Conformational Differences in Major Histocompatibility Complex–Peptide Complexes Can Result in Alloreactivity

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Summary

Mutations within the class I major histocompatibility complex (MHC) molecule that affect a peptide binding can result in strong allogeneic responses. It is believed this reflects, in part, binding of a different set of endogenous peptides by each MHC molecule. We have examined the representation of allopeptides recognized by K^b-specific cytotoxic T lymphocytes (CTL) clones among targets that express either the K^b or the K^{bm8} mutant. These class I molecules mutationally differ by several residues at the base of the peptide binding groove resulting in lack of recognition of bm8 targets by most K^b-specific CTL, and in strong mutual alloreactivity. Since these differences involve pockets in the base of the peptide binding groove that are presumed to contribute to the affinity of peptide binding, and there is evidence for differences in peptide binding by the mutant and wild type molecule, it was considered most likely that alloreactivity was due to binding of different sets of peptides by each of these molecules. Surprisingly, the allopeptides recognized by K^b-specific clones from a variety of responders, including bm8, are often found associated with both the wild type and mutant class I molecules. Although for some allopeptides the amount of peptide normally found associated with bm8 is less than that associated with K^b, reactivity could not be restored by increasing the amount of the relevant peptide. Thus, the basis for much of the alloreactivity observed in this particular mutant and wild type combination is not the presence or absence of the relevant allopeptide but rather the different conformation adapted by the peptide–MHC complex. These results allow us to conclude that strong alloreactive responses can result from T cell recognition of conformational differences between the stimulation and responder MHC molecules.

MHC class I molecules bind short peptide ligands representative of endogenously synthesized cellular proteins (1, 2). Formation of a stable MHC–peptide complex usually requires that the peptide sequence contain an appropriate motif that provides complementarity with the peptide binding cleft of the MHC molecule at certain key anchor positions (3–9). However, this constraint is sufficiently unrestrictive such that 100's–1,000's of different peptide sequences are able to bind each allelic form of MHC (10, 11).

It is believed that the T cell receptor interacts with the upper surfaces of the MHC α-helices and accessible portions of bound peptide (12). The high frequency of alloreactive T cells is the result of the large number of antigenic MHC–peptide complexes that are available for recognition (13–16). However, the basis for antigenicity of each of the various complexes may differ. Although allogeneic differences on class I that involve residues that interact directly with the TCR can be immunogenic (17), most allelic variation involves MHC residues that interact with the peptide rather than TCR (18). A sequence difference between the responder and the stimulator within the antigen binding groove could result in binding of a different set of peptides by each class I molecule, thereby leading to reciprocal antigenicity. It is also possible that a qualitative difference in peptide expression could lead to antigenicity. For example, if we consider that self-tolerance of a particular self-peptide–MHC complex only eliminates clones bearing TCRs with high affinity, it is possible that by increasing the number of these same peptide–MHC complexes, some T cells whose receptor affinity was sufficiently low to avoid deletion during thymic development may now be stimulated. Alternatively, MHC molecules that differ in their peptide binding grooves may bind the same peptide yet could differ with respect to the conformation of the MHC–peptide complex. In a recent report involving recognition of an ovalbumin peptide, it was found that a change in a peptide pointing into the peptide binding groove abolished T cell recognition, presumably by altering the conformation of the complex (19). It is possible that a change in the base of the

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peptide binding groove of class I could similarly result in a conformational change in the complex that results in antigenicity.

Most alloreactive responses involve MHC differences that potentially effect both the α-helices and peptide binding groove of the MHC. To evaluate the relative contribution to an allogeneic response of the possibilities considered above that relate to peptide binding, we have investigated the basis for allorecognition between two murine strains that mutationally differ from one another at residues at the base of the peptide binding groove (20). The sequence differences between K^b and the K^{bm8} mutant are confined to residues 22–24 and 30, all of which are at the base of the peptide binding groove. Based on the x-ray crystal structure of K^b (7–9), both 22 and 24 are involved in formation of pockets that accommodate peptide residues, thereby contributing to the affinity of interaction. As anticipated for a mutation that effects peptide binding, the majority of K^b-specific CTL do not recognize the bm8 molecule. Also, these strains demonstrate a strong reciprocal allogeneic response. Analysis of the contribution of individual residues that differ between K^b and the K^{bm8} mutant to allorecognition (21) and peptide binding (22) have concluded that residue 24 is dominant in determining the bm8 phenotype.

