Part I
Antigen Presentation in the Immune System

Antigen Presenting Cells: From Mechanisms to Drug Development. Edited by H. Kropshofer and A. B. Vogt

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Some Old and Some New Findings on Antigen Processing and Presentation

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1.1
Introduction

This chapter summarizes our investigations on antigen processing and presentation using the model protein antigen hen-egg white lysozyme (HEL). It covers mostly the work from our laboratory and does not contain a comprehensive analysis of the work of others, who are credited in the original papers and reviews from the laboratory. This chapter is based on recent ones on the same topic [1, 2].

Our research for many years has centered on the symbiotic relationship between the phagocyte or antigen presenting cell (APC) system and the T cells. Using HEL has several advantages for studying antigen processing by the class II-MHC molecules, which has been our central focus: first, HEL is one of the best studied proteins at a biochemical level; second it is a strong antigen for many strains of mice (we use the strains bearing the k haplotype, and have examined, particularly, I-A^k molecules, referred to here as Ak); and, third, it has been extensively analyzed immunologically [3–5].

Our initial studies on antigen processing analyzed for the first time how phagocytes handled an antigen that was recognized by CD4 T cells. We first examined the bacterium *Listeria monocytogenes* in short-term interactions between T cells and peritoneal macrophages containing *Listeria*. It was established that antigen recognition by T cells required an APC, which needed to internalize the antigen (*Listeria*) into acidic vesicles, after which an epitope was presented on its cell surface [6, 7]. This peptide associated with an MHC molecule allowed T cells to bind to the macrophage.

Biochemical analysis of processing was extended using HEL, leading to several fundamental observations. Early studies [5] and those of Grey's laboratory using ovalbumin [8] were the two systems that defined the basic parameters of antigen processing using purified proteins. These early studies went against the long-standing paradigm that protein antigens were recognized before catabolism by the host (which is what happens with the B cell, as we now understand, but not with the T cell system, reviewed in Ref. 1). Moreover, the finding that antigen was immunogenic only when associated with, presented by, phagocytic cells was unexpected [9].

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1.2 HEL Processing

HEL is processed by APC, and suffers denaturation and unfolding. Peptides of HEL or unfolded HEL can bind to the class II MHC molecules, in our case to Ak molecules. In fact, the interaction of Ak molecules with the chemically dominant peptide of HEL, that found in the trypsin fragment from residues 46-61, was the first direct documentation that MHC molecules were peptide-binding molecules [10, 11]. It was also demonstrated that the MHC molecules bind to autologous peptides, and do not make the self–non-self discrimination [11]. Progress made in understanding the biochemistry and biology of antigen processing led into a parallel examination of how autologous proteins were presented in autoimmunity.

In APC, HEL needs to be taken into a deep vesicular compartment to be unfolded and processed [12]. With HEL the evidence points to its reduction followed by the selection and assembly of the various peptide segments by the Ak molecules, with the catalytic help of the H-2 DM molecules (referred as DM) in such a deep vesicular compartment. To test this scenario, HEL was encapsulated in liposomes of different chemical compositions. These liposomes would release their content in either deep, highly acidic, vesicles, or in recycling, lightly acidic, vesicles: very strong presentation took place when the peptides were encapsulated in liposomes made of phosphatidylcholine and phosphatidylserine that were disrupted, releasing their cargo, only in a late, highly acidic vesicle – see the next section [13–15]. References 16 and 17 discuss the importance of protein reduction. In toto, our findings, both in macrophages and dendritic cells (DC), and those of other investigators, indicate that the assembly site of the peptide-MHC complex is an endocytic vesicle that receives newly synthesized Ak molecules bound to the invariant chain, and that also contains the auxiliary molecules DM.

As a result of processing, several peptides are selected and displayed on Ak molecules; all the Ak-bound HEL peptides were identified by combinations of cellular, immunological and biochemical analysis. Table 1.1 summarizes the characteristics of HEL peptides presented by the class II MHC molecules [18–22]. As can be noted, the selection of peptides by Ak is dominated by one family centered on the 52-60 segment and which can occupy up to 10% of the bound peptides. As discussed below, the 52-60 segment is flanked, usually by four residues on the amino side and two on the carboxy-end.

Ak bound peptides were examined by electrospray tandem mass spectrometry (MS), including sequence analysis of the peptide mixture (Figure 1.1). Although peptides bound to MHC were first isolated and their general features established by bulk sequencing [23], MS is now the best approach for chemically isolating and characterizing MHC-bound peptides – an approach strongly emphasized by Donald Hunt, see Refs. 24–26. (Our MS operation is run by our colleague Michael Gross and his staff in our Department of Chemistry.) For chemical analysis we mostly used the C3F.6 B lymphoma line, a variant of the M12 line transfected with the alpha and beta chains of Ak molecules. Chemical analysis was facilitated by examining lines transfected with a membrane form of HEL. These

Table 1.1 Major peptides of HEL presented by A^k molecules.[a]

			Binding IC-50 (μM)	Amounts (10^6pmol)
48-63	=	D G S T **D** Y G **I** L **Q** **I** N **S** R W W	0.04	404
31-47	=	A A K F E **S** N F **N** T **Q** **A** T **N** R N T	0.9	7
20-35	=	Y R G Y **S** L G **N** W **V** **C** A **A** K F E	1.4	2
114-129	=	R C K G T **D** V Q **A** W **I** **R** G **C** R L	5.0	<1

a) Indicated are the four major families of peptides selected by A^k of APC. Core sequences are underlined. Flanking residues most frequently identified are indicated. MHC contact residues are in bold. Binding was done using purified A^k molecules obtained from recombinant baculovirus. Amounts were estimated by ELISA assays using monoclonal antibodies. The main anchor residue of 48-63 is Asp 52. In 31-47 and 20-35, it is the asparagines at P4. Peptide 114-129 has the aspartic acid at P1 as the main anchor, but it contains hindering residues at P7 and P9.

