Anchoring Pockets in Human Histocompatibility Complex Leukocyte Antigen (HLA) Class I Molecules: Analysis of the Conserved B ("45") Pocket of HLA-B27

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Summary

Dissection of the peptide binding grooves of seven subtypes of human histocompatibility leukocyte antigen (HLA)-B27 into the six specificity pockets defined by the 2.6-Å structure of HLA-A*0201 revealed just one pocket, the B ("45") pocket, that is conserved among all the HLA-B27 subtypes. Functional studies of mutant HLA-B*2705 molecules with point substitutions in residues of the B pocket show that this structure, and the glutamine residue at position 45 in particular, plays a critical role in cell surface expression, peptide binding, and in the presentation of both exogenous and endogenous peptides by HLA-B*2705. We predict that the B pocket of HLA-B*2705 interacts with an amino acid side chain that anchors peptides in the binding groove, and that this peptide motif is present in most endogenously processed peptides that bind to all seven subtypes of HLA-B27.

HLA class I molecules bind and present antigenic peptides to CTLs (1, 2). Bound peptides form an integral part of the molecule and facilitate stable expression at the cell surface (3, 4). Antigenic peptides eluted from murine H-2 class I molecules and HLA-A*0201 have been sequenced. These analyses reveal that naturally processed peptides are generally heterogenous in character but restricted in length to eight or nine amino acid residues. Furthermore, for any given allele certain peptide residues show limited heterogeneity, allowing the definition of allele-specific sequence motifs in terms of these "anchor" residues (5–8). Examination of the structure of antigenic peptides, represented as "extra density" in the x-ray crystallographic solutions for HLA-A*0201 and A*6801, suggests that the peptide amino acid side chains are positioned in the class I molecule to interact with specificity pockets that extend from the peptide binding groove (9–11).

In light of these findings, we reasoned that peptide anchor residues must interact with class I molecules through specificity pockets that are optimized to bind their side chains, and other residue side chains may interact with pockets that are less discriminating. Pockets binding anchoring side chains would be specialized structures within the peptide binding groove that might be maintained during evolution because of their important functional role. To test this hypothesis, we first examined the primary structures of 61 HLA class I molecules and mapped their polymorphisms into the six specificity pockets revealed in the peptide binding groove of the 2.6-Å refined structure of HLA-A*0201 (11, 12). This analysis demonstrated that all six pockets contain highly polymorphic residues and that there is little tendency for major alleles to share similar pocket structures (data not shown). We then chose to examine the subtypes of major alleles, as products of relatively recent evolutionary change.

We report our findings with respect to seven HLA-B27 subtypes (12–14). Sequence comparison reveals eleven residues that are polymorphic among the subtypes, and these map to five of the six specificity pockets. One pocket, the B ("45") pocket, is totally conserved among all HLA-B27 subtypes. Further analysis by site-directed mutagenesis shows this region, and the glutamine residue at position 45 in particular, play a critical role in cell-surface expression, peptide binding, and antigen presentation by HLA-B*2705. We predict that the B pocket binds an anchor residue found in most HLA-B*2705 binding peptides, and that this represents a peptide motif that is likely to be shared by all the HLA-B27 subtypes.

Materials and Methods

Cells. PBL were obtained from Caucasian HLA-B*2705-positive male volunteers, and EBV-transformed B cell lines were made for use as autologous feeder cells in the generation of peptide-specific CTL lines. HLA types of CTL lines used were: RB (A2,32;

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B*2705,8; Cw2), DB (A25,32; B*2705,18), and WG (A3,A23; Bw50,Bw55; Cw3,Cw6). Other EBV lines used were: JY (A2,2; B7,7;), BL-EBV (A1,2;B7,37), HOM2 (A3; B*2705), JESTHOM (A2; B*2705), C1R (–, –, Cw4), B27C1R (–, B*2705, Cw4), LH (A24; B*2701,8; Cw1,7), PG (A11,31; B*2705,35; Cw2,4), WE1 (A11,24; B*2704,w62; Cw6), and LIE (A2,11; B*2706,5; Cw3).