Despite these differences, our results indicate that peptides recognized by K^b-specific CTL are often present in both the mutant and wild type molecule. This suggests that the basis for differences in CTL recognition of these particular class I molecules is often not the presence or absence of a particular allopeptide, but rather the conformation of the peptide–MHC complex.

Materials and Methods

Mice. C57BL/6 and B6.-H-2^{bm8} mice used in these studies were obtained from the breeding colony of The Scripps Research Institute.

Cell Lines. K^b-specific CTL clones and T2-K^b cells used as targets are as previously described (16). The VSV-N-specific CTL line used in these studies was obtained from Dr. Nancy Hosken and Dr. Michael Bevan (Howard Hughes Medical Institute, University of Washington, Seattle, WA) (23). T2-K^{bm8} targets were prepared by transfection of T2 cells with a mixture of pSVneo DNA (2 μg) and 10 μg of plasmid DNA containing the K^{bm8} cDNA gene obtained from Dr. Per Peterson of this institute. Transfection was performed as previously described using a Gene Pulser [230 mV; 960 mfd; Bio-Rad Laboratories, Richmond, CA; (24)]. Cells were immediately cloned in media containing G418 at 800 μg/ml (effective concentration). The clone used in these studies expresses threefold less K^{bm8} than the amount of K^b expressed on T2-K^b cells as determined by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) using the K^b-specific monoclonal antibody, Y3 (25).

Peptides. Peptides were prepared from either spleen cells or tumor cells using a modification of the method of Van Bleek and Nathenson (26) as previously described (25). Briefly, cells were lysed at a concentration of 10^8 cells/ml in buffer containing 0.5% NP-40 and cleared of nuclei and unlysed cells. K^b or K^{bm8} molecules were bound to protein A-Sepharose beads (1 ml packed volume beads for 10^9 cells) to which the Y3 mAb was coupled using 1 mg antibody/1 ml beads. Lysate and beads were incubated for 1 h at 4°C after which beads were removed and washed three times before resuspension in 2 ml 0.2% trifluoroacetic acid. Beads were discarded, and the eluate passed through a Centricon-10 (Amicon, Beverly, MA) filter to remove high molecular weight material, lyophilized, and resuspended in 1 ml distilled water (or the indicated volume). A synthetic peptide (GYVYQGLKSGN) representing the epitope recognized by VSV-N–specific CTL was kindly provided by Dr. Nancy Hosken. Derivation of HPLC purified allopeptide recognized by bm8.28 represents fraction 31 in the Y3 purified K^b peptide from EL4 described in reference 25.

Cytotoxicity Assays. The indicated amount of peptide was incubated with ^{51}Cr-labeled target cells for 30 min in a volume of 100 μl RPMI 1640 before addition of the indicated number of effector cells in media containing 10% FCS. Cytotoxicity assays were performed as previously described unless otherwise specified (16).

Results

As previously reported, and demonstrated in Table 1, the majority of CTL clones specific for K^b do not recognize bm8 targets (27). Also, a vigorous CTL response is obtained when bm8 T cells are stimulated with K^b stimulators (28). The simplest explanation for such allorecognition would be that not all peptides capable of binding K^b also bind the bm8 mutant; therefore bm8 mice would not be tolerant of these K^b–peptide complexes. If this were the case, then a pool of peptides extracted from K^b but not from bm8 should contain peptides required for recognition by K^b-specific CTL clones.

To test this hypothesis, peptides were extracted from ei-

Table 1. *Lysis of B6 and bm8 Targets by H-2K^b*–specific CTL Clones

Clone	Effector/target ratio	Percent specific lysis*	
		bm8	B6
bm8.10	10	4	100
	1	4	100
bm8.28	10	0	98
	1	0	78
72	10	0	98
	1	0	87
m6	10	0	49
	1	0	13
13	10	0	60
	1	0	8
m37	10	38	37
	1	22	21

* Targets were Con A blasts prepared from bm8 or B6 spleens.