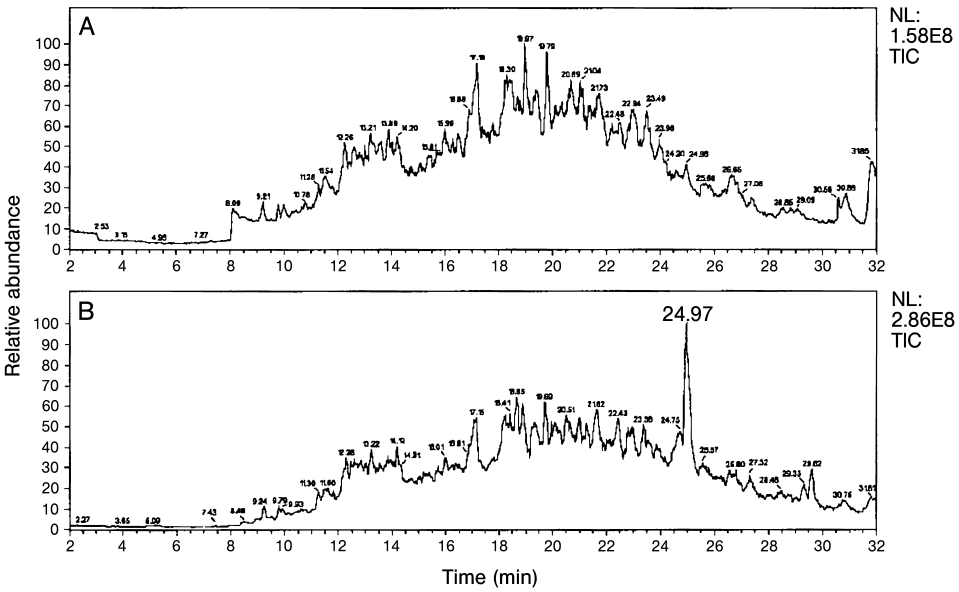

Figure 1.1 Peptides were isolated from Ak molecules of the C3.F6 APC line. (A) cultured with HEL; (B) cultured with HEL. Note the many peaks of autologous peptides in both parts; and the prominent peak of the chemically dominant 48-62 peptide in (B). (From Ref. 21, with permission.)

lines handled HEL identically, as if the soluble protein had been offered as an exogenous protein. The lines have the advantage that the mHEL gene can be mutated, allowing different residues in peptide selection and presentation to be

evaluated. Peptides were also analyzed from the standard APC of HEL transgenic mice with identical results.

Our studies were facilitated by using monoclonal antibodies to peptides or to peptide–MHC complexes as capture reagents. We used monoclonal antibodies against the main core segment of the MHC-bound peptides of HEL [20–22], or to specific peptide-MHC complexes [27]. An important first use of these reagents was to quantitate the amounts of peptide bound to Ak using ELISA techniques [28]: bound peptides were released from the isolated Ak molecules from APC and their amounts were estimated by ELISA, by inhibition assays using standards of peptides. Figure 1.2 shows an example of quantitating one of the minor peptides from HEL. This allowed us for the first time to estimate the exact amounts displayed by APC of the various peptides. A second use was as an antigen capture device for the identification of peptides. Peptides released from the class II molecules were bound to the antibodies attached to Sepharose particles and then released into the MS [op. cit.].

Most class II MHC-bound peptides are presented as families having a core nine amino acid segment plus flanking residues that vary both at the amino and carboxy termini. The peptides vary from 14 up to 20+ residues long [1, 20–22, 24–26]. The variation in length of flanking residues determines the number of peptides in a family. The "core" segment is responsible for the specificity of the interaction: usually amino acid side chains of the peptide stretched in the binding groove establish contact with four or five pocket sites, an issue well established by the X-ray crystal structure of several peptide-class II MHC complexes [29–37]. Depending on the particular MHC genotype, peptides are selected that contain favorable motifs for interaction. Flanking residues contribute to binding energy by adding to the conservative interactions between the main chain of the peptide and the surrounding hydrogen network (reviewed in Ref. 29).

The peptide segment selected by, and bound to Ak molecules, is protected from catabolism, an issue first shown by Paul Allen [38; see also 39]. Indeed the 48-62-Ak complex was highly resistant to proteolytic enzymes, and was also resistant to SDS denaturation (in SDS-PAGE examinations) [40] (Figure 1.3). But the MHC-bound peptide can be subjected to amino and carboxypeptidases that trim the overhanging portions close to the class II combining site. Notably, the naturally selected peptides containing amino terminal prolines are the longest bound to MHC molecules [41]. Prolines, because of the structure of their peptide bonds formed by their amino group, block exopeptidases. We examined this issue directly by making mutations in the HEL molecules at the sites flanking the 48-62 segment [42]. Most of the 52-60 family have four flanking residues on the amino terminus starting at amino acid 48 (Table 1.1); by placing a proline at 48, which is P-4, the resulting peptide bound to Ak was extended by one or two residues. The nature of the aminopeptidase has not been determined.

