Comparison of Peptides Bound to Spleen and Thymus Class II

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Summary

In the past we and others have suggested that positive selection of developing thymocytes may depend upon interaction between the $\alpha\beta$ receptors on these cells and major histocompatibility complex (MHC) proteins bound to peptides found uniquely in the selecting tissue, thymus cortical epithelium. To test this hypothesis, peptides were isolated from MHC class II proteins of spleen, thymus cortical plus medullary epithelium, or thymus cortical epithelium alone. The results showed that the major peptides bound to class II on thymus cortical epithelium were also associated with spleen class II. Some peptides could only be detected in isolates from spleen, probably because of differences in the distribution or uptake of the donor proteins between spleen and thymus. Thus, although we found some tissue-specific distribution of self-peptides, our data suggest that there are no fundamental differences among these tissues in the occupancy of class II MHC by self-peptides. These results limit hypotheses which depend on a specialized mechanism of peptide generation and/or MHC class II loading to account for the positive selection of T cells on thymic cortical epithelium.

Many peripheral T cells bear antigen receptors made up of α and β chains. These receptors are encoded by sets of genes which rearrange randomly during T cell development, such that any given mature T cell may bear 1 of $\sim 10^{12}$ possible receptors on its surface. Even though the receptor genes of an animal encode a very large number of possible combinations, it is known that not all receptors are expressed on the mature T cell pool of any given animal. In large part this is because the allowed repertoire of receptors on mature T cells is restricted by two different phenomena which occur during T cell development. These are the events which accompany positive and negative selection of thymocytes (1–3).

Both positive and negative selection involve interaction between the TCRs on developing thymocytes and MHC plus peptide complexes expressed by cells in the thymus cortex (3–6). Both events may also involve accessory molecules, CD4 and/or CD8 on the thymocyte surface (7–10). In spite of their similarities, however, the two events have entirely opposite outcomes and a number of hypotheses have been put forward to solve this paradox. For example, one hypothesis suggests that tolerance (or mature T cell response) requires, on the one hand, a fairly high affinity reaction between the receptors on a developing thymocyte and MHC plus peptide, but that positive selection, on the other hand, might occur during a very low affinity reaction between the same two components. Thus, some thymocytes could be selected to mature by a reaction between receptor and self-MHC plus self-peptide which is too low to invoke the forces of tolerance. These thymocytes would then escape the thymus to form the mature, self-MHC–restricted pool of T cells in their hosts (11, 12).

Some years ago we and others suggested an alternate hypothesis, that positive selection might occur during reaction between TCRs on developing thymocytes and MHC molecules bearing a special set of self-peptides, found only in the thymus cortex (13, 14). The hypothesis stated that thymocytes at this stage of their development might be resistant to tolerance induction and that, by the time the thymocytes had become sensitive to tolerance, they would have moved out of the thymus compartment where these thymus-specific peptides were to be found.

Since these hypotheses were put forward many investigations of positive and negative selection have been reported. Some findings have supported the "low affinity" theory of positive selection. For example, several papers have shown that T cells can become reactive to self if they are provided with more, or functional CD8, acting, perhaps to promote

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cell–cell interaction (15–17). In another approach Duke has shown that T cells activated to attack cells bearing allogeneic MHC may also attack cells bearing self-MHC, but not "innocent bystander" cells (18).

In contrast, some evidence has also been collected that favors versions of the "altered peptide" theory. For example, we have shown that some T cells can react with the thymus cortical epithelial cells of their own host (19), and Murphy et al. (20) have shown that a particular self-peptide may be found at fairly high levels bound to I-A of spleen cells, but at much lower levels on I-A on thymus epithelium. Moreover, there are also suggestions that early, TCR^+, $CD4^+$, and $CD8^+$ thymocytes, unlike their later counterparts, are resistant to death induced by engagement of their TCRs (21).

The idea that unique selecting peptides are associated with thymus cortical epithelial MHC has recently been challenged, however, by two types of experiments. Two publications have shown that positive selection, of at least class I–restricted T cells, can be driven by MHC bound to foreign peptides, or to peptides from spleen (22, 23). Three other papers have shown that positive selection can be effected by reaction between the receptors on developing thymocytes and MHC proteins on nonspecialized cells such as fibroblasts or those derived from bone marrow (24–26). Both sets of results indicate that no unique properties are required of the selecting ligand in the thymus.

The recent description of methods for isolating and identifying peptides bound to MHC class I and class II (27–29) has made it possible to perform a direct test of the "altered peptide" theory of positive selection. The results of such a test, described here, show that many peptides are shared between thymus and spleen class II. Some are even shared between thymus cortical epithelial cells and spleen class II. Some peptides could only be found in spleen, probably because the proteins from which they were derived were at higher concentration in spleen than thymus. Overall, the results suggest that the major species of peptides bound to MHC on thymus cortical epithelial cells are also associated with MHC on spleen and thymus MHC molecules are neither predominantly empty nor occupied by a set of major peptides unique to the tissue.

Materials and Methods

Mice. 6-week-old C3H/HeJ female mice were purchased from Harlan Sprague Dawley Laboratories (Indianapolis, IN). ΔY mice were bred in the Animal Care Facility at National Jewish Center. Breeding pairs of the ΔY mice were the generous gift of Drs. Benoist and Mathis (Institut National de la Santé et de la Recherche Médicale, Strasbourg, France). These animals are transgenic for an E^k_α gene with a promoter region carrying a deletion of the Y box. The transgene is expressed only in thymus cortical epithelial cells and at low levels in B cells and macrophages (30). Consequently, in mice carrying class II genes of the s haplotype, the haplotype expressed in these mice, expression of the I-E protein is restricted to these tissues.