ther bm8 or B6 spleen cells and used in an attempt to reconstitute recognition of "empty" K^b molecules expressed by T2-K^b targets by two different K^b-specific CTL clones from bm8 responders, bm8.28 and bm8.10. As demonstrated in Fig. 1, *a* and *b*, these clones recognized a peptide found in both the bm8 and B6 peptide preparations. However, the amount of peptide found in the bm8 peptide pool was ~10-fold lower than in the K^b pool. In this same experiment, we also assessed the relative amount of peptide present in the K^b and bm8 peptide pools for a number of other K^b-specific CTL clones. Despite the fact that only one of these clones, m37, was capable of recognizing bm8 targets, it was observed that the majority of the allopeptides were present in both extracts. Most surprising was the observation that whereas clone m6 recognized K^b but not bm8, the peptide recognized by clone m6 was actually present at a higher concentration in the pool of peptides released from K^{bm8} than from K^b. Similar results were obtained using two other peptide preparations. Thus, the difference in recognition of bm8 and B6 targets was not solely attributable to the amount of a specific allopeptide.

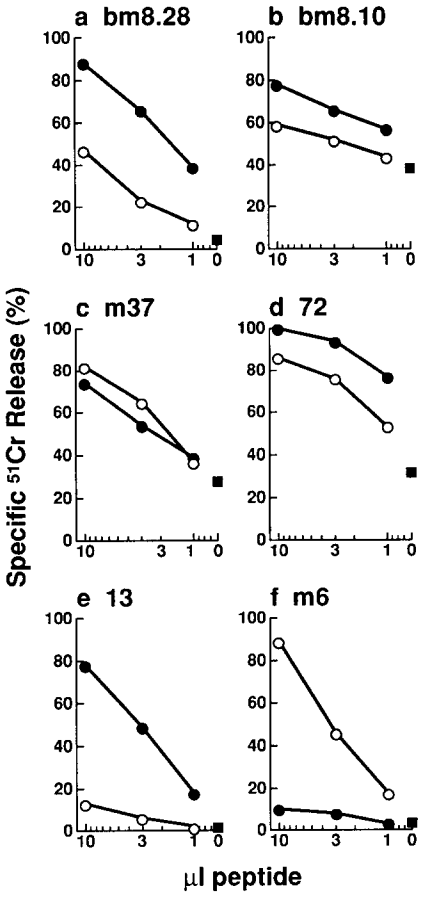
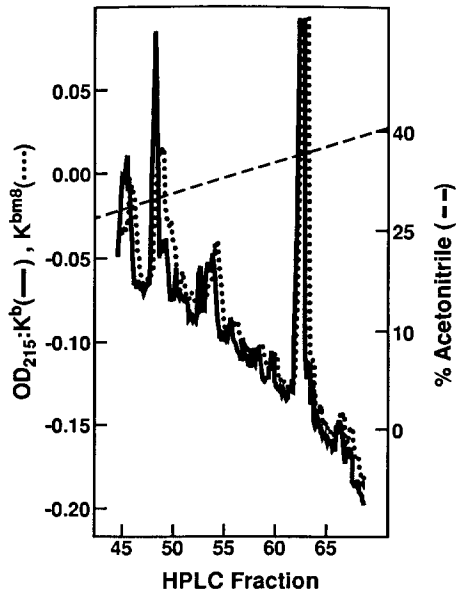


Figure 1. Recognition of allopeptides extracted from K^b and K^{bm8} molecules by K^b specific CTL. 2×10^5 Cr-labeled T2-K^b targets were incubated in a volume of 100 μl with the indicated volume of peptide, diluted to 2 ml, and distributed in microtiter wells (100 μl/well) that then received 10^5 effector cells of the indicated clone in a volume of 100 μl. (■) Lysis of T2-K^b in the absence of added peptide. (●) Lysis of T2-K^b in the presence of peptide extracted from K^b. (○) Lysis of T2-K^b in the presence of peptide extracted from K^{bm8}.

In general, alloreactive clones are usually highly specific in that they recognize a unique peptide or set of peptides presented by the MHC molecule (13–16). However, it was possible that different peptide species present in the K^b and K^{bm8} extracts were being recognized by the various T cell clones. To determine if the same peptide species in each of these complex peptide pools was responsible for allorecognition, peptide extracts were resolved by HPLC and individual fractions tested for their ability to promote lysis of T2-K^b targets. As demonstrated by the experimental results in Fig. 2, the dominant peaks of activity present in the K^b and K^{bm8} extracts recognized by three of the clones analyzed by this procedure (bm8.28, m37, and m6) migrated at the same position on HPLC, strongly suggesting that the same peptide was present in both extracts. Of interest, the major peaks of activity responsible for recognition by bm8.10 migrated at different positions in the K^b and K^{bm8} extracts. This suggests that for this particular clone, different peptide species were responsible for the lysis observed in the experiment described in Fig. 1, which utilized unfractionated pools of peptides. However, the fact that the position representing the major peak of activity in the one extract also represented a minor peak in the other extract suggests that both peaks were present in both extracts and that this, again, was a quantitative difference in the amount of binding to each molecule.