Mutagenesis and Transfection. HLA-B27 single residue mutants (see Table 3) were created by site-directed mutagenesis of the HLA-B*2705 gene obtained from Dr. Joel Taurog (University of Texas Southwestern Medical Center, Dallas, TX), as a 6.5-kb EcoRI fragment subcloned in pUC19. Three constructs were made. First, the HLA-B*2705 gene was shortened in the 3′ untranslated region by restriction enzyme digestion with BstEII and HincII (in the 3′ polylinker of pUC19), Klenow DNA polymerase I fill in, and subsequent religation. This served to shorten the gene to 3.9 kb and to remove an inconvenient second 3′ DraIII site. Next the exons encoding the leader sequence, α_1 and α_2 domains were subcloned as a 2-kb EcoRI/AvrII fragment into EcoRI/XbaI cut M13mp18 vector, and the exons encoding the α_3, transmembrane, and cytoplasmic domains were subcloned as a 2.2-kb KpnI/Ssp1 fragment into KpnI/HincII cut M13mp18. Mutagenesis was then effected on these fragments in M13mp18 by the method of Kunkel (15) using the Muta-gene in vitro mutagenesis kit (Bio-Rad Laboratories, Richmond, VA). Mutated α_1 and α_2 exons were subcloned back into the 3.9-kb HLA-B27/pUC19 construct on a 1.8-kb EcoRI/DraIII fragment, and mutated α_3 exons were subcloned as a 1.8-kb DraIII/BstXI fragment.

Mutant HLA-B27 genes were then subcloned into pHEBO (16) as a 4.0-kb HindIII (in the 3′ polylinker)/FspI (150 bp 5′ in the pUC19 sequence) fragment. pHEBO was prepared by digestion with SalI, blunt-ended with Klenow DNA polymerase I, and digestion with HindIII, before gel purification. Mutant genes were electroporated into C1R cells as previously described, and selection with Hygromycin was initiated 3 d later (17, 18). Mutant genes were verified by DNA sequencing.

FACS® Analysis. Levels of cell surface HLA class I expression of all cell lines was determined within 72 h of the performance of both peptide binding and CTL assays. This was done by FACScan® analysis, using fluorescein-conjugated antibodies W6/32 and PA2.6 (both monomorphic anti-HLA class I antibodies), ME1 (anti-HLA-B27, B7, and Bw42) and B27M1 (anti-HLA-B27) (19–21). In addition, CD8 expression by all CTL lines was verified by FACScan® using fluorescein-conjugated antibodies OKT4 (anti-CD4) and OKT8 (anti-CD8).

Synthetic Peptides. A peptide (SRYWAIRTRSGG) corresponding to the sequence of influenza A nucleoprotein (383–394) (NP1)[1] (22) was synthesized by the solid phase method using Fmoc chemistry.

Generation of Autologous CTL. PBL from the HLA-B27-positive individuals were stimulated in primary culture with 5 μg/ml of (NP1) peptide in RPMI 1640 supplemented with 2-mM L-glutamine, 10% FCS (Hyclone Laboratories, Logan, UT). After 1 wk, cultures were restimulated with 5×10^5 irradiated (10,000 rad) autologous EBV-transformed cells plus 5 μg/ml NP1, and were supplemented with T cell–conditioned medium every 7 d (23) with either the autologous EBV cell line or HOM2 plus NP1 peptide.

Generation of Allogeneic CTL. PBL from a normal donor WG were cultured with irradiated (10,000 rad) B27C1R cells in RPMI 1640 supplemented with 10% FCS and 2 mM glutamine. After 7 d, the cultures were restimulated with a different irradiated HLA-B27-positive EBV-transformed cell line and were supplemented with T cell conditioned medium (23). After 3 wk of culture, the line was cloned by limiting dilution (24).

Assay of CTL. CTL activity was measured using a standard 4-h ^{51}Cr release assay (23). Unless otherwise noted, target cells were incubated for 2 h with 200 μCi of ^{51}Cr. Targets were then washed three times, counted, and plated in the presence of 1 or 2 μg/ml NP1 for the duration of the assay.