$I\text{-}E^k$–expressing mice lacking a functional β_2-microglobulin (β_2m [31])[1] gene were the F2 progeny of a cross between β_2m^-, $H\text{-}2^b$ mice and B10.BR mice. Experimental mice were selected for lack of class I and lack of $I\text{-}A^b$ expression.

Preparation of Peptides from Class II. Spleens and thymuses were isolated from 100–375 mice for each class II preparation. These yielded between 10^{10} and 4×10^{10} cells. Tissue that could not be dispersed was included in the preparations of cells from thymus, in that the goal of the experiment was to isolate class II from thymus epithelial cells, which might not separate well into single cell suspensions. In each experiment 10^8 cells of each type were surface labeled with ^{125}I, to provide a trace label, and then added back to the bulk preparations. Cells were lysed with a buffer containing 2% NP-40, 50 mM iodoacetamide, and 100 μM PMSF. The lysate was cleared by spinning at 100,000 *g* for 2 h. Each lysate was then divided into thirds and passed sequentially over columns bearing 117/27 (anti-$I\text{-}A^k$), 14-4-4 (anti-$I\text{-}E^k$), or MKS4 (anti-$I\text{-}A^s$). The MKS4 column served as a nonspecific control for preparations from the $I\text{-}A^k$, $I\text{-}E^k$–bearing C3H mice. The 17/227 column served a similar purpose for the $I\text{-}A^s$, $I\text{-}E^s$–bearing ΔY cells. Each third of the preparations was passed over these columns in a different order to ensure that each column was equally exposed to material that might bind nonspecifically. The columns were then washed and eluted as described by Buus et al. (32). Elution was followed by monitoring ^{125}I counts. The eluates were immediately neutralized with 2 M Tris, pH 8.0, concentrated on prewashed microconcentrators (Centricon 30, Centrico, Inc., Northvale, NJ) and washed with 10 mM Tris, pH 6.5.

Peptides were eluted on the Centricon 30 microconcentrators with 2.5 M acetic acid as previously described (28, 32), and were then isolated by passage through prewashed microconcentrators (Centricon 10). The eluates were lyophilized and stored at −70°C.

SDS-PAGE Analysis of Class II Proteins. After the peptides had been eluted from the class II proteins, the quantity of these proteins was estimated by solubilizing the preparations in buffer containing 0.2 M Tris pH 6.8, 30% glycerol, and 4% SDS (sample buffer [33]). Samples of the solubilized protein were boiled for 5 min and run on SDS-PAGE in parallel with titrations of known amounts of standard proteins. The gels were silver stained and the amounts of class II protein estimated by comparison of the intensities of their bands with those of the protein standards.

In experiments not shown, the percentage of relatively unstable class II proteins in each preparation was estimated after solubilization of the proteins with 3 min incubation on ice in sample buffer containing 1% SDS. The percentage of associated $\alpha\beta$ chains in this preparation was then compared with that in a preparation treated conventionally before electrophoresis (34, 35).

HPLC Analysis of Class II–derived Peptides. Peptides were separated by HPLC reverse-phase chromatography using a 2.1 × 15-cm column (VYDAC C18, The Sep/a/ra/tions Group, Hesperia, CA) washed with 5 min of isocratic 0.1% TFA/water followed by a 0–60% linear gradient of 0.1% TFA/water versus 0.083% TFA/CH_3CN over a period of 60 min. Peptides were detected by absorbance at 214 nm.

Peptide Sequences. Fractions isolated by HPLC were sequenced by gas phase Edman degradation on a model 470A/120A protein microsequencer (Applied Biosystems, Inc., Foster City, CA). Data analysis was performed using Dynamax chromatography software (Rainin Instrument Co., Woburn, MA). The molar yield of each peptide was calculated from the concentration of one of the amino acids in the peptide corrected for losses estimated from yields ob-

[1] *Abbreviations used in this paper:* HEL, hen egg lysozyme; β_2m, β_2-microglobulin; PCC, pigeon cytochrome *c*.

tained when a known amount of a standard peptide was sequenced. The molar amounts of the class II proteins from which the peptides were derived were estimated by comparison with standards on silver-stained gels. Peptide and class II molar yields were used to calculate the percentage of each class II protein occupied by a given peptide. Genbank was searched to identify the probable protein sources of the peptides.

Peptide Synthesis. Synthetic peptides built to match some of the sequences isolated from class II were purchased from Macromolecular Resources (Fort Collins, CO) or prepared and purified in the Molecular Resource Center of the National Jewish Center. In some cases the NH_2-terminal amino acid of a particular peptide isolated from spleen or thymus was known, but the COOH-terminal end was unknown, because the sequence of the peptide fell below detectable levels before a well-defined COOH terminal could be identified. In these cases, synthetic peptides were built to match the natural peptide, starting at the known NH_2 terminal and continuing for the next 12–16 amino acids.

T Cell Stimulation Assays. The ability of the peptides identified in these experiments to bind to class II was assessed by their ability to block binding of known peptide ligands of the class II in question. This was done in T cell hybridoma stimulation assays as previously described. Antigen-presenting cells, CH12.1 or LS102.9, bearing class II I-A^k, I-E^k, or I-$A^{d,s}$, I-$E^{d,s}$, respectively (36) were fixed by 30 seconds incubation in 0.1% gluteraldehyde and then thoroughly washed. 10^5 of each of these cells were added to microtiter wells in 50 μl of balanced salt solution and various concentrations of inhibitor peptide and the mixture incubated at 37°C for 1 h. 10^5 T hybridoma cells and stimulatory peptides were then added to each well in 200 μl of culture medium. The mixture was incubated for 24 h at 37°C. The response of the T cell hybridomas was assessed by measurement of the amount of IL-2 that they secreted (37).