Considering that the concentration of the allopeptide recognized by bm8.28 was significantly lower in the pool of peptides extracted from K^{bm8} than K^b, it was possible that the reason bm8.28 was unable to lyse bm8 targets was the low level of presentation of this allopeptide. If this were the case, then it should be possible to obtain lysis of bm8 targets by providing a sufficiently high concentration of the relevant allopeptide to the K^{bm8} molecule.

Also, considering empty class I molecules such as those expressed on the processing deficient cell line T2 (29, 30) are far more receptive to exogenous peptide loading than molecules on normal cells, T2 cells were transfected with the K^{bm8} gene in order to obtain a target with empty bm8 molecules. The T2-K^{bm8} cells obtained expressed threefold less K^{bm8} than the amount of K^b expressed on T2-K^b as determined by FACS® analysis (data not shown). These cells were able to present the VSV-N peptide for recognition by an appropriate peptide-specific CTL line. However, as anticipated due to their somewhat reduced level of K^{bm8} relative to K^b, they required approximately sixfold more VSV-N peptide as compared with T2-K^b targets to achieve comparable levels of lysis (Fig. 3 *b*). In initial experiments, T2-K^{bm8} targets were incubated with the HPLC fraction containing the partially purified bm8.28 allopeptide extracted from K^{bm8} molecules. Although the peptide facilitated lysis of T2-K^b targets by clone bm8.28, no lysis of peptide pulsed T2-K^{bm8} was obtained (data not shown). Since there was 10-fold more allopeptide activity present in B6 than bm8, it would be preferable to use B6 as a source of peptide from which to purify large quantities of the bm8.28 allopeptide. To this end, the allopeptide was purified from 10^{10} B6 cells. As demonstrated



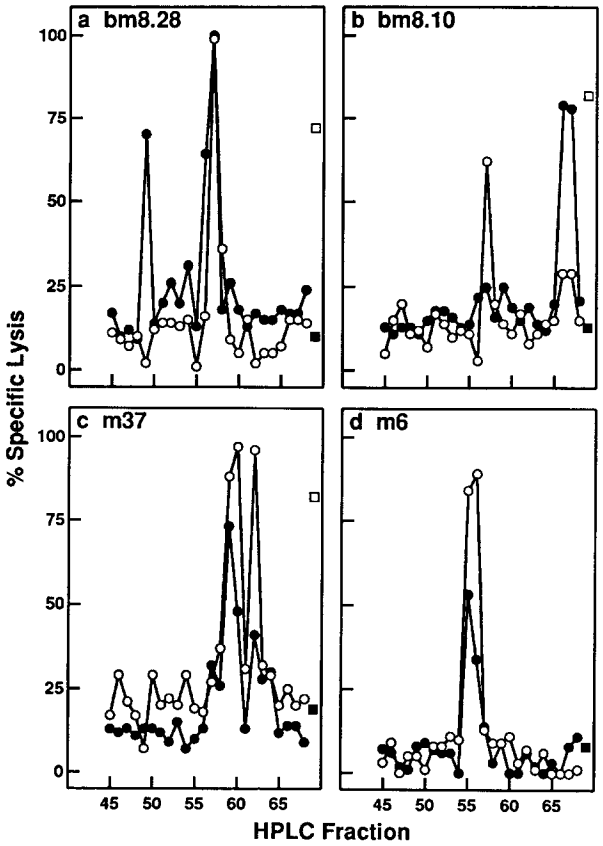


Figure 2. HPLC purification of the allopeptides recognized by K^b specific CTL clones eluted from K^b and K^{bm8} molecules. Peptides eluted from the indicated source were fractionated by HPLC as previously described (25). The indicated CTL clones were used to assay K^b fractions (●) or bm8 fractions (○) incubated with T2-K^b target cells at an effector to target ratio of 10:1. Lysis of EL4 targets (□) served as a positive control and lysis of T2-K^b in the absence of peptide (■) served as a negative control.

