Brief Definitive Report

Peptide-induced Conformational Changes in Class I Heavy Chains Alter Major Histocompatibility Complex Recognition

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Summary

Small peptides, derived from endogenous proteins bind within the antigen binding groove created by the β-pleated sheets and α helices of the α1 and α2 domains of the class I molecule of the major histocompatibility complex (MHC). However, the precise role of peptide in class I MHC conformation remains unclear. Here, we have shown that, in at least some instances, changes induced in the MHC molecule by the binding of distinct peptides can be identified as specific alterations in serological epitopes expressed on the class I protein. The nature of specific peptides expressed by class I-bearing cells may, therefore, have a dramatic influence on T cell development, self-tolerance, and alloreactivity.

In recent years, tremendous progress has been made in elucidating the molecular aspects of MHC class I–peptide interaction. The structural analyses of crystallized HLA class I molecules have included A2, Aw68, and B27 (1–3). In all instances, small peptides have been observed within the cleft formed by the α helices and β-pleated sheet constituted by the α1 and α2 domains. Recent studies have shown that optimal peptide binding to the murine class I antigen, H-2K^b, occurs with small peptides (8–9) amino acids long, peptides containing a leucine at the COOH terminus, and amino acids with aromatic side chains (phenylalanine or tyrosine) at the 5th (8 mer) or 6th (9 mer) position (4–6). These amino acid residues provide the anchor points for the peptide in the cleft. The recent solution of the x-ray crystal structure of a single MHC class I molecule, H-2K^b, occupied by distinct peptides, suggested that these peptides may influence MHC class I conformation (7).

We, therefore, examined the role of peptide in MHC conformation using the mutant cell lines RMA-S and T2. These cell lines are class I-deficient somatic variants of RMA (mouse) and B-LCL721 (human) tumor cells due to defects in the transporter proteins, transporter associated with antigen processing (TAP), necessary for providing processed peptides for binding to the newly synthesized class I molecules (8–12). The addition of preprocessed antigenic peptides to these mutant cells results in stable assembly and cell surface expression of class I predominantly bound to the added peptide.

In the present experiments, we studied the effects of specific peptides on class I recognition of H-2K^b–specific alloantibodies. The peptide transporter mutants, RMA-S and T2-K^b/D^d, were pulsed with various K^b binding peptides. Class I molecules bound by the different peptides were distinguishable by mAb binding. These results suggest that, in at least some instances, MHC conformation as detected by serological epitopes may depend on the peptide bound to the class I molecule.

Materials and Methods

Tumors. The Rauscher virus-transformed lymphoma cells, RMA and RMA-S (13), were a gift from Dr. K. Kärre (Karolinska Institute, Stockholm, Sweden). The human T cell lymphomas, C1R and B-LCL721.174 × CEM, (T2 [14]), were the kind gift of Dr. P. Cresswell (Yale University, New Haven, CT). The T2 cells were transfected with a chimeric MHC class I gene, K^b/D^b (i.e., exons 2 and 3 encoding the α_1 and α_2 domains of K^b ligated to exon 4 encoding the α_3 domain of D^d; kindly provided by Dr. T. Potter (National Jewish Hospital, Denver, CO) by electroporation.

Peptide Preparation and Binding Assays. The majority of the peptides were prepared by solid-phase techniques using the F-moc method, or by the University of Chicago Peptide Synthesis Laboratory by the T-boc method, and analyzed by HPLC for purity. Amino acid analyses were performed to assure appropriate amino acid composition. The peptides used in the present studies included: OVA 257-264 (SIINFEKL [15]); influenza nucleoprotein 1968 (FLU NP) 345-360 (SFIRGTKVSPRGKLST [16]); vesicular stomatitis virus nucleoprotein (VSV NP) 52-59 (RGYVYQGL [17]); and Sendai virus nucleoprotein 322-330 (APGNYPAL; SV8 NP) or 321-

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330 (FAPGNYPAL; SV9 NP) (18). In peptide-pulsing experiments, tumor cells at a concentration of 5×10^5/ml, were incubated overnight at 37°C with individual peptides (100 μg/ml) before assay.

mAb The MHC H-2K^b–specific mAbs used were as follows: 5F1-2-14 (α2 [19]); EH-144 (α1 [20]); AF6-88.5 (21); 100-30 (22); and Y-3 (23). In addition, the α3-specific, H-2D^d mAb, 34-2-12 (24), were used in some experiments as a means to determine absolute levels of the product of the exon-shuffled H-2K^b/D^d gene. Cell surface expression of H-2K^b was studied by flow cytometry using a FACScan® (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Results and Discussion