Binding Assay. Peptide NP1 was iodinated by the Chloramine T method and free ^{125}I separated from the iodinated peptide by means of a Sep-pak C18 column as previously described (25). In the experiment shown in Fig. 2 *a*, 200 μg NP1 was iodinated with 6.5 mCi ^{125}I and in that shown in Fig. 2 *b*, 20 μg was iodinated with 2 mCi ^{125}I.

^{125}I-NP1 peptide was added to 500-μl aliquots of 2.5×10^6 cells and incubated for 6 h at 37°C. The cells were then washed, lysed, and the HLA-class I molecules immunoprecipitated with the W6/32 antibody as previously described (25). The amount of bound peptide was then assessed by measuring the ^{125}I radioactivity coprecipitated with the HLA class I molecules using a 1260 Multi gamma counter (LKB Instruments, Inc., Gaithersburg, MD).

Results

Conservation of the B Specificity Pocket among HLA-B27 Subtypes. The 2.6-Å refined crystallographic structure of HLA-A2 reveals six specificity pockets (labeled *A–F*) located in the peptide binding groove, which are thought to interact with amino acid side chains of bound peptides (Fig. 1 *a*) (11). Comparison of the sequences of the known HLA-B27 subtypes shows that variation between the subtypes occurs in five of the six pockets: A, C, D, E, and F (Table 1). In contrast, the 10 residues comprising the core of the B pocket (positions 9, 24, 34, 45, and 67) and its environs (positions 7, 63, 66, 70, and 99) are totally conserved among the subtypes (Table 2) and the HLA-B27 combination of 10 residues is not found in the B pocket of any other HLA class I sequence. This finding is significant as the B pocket is highly polymorphic in all classical class I heavy chains, especially in the products of the HLA-B locus, strongly suggesting that the B pocket has been actively conserved throughout the recent evolution of HLA-B27 (Table 2).

Mutagenesis of the B Pocket of HLA-B27. Conservation of the B pocket among subtypes suggests its structure is vital to the function of the HLA-B27 molecule. To test this hypothesis, HLA-B27 point mutants with changes at positions 9, 45, and 67 in the B pocket and position 70 at its rim, were derived. Further mutants with substitutions at positions 71 and 77 (α_1 domain), 97 (α_2 domain), and at position 227 (α_3 domain) were included for comparison (Table 3). In each case, the amino acid introduced by mutagenesis was one that occurs naturally in other HLA class I molecules (see Table 2). Mutant genes were electroporated into C1R cells (17), and expression assessed with the monomorphic anti-HLA class I mAb W6/32, as well as with the anti–HLA-B27 antibodies ME1 and B27M1 (19–21). Comparable expression of each mutant was detected with all three antibodies, with the exception of the 45K mutant, which was detected at levels 12–15-fold lower than wild-type with ME1 and was

[1] *Abbreviation used in this paper:* NP1, influenza A nucleoprotein.

