peptides, again consisting of a core with variations in the amino or carboxy ends. This set of peptides is represented by 1/30 or so of the amounts of the 48–62/63 dominant series (Figure 8). These peptides' major difference is that their binding affinity for I-A^k molecules is considerably less. Thus, there may be a relationship, perhaps not unexpected, between peptide display and binding affinity.

Two Different Conformations of a Single Peptide-MHC Complex?

The issue of cryptic or hidden determinants of a protein is an important consideration for our understanding of im-

munogenicity. Cryptic determinants⁶⁶ are defined as those not revealed by normal processing of the protein: T cells are elicited by immunizing with the peptide derived from the protein antigen; these T cells react when APC are fed the peptide (ie, the peptide binds to the class II MHC molecules) but do not react with the APC exposed to the protein; thus, the peptide should not be processed.

Our recent results have brought another dimension and interpretation to this issue of cryptic determinants.^{67,68} On examination of epitopes of HEL presented by I-E^k we isolated a peptide (residues 84–96) that was expressed at high levels, ie, it was dominant, and yet had been claimed to be cryptic. The explanation for this apparent conflict was on the T cell used in the assay. T cells raised by immunizing with HEL would recognize APC fed with HEL or the peptide 84–96. However, many of the T cell clones raised by directly immunizing against the 84–96 epitope recognized I-E^k complexed to 84–96 but did not recognize the same complex after processing of HEL!

Details of this phenomenon were very apparent when examining the 48–62 epitope presented by I-A^k

Figure 7. Identification of minor HEL peptides by immunoaffinity capture. The peptides bound to I-A^k from APC either not fed, or fed, with HEL were isolated and passed through a column containing an antibody to the 34–45 sequence. The peptides were then released and examined by tandem mass spectrometry. No peptides were identified from the column to which the I-A^k extract from APC not cultured with HEL (A and C). A series of peptides could be identified from the 31–49 family; the precise sequence was then identified. Reprinted with permission from ref 65, copyright 1998 American Association of Immunologists.

(Figure 9). Indeed, on immunizing with 48–62, Nick Viner and Brian Deck found that about one-half of the clones recognized only APC pulsed with 48–62.⁶⁸ The other half did not recognize APC fed with HEL in which the 48–62 was expressed, a result of the intracellular processing of

HEL as detailed in the previous sections. The T cells that recognized only exogenous 48–62 have been termed “type B” in contrast to those that recognize 48–62 resulting from intracellular processing of HEL, which we term “type A” (Table 3).

Figure 8. The peptides identified in Figure 7 are weak binders to I-A^k. These two figures compare the binding to I-A^k of the dominant 48–61 and minor 31–47 HEL determinant (A): about 30-fold more of the latter is required to inhibit binding to the standard peptide. B shows that the strong complex of I-A^k–48–61, but not that with 31–47, is resistant to SDS-PAGE. Reprinted with permission from ref 65, copyright 1998 American Association of Immunologists.

The type B response is not directed to secondary modifications of the peptide, because the clones recognize a tryptic peptide of HEL (residues 48–61). Very importantly, both clones recognize the same sequence. Indeed, we have isolated the 48–62 peptides derived from intracellular processing of HEL, and now tested whether type B clones recognize it when offered to APC now as an exogenous peptide: this is indeed the case.

Type B clones should not be confused with clones that are directed to peptides modified after their handling by APC. Indeed, some peptides during their internalization by APC can be cleaved and recognized now in a different chemical form from the native exogenous peptide. There are some T cells that actually recognize these modified

exogenous peptides. For example, one of our infrequent T cell clones, the 2A11 clone, is directed to short sequences derived from the 48–62 peptide. The clone reacts poorly with long peptides but optimally with 52–61.

What is the explanation of the A and B clones? Our interpretation is that there are two conformational states of the I-A^k–48–62/63 complex, and that these conformations are caused by differences in the site and conditions of assembly of the complex. The type A complex assembles in an acid-rich compartment on nascent class II molecules. The exogenous complexes assemble on class II molecules found on plasma membrane or light endosomes, at a higher pH (see Figure 5).