To assess class I conformational features, the binding of a panel of mAbs specific for H-2K^b was examined on wild-type, RMA, and mutant, RMA-S tumor lines in the absence or presence of distinct exogenous peptides. As seen in Fig. 1, four anti-H-2K^b mAbs reacted with the wild-type RMA cells whereas RMA-S cells, when incubated at 37°C, expressed little H-2K^b. In contrast, overnight incubation of RMA-S at room temperature upregulated K^b expression (*right*). However, examination of the relative binding of the different mAbs to RMA-S RT revealed that 100-30 bound proportionally greater than 5F1-2-14 or Y-3, a serological pattern different from that seen on RMA.

The serological profile of K^b molecules on RMA-S pulsed with exogenous peptides was also significantly different than RMA. The serology of RMA-S cells pulsed with SV8 NP, SV9 NP, and FLU-NP peptides was similar to the RMA-S RT cells as 5F1-2-14 bound relatively poorly, if at all (SV9 NP), to these cells, and 100-30 reacted strongly. In contrast, K^b molecules expressed by RMA-S cells incubated with the OVA peptide or VSV NP peptide reacted strongly with the 5F1-2-14 mAb. In addition, there was virtually no binding of the 100-30 mAb to OVA-pulsed RMA-S cells and weak binding to VSV NP-pulsed RMA-S. The relative differences in the K^b reactivity were not due to antibody titer or isotype, as shown in Fig. 2. All of the antibodies bound at different saturating levels on each cell type. For instance, plateau binding of 5F1-2-14 occurred with a mean fluorescence (MF) of 325 on OVA-pulsed RMA-S cells, but only MF 50 on SV8 NP-pulsed RMA-S. In contrast, 100-30 bound with a MF of 125 and 15 on SV8 NP- and OVA-pulsed RMA-S cells, respectively. The differences in maximal antibody binding, combined with the dramatically different slopes of the titration curves, suggested that the same mAb has different affinities for the K^b molecule associated with different peptides. It is interesting that another mAb, AF6-88.5, whose K^b binding was previously reported to be murine β_2M dependent (25), and did not react with human β_2M-associated human C1R-K^b cells (data not shown), also demonstrated different binding activity on K^b molecules loaded with the different peptides. RMA-S cells pulsed with OVA, VSV NP, and SV9 NP bound AF6-88.5 well, whereas RMA-S RT, RMA-S plus SV8 NP, and RMA-S FLU-NP (data not shown) bound it poorly. Therefore, it is likely that the AF6-88.5 binding is dependent on both murine β_2M association and peptide-induced class I conformation, perhaps at the site where β_2M interacts with the α1 domain of K^b (26).

To determine the absolute binding of the K^b-specific mAbs, T2 cells were transfected with a chimeric K^b/D^d construct encoding the α1 and α2 domains of K^b and the α3 domain of D^d. This chimeric MHC molecule can be identified by an α3-specific mAb, 34-2-12, that is not influenced by peptide binding. As seen in Fig. 3 *a*, the pattern of mAb binding varied significantly among the peptide-pulsed T2-K^b/D^d tumor lines. Anti-H-2D^d binding increased equivalently on transfectants pulsed with SV9 and OVA peptides (*dashed lines*). Furthermore, there was a dramatic difference in the reactivity of 5F1 (*solid lines*) and 100-30 (*dotted lines*) between the two peptide-pulsed cell populations. Similar dramatic differences were observed when K^b-specific mAb reactivity was normalized to the α3-specific mAb staining (Fig. 3 *b*). Two conclusions can be drawn from this data. First, the staining of peptide-pulsed K^b/D^d–transfected T2 cells was remarkably similar to the RMA-S cells cultured

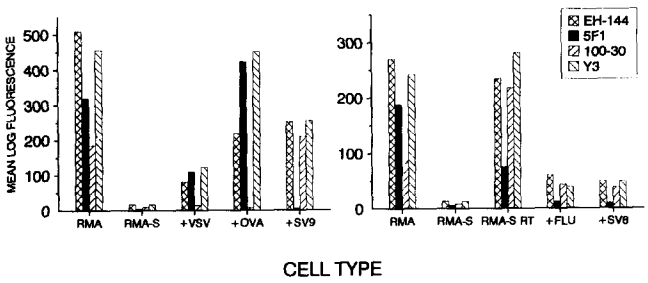

Figure 1. Serological analysis of peptide-pulsed RMA-S cells. Tumor cells, pulsed overnight with individual peptides (100 μg/ml), were incubated for 30 min with saturating amounts of mAb followed by counterstaining reagent. Flow cytometry analyses were performed on 10,000 cells gated by size and propidium iodide exclusion to eliminate dead cells. Data is shown as the mean log fluorescence of the cell population.

