Cutting Edge: A Single MHC Anchor Residue Alters the Conformation of a Peptide-MHC Complex Inducing T Cells That Survive Negative Selection

Daniel A. Peterson, Richard J. DiPaolo, Osami Kanagawa and Emil R. Unanue

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We generated transgenic mice that expressed hen egg-white lysozyme (HEL) under a class II MHC promoter. The A7 line expressed HEL with a point mutation in the Asp\textsuperscript{52} residue, the main anchor amino acid responsible for the selection of the chemically dominant family of peptides (52–60) by I-A\textsuperscript{k} molecules. Mice expressing HEL with Ala\textsuperscript{52} were completely unresponsive when immunized with the same protein, i.e., HEL A52. However, the same mice immunized with wild-type HEL elicited T cells that recognized a conformation of the 52–61 core sequence uniquely different between Asp\textsuperscript{52} and Ala\textsuperscript{52} containing peptides. Importantly, some T cells also recognized the HEL A52 peptide given exogenously but not the same peptide processed from HEL A52 protein. Thus, a core MHC anchor residue influences markedly the specificity of the T cells. We discuss the relevance of these findings to autoimmunity and vaccination with altered peptides. *The Journal of Immunology*, 2001, 166: 5874–5877.

We are examining the chemical features of a protein or peptide Ags that determines their ability to shape the CD4 T cell repertoire. The chemically dominant peptide family of hen egg-white lysozyme (HEL)\textsuperscript{3} selected by I-A\textsuperscript{k} centers on the 52–60 core segment, which has 48–63 as a major member (DGSTDYGILQINSWW). The binding of the 48–63 family of peptides to I-A\textsuperscript{k} is of relatively high strength, forming a very stable complex that is protected from lysosomal proteolysis and that persists with a long half-life in APC (1–3). The aspartic acid at residue 52 (D52) of 48–63 is responsible for these properties; it provides a tight interaction in the P1 pocket (2–4). The Ala\textsuperscript{52} substitution in 48–63 of HEL results in two changes: a decrease in binding strength and a change in the display of the solvent exposed residues. A mAb that is specific for the I-A\textsuperscript{k}-48–63 D52 complex did not recognize the peptide-MHC complexes made with the 48–63 A52 peptide (5). An additional dimension to understanding subtle conformational changes induced by substitution of the MHC anchors relates to our previous identification of the type A and B conformation of a peptide-MHC complex (6, 7). Addition of a peptide to APC produced peptide-MHC complexes that were structurally unique (which we refer to as type B) from those containing the identical linear peptide but generated from the processing of intact protein (which we refer to as type A).

To study the impact of the A52 mutation on Ag presentation and on T cell tolerance to HEL, we generated the A7-transgenic mice that expressed HEL with the Asp to Ala change at residue 52 (HEL A52) in a membrane-tethered form in all APC. We dissect the T cell repertoire’s ability to distinguish among the various conformations of a peptide-MHC complex.

**Materials and Methods**

**Mice**

The 3A9 TCR-transgenic mouse, WT-mHEL (expressing wild-type HEL), mice have been previously reported (7, 8). The A7-transgenic mouse (expressing HEL A52) was generated by injecting C57BL/6 (B6) oocytes with a cDNA gene for chimeric HEL (A52)-L\textsuperscript{d} fusion protein under the immediate-early \(\alpha\) promoter (9, 10). Of four founder lines of HEL A52-transgenic mice, we used the A7 that expressed HEL A52 on nearly 100% of APC and had similar levels of expression when compared with WT-mHEL mice. The mice were generated, back-crossed (six generations to B10.BR, then intercrossed), and maintained as previously described for the WT-mHEL (7).

**Proteins and peptides**

Wild-type HEL (i.e., HEL with D52) was obtained from Sigma (St. Louis, MO) and purified further in our laboratory over a BioRex 70 column (Bio-Rad, Hercules, CA). HEL with the D52A mutation, termed HEL A52, was obtained from culture supernatants of baculovirus-infected SF-9 cells and purified by immunoaffinity. Two peptides were produced by F-moc technology: wild-type 48–63 (labeled here as 48–63 D52) and the 48–63 peptide with the D52A mutation (labeled here as 48–63 A52). Peptides were synthesized and analyzed as described previously (7).

**T cell assays**

To examine for the presentation of the 48–63 peptide, titrating numbers of splenocytes from the HEL-transgenic mice (WT-mHEL or A7) were incubated with 10\textsuperscript{4} lymph node cells per well from 3A9-transgenic mice in 96-well U-bottom plates. Proliferation was assessed by incorporation of...
[H]thymidine by pulsing the cultures during the last day of a 4-day culture.