We have speculated along two lines on the biological consequences of having type B complexes. First, the phenomenon is important in the context of vaccination. If peptide vaccines will give rise to an abundant number of type B T cells, then these vaccines may not be effective, in that many of the T cells will not recognize the natural product. Second, type B T cells directed to peptides from autologous proteins may not be deleted in the thymus, escaping the normal purging of autoreactive T cells. That is to say, the thymic APC will present peptides derived from intracellular processing, allowing the type B T cells directed to their unique conformations to peripheralize. However, in conditions of inflammation the autologous protein could be proteolyzed, producing peptides that bind to APC to produce the type B conformation. We are currently evaluating both of these issues.

A Difference with I-A^{g7}: the Class II MHC Molecule of NOD Mice

The NOD mouse spontaneously develops type I or insulin-dependent autoimmune diabetes mellitus. The main gene responsible for the diabetic trait in both humans and NOD mice is one that encodes for a particular allele of class II MHC molecules.^{69,70} In the mouse the I-A^{g7} molecule was first cloned and analyzed by Hugh McDewitt and his associates.⁷⁰ I-A^{g7} is made up of an α^d chain coupled to a unique β chain allele, the β^{g7} chain. The β^{g7} has a serine residue at position 58 of β^{g7} instead of aspartic acid, like all other alleles. The β^{g7} forms an ion pair with the α^{79} arginine residue, but this is lacking in I-A^{g7} and in HLA-DQ diabetogenic alleles.⁶⁹ Our experiments have all been done in collaboration with Osami Kanagawa and with the contribution of Eugenio Carrasco and Jun Shimizu. Initially Shimizu was able to isolate CD4 T cells from islets of diabetic NOD mice.⁷¹ With these cells in culture he was able to isolate islet APC and to show that they contained diabetogenic antigen, ie, they stimulated his diabetic clones.⁷²

Our surprise came when Carrasco proceeded to isolate and analyze I-A^{g7} molecules for peptide binding.⁷³ I-A^{g7} molecules were very weak peptide-binding MHC molecules. Their binding to different peptides known to elicit CD4 T cells was very poor, with a very fast off-rate. This fast off-rate correlated with the response to peptides in culture. Although the CD4 T cells responded to continuous exposure of APC to peptides in the culture, this

Figure 9. Two sets of 48–62-reactive T cells can be identified: type A and type B. Each set was tested on HEL, a tryptic peptide from residues 46 to 61, and with a 48–61 synthetic peptide. Type A T cells are induced by immunization with HEL, react with 48–62 derived from intracellular processing and from 48–61 offered to the cell as either a synthetic or a tryptic peptide. Type B T cells are induced by immunization with the peptide and react only with the peptide, not with the 48–62 derived from processing. Reprinted with permission from *J Immunol* 156:2365–2368, copyright 1996 American Association of Immunologists.

was not the case with peptide-pulsed APC: the T cell response was rapidly lost as the finite amount of bound peptide rapidly dissociated. A second correlation was with the time of persistence of I-A⁹⁷ on the membranes of APC which was short, about 1/3 that of other class II molecules, like I-A^k. This fast off-rate also applied to the diabetogenic peptides.

The poor peptide-binding property of I-A⁹⁷ is also evidenced by examining I-A⁹⁷ molecules from APC metabolically labeled with radioactive amino acids. Close to 100% of the I-A⁹⁷ are SDS-unstable, an indication that they are empty or contain very weak peptides that rapidly dissociate (Figure 2). These observations are also true for I-A⁹⁷ molecules isolated from islet APC.

This weak peptide-binding property of I-A⁹⁷ is puzzling and clearly applies to all peptides so far analyzed. The

issue that we have raised is whether there is a relationship between this trait and autoimmune diabetes.⁷⁴ In recent studies Osami Kanagawa isolated lymph node CD4 T cells from mice having I-A⁹⁷ molecules and quantitated the number of autoreactive T cells. Using limiting dilution analysis, he found a marked increase in their number. This has led us to postulate that the weak I-A⁹⁷-binding trait correlates with a defect in thymic negative selection process.