T cell hybridomas used were as follows: kLy-4.3, specific for hen egg lysozyme (46-61) (HEL [46-61])/I-A^k (38); h4Ly-50.5, specific for HEL (46-61)/I-A^k (38); 5KC-73.8 (39) and 2B4.6 (40) specific for pigeon cytochrome *c* (88-104) (PCC [88-104])/I-E^k; t3C-32.9 specific for PCC (88-104)/I-E^s (unpublished); DO-11.10/S4.4 specific for chicken ovalbumin (323-339 [OVA (323-339])/I-A^d (41). Stimulatory peptides were as described for each hybridoma.

In Vivo Responses to Peptide. B10.BR or H-2^k animals lacking a functional β_2m gene (31) were immunized with 20 μg of β_2m (31-43) in CFA in the base of the tail. 7–9 d later draining lymph node cells were harvested and cultured for 4 d in Click's medium containing the same peptide at 100 μg/ml. Dividing cells were then expanded for 3 d in IL-2 before fusion with a variant of BW 5147 lacking functional TCR α and β chains. Hybrids were selected as previously described. T cell hybridomas were screened for response to the I-E^k–bearing, β_2m-expressing B cell lymphoma, CH12.1, in the presence or absence of additional β_2m peptide.

Results

We wished to compare the spectrum of peptides bound to MHC proteins on the tissue responsible for positive selection, thymus epithelium, and on peripheral tissues in the same animal. We chose to work with MHC class II proteins, for which the predominant source in thymus is the thymus epithelium. Peptides from class I proteins could not be used because class I is expressed in mouse thymus not only on epithelial cells but also on thymocytes. Therefore epithelial-derived class I could not be examined without extensive prior tissue purification. The low yields resulting from such purification would have made peptide analysis of the type reported here impossible.

Peptides Bound to Class II on Spleen and Thymus Epithelial Cells. Peptides were isolated from the class II proteins, I-A^k and I-E^k, prepared from spleen or thymus of C3H/HeJ animals. In the normal animals used in these experiments most of the class II in spleen is borne on resting B cells. As noted above, most of the class II in thymus is expressed on thymus cortical or medullary epithelium (42). The idea that class II is derived from different cell types in the two tissues is supported by the finding that the α chains of I-A or I-E purified from thymus all had somewhat faster mobilities on SDS-PAGE than those purified from spleen and, in fact, gave two bands on SDS-PAGE analysis rather than the one found in spleen, as illustrated in Fig. 1. These differences are due to the fact that class II α chains are glycosylated differently in spleen and thymus, since, after *N*-glycanase treatment, class II proteins from spleen and thymus ran identically (data not shown).

Peptides were eluted from the I-A or I-E preparations and separated by HPLC. The absorbances at 214 nm of the HPLC runs are shown in Fig. 2. Some of the largest peaks were found in all runs, including those of control preparations. These peaks contained no peptides as determined by Edman degradation (not shown) and were therefore probably caused by solvents or chemicals extracted from the Centricon microconcentrators. Other peaks were common to I-A isolated from either spleen or thymus, but were not found in the control runs, nor in the peptide preparations isolated from I-E. Conversely, many of the I-E peaks were shared between spleen and thymus but were not found in the I-A preparations. These peaks, which did turn out to contain peptides, indicate that some of the peptides bound to I-A or I-E in spleen or thymus probably are common to the same class II molecule found in different sites. These peaks also suggest that I-A and I-E do not bind the same spectrum of peptides, not surprisingly. A few peptide peaks were found in spleen but not thymus. These may indicate different abilities of thymus and spleen to capture or produce MHC-bound self-peptides.

Some of the peaks identified in these HPLC runs were sequenced and their yields estimated by comparison of the molar yields of the peptides with the known concentration (estimated from silver stained gels) of the donor class II protein.

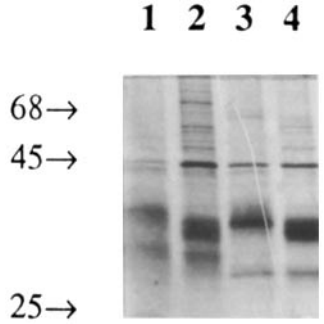
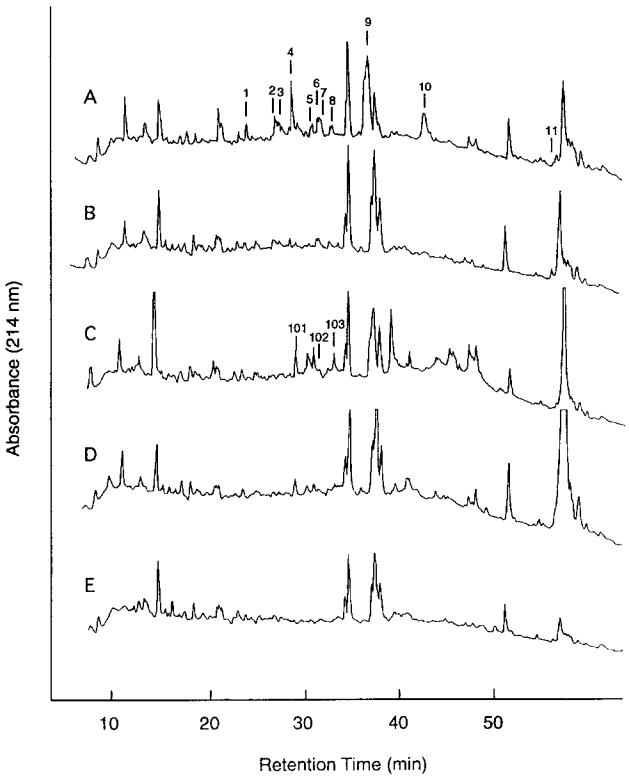


Figure 1. SDS-PAGE analysis of class II isolated from spleen and thymus. I-A^k and I-E^k were isolated from the thymuses and spleens of 100 C3H/HeJ animals as described in Materials and Methods. 1/100th of each preparation was run in SDS on 12% PAGE and silver stained. Preparation sources were as follows: lane *1*, I-E^k, spleen; lane *2*, I-E^k, thymus, lane *3*, I-A^k, spleen; lane *4*, I-A^k, thymus. The molecular masses of markers in kilodaltons are noted on the figure.