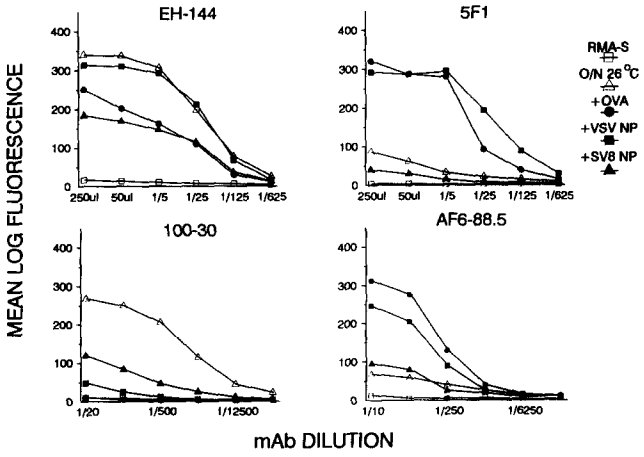

Figure 2. Titration of mAbs on peptide-pulsed RMA-S cells. Peptide pulsing of RMA-S cells was performed as in Fig. 1. Various dilutions of mAb were added and binding assessed as above.

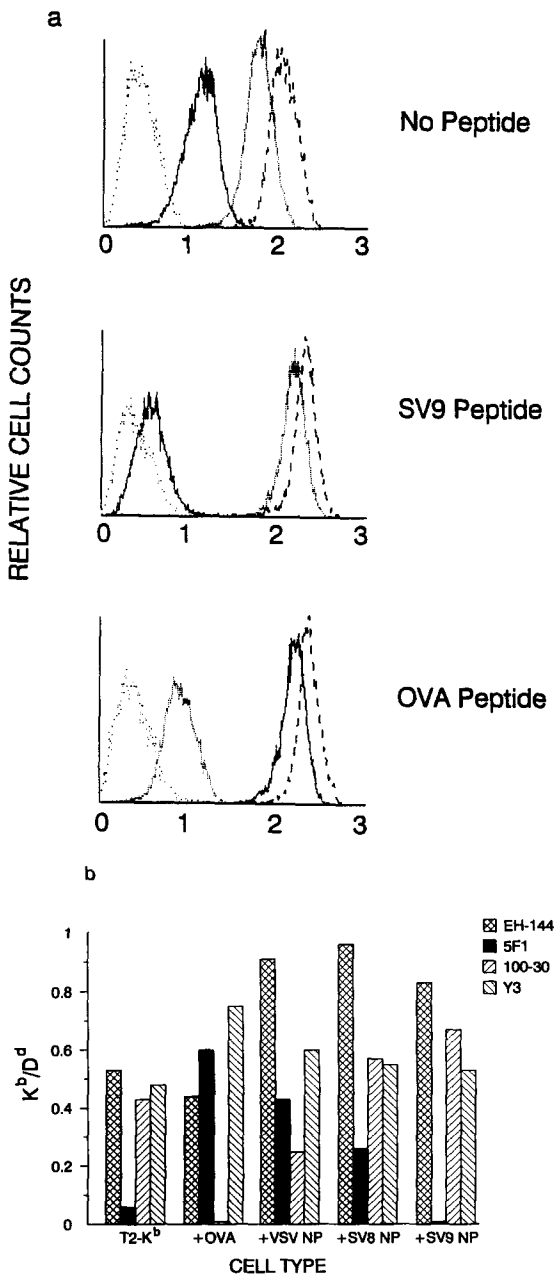

Figure 3. Serological analyses of peptide-pulsed T2-K^b/D^d cells. (*a*) FCM analyses were performed as in Fig. 2 on T2-K^b/D^d cells pulsed with individual peptides overnight. Data is depicted as histograms of 10,000 cells. Background staining with FITC-coupled anti-mouse Ig is shown (*spread dots*). Cells were stained with 5F1-2-14 (*solid lines*), 100-30 (*dotted lines*), and 34-4-12 (anti-D^d, *dashed lines*). (*b*) K^b/D^d staining ratios were determined by dividing the maximal mean log fluorescence of the individual anti-K^b mAbs by the maximal mean log fluorescence of the α3-specific, anti-D^d mAb, 34-2-12. In this experiment, 34-2-12 stained unpulsed T2-K^b with a mean log fluorescence of 500, whereas the mean fluorescence of peptide-pulsed K^b ranged from 1,300 to 1,500.