Thus, for peptides that trigger autoimmune reactions, the situation may be different from the situation found with foreign peptides, as described in the HEL studies. In those studies, high-affinity binding peptides may be key, driving the selection of T cells. With self-peptides, other considerations come into play that concern the status of autoreactive T cells. In the I-A⁹⁷ example, the peptides

Table 3. Type A and B T Cells

	Type A	Type B
Reactivity	Immunization with HEL or 48–62 peptide	Immunization with 48–62 peptide
1. APC + HEL	+	–
2. APC + 48–62	+	+
3. APC + tryptic peptide	+	+
4. APC + covalently bound 48–62	+	–
5. APC + extracted 48–62	+	+

The two patterns of reactivity of the 48–62 type of T cells. In item 3, APC were fed a tryptic peptide 46–61. Results indicated that the type B T cells do not react with modifications of the synthetic peptide (item 2). In item 4, the APC express β chain with a covalently linked peptide produced as initially described by Kappler's laboratory (Ref. 60). In item 5, HEL 48–62 was extracted from I-A molecules of APC fed with HEL. It was purified and then given to APC. It indicates that the Type B T cells do not recognize modifications of the natural peptide, i.e., negative in item 1 but positive in item 5.

that drive to the autoimmune state may, in fact, be those that bind very poorly and allow for self-T cells to escape purging in the thymus. A situation akin to that of the I-A⁹⁷ was also described by Wraith for a myelin basic protein peptide. Likewise, this group speculated on the relationship between poor peptide binding and autoimmune disease.⁷⁵

Summary

We have reached the stage where immunogenicity of protein antigens can be defined precisely at a biochemical and cellular level and where important quantitative parameters can be defined. The immunogenicity of T cell epitopes has been particularly difficult to define because of the added complexity resulting from the need for a first step for processing, and peptide interaction with MHC proteins. We have shown in this review some of the advances brought about using HEL as a model protein and the rewards of applying biochemical criteria to antigen presentation. The recent results with I-A⁹⁷, a diabetogenic class II MHC molecule, strongly indicate the complexities of autoimmunity and the urgent need to continue applying biochemical features to explain self-immunization.

Acknowledgments

This paper highlights the work presented at the 1998 meeting of the American Society for Investigative Pathology on the occasion of the Rous-Whipple Award. I thank the National Institute of Allergy and Infectious Diseases of the National Institutes of Health for their support of this research. This work represents the efforts of many colleagues—graduate students, postdoctoral fellows and faculty—to whom I am deeply grateful. The efforts of our recent laboratory members have been cited in the text.