The results of these analyses, for I-A^k– and I-E^k–derived peptides, respectively, are shown in Tables 1 and 2. Since sequencing starts at the NH_2-terminal amino acid, the NH_2-terminal end of each peptide could be unequivocally identified. Because only very low amounts of the peptides were isolated, however, often we could not be certain whether the loss of readable sequence corresponded to the end of a peptide or not. Even so, most sequences contained 14 or 15 amino acids and were therefore similar in length to peptides found by others bound to class II isolated from cultured cells (28, 29).

Genbank was searched for the likely sources of the peptides. Of the 17 sequences, 14 came from known proteins. In all cases the peptide sequences matched those of the mouse

Figure 2. HPLC separations of peptides isolated from spleen or thymus I-A or I-E. Peptides were isolated from spleen and thymus preparations of I-A^k and I-E^k or material isolated on a control column and fractionated by reverse-phase HPLC as described in Materials and Methods. Shown are the absorbances at 214 nm of typical peptide HPLC separations. Splenic peaks that gave informative sequences are numbered from 1 to 11 for I-A^k and from 101 to 103 for I-E^k. Peaks eluting at the following minutes gave no sequence: I-A^k spleen, 17.8, 34.4, 36.4, 37.4, 38.7, 42.5, 46.4, 51.7, 53.2, 55.3; I-A^k thymus, 18.7, 31.5, 33.6, 34.3, 35.9, 50.5; I-E^k spleen, 18.4, 44.5; I-E^k thymus, 48.3. (*A*) I-A^k spleen; (*B*) I-A^k thymus; (*C*) I-E^k spleen; (*D*) I-E^k thymus; (*E*) control thymus.

Table 1. *Peptides Bound to I-A^k in C3H/HeJ Mice*

Fraction	Peptide	Possible donor	Percent of MHC occupied in Spleen	Thymus
1	xQLGAQNEMLxPV	Unknown	0.6	<0.1
2	a vvKKGTDFQLNQLE	Transferrin 100-	2.5	0.3
3	k kGTDFQLNQLEGKKG	Transferrin 103-	2.5	0.2
4	e YVRFDSFVGEYRAVT	A^k_β 37-51	9.3	1.1
4	xPLALQFAELPVNKG	Unknown	0.5	0.2
5	xPIDDGGIEA	Unknown	0.4	<0.1
6	e eNLRFDSDVGEFRAV	E^k_β 33-	1.6	nd
7	y EDENLYEGLNLDDcSMYE	MB1 177-	9.2	<0.1
8	e y i LYNKG I MGEdSYPY	Cathepsin H 77-	3.5	0.2
9	r sYLDAwVcEQLAT	Fcε Receptor II 298-	1.0	<0.1
10	n serHFVHQFQPFcyF	A^k_β 3-	0.5	<0.1
11	h QFQPFcYFTNT	A^k_β 10-	4.3	<0.1

Peptides were separated and sequenced as described in Materials and Methods. The molar yield of each peptide was calculated from the concentration of one of the amino acids in the peptide (underlined) corrected for losses estimated from yields when a known amount of standard peptide was sequenced. The molar amount of donor I-A^k was estimated by comparison with standards on silver-stained gels. Sequences are shown with amino acids identified by sequencing in capital letters, and those derived from the known sequence of the probable donor protein in lower case. Also shown in lower case, derived from the probable protein donor, is the amino acid immediately preceding the NH_2-terminal amino acid of the identified peptide. Unidentifiable amino acids in peptides of unknown origin are shown as "x." Fractions indicated as "nd" were not sequenced in the thymus preparation. We were not sure at which point peptides designated with an NH_2-terminal amino acid number only ended. Peptides for which we were confident of the COOH-terminal amino acid are designated with both an NH_2 and COOH-terminal amino acid number.

Table 2. *Peptides Bound to I-E^k in C3H/HeJ Mice*

Fraction	Peptide	Possible donor	Percentage of MHC Occupied in	
			Spleen	Thymus
101	f hPpH I E I QMLKNG	β_2m 31-	3.5	2.8
101	y vNKE I QNAVQGVK	C cyt inh 41-	1.9	3.4
102	f HPPH I E I QMLKNGKK I P	β_2m 31-47	5.2	nd
103	f dNRMVNHF I AEFKRK	Cognate HSP70 234-	2.0	2.0
103	s tPTLVEAARNLGRVG	Serum albumin 347-	1.1	2.6

Peptides were analyzed and are presented as described in Table 1. Abbreviations: C cyt inh, complement cytolysis inhibitor; HSP70, heat shock protein 70.

protein identified as the likely donor except for the two peptides thought to be derived from transferrin. The sequence of the mouse transferrin gene has not been published. However the peptides we found are very similar to sequences in human transferrin (VVKKDSGFQMNQLRGKK) and pig transferrin (VVKKGSNFQWNQLQGKR). The peptide identified as from cathepsin H is identical in sequence to a peptide from rat cathepsin H. The homologous mouse protein has not been sequenced.

Of the 10 known donor proteins, 9 are found in the cellular compartments thought to be the sources for class II–bound peptides; i.e., they are plasma membrane or exogenous proteins. The cellular site of the 10th protein donor, cognate hsp70, is less certain. It has recently been recognized, however, that a cognate form of hsp70 is involved in the transport of proteins into lysosomes, in which case the hsp70 peptide we found bound to I-E^k may be lysosomal in origin (43).