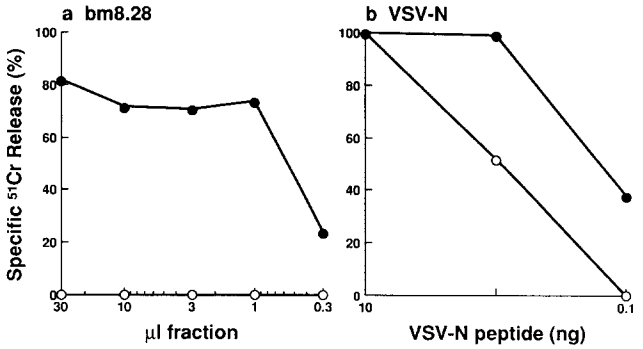


Figure 3. Recognition of T2-K^{bm8} by VSV-N specific CTL and clone bm8.28. The allopeptide recognized by bm8.28 is HPLC fraction 31 obtained from EL4 tumor cells as described in ref. 25. The indicated volume of fraction 31 (*a*) or amount of VSV-N peptide (*b*) was incubated with either T2-K^b (●) or T2-K^{bm8} (○) targets as described in Fig. 1 and cells were used as targets for the indicated CTL.

in Fig. 3 *a*, as little as 0.3 µl of this peptide preparation was sufficient to detect lysis of T2-K^b targets by the bm8.28 CTL clone. In contrast, we were unable to obtain lysis of T2-K^{bm8} targets using as much as 100-fold more peptide.

Since we could not obtain lysis by clone bm8.28 of T2-K^{bm8} cells, we wished to ascertain that these targets were indeed binding the relevant allopeptide. T2-K^{bm8} cells were first incubated with the HPLC fraction that contained the bm8.28 allopeptide, cells were washed, and the K^{bm8} molecules purified for the purpose of reextracting bound peptide. This same procedure was followed using T2-K^b cells, and the peptides extracted from each were incubated with T2-K^b targets. As demonstrated in Fig. 4 *a*, the amount of allopeptide that was bound by T2-K^{bm8} was only about 6–10-fold less than the amount that had bound T2-K^b. Thus, the amount of peptide bound by T2-K^{bm8} targets should have been sufficient to obtain lysis had the bm8.28 clone been

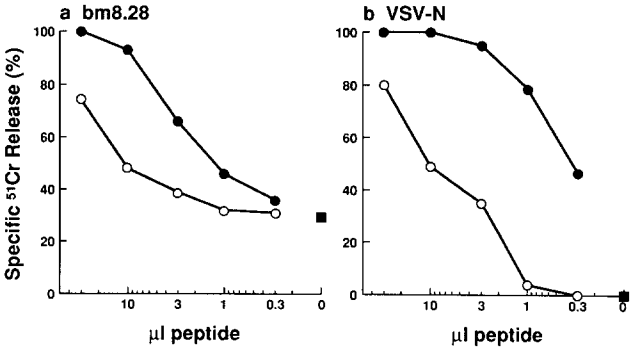


Figure 4. Reextraction of peptides from T2-K^{bm8} cells. 20 µl of the HPLC fraction containing the allopeptide recognized by bm8.28 (*a*) or 0.1 µg of the VSV-N peptide (*b*) was incubated with 10^7 T2-K^{bm8} (○) or T2-K^b (●) cells for 3 h at 37°C. Peptides were extracted from K^b or K^{bm8} molecules as described under Materials and Methods and dissolved in 100 µl of PBS. The indicated volume of reextracted peptide was added to ^{51}Cr-labeled T2-K^b targets and detected using either bm8.28 (E/T ratio 20:1) or VSV-N–specific CTL (E/T ratio 10:1) in a 6-h ^{51}Cr-release assay. Lysis of T2-K^b in the absence of peptide (■) served as a negative control.

capable of recognition of the K^{bm8}-allopeptide complex. When this same protocol was followed using the VSV-N peptide, it was found that 10–30-fold less peptide bound T2-bm8 than T2-K^b (Fig. 4 *b*). Considering that both targets are recognized by VSV-N–specific CTL, this again suggests that there should have been adequate amounts of the bm8.28 allopeptide to achieve recognition and lysis.