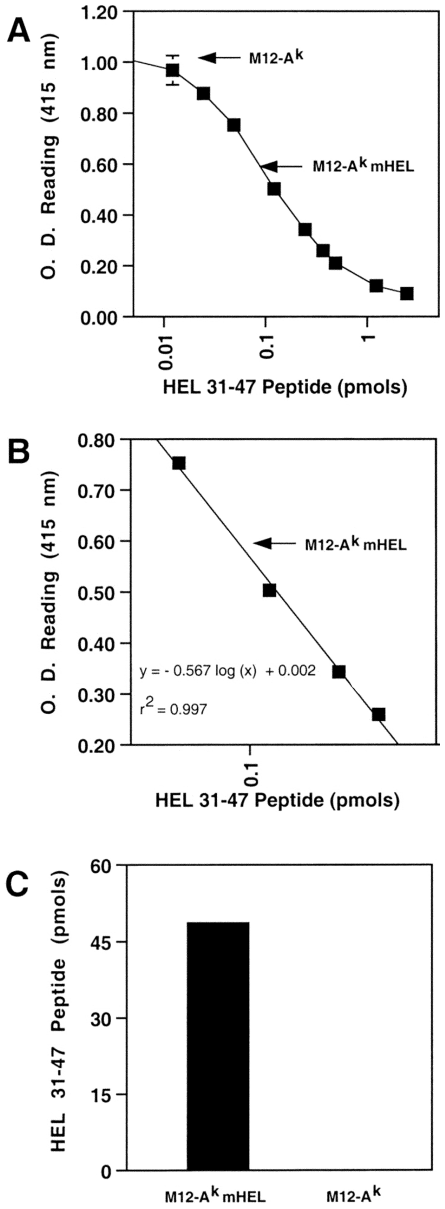

Figure 1.2 Quantitation of the 31-47 family of HEL peptide using specific anti-peptide monoclonal antibodies. (A) Actual results with the anti-31-47 antibody binding to the peptide attached to the plate in the presence of increasing amounts of soluble peptide as a competitor. (B) Calibration isotherm from which the amounts of peptide in the test samples (arrow) can be determined (C). (From Ref. 20, with permission.)

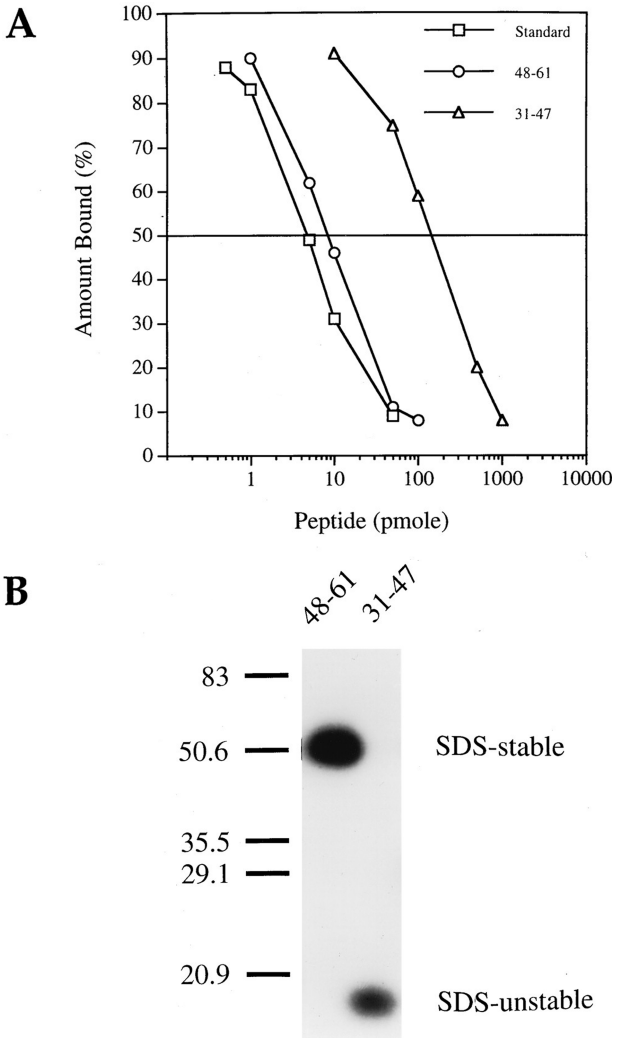

Figure 1.3 Binding features of the chemically dominant peptide 48-61 (i. e., (–○–)) and the minor peptide 31-47. (A) The inhibitory curve when purified Ak molecules are bound to a standard radioactive peptide. Each curve indicates the amounts bound in the presence of unlabeled peptide; a standard unlabeled, and the two HEL peptides. 48-61 is a strong binding peptide as evidenced by the amounts required to inhibit by 50%, which are much less than those required from 31-47. (B) Indication that the complex of 48-61 with Ak is SDS-resistant, whereas that with 31-47 readily dissociates. (From Ref. 20, with permission.)