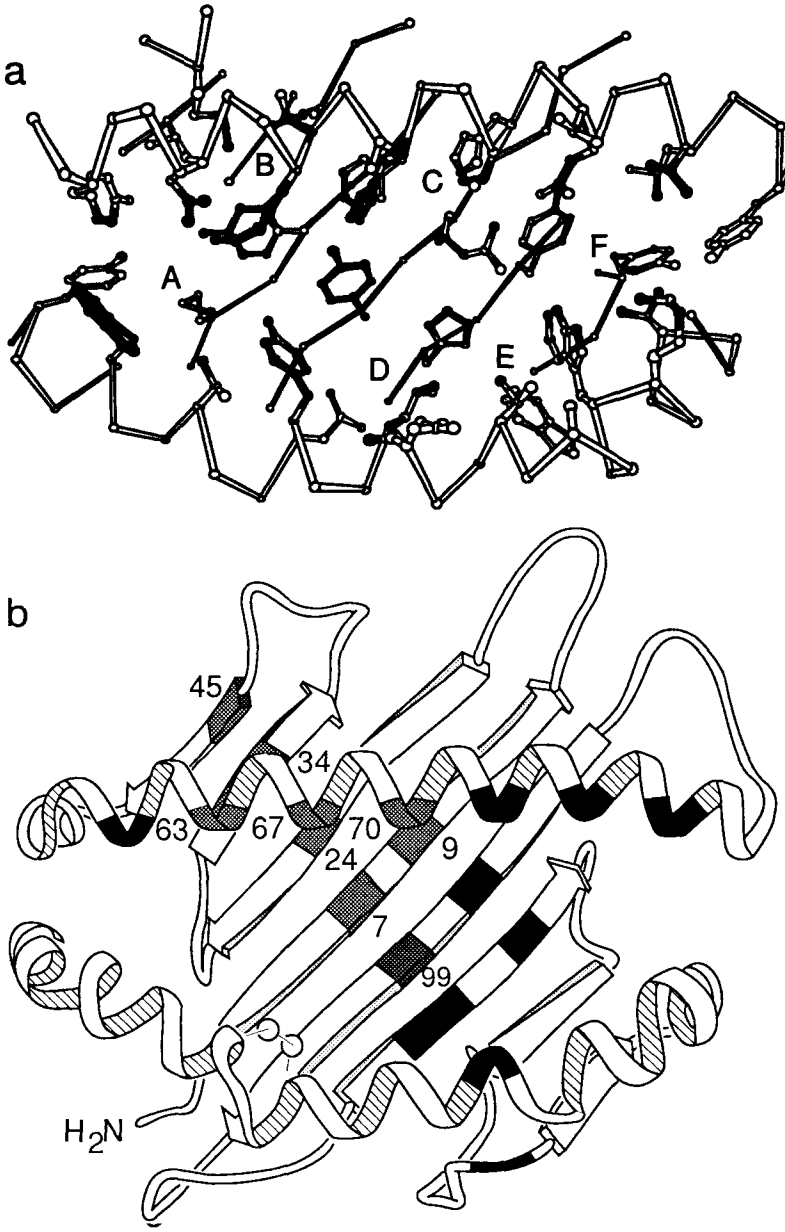

Figure 1. Mapping the polymorphic and conserved residues of the HLA-B27 subtypes in terms of the specificity pockets described in the x-ray crystallographic structure of HLA-A*0201. (*a*) Specificity pockets *A–F* of HLA-A*0201, as described by Saper et al. (reproduced from *J. Mol. Biol.*, 1991, by copyright permission of Academic Press Limited, 219:277–319). (*b*) HLA-B27 subtype polymorphisms (*solid* areas) and B pocket conserved residues (*shaded* areas) mapped onto the ribbon structure of HLA-A*0201, as described by Bjorkman et al. (9).

undetectable with B27M1, and the 67Y mutant, which was not detectable with any of the antibodies (Table 3). Electroporation was repeated on at least three occasions with similar results. Lysine at position 45 occurs naturally in many HLA-B locus products including HLA-B*4001, B*4002, B*4101, B*4401, B*4701, and B*4901, and tyrosine at position 67 occurs in HLA-B*0701, B*4201, and B*4601 (12). Clearly, expression of the HLA-B27 molecule is sensitive to mutations within the B pocket, however the finding that the 45M, 67A, and 67S mutants were expressed demonstrates that some mutations are permissible at these positions.

Influenza Nucleoprotein Peptide (383–394) Binding to Mutant HLA-B27 Molecules. NP1 (383–394) is recognized by CTL when bound and presented by HLA-B*2705 molecules (22). Furthermore, we have shown that radioiodinated-NP1 binds directly to a large proportion of cell surface HLA-B*2705 molecules in a peptide- and allele-specific fashion in culture (25). We therefore tested the effects of point mutations in the B pocket of HLA-B*2705 on the binding of the NP1 peptide. The results show that all mutations within the B pocket (9Y; 45M; 67A; and 67S) diminish peptide binding (Fig. 2). These effects were seen at two different peptide concentrations – 5 μg/ml (Fig. 2 *a*) and 50 μg/ml (Fig. 2 *b*), and >90% inhibition of binding was observed. The 70Q mutation at the rim of the B pocket, and also the 227K and 77S mutations had no effect on peptide binding. The 97R mutation, on the other hand, also decreased binding by >90%. Surprisingly, the mutation at position 71, a residue