Discussion

There is now ample evidence that the majority of alloreactive CTL are as peptide-specific in their recognition of class I as conventional antigen-specific CTL (reviewed in 13). The heterogeneity of peptides that bind a single class I molecule can readily explain the high frequency and receptor diversity of an alloresponse. The basis for the antigenicity of these complexes is the subject of this investigation. The naturally arising K^b mutants always involve one or more amino acids that affect peptide binding. The bm8 mutant is particularly striking in this regard, since its mutations are confined exclusively to residues involved in peptide binding as opposed to the possibility of direct interaction with the TCR (residues 22, 23, 24, and 30). In particular, residue 24 is most involved in determining the bm8 phenotype as defined by both allorecognition and peptide binding (21, 22). In contrast, many of the other K^b mutants are altered not only in residues that affect peptide binding, but also in residues that could alter interactions with the TCR (18). Unlike these other naturally arising K^b mutants, the only way the bm8 mutant could stimulate K^b responders would be through its effect on peptide binding as opposed to an effect on portions of the MHC that interact with the TCR. Indeed, in a recent analysis of the endogenous peptides bound by K^b and K^{bm8}, it was found that significant differences in the HPLC profile of bound peptides was apparent (20). Our goal in this study was to learn more about the contribution of such peptide differences to this alloresponse. In particular, was it due to the quantitative or qualitative differences in the peptides bound by these molecules, or alternatively, to conformational differences in the appearance of the peptide–MHC complex as viewed by the TCR?

Our results suggest that the most frequently observed difference in the ability of K^b and K^{bm8} to bind peptide is quantitative rather than qualitative. In most cases in which a particular allopeptide is sought, it can be found on both K^{bm8} and K^b. This is similar to what has also been observed for the binding of K^b restricted minor antigens, which are not recognized in association with bm8, yet are found in mixtures of peptides extracted from bm8, albeit at reduced level (31). It is important to note, this reduced level of binding does not necessarily account for lack of recognition in association with bm8. For example, the VSV-N peptide is recognized when presented by either K^b or K^{bm8}, yet it binds K^{bm8} at least 10-fold less well. A dramatic example of a situation in which the amount of peptide bound does not predict T cell recognition is exhibited by the alloreactive clone, m6. This clone recognizes K^b but not bm8 targets, yet the amount of peptide that can be extracted from K^b expressing cells is much less than from K^{bm8} expressing cells. Taken together, these results suggest that although many peptides that bind K^b are reduced in their ability to bind K^{bm8}, this is not necessarily the reason for lack of recognition. It appears the number of peptide molecules bound per cell is usually more than adequate for T cell recognition, when the target expresses wild type K^b. Rather, it is the fact that bm8–peptide complex is not recognized by the TCR. Considering that there can be no sequence differences in either the peptide or MHC that can explain such lack of interaction with the TCR, it must be concluded this is due to conformation differences that are indirectly attributable to the bm8 mutation.

In a recent report, Chen et al. (19) demonstrated the significance of conformational alteration of a peptide–MHC complex to recognition by OVA-specific CTL. In this study it was found that by changing a residue in the peptide that interacted with pocket B on K^b it was possible to eliminate recognition by OVA-specific CTL. Our results demonstrate that alteration in the MHC can have a similar conformational effect leading to ablation of T cell recognition. We have further demonstrated that such a conformational alteration can be responsible for the stimulation of alloreactive T cells.

There have been several reports concerning the possibility that conformational changes in the MHC per se could lead to allorecognition (32–34). It has been observed that high concentrations of synthetic peptide could restore recognition of empty class I by some allospecific CTL, thereby suggesting specific peptide was not required for recognition, but rather the conformation of the MHC molecule induced by peptide (34). Considering that the number of MHC–peptide complexes required to achieve such cross-reactive recognition is much higher than occurs normally for most endogenously derived self-peptides, it is not yet known to what extent this type of mechanism may contribute to normal alloresponses. The affinity required for cross-reactive recognition is likely to much lower than the affinity required for initial stimulation of a T cell.

Our results do not allow us to distinguish whether the conformational difference responsible for loss of T cell receptor recognition of K^{bm8} is exhibited by the peptide, the MHC, or both. In view of the fact that the conformational epitopes described in this report are dependent on the presence of a specific allopeptide, and that it is unlikely the K^{bm8} and K^b molecules differ conformationally in their α-helical regions, it seems most likely the relevant conformational differences involve the peptide. Further understanding of the structural consequences of the bm8 mutation and how it effects the conformation of a particular peptide–MHC complex awaits x-ray crystallographic information.

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