1.3
Selection of Peptide Segments of HEL

The chemically dominant HEL peptide epitope is that having the 52-60 residues as a core segment (Table 1.1): DYGILQINS. The family usually encompasses peptide starting at residue 48 and ending with one or two tryptophans at residues 62 or 63 (in about equal amounts). This family is the chemically dominant family selected during processing (Figure 1.1). The main interaction responsible for binding involves the aspartic acid at P1, residue 52, which establishes an ion pair with residue arginine 62 in the alpha chain that forms the base of the pocket 1 [19, 34]. An indication of the importance of the P1 interaction is that a peptide made only of alanines, except for an acidic residue for P1, will bind as strongly as 48-62 to Ak molecules [19]. Similar results were obtained by Jardetzky, Wiley and colleagues: a peptide made of alanines, except for a tyrosine at P1 was responsible for binding to HLA DR2 molecules [43]. For the HEL 48-62 family, the other MHC-contact amino acids, at P4, P6, P7 and P9, did not contribute much binding energy, provided the strong driving interaction was at P1.

Importantly, changes in the Arg 62 to alanine of the alpha-k molecules, or the HEL 52 residue also to alanine, abolished the extensive selection of this peptide family during processing. Thus, the high level of selection is dominated by a single amino acid of the peptide in which its side chain has a highly favorable interaction with one of the allelic sites in the Ak binding groove.

Another major consideration both in the binding and in the selection of HEL peptides was the presence of negative or hindering residues at the auxiliary positions, which weakened the interaction with the P1 favorable site [44]. Although other groups had given evidence of hindering residues [45, 46], their strong influence became very evident in our analysis of the selection of various HEL segments, particularly of the 52-60 family (Figures 1.4 and 1.5). For example, only four peptides were presented of 21 potential peptides having an aspartic acid to interact at the P1 site. Not only did we find single residues that were strongly hindering, but we found combinations of them that together affected the binding. Glycines were particularly strong negative residues, probably by causing entropic

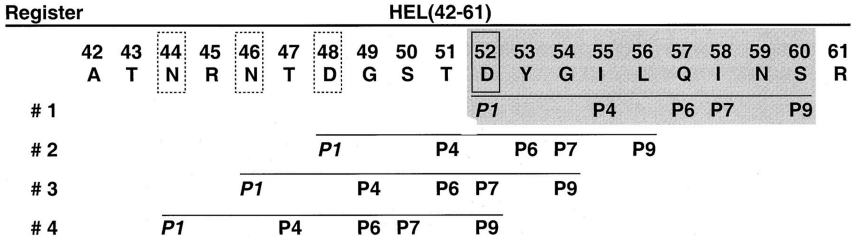

Figure 1.4 Segment of 52-60 is selected from a long stretch of the HEL molecule. Four possible registers are indicated here but only register # 1 is used. MHC contacts of P4, P5, P7 and P9, of register # 2, # 3, or # 4 were substituted instead of those in register # 1 in the sequence of 52-60: no binding was found (Figure 1.5B). (From Ref. 44, with permission.)

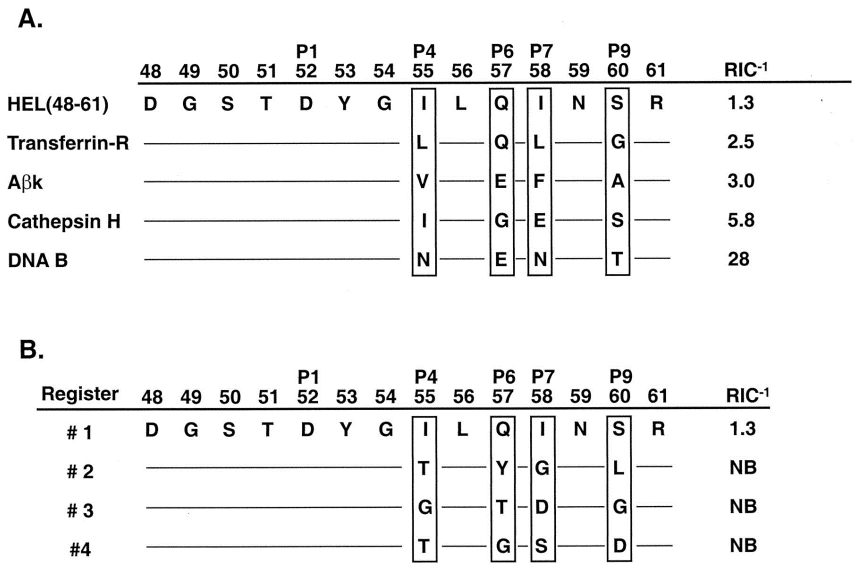

Figure 1.5 Importance of the "auxiliary" binding sites for the 52-60 segment binding. Peptide 48-61 binds well to Ak; note the binding results on the last columns (the lower the number, the better the binding). (A) Secondary residues in the indicated peptides were substituted in 48-61. All allow for the binding, which was similar to that of the peptide, all of which have aspartic acid at P1. (B) Putative registers 2–4 shown in Figure 1.4 inhibit the binding (NB). (From Ref. 44.)

disorganization at the binding sites. Figures 1.4 and 1.5 show the results of experiments in which the residues corresponding to P4, P6, P7 and P9 of different peptides were placed instead of those of the 52-60 sequence. For example, Figure 1.4 shows the sequence of HEL, from residue 42 to 61. Of the four possible binding registers, only one was used, and this was as a result of hindering amino acids.