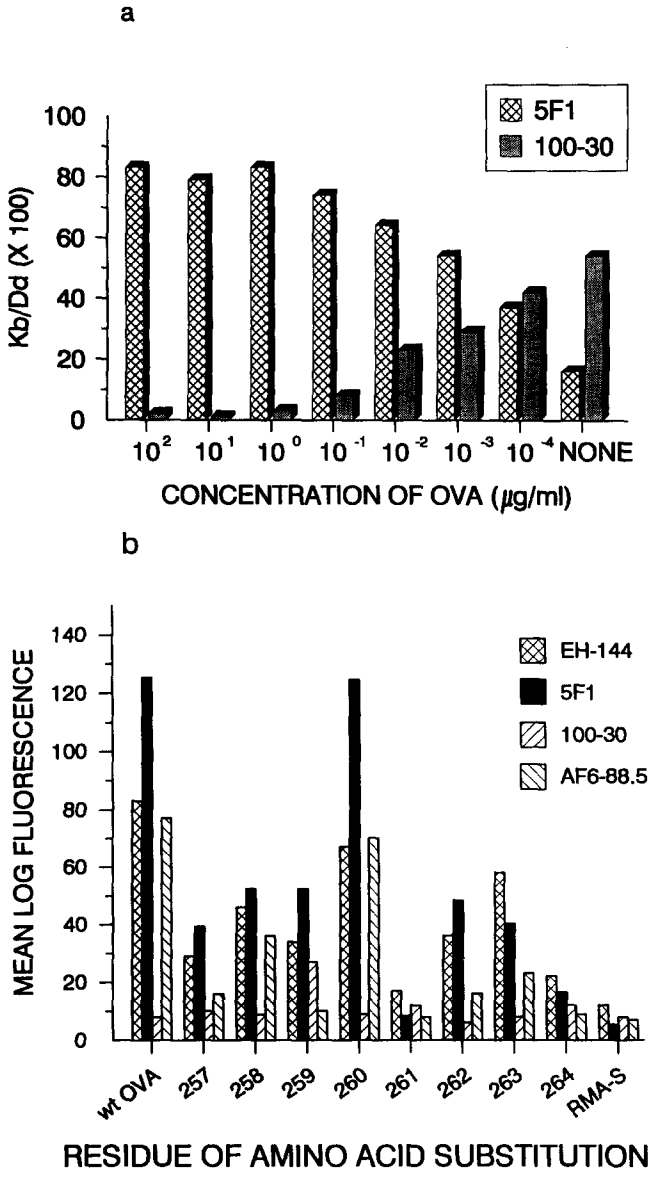

Figure 4. Titration of OVA peptide T2-K^b/D^d. (*a*) T2-K^b/D^d tumor cells were incubated overnight with varying concentrations of peptide, washed and analyzed using different mAbs including 5F1-2-14, 100-30, and 34-2-12. K^b/D^d staining ratios were determined by dividing the maximal mean log fluorescence of the individual anti-K^b mAbs by the maximal mean log fluorescence of the α3-specific, anti-D^d mAb, 34-2-12 as above. (*b*) Flow cytometry analysis of RMA-S cells pulsed with mutated OVA peptides. Individual amino acid numbers represent the alanine-substituted residue in the OVA peptide. Cells were incubated overnight with saturating amounts of peptide. mAb staining of peptide-pulsed cells is depicted as mean log fluorescence.

with the different peptides. For instance, the absolute binding of 5F1-2-14 was much more efficient on OVA-pulsed cells than on SV9 NP cells. In contrast, 100-30 did not bind OVA-pulsed cells at all, but bound very well to SV9 NP-pulsed cells. In fact, these serological alterations were observed in as little as 1-h incubation with peptide (data not shown). Second, recent studies have shown that the nonamer peptide of the Sendai NP binds 1,000-fold better than the octamer. Yet, with the exception of 5F1-2-14 binding, which was weak on SV8 NP-pulsed RMA-S but nonexistent on SV9 NP-pulsed cells, the mAb binding pattern observed with SV9 and SV8

NP was similar, whereas mAb-binding to SV8 NP- and OVA-pulsed cells was quite dissimilar. Thus, the differences in serology of the K^b molecule loaded with different peptides did not correlate with peptide length or affinity.