Some of the class II–bound peptides, for example, transferrin 100- and transferrin 103-, were overlapping. This phenomenon has been observed before (28, 29) and probably reflects differential processing of peptides which are bound by the same core sequence to class II. The fact that class II–bound peptides can extend beyond the MHC proteins to varying degrees probably accounts for the fact that it is difficult to perceive a motif in the sequences of the peptides we found bound to I-A^k (Table 1). Presumably the sequences of these peptides does not necessarily reflect the register of the peptides in the class II cleft.

In spite of the differences in register, a motif could be detected for the peptides bound to I-E^k (Table 2). This motif, which has been noticed in the past by others (44, 45), is illustrated in Table 3. Results presented here and elsewhere show that I-E^k binding requires a hydrophobic aliphatic residue near the NH_2 terminus of the peptide. An amino acid which can function as a hydrogen bond donor/acceptor is often found adjacent to this position. Glutamine or asparagine are usually found five residues COOH terminal to the hydrophobic amino acid and a lysine residue is usually found four or five residues further down the peptide.

5 of the 16 peptides bound to class II^k contained a proline at position 2. This finding suggests that a peptidase with specificity for peptide sequences with this property might be involved in the production of some class II–bound peptides.

In these studies we sequenced virtually every fraction eluted from the HPLC. Although snippets of sequence were obtained at other positions, the peptides described here represent by far the most abundant found bound to I-A^k and I-E^k. Since the found peptides account for ~35% of I-A^k and 15% of I-E^k this suggests that the peptides bound to the remaining 65% and 85% of I-A^k and I-E^k, respectively, must be at much lower abundance than the ones we could sequence, i.e., they each must occupy <0.1% of the grooves of the relevant class II protein. This indicates that each class II protein in vivo is probably occupied by >100 peptides, and perhaps by as many as the 2,000, as has been suggested by some other groups (29). Whether the low abundance peptides are at high enough concentration to affect the repertoire of T cells in the animal remains to be established.

Table 3. *Motif for Peptides Bound to I-E^k*

Peptide	Sequence
β_2m 31-47	HPPH I E I QMLKNGKK I P
C cyt inh 41-53	VNKE I QNAVQGVK
HSP70 234-248	DNRMVNHF I AEFKRK
MSA 347-361	TPTLVEAARNLGRVG
PCC 88-104	KAERADL I AYLKQATAK
MCC 88-104	ANERADL I AYLKQATK
Lambda rep 12-26	LEDARRLKA I YEKKK
HEL 81-96	SALLSSD I TASVNCAK

Peptides from this and other studies were aligned according to the I-E^k binding motif. Underlined residues are those suggested to bind to I-E^k by Jorgensen et al. (45), whereas those conserved throughout the sequences determined in this study are shown in bold. The ability of the PCC, moth cytochrome *c* (MCC), lambda repressor (Lambda rep), and HEL peptides to bind to I-E^k has been described in references 56 and 57.

Many of the peptides had the same HPLC elution position and sequence regardless of whether they came from spleen or thymus class II, suggesting that the pathways for protein processing are similar in the two tissues. There were some differences in the peptide profiles from the two tissues, however. Some of the discrepancies were due to differential donor protein expression in the two tissues since two of the peptides that were unique to spleen were derived from hematopoetic or B cell–specific proteins, Fcε receptor II (46) and MB1 (47), a member of the surface immunoglobulin complex. Other differences may have been due to differential uptake of the donor protein by cells in the two tissues. Transferrin peptides, for example, were readily apparent in isolates from spleen class II, but barely discernable in thymus isolates. Growing cells take up more transferrin than resting cells do, but since neither the thymus epithelium nor the spleen cells in the noninfected mice used in these experiments are rapidly dividing, differential growth rates in the two tissues probably do not account for the differential transferrin peptide distributions. Perhaps resting thymus epithelial cells take up even less transferrin than resting B cells do, and this explains the difference.

Overall, these results showed that splenic B cells and thymus epithelial cells have the capacity to make many of the same peptides. There may be some differences in their class II peptide profiles, however, usually because of differential availability of the donor proteins.

Isolation of Peptides from Class II on Thymus Cortical Epithelial Cells. In normal mice class II is expressed on epithelial cells in both the thymus cortex and medulla. Staining experiments show that medullary cells probably bear >50% of the class II in thymus because, even though they are fewer in number, they bear much more class II per cell than do cortical epithelial cells (42). It is therefore possible that the analysis described above did not reveal the peptides bound to the class II proteins actually involved in positive selection, those on cortical epithelial cells.

We therefore repeated the experiment described above using thymus and spleen from ΔY mice. Unusual tissue expression of the transgenic *I-Eα* gene in these animals leads to expression of I-E on thymus cortical but not on thymus medullary epithelium (30). I-E is also expressed on bone marrow–derived cells in these animals, at ~30% the level of normal mice. Therefore nearly all the I-E isolated from the thymuses of ΔY mice is derived from thymus cortical epithelium.

The ΔY *I-Eα* transgene was backcrossed in to B10.S(7R) mice. The resulting animals expressed I-E^s with a tissue distribution characteristic of ΔY. I-E^s was isolated from the spleens and thymuses of these animals. A low yield of I-E was obtained from both tissues, a result which reflects the low level expression of I-E. Like the class II α chains isolated from normal animals, the α chains of I-E isolated from thymus ran more quickly on SDS-PAGE than did the α chains isolated from spleen (data not shown). This property is therefore a characteristic of class II expressed on all epithelial cells in the thymus.

Peptides were eluted from I-E from both tissues, and analyzed as described above. Few peptides were unequivocally identified and the sequences appeared more contaminated with minor species than those obtained from normal mice. This was in part due to the fact that, because of expected low yields, many more mice were used in for the ΔY preparations than for the normal mouse isolations. Perhaps this led to higher background contamination with peptides produced in tissues during protein degradation unrelated to class II binding. For example, peptides derived from actin were found at several positions in several of the ΔY isolations. Binding experiments similar to those described below showed that these actin peptides had no affinity for I-E^s, and therefore they were probably present as contaminants rather than as genuine class II associates.