Finally, to note in Table 1.1, the other two peptide families 20-35 and 31-45 did not use an acidic residue at P1: their main MHC anchor was the asparagine at P4. Both these sets of peptides bound with lower affinities to Ak and were selected to a much lesser degree. Peptide 114-129 showed a weak interaction, despite the favorable aspartic acid at P1, due to two unfavorable residues, the arginine at P7 and the cysteine at P9: changing them to alanines restored high-affinity binding (our unpublished data with Ravi Veraswamy).

Thus, high-affinity interactions result from favorable residues in the core segments of the peptide, together with the absence of negative or hindering amino acids, plus the contributions of the peptide backbone with conserved residues along the binding groove [reviewed in Ref. 29]. The findings with HEL peptide selection are mimicked by the analysis of natural peptides bound to Ak molecules in which a very high number contain acidic residues at P1.

In most bound peptides, three amino acids are potential contacts with the T cell receptor (TCR); for the 52-60 segment Tyr 53, Leu 56 and Asn 59 are the TCR contacts; substitution of any of them results in a loss of T cell responses [47, 48].

No evidence was found of intramolecular competition among different segments of the HEL molecules (Figure 1.2). For example, take the processing of the HEL segments 31-47 of low binding strength, and 48-62, the highest affinity segment [21]. In the normal selection the family of 31-47 ends at residue 47 while the family of 52-60 starts at 48: there is no overlap between most family members. If proline is placed at residue 48, then the peptides identified by MS have substantial overlap in their sequence. However, despite this overlap there was no effect in the *amounts* of each segment selected chemically by the APC. These results are supported by an examination of HEL in which the 52-60 segment is not selected because of introduction of hindering residues. Such an HEL did not affect the amounts of minor HEL peptides of lower affinities, i.e., the dominant 48-62/3 segment was not competing out the minor, and each were selected from different molecules [21].

1.4 HEL: Conformational Isomers

Chemical analysis of naturally presented peptides from HEL led to the identification of conformational isomers of a peptide-MHC complex. Our initial observations challenged the concept of peptide determinants termed "cryptic" or hidden in the native protein, i.e., determinants that were not processed.

Several groups had indicated that CD4 T cells directed to HEL peptides did not recognize HEL presented by APC, leading to the interpretation that these peptides were not generated during processing [49]. However, our biochemical studies proved directly that such peptides from the processing of HEL were selected and bound to MHC molecules [50]. To reconcile these conflicting findings, we focused on the chemically dominant 48-62 epitope of HEL described above and found two sets of CD4 T cells to that epitope [51]. One set recognized both the peptide derived from HEL processing as well as the peptide 48-62 peptide given exogenously to the APC. These are the conventional T cells that we termed type A. In contrast, the type B T cells recognized only the exogenous peptide but not the peptide derived from the processing of HEL! Table 1.2 summarizes the various experimental manipulations that led to the characterization of type A and B peptide-MHC complexes.

Table 1.2 Type A and B complexes.[a]

APC Status	Antigen	Type A	Type B
Live	Native HEL	+	–
Live	Synthetic 48-61 peptide	+	+
Live	Tryptic peptide 48-61	+	+
Live	Extracted peptide from APC	+	+
Live	Covalent peptide-MHC complex	+	–
Live	Denatured HEL	+	+
Fixed[b]	Native HEL	–	–
Fixed[b]	48-62	+	+
Fixed[b]	Denatured HEL	+	+
Chloroquine-treated live[c]	Native HEL	–	–
Chloroquine-treated live[c]	48-62	+	+
DM-deficient[d]	Native protein	–	–
DM-deficient[d]	Peptide	+	+
Soluble I-$A^{k\,(e)}$	Covalent peptide-MHC complex	+	–
Soluble I-$A^{k\,(e)}$	Peptide-exchanged complex	+	+

a Indicated are the various experimental manipulations. Live APC included peritoneal macrophages, spleen DC, or B cells.
b Fixation in paraformaldehyde, which inhibits processing but allows for peptide exchange at the cell surface.
c APC also treated with chloroquine to inhibit intracellular processing.
d Refers to APC from mice with genetic ablation of the DM gene.
e Soluble A^k refers to baculovirus purified molecules.

The differences were neither explained by contaminants in the preparations of synthetic peptide nor by post-translational changes [52, 53]. Moreover, the peptide could be extracted from the class II molecules of APC, after HEL processing, and offered to the type B T cells, which then recognized it. In addition, recombinant complexes in which the 48-62 peptide was covalently linked to Ak molecules only stimulated the type A sets, while complexes formed by peptide exchange stimulated both subsets [53]. Presentation of the peptide by the type B complex was resistant to chloroquine, and did not require any processing, since fixed APC were able to present it, indicating that the peptide did not require further processing in acid vesicles [48]. Thus, the two T cells recognized the *identical* linear sequence. The mode of processing and assembly of the peptide was responsible for the difference in recognition, as will be explained below.