Limiting dilution analysis (LDA) was done as previously reported. Mice were immunized with 10 nmol of HEL emulsified in CFA (Difco Laboratories, Detroit, Michigan) s.c., and 7 days later the draining lymph nodes were removed and placed into the LDA using the methods previously described (7, 11). For the cloning of HEL-specific T cells, the spleens of WT-mHEL-transgenic mice were used as a source of APCs. The growth-positive wells of the LDA were expanded and then tested for peptide specificity. To determine the specificity of the T cells, clonal wells from the LDA were expanded and then split into three to six wells and stimulated by adding 5 × 10^5 APCs plus the relevant Ag, in the absence of exogenous IL-2, in U-bottom 96-well plates. We directly measured T cell proliferation by thymidine incorporation. Ag-specific T cells were identified by having thymidine incorporation >2-fold above background. T cells were tested for their response to the peptides 48–63 D52 and 48–63 A52 and a mixture of the HEL peptides 18–33, 31–47, and 115–129 (7).

Results and Discussion

We used the 3A9 T cell that recognizes both peptides 48–63 D52 and 48–63 A52 (Fig. 1) to detect HEL in APC from the A7-transgenic mice. As shown (Fig. 1), APC from the WT-mHEL mice presented this epitope better than APC from the A7 line. The 48–63 epitope is the predominant epitope in APC from the WT-mHEL mice, with as much as 10% of the class II loaded containing this single peptide family (5, 7, 12). In our preliminary results, the amount of 48–63 A52 peptide presented from HEL A52 is 1/10th of that presented from HEL, but direct biochemical estimates of the amounts of 48–63 A52 have not been made.

When A7 mice were immunized with 10 nmol of recombinant HEL A52 (the Ag identical with the transgenically expressed protein), we found that there was no response in the LDA assay testing on the APC from the same A7 mice, i.e., with HEL A52. Tolerance to HEL A52 protein was complete. This was in agreement with the results from our previous studies immunizing WT-mHEL mice with HEL (7). Therefore, a priori, peptide characteristics like binding strength, SDS stability, and half-life of the peptide-I-Ak complex (which are very different between 48–63 D52 and 48–63 A52), do not absolutely limit tolerogenicity of an epitope.

In contrast, when the same transgenic A7 mice were immunized with HEL (an Ag that differs from the self protein by just one amino acid), there was an interesting result. In this case a response was elicited but focused entirely on the 48–63 segment of the HEL protein. A representative LDA is shown (Fig. 2A): 1 in 2000 lymph node cells responded to HEL in B10.BR mice, similar to our previously published results (7). Of these, 1 in 9000 cells were specific for the 48–63 epitope, around 25% of the total HEL response.

![Figure 1](http://www.jimmunol.org/DownloadedFrom/5875/FIGURE%201.png)

**FIGURE 1.** Demonstration of 48–63 presentation by the stimulation of the 3A9 T cells. *Left,* APC from WT-mHEL- and A7-transgenic mice were cultured at titrating doses with lymph node cells from 3A9 TCR-transgenic mice. B10.BR mice were used as negative controls. The peptide was presented by APC from both transgenic mice. *Right,* Titrating doses of the 48–61 D52 and 48–61 A52 peptides were added to cultured LN cells from 3A9 TCR-transgenic mice.

![Figure 2](http://www.jimmunol.org/DownloadedFrom/5875/FIGURE%202.png)

**FIGURE 2.** Precursor frequency determination of A7 and B10.BR mice immunized with HEL. *A,* A representative LDA: LN cells from mice immunized with 10 nmol HEL were placed in limiting numbers (as indicated on the x-axis) into 96-well plates. The corrected precursor frequency of HEL-specific cells is displayed in the figure. There was roughly a 30-fold decrease in HEL precursor frequency (from 1:2,000 to 1:60,000). *B,* Results from four experiments as determined in A are summarized in this histogram. The precursor frequency is plotted on the x-axis (n/d, not determined).

![Figure 3](http://www.jimmunol.org/DownloadedFrom/5875/FIGURE%203.png)

**FIGURE 3.** Specificity of 48–63-reactive T cells from B10.BR and A7 mice. *A,* Growth positive wells from the LDA were expanded and tested for their ability to proliferate in response to different Ags. All T cells that responded to HEL and 48–63 D52 were also tested for reactivity to HEL A52 and 48–63 A52. To determine responsiveness to HEL or HEL A52, T cells were stimulated with 5 × 10^5 APC from the respective transgenic mice or T cells were stimulated with 5 × 10^5 APC and 5 μM of the indicated peptide. Representative clones from both B10.BR and A7 mice are shown. *B,* Dose response of A7-derived T cells to various Ags. Selected T cells were expanded and stimulated with 5 × 10^5 APC plus titrating concentrations of Ag.
In A7-transgenic mice the response was 1 in 60,000 cells responding to HEL, but in this case all were specific for the 48–63 epitope. The frequency of HEL-reactive T cells in four independent experiments is shown (Fig. 2B). Thus, in A7 mice the tolerance to the epitopes other than 48–63 was complete. However, there was still a 48–63 epitope-specific response that could be detected, although the frequency was reduced.