Table 4 contains the sequences and yields of peptides that we are confident were bound to I-E^s and one peptide that we think is probably a contaminant (see below). Two peptides were isolated from I-E^s on both spleen and thymus cortical epithelium in relatively high amounts. Genbank searches showed that both of these peptides were derived from MHC-associated proteins, one from L^d and the other from β_2m. The latter peptide was identical in HPLC elution profile and sequence to the β_2m peptide isolated from I-E^k. Pigeon cytochrome *c* 88-104 also binds to I-E of both the k and s haplotypes, so perhaps these class II proteins share some requirements for peptide binding.

Some other sequences were present in low amounts, and complete sequences could not be derived, nor could donor proteins be identified. These sequences were difficult to identify because, of course, each fraction of the HPLC peptide separations contained many peptides, most in very low quantities. Therefore amino acid sequence analysis of many of the peptide HPLC fractions yielded from one to four amino acid calls at each position. Nevertheless, for some fractions, particular amino acids were found at the same position upon sequencing the same peptide HPLC fraction of several independent peptide preparations. Sequences identified in this way are shown in Table 4. Although we cannot be sure that each sequence represents the product of a single peptide, we are sure that the amino acids shown and their positions in their donor peptides are characteristic of peptides eluting in that position on the HPLC. Several of these sequences could be found in both spleen and thymus although they were in lower amounts in the thymus preparations, probably because less I-E could be purified from this tissue.

One peptide of this type was found only in peptide preparations from spleen. We do not know whether this was because the donor protein for this peptide was expressed at higher levels in spleen than in thymus or simply because of the small amounts of I-E, and therefore I-E–derived peptides, which could be isolated from thymus.

Occasionally sequences were present in thymus but not spleen. These included the NH_2-terminal sequence of histone 2B (Table 4) which was found in two independent preparations of I-E^s from ΔY thymuses but not from spleen. However, it is likely that this peptide was simply a contaminant in the preparations because the peptide does not bind I-E^s

Table 4. *Peptides Bound to I-E^s in ΔY Mice*

Fraction	Peptide	Possible donor	Percentage of MHC occupied in	
			Spleen	Thymus
47-48	XXXLK I XXKG	Unknown	0.5–1.3	0.6
58-61	HPPH I E I QMLKNGK	β_2m 31-44	2.4	>1.7
60-63	EGECVEWLHRYKNGNA	L^d 160-175	2.7	>4.9
61-62	XXQ I LXLXGK	Unknown	1.4	<0.1
88-90	X I MPFLKLFPG	Unknown	0.6	0.1–1.0
99-100	pEPAKxAPApK	Histone 2B 1-	<0.1	0.2–1.2

Peptides were isolated and fractionated as described in Materials and Methods and in the footnote to Table 1. 0.5-ml fractions were collected from the HPLC separation and fractions were numbered from the time of injection of the peptide preparation into the HPLC loop. Amino acids shown were found reproducibly in peptides eluting in the fractions indicated from at least two independent isolates of peptides bound to I-E^s in the spleens or thymuses of ΔY mice. Multiple residues were used to determine the amount of peptide applied to the sequencer. In some cases a range of relative abundances, found in the different preparations analyzed, is given.

in inhibition experiments (see Fig. 4 below), and one might expect histone fragments to be at high concentration in tissues containing many dying cells, such as the thymus.

Overall, there was no indication from the few peptides we were able to identify that the peptides bound to thymus cortical epithelial class II were substantially different from those bound to class II of spleen. Also, there was no indication that class II on thymus cortical epithelium might be empty. This conclusion was supported by preliminary experiments (not shown), which demonstrated that the percentages of unstable I-E proteins in spleen and thymus of ΔY mice were similar (34, 35).

Demonstration That the Peptides Found in These Analyses Bind to Class II. In order to prove that the peptides we had identified were indeed derived from the antigen-binding clefts of class II, some of the peptides were synthesized and tested for their ability to bind to the clefts of the appropriate MHC protein in antigen presentation inhibition assays (47a). As shown in Fig. 3 nearly all the peptides so tested had activity in such assays. For example, A$^k_\beta$ 3-17 or 37-52 blocked recognition of I-A^k plus HEL (46-61) by a T cell hybridoma but had no effect on recognition of PCC (88-104) by another hybrid. The I-E^k–associated β_2m (31-45) had the reverse activity.

As expected, the β_2m peptide also inhibited the response of a T cell hybridoma specific for PCC (88-104) plus I-E^s (Fig. 4), demonstrating that the β_2m peptide could bind to this allele of I-E. Likewise, the L^d peptide isolated from ΔY animals inhibited the same hybrid. The histone 2B fragment described in Table 4 had no inhibitory activity in these experiments (data not shown). A peptide built to match a presumed leader sequence for Vβ7 was used as a negative control in the experiments shown in Fig. 4.