Zheng Pu and Javier Carrero went on to identify the T cell receptor gene segment usage of multiple type A and B T cell hybridomas, and found no skewing in either subset. However, type B T cells displayed unique recognition of the TCR-contact residues. As mentioned above, the three TCR contact residues for most if not all type A T cells are at P2 (tyrosine), P5 (leucine), and P9 (asparagine). Substitution of any of them by alanines resulted in complete loss of the response. Type B T cells segregated into two subsets: *type B-long* required contact with the P5 and P8 side chains and were indifferent to substitutions at P2. While t*ype B-short* T cells required contact with the P2 and P5 side chains and were indifferent to substitutions at P8. Type B-short T cells responded to a peptide consisting only of the P1–P7 residues [48].

Two sets of data led to the conclusion that the T cells differed in their unique recognition of conformational isomers of the same peptide-MHC complex in which the DM molecule played the pivotal role. Zheng Pu in our group developed an assay system in which the 48-62-Ak complex was formed in vitro either in the presence or absence of DM molecules. Both purified molecules were isolated from cultures of insect cells infected with recombinant baculoviruses (Figure 1.6). Peptide 48-62 was incubated with Ak molecules, and the complex was isolated and cultured with T cells. Both type A and B T cells responded equally well to the complex. Addition of DM increased the amount of peptide bound to Ak. But the addition of DM to the peptide-Ak complex had no effect on the response of type A T cells. However, type B response was completely eliminated. In a different manipulation, complexes were formed first and then incubated with DM briefly.

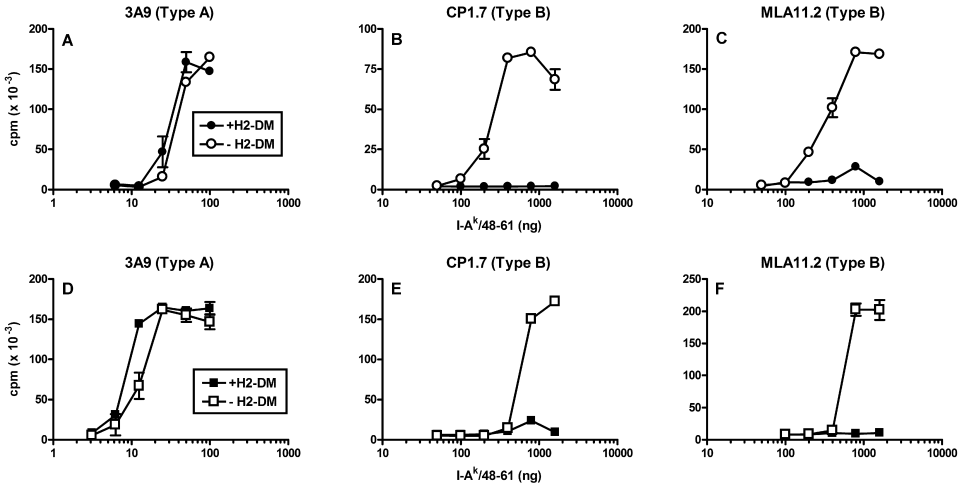

Figure 1.6 Formation of type A and B complexes in vitro, in the presence or absence of DM molecules. (A)–(C) Ak molecules were incubated with 48-62 in the presence or absence of DM. The complex was then isolated and incubated with one type A T cell (3A9) or two type B T cells (Cp1.7 and MLA11.2). (D)–(F) Ak molecules were first incubated with 48-61, after which Dm molecules were added. See the text for an explanation. (From Ref. 15.)

Such addition of DM also eliminated the type B conformer. This result indicated that the editing function of DM was exerted on previously formed complexes and not during assembly.

Scott Lovitch set out to evaluate the involvement of DM in the APC by using the system of liposome-mediated antigen delivery. Peptides were encapsulated in liposomes of different chemical composition to be delivered to either late endocytic vesicles, the sites of loading of peptides generated through processing of the protein, or to early endocytic vesicles, where exogenous peptide loading occurs.

Type A T cells responded to peptide delivered to both compartments, although targeting of peptide to late vesicles resulted in a markedly enhanced response; in contrast, type B T cells did not respond to peptide delivered to late vesicles, and responded only to peptide delivered to early endosomes [48]. DM molecules eliminated the type B complex in late vesicles. However, in DM-deficient APC, type B T cells responded to peptide delivered to late vesicles, in contrast to the results with wild-type APC.

We concluded that DM molecules are active in late vesicles, but essentially inactive in early endosomes and at the cell surface; furthermore, DM edits the repertoire of class II MHC-bound peptides, favoring high-affinity epitopes [54–58]. We reasoned that DM, likewise, was eliminating flexible conformers and favoring stable ones.

We envisage the following scenario: a globular protein such as HEL requires reduction and denaturation for processing; as a result, peptide-MHC loading only occurs in late endocytic vesicles. Here, protein antigens are unfolded and degraded, and peptides complex with nascent MHC class II molecules; DM then edits the conformation of the complex so that only the most stable conformer, type A, emerges and is exported to the cell surface (Figure 1.7A). In contrast, loading of exogenous peptides occurs in early endocytic vesicles, or at the cell surface, by exchange with weakly-bound peptides. Due to the absence of functional DM in these compartments, the flexible type B conformer forms in addition to the type A conformer (Figure 1.7B).