A dose titration of OVA peptide on T2-K^b/D^d showed that overnight incubation with as little as 10^{-3}–10^{-4} μg/ml of OVA peptide resulted in a diminution of 100–30 mAb binding and a concomitant increase in 5F1-2-14 binding (Fig. 4 *a*). A more detailed analysis of the influence of the OVA peptide on K^b serology was performed using OVA peptides in which individual amino acids were substituted with alanine residues. As illustrated in Fig. 4 *b*, OVA peptides containing substituted alanines at positions 261 or 264 did not increase K^b expression. In previous studies by Falk et al. (4, 27), residues 5 (amino acid 261) and 8 (amino acid 264) were shown to be critical residues for peptide binding to K^b. Thus, it is not surprising that these alanine substitutions affected MHC binding. In contrast, significant increases in H-2K^b expression was observed with all other alanine-substituted peptides. The reactivity of four different mAbs on OVA-pulsed RMA-S cells was not significantly effected by several individual alanine substitutions including: OVA peptide-serine 257 → alanine 257 (OVA-S257A), OVA-I258A, OVA-N260A, or OVA-E262A. However, substitution of the isoleucine 259 with an alanine resulted in diminished AF6.88-5 staining and increased 100-30 staining. Residue 259 is thought to be an MHC contact residue (5, 7). Therefore, substitution of an isoleucine with an alanine may alter peptide–MHC contact.

One implication of the serological analysis of the alanine-substituted peptide is that, in addition to direct effects of peptide binding, there may be allosteric effects as well. Several years ago, in collaboration with Ajitkumar et al., we demonstrated that single point mutations in amino acids 162 or 166 of the α2 domain had profound effects on the 5F1-2-14 epitope (28). Based on recent crystal structure analyses, the NH_2-terminal residues of the peptide localize near these amino acids. These observations suggest that many of the serological changes observed using different peptides depend on the NH_2-terminal residues of the peptide which vary substantially among OVA, VSV NP, and SV NP. In fact, the difference in 5F1-2-14 binding between SV8 NP and SV9 NP is a direct result of the NH_2-terminal phenylalanine. However, we also observed that a single amino acid change in residue 7 of the OVA peptide (OVA-K263A) decreased 5F1-2-14 binding (Fig. 4 *b*), suggesting possible allosteric effects on mAb binding.

In conclusion, the data cannot easily distinguish whether the serological changes observed result solely from conformational changes in the α helices or associated side chains, or from the steric effects of peptide binding. The 100-30 and 5F1-2-14 epitopes have totally distinct patterns of binding. This does not appear to be a steric effect of peptide binding since 5F1-2-14 does not effectively bind to "empty" K^b molecules (Fig. 3). By comparison, the 100-30 mAb binds effectively to empty K^b and SV-pulsed K^b, but not OVA-pulsed K^b. More importantly, recent x-ray crystallographic studies by Fremont et al. (7) illustrate the potential conformation changes that result from the binding of different peptides to the same MHC class I molecule.

Finally, the likelihood that class I MHC molecules can exist in different conformations on the cell surface has profound implications for allorecognition. Although a number of studies have suggested that many alloreactive cells are peptide specific, recent studies by several groups support the idea that some alloreactive clones recognize MHC conformational epitopes, since some of the alloreactive CTL examined recognized peptides present in several distinct peptide peaks after HPLC separation of peptides eluted from class I MHC, "naked" MHC (29, 30), or class I loaded with distinct peptides (31; and Bluestone, J. A., S. Jameson, K. Arunan, and R. Dick II, manuscript in preparation). These results support the notion that at least some alloreactive T cells recognize class I in a peptide-dependent but not peptide-specific manner, and that the nature of the peptide that occupies the MHC groove can alter allorecognition of foreign class I MHC. If, indeed, the nature of the peptide within the MHC cleft can influence TCR recognition, then subtle peptide-driven changes in MHC structure would impact not only on antigen-specific responses, but also on T cell development, self-tolerance to tissue-specific antigens, and alloreactivity.

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