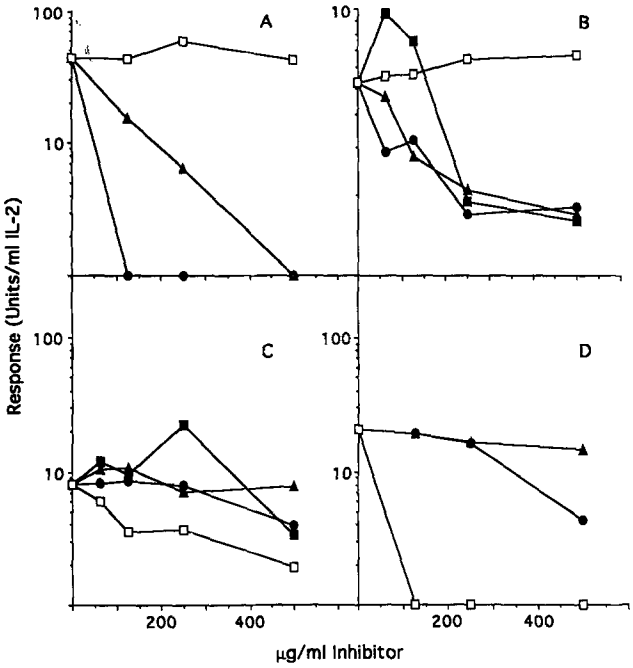


Figure 3. Peptides isolated from I-A^k or I-E^k bind to these proteins. Peptides were synthesized to match some of the sequences identified in Table 1. In each case the peptides started at the known NH_2 terminal of the sequenced peptide and continued for the next 15 amino acids. The peptides were titrated for their ability to interfere with presentation of stimulatory peptides by fixed CH12.1 cells to T cell hybridomas (Materials and Methods). Responding cells and stimulatory peptides were as follows: (*A*) kLy-4.3 plus 60 μg/ml HEL (46-61); (*B*) h4Ly-50.5 plus 60 μg/ml HEL (46-61); (*C*) 5KC-73.5 plus 2 μg/ml PCC (88-104); (*D*) 2B4.6 plus 60 μg/ml PCC (88-104). Inhibitor peptides were: Aβ^k (3-17) ■; Aβ^k (37-52) ●; Fcε II receptor (298-310) ▲; β_2m (31-45) □.

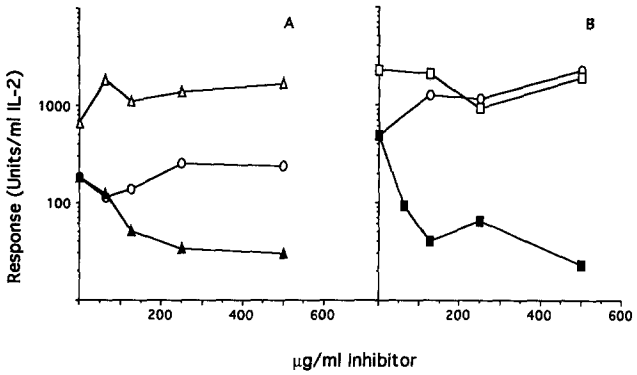


Figure 4. Two peptides associated with I-E^s on thymus cortical epithelium and spleen do bind I-E^s. The peptides β_2m (31-45) and L^d (160-176) were titrated for their ability to inhibit presentation of PCC (88-104) to the PCC (88-104)/I-E^s–specific T cell hybridoma, t3C-32.9. Controls included evaluation of the effects of a nonspecific peptide on the response of this hybrid, and evaluation of the effects of the two I-E^s–associated peptides on the responses of T cells to peptides bound to I-A^k or I-A^d. Cultures were set up as described in Materials and Methods and the legend to Fig. 3 and included the following: (*A*) t3C-32.9 plus fixed LS-102.9 cells plus 40 μg/ml PCC (88-104) plus β_2m (31-45) ▲, Vβ7 leader peptide O; DO-11.10/S4.4 plus fixed LS-102.9 cells plus 20 μg/ml OVA (323-339) plus β_2m (31-45) △; (*B*) t3C-32.9 plus fixed LS-102.9 cells plus 20 μg/ml PCC (88-104) plus L^d (160-176) ■, Vβ7 leader peptide O; kLy-4.3 plus fixed CH12.1 cells plus 62.5 μg/ml HEL (46-61) plus L^d (160-176) □.

Mice Are Tolerant to Class II–bound Peptides. Mice have been produced which do not express β_2m. These animals, which originally bore H-2^b, were crossed with B10.BR mice and F2 animals were selected which were homozygous for the "knocked out" β_2m gene and for H-2^k. These animals, and normal B10.BR animals, were immunized with β_2m (31-45) in CFA. T cells were isolated from the mice, expanded with antigen in vitro, and converted into T cell hybridomas. As shown in Table 5, 38% of the T cell hybrids obtained from β_2m$^-$ mice responded to the β_2m peptide, and, in fact, responded to an I-E^k–expressing B cell lymphoma in the absence of added peptide. Presumably the lymphoma expressed the peptide/I-E combination naturally. By contrast, none of the hybridomas from B10.BR mice responded to the lymphoma cells in the presence or absence of added β_2m peptide. Therefore the normal animals were indeed tolerant to a peptide/class II combination which is expressed on their own cells.

Table 5. *Normal I-E^k Expressing Mice are Tolerant to β_2m (31-45)*

Mice	Percent reactive T cell hybrids (No. tested)
B10.BR	0 (38)
B10.BRβ_2m$^-$	38 (216)

B10.BR or B10.BRβ_2m$^-$ mice were immunized with β_2m (31–45) in CFA. T cells from the draining lymph nodes were later stimulated with the peptide in vitro, expanded in IL-2 and fused. Hybridomas were assayed for their response to I-E^k plus the β_2m peptide.

Discussion

All theories of positive and negative selection have to explain both the skewing of the selected T cell repertoire toward foreign peptides presented by the selecting MHC allele and the failure of the mature selected T cells to react with the selecting MHC molecule complexed with any of the self-peptides found in the periphery. The "affinity hypothesis" suggests that some mechanism in the thymus allows immature T cells to register TCR interactions with MHC/self-peptide complexes at affinities that are below the threshold capable of tolerizing or triggering a more mature T cell. The "altered peptide hypothesis" proposes that the selecting MHC ligands in the thymus are unique to that tissue, either because the MHC molecules in that tissue are not bound to peptides at all or because they are occupied by peptides unique to the thymus.