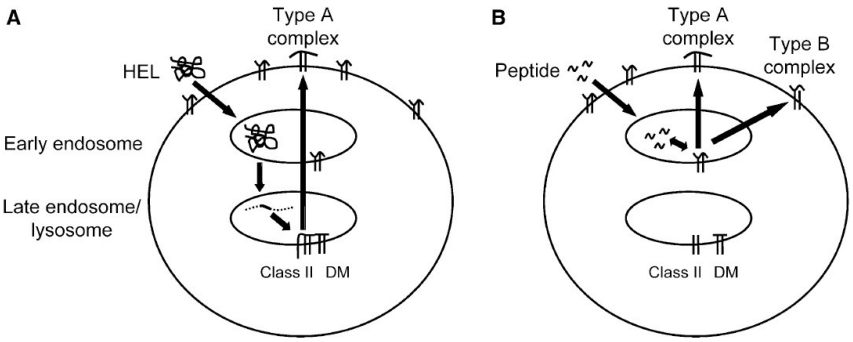

Figure 1.7 Model for the formation of distinct conformers of a peptide-MHC complex. (A) Processing of an intact protein affords a peptide-MHC complex in late endosomal vesicles under the influence of DM molecules, giving only type A complexes. (B) However, peptides can load by exchange in recycling vesicles without the editing role of DM and can generate various isomers. (From Ref. 15.)

1.4.1 Biology of Type B T Cells

The frequency of type B T cells in mice immunized with 46-61 in complete Freund's adjuvant varied from 30 to 50% of the responding T cells [53]. This was measured by limiting dilution analysis of cells in the draining lymph node. Thus the type B T cells did not represent a rare or aberrant T cell, but a significant component of the T cell repertoire. Very importantly, transgenic mice expressing HEL as a membrane-linked protein in their APC showed complete deletion of type A T cells. However, the type B T cells were not deleted, indicating that they had escaped negative selection. The HEL transgenic mice did not respond to immunization with native HEL (which induces mostly type A T cells), but responded to immunization with the peptide. Instead of representing the 30–50% of the anti-48-62 repertoire, type B now represented 100%.

To determine whether such self-reactive type B T cells were present in the normal T cell repertoire of non-transgenic mice, Scott Lovitch immunized B10.BR mice with an abundant autologous peptide derived from the beta chain of I-A^k; this particular peptide was shown by Kappler and Marrack and by us to be present on ~10% of MHC molecules in the spleen and abundantly present in the thymus [18, 19, 59]. Immunization with this peptide did, indeed, result in priming of type B T cells. That the naturally processed peptide was bound to Ak but the T cells failed to recognize it was proven by isolating it from the Ak molecules of the APC and then offering it as an exogenous peptide. Under these circumstances, the peptide was recognized [60].

Thus, naive type B T cells reactive to some self-peptides are found – but does their activation result in autoimmune pathology? Teleologically speaking, type B T cells are unlikely to have evolved to induce autoimmunity. Because of their abundance, we suggest that they represent a specific mode of recognition of exogenous peptides with biological importance, perhaps in antimicrobial immunity. Peptides from processed microbes (or dead and dying infected cells) may reach the extracellular milieu and in this way amplify the repertoire of the anti-microbial T cells. In this context, type B T cells that preferentially recognize short peptides suggest a preference for peptides generated under conditions of high proteolytic activity at inflammatory sites. Recently, Scott Lovitch generated mice with transgenic type B T cell receptors, which should prove extremely useful in investigating the mechanisms whereby peptides are released from proteins to become the type B epitopes.

Other laboratories have reported on T cell reactivity that could be explained by conformational differences. Janeway's group studied the presentation of the dominant peptide derived from Eβ chain presented by I-A^b. They identified T cells that appeared to respond to distinct forms of the complex and that varied in their susceptibility to negative selection in strains of mice that express Eβ [61–65]. Ward's group identified different specificities in the T cell response to the acetylated 1-11 peptide of myelin basic protein (MBP) presented by I-A^u explained by conformational differences [64]. Similar observations were made with respect to the MHC

class I allele HLA-B27 [65]. Finally, McConnell's group presented biochemical evidence for conformational isomers of cytochrome peptides [66].

1.5 Negative Selection and Peripheral Activation to HEL Peptides

The relationship between antigen presentation and an ensuing T cell response was addressed in the framework of the biochemical analysis on MHC-bound peptides. The biology of the T cell response to HEL was studied in two biological contexts. The first was central thymic tolerance: we examined how HEL as a self-protein affected T cell development and the response, particularly in situations where TCR transgenic anti-HEL T cells were present. The second issue was the T cell response after immunization of mice with HEL in adjuvant, focusing on their clonal distribution following immunization with HEL in Complete Freund's Adjuvant (CFA). The main issue was to understand how T cell clonal distribution correlated with 300-fold differences in peptide display [28].