References

1. Unanue ER, Allen PM: The basis for the immunoregulatory role of macrophages and other accessory cells. *Science* 1987, 236:551–557
2. Unanue ER: Studies in *Listeriosis* show the strong symbiosis between the innate cellular system and the T cell response. *Immunol Rev* 1997, 158:11–25
3. Buchmeier NA, Schreiber RD: Requirement of endogenous interferon- γ production for resolution of *Listeria monocytogenes* infection. *Proc Natl Acad Sci USA* 1985, 82:7404–7408
4. Lenschow DJ, Walunas TL, Bluestone JA: CD28/B7 system of T cell costimulation: *Annu Rev Immunol* 1996, 14:233–258
5. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy SM: Development of Th1 CD4⁺ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 1993, 260:547–549
6. Dalton DK, Pitts MS, Keshav S, Figari I, Bradley A, Stewart TA: Multiple defects of immune cell function in mice with disrupted interferon- γ genes. *Science* 1993, 259:1739–1742
7. Huang S, Hendricks W, Althage A, Hemmi S, Buethmann H, Kamji R, Vilcek J, Zinkernagel R, Aguet M: Immune response in mice that lack the interferon- γ receptor. *Science* 1993, 259:1742–1745
8. Mackaness GB: Cellular resistance to infection. *J Exp Med* 1962, 116:381–390
9. Ziegler K, Unanue ER: Identification of a macrophage antigen-processing event required for I-region-restricted antigen presentation to lymphocytes. *J Immunol* 1981, 127:1869
10. Ziegler K, Unanue ER: Decrease in macrophage antigen catabolism by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells. *Proc Natl Acad Sci USA* 1982, 78:175
11. Allen PM, Unanue ER: Differential requirements for antigen processing by macrophages for lysozyme-specific T cell hybridoma. *J Immunol* 1984, 132:1077
12. Buus S, Sette A, Grey HM: The interaction between protein-derived immunogenic peptides and Ia. *Immunol Rev* 1987, 98:115–141
13. Shimonkevitz R, Kappler J, Marrack P, Grey H: Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J Exp Med* 1983, 158:303–316
14. McDevitt HO, Chinitz A: Genetic control of the antibody response: relationship between immune response and histocompatibility (H-2) type. *Science* 1969, 163:1207–1210
15. Benacerraf B: Role of MHC gene products in immune regulation. *Science* 1981, 212:1229–1231
16. Rosenthal AS, Shevach EM: Function of macrophages in antigen recognition by guinea pig T lymphocytes. *J Exp Med* 1973, 138:1194–1199
17. Katz DH, Hamaoka T, Dorf ME, Maurer Ph, Benacerraf B: Cell interactions between histoincompatible T and B lymphocytes. IV. Involvement of immune response (Ir) gene control of lymphocyte interaction controlled by the gene. *J Exp Med* 1973, 138:734–740
18. Zinkernagel RM, Doherty PC: Activity of sensitized thymus-derived lymphocytes in lymphocytic choriomeningitis reflects immunological surveillance against altered self components. *Nature* 1974, 251:547–552
19. Babbitt BP, Allen PM, Matsueda G, Haber E, Unanue ER: Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* 1985, 317:359
20. Babbitt BP, Matsueda G, Haber E, Unanue ER, Allen PM: Antigenic competition at the level of peptide-Ia binding. *Proc Natl Acad Sci USA* 1986, 83:4509–4513
21. Luescher IF, Allen PM, Unanue ER: Binding of photoreactive lysozyme peptides to murine histocompatibility class II molecules. *Proc Natl Acad Sci USA* 1988, 85:871–874
22. Watts TH, Brian AA, Kappler JW, Marrack P, McConnell HM: Antigen presentation by supported planar membranes containing affinity-purified I-A^d. *Proc Natl Acad Sci USA* 1984, 81:7564–7568
23. Buus S, Sette A, Colon SM, Miles C, Grey HM: The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science* 1987, 235:1353–1358
24. DeLisi C, Berzofsky JA: T-cell antigenic sites tend to be amphipathic structures. *Proc Natl Acad Sci USA* 1985, 82:7048–7052
25. Lamb JR, Rees ADM, Bal V, Ikeda H, Wilkinson D, De Vries RRP, Rothbard JB: Prediction and identification of an HLA-DR-restricted T cell determinant in the 19-kd protein of *Mycobacterium tuberculosis*. *Eur J Immunol* 1988, 18:973
26. Rammensee H-G, Friede T, Stevanovic S: MHC ligands and peptide motifs: first listing. *Immunogenetics* 1995, 41:178–228
27. Townsend ARM, Rothbard J, Gotch FM, Bahadur G, Wraith D, Michael AJ: The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 1986, 44:959–968
28. Bjorkman PJ, Saper B, Samraoui B, Bennett WS, Strominger JL, Wiley DC: The foreign antigen binding site and T cell recognition regions of class I histocompatibility regions. *Nature* 1987, 329:512
29. Stern L, Brown G, Jardetsky T, Gorga J, Urban R, Strominger J, Wiley D: Crystal structure of the human class II MHC protein HLA-DR1 complexed with an antigenic peptide from influenza virus. *Nature* 1994, 368:215
30. Donermeyer DL, Allen PM: Binding to Ia protects an immunogenic peptide from proteolytic degradation. *J Immunol* 1989, 142:4101–4109
31. Falk K, Rötzschke O, Rammensee H-G: Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature* 1990, 348:248–251
32. Chicz RM, Urban RG, Lane WS, Gorga JC, Stern LJ, Vignali DAA, Strominger JL: Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. *Nature* 1992, 358:764–768
33. Buus S, Sette A, Colon SM, Grey HM: Autologous peptides constituting