Both of these hypotheses have advantages and disadvantages in explaining the existing data. For example, the affinity hypothesis, on the one hand, obviates the need both for a specialized MHC bearing cell and for a temporal order for positive and negative selection, because the outcome of the signaling is controlled by the quality of the TCR/MHC interaction. On the other hand, the altered peptide hypothesis does not require a special signaling mechanism mediated by very low affinity TCR/MHC interactions. It has not yet been possible to design an experiment to distinguish definitively between these hypotheses. The difficulties are due both to our inability to measure the affinity of the selecting TCR/MHC interaction directly and to our lack of knowledge of the precise nature of the selecting MHC ligand. Consequently, most recent experiments, such as those presented here, serve to place limitations on these hypotheses rather than eliminate one altogether.

In order to examine the altered peptide hypothesis we compared the peptides associated with class II derived from spleen and thymic cortical and medullary epithelium. Examination of peptides bound to I-E^s in ΔY mice showed that class II on thymus cortical epithelium certainly has bound peptides. The six identified peptides accounted for ~8% of the peptides binding sites on I-E regardless of whether the MHC protein was isolated from spleen or thymus. These results showed that MHC proteins on selecting tissue are not occupied by a few dominant thymic-specific peptides. The fact that the percent occupancy by the few peptides we could identify was the same for spleen and thymus cortical I-E suggests that most cortical I-E molecules are bound to peptides. We found no indication that a substantial amount of this material is empty. Finally, the peptides identified in spleen and thymus had the same sequence and eluted at the same positions on reverse-phase HPLC; thus spleen cells and thymus cortical epithelial cells are able to process these proteins into

the same fragments. We conclude that (*a*) thymic cortical epithelial cells are similar to peripheral class II–bearing cells in their ability to generate and load peptides into class II and (*b*) thymic cortical epithelial class II is not occupied by a set of dominant peptides different from that found on spleen cells.

These results obviously place limitations on the altered peptide hypothesis, while not eliminating it entirely. For example, one could still make the ad hoc argument that only some class II on thymic cortical epithelium is loaded with peptides similar to those on peripheral class II, but that the rest is empty or occupied by peptides derived from normal thymus-specific proteins or even special thymic selecting peptides. In order to eliminate this possibility altogether, the majority of the peptides in thymic class II would have to be sequenced, a task impossible with current technology.

There is other recent evidence that argues against the altered peptide hypothesis. Several groups, including ourselves, have now shown that positive selection can occur during reaction between receptors on developing thymocytes and bone marrow–derived cells, or fibroblasts, in the thymus (24–26). The overall finding of experiments of this type is that nonspecialized cell types can present MHC for positive selection providing those cells bear the MHC protein in question, and are present in the thymus cortex. Such a conclusion indicates that production of MHC proteins bearing a specialized set of peptides is not a prerequisite for positive selection. Others have shown that MHC class I proteins bearing synthetic peptides, or pooled peptides isolated from spleen, can participate in positive selection (22, 23), again suggesting that special peptides are not required for this function.

If nonspecialized cells bearing peptide/MHC complexes found also in the periphery can drive positive selection, then the thymocyte itself must determine whether interaction between a particular TCR/MHC pair will result in positive or negative selection. The affinity hypothesis attempts to explain the phenomenon along these lines by proposing that the thymocyte can detect the affinity of the TCR/MHC interaction (11). Recent evidence supports this idea. For example, several groups have shown that increases in the levels of accessory molecules such as CD8 can convert events that normally lead to positive selection into those that cause tolerance, i.e., an increase in the avidity with which the thymocyte reacts with target cells converts positive into negative selection (15, 16).

If positive selection does involve a low affinity reaction between the thymocyte receptor and MHC, how does the thymocyte detect this and distinguish it from a high affinity reaction? If no other molecules are involved, then thermodynamics dictate that high and low affinity reactions are distinguished by two features. First, at any given concentrations of receptor and ligand, fewer receptors will be engaged at any one time if the reaction is of low, rather than of high, affinity. Secondly, regardless of concentration, the half-life of a given low affinity receptor/ligand complex will be shorter than that of a high affinity complex. Therefore the thymocyte could detect the affinity of the reaction between its receptor and peptide/MHC by measuring either the number of receptors engaged at any one time and/or the time of engagement of any given pair. Of these two differences perhaps the more reliable indicator is the time of engagement, because the number of receptors engaged at any one time is concentration dependent, and the concentration of a particular self-peptide/MHC complex may vary from tissue to tissue, as illustrated in this paper.

In support of this idea, the dissection of the signaling pathway from the TCR has suggested a complex mechanism with an accumulating signal built up as various intracellular and surface molecules are pulled into an aggregating TCR/MHC complex (48). Low affinity TCR/MHC interactions might dissociate before a fully aggregated complex forms resulting in a partial set of signals. In the developing thymocyte this may determine positive versus negative selection (49). Similar ideas have been suggested to account for the differential effects of agonist and antagonist peptides on mature T cells (50–54) and affinity maturation of antibody responses (55).

Finally, several groups have analyzed the peptides bound to class I and class II proteins on cultured cells and there are a few reports of the peptides bound to class I MHC in normal mice (27–29). So far, however, there has been no description of the peptides bound to class II molecules in animals. Moreover, there has been no published analysis of the differences in peptides bound to class II on different tissues. The matter is of some interest, because many autoimmune reactions are thought to be driven by class II–restricted T cells that recognize tissue-specific peptide class II complexes. The data in this paper show that this is possible. Analysis of peptides derived from class II on spleen cells and thymus epithelial cells revealed that, although many peptides were shared between the two sources, some peptides were found in one tissue only. This was usually due to the fact that the donor proteins for these peptides are well expressed in one tissue (the spleen) but not the other (the thymus). Thus class II proteins in different tissues are bound, to some extent, by different peptides, and tissue specific attack by class II–restricted autoreactive T cells based on this differential peptide distribution is a possibility.

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