To understand the biology of self-antigen presentation, Dan Peterson and Rich DiPaolo first measured the in vitro response of transgenic T cell receptor 3A9 cells that recognizes the complex of the 48-62-Ak. As few as 2–3 complexes per APC [67] deleted double positive thymocytes while 100-fold more complexes were required to activate single positive mature T cells [see also Ref. 68]. The highly efficient process of deletion was confirmed in vivo when transgenic mice expressing membrane HEL driven by the class II promoter were immunized with HEL and the clonal response to its various peptides examined. HEL expression in the thymic APC negatively selected T cells against all HEL displayed peptides [53], even against those with display levels 300-fold less than the chemically dominant 48-63 epitope.

We postulated a "biochemical margin of safety" for self-peptides: thymocytes required fewer peptide-MHC complexes to be deleted than mature T cells needed for their activation. This ensures that a mature T cell that escapes negative selection must see high levels of peptide-MHC complex in the periphery to become spontaneously activated. However, conditions of inflammation may circumvent the need for high-level peptide-MHC to attain peripheral activation [69].

We are evaluating the "biochemical margin of safety", in models of autoimmune diabetes in which HEL is expressed on pancreatic beta cells that express HEL as a membrane protein under the rat insulin promoter [70]. The first transgenic mouse, developed by Goodnow, called ILK-3, had about two million mHEL molecules per beta cells. A cross of the 3A9 TCR transgenic to the RIP-mHEL mice resulted in the development of diabetes [70, 71]. The 3A9 x ILK-3 double transgenic mice developed diabetes, despite extensive negative selection and limited number of T cells in the lymph nodes [71]. Diabetes was likely caused by the high level of HEL in the beta cells (2×10^6 molecules per cell), and its effective cross presentation by APCs in the draining peri-pancreatic lymph node These studies, first by my colleague Rich DiPaolo and most recently by Craig Byersdor-

fer, suggested that 48-63-Ak complexes presented in a localized fashion exceeded the peripheral "margin of safety" and activated the T cells, beginning the autoimmune process.

1.6 Response to HEL Immunization in the Draining Lymph Node

We studied the clonal distribution of T cells following immunization with HEL in adjuvants by using a sensitive limiting dilution assay for measuring T cell responses [72]. The number of HEL reactive T cells was directly proportional to the amounts of HEL used for immunization. However, a strikingly poor correlation was found between the amount of each epitope displayed by Ak molecules of APC and the *relative* distribution of the reactive T cells following immunization [73]. For example, while the 52-60 peptide family was displayed in very high numbers the T cell clones to it were about the same as for the 20-35 and 114-129 peptide families, which are displayed at considerably lower amounts. Even when immunizing with 100-fold lower levels of HEL as immunogen, the relative reactivity to the four characterized HEL epitopes remained consistent.

Immunization experiments were performed in mice lacking CD40 [74], B7-1 & B7-2 concurrently [75], or in mice treated with an antibody to block CTLA-4 [76], to investigate the role of co-stimulatory molecules. The total anti-HEL response was lowered to varying degrees in the gene knockout mice, but the relative distribution (i.e., percentage of clones found against each epitope) did not change [77]. No one epitope was more or less sensitive to the effects of either co-stimulation or lowered antigen levels. Neither was there an influence of CIITA (Table 1.3).

Table 1.3 Distribution of T cells in lymph nodes of mice immunized to HEL.

Dose			% Specificity			
Mouse	**(nmol)**	**Frequency**	**48-63**	**31-47**	**18-33**	**115-129**
B10.BR	10	1/5000	30	9	17	25
B10.BR	1	1/9500	38	9	15	17
B10.BR	0.1	1/50 000	37	3	17	11
B10.BR	10	1/5000	30	9	17	25
$CD40^{-/-}$	10	1/12 600	45	7	21	27
$B7\text{-}1/B7\text{-}2^{-/-}$	10	1/64 500	37	16	18	8
Anti-CTLA-4	1	1/32 000	25	5	11	20
Control IgG	1	1/27 500	32	5	17	25

This table summarizes experiments published in Refs. 73 and 77.

The APC responsible for presentation of HEL in vivo was examined by tracing radiolabeled HEL into the draining lymph node [78]. The major subsets of cells bearing HEL at 24 h were $CD11c^+$ or $CD11b^+/CD11c^-$ (i.e., DCs and macrophages). At the peak times of presentation, 24–96 h post immunization, the HEL was highly concentrated in a small percentage of DC and macrophages (ca. 1%). This highly efficient concentration indicated that each APC had enough HEL to present all the different peptide-MHC, albeit at widely different densities. If the total HEL had been distributed among most of the APC of the node, rather than concentrated in a few, it would have been mathematically and biochemically impossible to present the minor peptides.

Following high-dose immunization, B cells were found to be positive for 48-62-Ak complexes and functionally capable of presenting HEL peptides, but only following high-dose immunization. The role of B cells is likely to be as an accessory APC, amplifying the response initiated by DCs.

The question as to why immunodominance (the distribution of the reactive T cells) did not correlate with chemical dominance (hierarchy of epitope presentation) remains unresolved. The leveling of the clonal distribution could be the result of (i) a complex interplay between negative and positive co-stimulatory molecules and/or cells not yet investigated; (ii) cooperativity among activated T cell clones, shown in one example in the CD4 response [79], but argued against with respect to CD8 T cells [80]; or (iii) a difference in the primary repertoire of reactive T cells that is then expanded during immunization. All three areas are actively being pursued in the laboratory.

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