- tively occupy the antigen binding site on Ia. *Science* 1988, 242:1045–1047
34. Rudensky AY, Preston-Hurlburt P, Hong SC, Barlow A, Janeway CA Jr: Sequence analysis of peptides bound to MHC class II molecules. *Nature* 1991, 353:622–627
35. Hunt DF, Henderson RA, Shabanowitz J, Sakaguchi K, Michel H, Sevilir N, Cox AL, Appella E, Engelhard VH: Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* 1992, 255:1261–1263
36. Hunt DF, Michel H, Dickinson TA, Shabanowitz J, Cox AL, Sakaguchi K, Appella E, Grey HM, Sette A: Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-A^d. *Science* 1992, 256:1817–1820
37. Allen PM, Matsueda GR, Evans RJ, Dunbar JB. Jr, Marshall GR, Unanue ER: Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. *Nature* 1987, 327:713–715
38. Gammon G, Shastri N, Cogswell J, Wilbur S, Sadegh-Nasseri S, Krzych U, Miller A, Sercarz E: The choice of T-cell epitopes utilized on a protein antigen depends on multiple factors distant from, as well as at, the determinant site. *Immunol Rev* 1987, 98:53–73
39. Nelson CA, Roof RW, McCourt DW, Unanue ER: Identification of the naturally processed form of hen egg white lysozyme bound to the murine major histocompatibility complex class II molecule I-A^k. *Proc Natl Acad Sci USA* 1992, 89:7380–7383
40. Nelson CA, Viner NJ, Unanue ER: Appreciating the complexity of MHC class II peptide binding: Lysozyme peptide and I-A^k. *Immunol Rev* 1996, 151:81–105
41. Lorenz R, Allen PM: Processing and presentation of self-proteins. *Immunol Rev* 1988, 106:115–127
42. Marrack P, Ignatowicz L, Kappler JW, Boymel J, Freed JH: Comparison of peptides bound to spleen and thymus class II. *J Exp Med* 1993, 178:2173–2183
43. Nelson CA, Viner NJ, Young SP, Petzold SJ, Unanue ER: A negatively charged anchor residue promotes high-affinity binding to the MHC molecule I-A^k. *J Immunol* 1996, 157:75–762
44. Malnati MS, Marti M, LaVaute T, Jaraquemada D, Biddison W, DeMars R, Long EO: Processing pathways for presentation of cytosolic antigen to MHC class II-restricted T cells. *Nature* 1992, 357:702–704
45. Nelson CA, Petzold SJ, Unanue ER: Peptides determine the lifespan of MHC class II molecules in the antigen-presenting cell. *Nature* 1994, 371:250–252
46. Germain RN, Hendrix LR: MHC class II structure, occupancy and surface expression determined by post-endoplasmic reticulum antigen binding. *Nature* 1991, 353:134–139
47. Dornmair K, Rothenhäusler B, McConnell HM: Structural intermediates in the reactions of antigenic peptides with MHC molecules. *Cold Spring Harb Symp Quant Biol* 1989, 54:409–416
48. Nelson CA, Petzold SJ, Unanue ER: Identification of two distinct properties of class II major histocompatibility complex-associated peptides. *Proc Natl Acad Sci USA* 1993, 90:1227–1231
49. Stern LJ, Wiley DC: The human class II MHC protein HLA-DR1 assembles as empty $\alpha\beta$ heterodimers in the absence of antigenic peptide. *Cell* 1992, 68:465–477
50. Carson RT, Vignali KM, Woodland DL, Vignali DA: T cell receptor recognition of MHC class II bound peptide flanking residues enhances immunogenicity and results in altered TcR V region usage. *Immunity* 1997, 7:387–400
51. Dadaglio G, Nelson CA, Deck MB, Petzold SJ, Unanue ER: Characterization and quantitation of peptide-MHC complexes produced from hen egg lysozyme using a monoclonal antibody. *Immunity* 1997, 6:727–738
52. Lee P, Matsueda GR, Allen PM: T cell recognition of fibrinogen: a determinant on the A α -chain does not require processing. *J Immunol* 1988, 140:1063–1068
53. Harding CV, Collins DS, Slot JW, Geuze HJ, Unanue ER: Liposome-encapsulated antigens are processed in lysosomes, recycled, and presented to T cells. *Cell* 1991, 64:393–401