We examined the specificity of 192 clones from B10.BR and 60 clones from A7 mice, both immunized with HEL. We describe three specificities within the 48–63 epitope reactive repertoire, examples of which are shown in Fig. 3. First, in the B10.BR mice, about half of the T cells had the same specificity as the 3A9 T cell (Fig. 1B). They responded to HEL protein, and the 48–63 D52 peptide, as well as the HEL A52 protein and 48–63 A52 peptide (see clone B10.201 in Fig. 3, summarized in set 1 in Fig. 4). Therefore, these T cells recognize a common conformation of the 48–63 I-A^b complex produced from either having an Asp or Ala in the P1 anchor site, residue 52. None of the T cells from the immunized A7 mice responded with this pattern of reactivity (Fig. 4). Thus A7 mice are fully tolerant to the shared conformation induced with either D52 or A52 as the P1 anchor.

Second, in B10.BR mice, ~20% of the T cells recognized a unique conformation induced by the D52 residue (Fig. 4). These T cells did not recognize the 48–63 A52 peptide presented from either intact protein or given exogenously (note clones B10.226 and A7.146 of Fig. 3A, clone A7.46 of Fig. 3B, and set 2 of Fig. 4). In A7 mice, these T cells represent the majority, ~70% of the T cells. Thus the majority of the T cells in A7 mice that escaped the strong negative selection pressure in the thymus were directed to the unique conformation represented in the 48–63 D52 ligand.

Previous studies indicated that immune responses could be made to the self epitope that were absent in vivo as a result of a mutation in a self protein (13, 14). Gapin et al. demonstrated that ablation of the presentation in a single epitope of HEL in I-E^k expressing mice resulted in a complete lack of tolerance to it (13). Our results add an additional dimension to this general phenomenon by emphasizing how subtle changes by a single unexposed anchor residue influenced T cell recognition. After all, the solvent exposed residues in both of our peptides are identical. The only differences must be in their display, which is brought about by a single amino acid change in a key anchor residue P1. The conformational change may not be limited to the peptide side chain, but could represent a change in the conformation of the class II molecule.

The third T cell specificity found in both B10.BR and A7 mice recognized APC that were presenting 48–63 D52, either from giving the peptide exogenously or from processing of the HEL protein. These T cells did not recognize HEL A52 protein. However, the unique feature of this set of T cells was that they recognized APC given 48–63 A52 peptide exogenously. Examples of this type of T cells are shown (note clones B10.339 and A-7.146 in Fig. 3A, A7.113 in Fig. 3B, and set 3 of Fig. 4). T cells of this specificity made up ~30% of both the B10.BR and A7 repertoire. The specificity of this third set of T cells indicates that there is enough similarity between the endogenously processed form (type A) of the 48–63 D52 peptide and the exogenously added peptide form (type B) of the 48–63 A52 peptide to allow T cells primed with the former to recognize the latter.

Sets 2 and 3 were separated on the basis of an all or none response to 5 µM 48–63 A52. Selected T cells from set 2 that were expanded and tested with up to 30 µM peptide were still unresponsive. We had previously found in T cells from B10.BR mice that there is a wide range in preference for different ligands (7). Therefore, some of the T cells that were unreactive at the concentrations of Ags used for classification may respond at higher doses; however, this does not change their overall preference for one conformation over the other.

We argued previously that peptides added exogenously to APC can generate peptide-MHC conformations antigenically distinct from the identical peptide-MHC complexes formed from the processing of native protein (6, 7). We separated the T cell response into those that respond to both peptide added exogenously and/or to the protein (type A conformer) and those that only respond to the exogenously added peptide (type B conformer). We have proposed (7) that because these type B T cells were not entirely deleted by negative selection, they could participate in autoactivity at sites of inflammation where self peptides could be produced by the proteolytic environment (15). Our results here present the same situation in that type B T cells have again been found to escape negative selection and could also recognize a self peptide and potentially be activated at inflammatory locales. However, in contrast to our first report, these present findings add another unique mechanism of generating such cells, which is by immunizing with a closely related protein (and not peptide). We envision a scenario in which an encounter with a foreign Ag that is highly related to a self protein (16, 17) (in our case HEL given to HEL A52 transgenic mice) might prime these T cells and lower their threshold of activation (18). Such primed type B T cells could then react with smaller levels of self peptides generated by inflammation.

The importance of very subtle chemical differences in modulating the T cell repertoire cannot be underestimated as these and other results attest (19). The biological significance of these results in regard to vaccination and the identification of self-reactive T cells needs to be determined. Furthermore, our results indicate that changing anchor residues of peptides for vaccination purposes (20–23) would result in an unknown proportion of the repertoire of T cells that does not recognize the conformation of the native epitope.

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References