54. Collins DS, Unanue ER, Harding CV: Reduction of disulphide bonds within lysosomes is a key step in antigen processing. *J Immunol* 1991, 147:4054–4059
55. Parra-Lopez CA, Lindner R, Vidavsky I, Gross M, Unanue ER: Presentation on class II MHC molecules of endogenous lysozyme targeted to the endocytic pathway. *J Immunol* 1997, 158:2670–2679
56. Pierre P, Mellman I: Exploring the mechanisms of antigen processing by cell fractionation. *Curr Opin Immunol* 1998, 10:145–153
57. Cresswell P: Invariant chain structure and MHC class II function. *Cell* 1996, 84:505–507
58. Lindner R, Unanue ER: Distinct antigen MHC class II complexes generated by separate processing pathways. *EMBO J* 1996, 15: 6910–6920
59. Nelson CA, Vidavsky I, Viner NJ, Gross ML, Unanue ER: Amino-terminal trimming of peptides for presentation on major histocompatibility complex class II molecules. *Proc Natl Acad Sci USA* 1997, 94:628–633
60. Kozono H, White J, Clements J, Marrack P, Kappler JW: Functional soluble major histocompatibility complex proteins with covalently bound peptides. *Nature* 1994, 369:151–153
61. Fremont D, Hendrickson W, Marrack P, Kappler J: Structures of an MHC class II molecule with covalently bound single peptides. *Science* 1996, 272:1001–1005
62. Fremont DH, Monnaie D, Nelson CA, Hendrickson WA, Unanue ER: Crystal structure of I-A^k in complex with a dominant epitope of lysozyme. *Immunity* 1998, 8:305–317
63. Jardetzky T, Gorga J, Busch R, Rothbard J, Strominger J, Wiley D: Peptide binding to HLA-DR1: A simple polyaniline peptide retains MHC binding. *EMBO J* 1990, 9:1797–1804
64. Nelson CA, Viner N, Young S, Petzold S, Benoist C, Mathis D, Unanue ER: Amino acid residues of the I-A^k α -chain required for the binding and stability of two antigenic peptides. *J Immunol* 1996, 156:176–182
65. Gugasyan R, Vidavsky I, Nelson CA, Gross ML, Unanue ER: Isolation and quantitation of a minor determinant of hen egg white lysozyme bound to I-A^k by using peptide-specific immunoaffinity. *J Immunol* 1998, 161:6074–6083
66. Lehmann PV, Forsthuber T, Miller A, Sercarz EE: Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 1992, 358:155–157
67. Viner NJ, Nelson CA, Unanue ER: Identification of a major I-E^k-restricted determinant of hen egg lysozyme: Limitations of lymph node proliferation studies in defining immunodominance and crypticity. *Proc Natl Acad Sci USA* 1995, 92:2214–2218
68. Viner NJ, Nelson CA, Deck B, Unanue ER: Complexes generated by the binding of free peptides to class II MHC molecules are antigenically diverse compared with those generated by intracellular processing. *J Immunol* 1996, 156:2365–2368
69. Todd JA, Bell JI, McDevitt HO: HLA antigens and insulin-dependent diabetes. *Nature* 1988, 333:402–411
70. Acha-Orbea H, McDevitt HO: The first external domain of the nonobese diabetic mouse class II I-A β chain is unique. *Proc Natl Acad Sci USA* 1987, 84:2435–2441
71. Shimizu J, Kanagawa O, Unanue ER: Presentation of β -cell antigens to CD4⁺ and CD8⁺ cells of non-obese diabetic mice. *J Immunol* 1993, 151:1723–1730
72. Shimizu J, Carrasco-Marin E, Kanagawa O, Unanue ER: Relationship between β cell injury and antigen presentation in NOD mice. *J Immunol* 1995, 155:4095–4099
73. Carrasco-Marin E, Shimizu J, Kanagawa O, Unanue ER: The class II MHC I-A^{g7} molecules from non-obese diabetic mice are poor peptide binders. *J Immunol* 1996, 156:450–458
74. Kanagawa O, Martin SM, Vaupel BA, Carrasco-Marin E, Unanue ER: Autoreactivity of T cells from nonobese diabetic mice: An I-A^{g7}-dependent reaction. *Proc Natl Acad Sci USA* 1998, 95:1721–1724
75. Fairchild PJ, Wildgoose R, Atherton E, Webb S, Wraith DC: An autoantigenic T cell epitope forms unstable complexes with class II MHC: a novel route for escape from tolerance induction. *Int Immunol* 1993, 5:1151–1158