Table 1. *HLA-B27 Subtype Polymorphisms Mapped to Specificity Pockets as Defined in the 2.6-Å Refined Structure of HLA-A2*

	Amino acid residues										
Pocket	59 A	74 C	77 F	80 F	81 F	97 C/E	113	114 D/E	116 F	131	152 E
B*2701	Tyr	Tyr	Asn	Thr	Ala	Asn	Tyr	His	Asp	Ser	Val
B*2702	–	Asp	–	Ile	–	–	–	–	–	–	–
B*2703	His	Asp	Asp	–	Leu	–	–	–	–	–	–
B*2704	–	Asp	Ser	–	Leu	–	–	–	–	–	Glu
B*2705	–	Asp	Asp	–	Leu	–	–	–	–	–	–
B*2706	–	Asp	Ser	–	Leu	–	–	Asp	Tyr	–	Glu
B*27"HS"	–	Asp	Asp	–	Leu	Ser	His	Asn	Tyr	Arg	–

The table displays only the residues that differ among the seven subtypes of HLA-B27, and the pockets (*A–F*) to which they map in the 2.6-Å refined structure of HLA-A2.

that is not thought to directly contribute to the peptide binding groove, reproducibly reduced binding by as much as 70%.

*NP1-specific CTL Lysis of HLA-B*2705 Point Mutants.* We next tested whether recognition of HLA-B27 by NP1-specific CTL was similarly affected by point mutations in the B pocket. CTL lines generated from HLA-B*2705-positive donors were shown to be both peptide- and allele-specific by their requirement for the presence of NP1 for lysis, and their inability to lyse cell lines bearing other HLA-B27 subtypes (HLA-B*2701, 02, 04, and 06) (Fig. 3).

Peptide-specific lysis of cells expressing point mutants of HLA-B*2705 was tested using CTL lines from two donors, RB and DB (Fig. 4). The 67A and 67S mutants, which did not bind peptide (Fig. 2), were not lysed by NP1-specific CTL. The 70Q mutation at the rim of the B pocket, which did not affect the extent of NP1 peptide binding, reduced lysis by both CTL lines by about 50%. The 9Y and 45M mutants, which bound NP1 peptide poorly, were lysed about 50% as well as wild-type HLA-B*2705–bearing cells by the DB CTL line. This latter result suggested that the 45M and 9Y mutations do not abrogate binding, but reduce it to below the limit of detection in the binding assay, which we estimate to be about 1,000 peptide molecules/cell. This hypothesis was tested in two ways. First, we examined the requirement for the presence of NP1 peptide throughout the 4-h time period of the CTL assay. Cells expressing the 45M and 9Y mutants and control target cells were cultured overnight in the presence of NP1 peptide to allow a steady-state level of peptide binding to be reached, and then washed thoroughly before adding CTL and, to half of the assay, additional NP1 peptide. Cells expressing wild-type HLA-B*2705 were lysed equally well, regardless of the presence or absence of NP1 during the CTL assay, and both the 45M and 9Y mutants were lysed less well when NP1 was not included throughout the 4-h duration of the ^{51}Cr release assay (Fig. 5). Second, titration revealed that optimum lysis of cells expressing the 45M and 9Y mutants of HLA-B*2705 required, respectively, 50 and 20 times more NP1 peptide in the assay, when compared with wild-type HLA-B*2705 (data not shown). These results indicate the kinetics of dissociation and/or association of the NP1 peptide with the wild-type and mutant HLA-B27 molecules are significantly different, with the mutants having a lower affinity for peptide. This is compatible with the conclusion that NP1 binds to the mutant HLA-B27 molecules at a level that is sufficient to trigger CTL, but is undetectable in the binding assay (29, 30).

The 45M mutation had a surprising effect on recognition of HLA-B27 by the RB CTL line. This CTL line lysed cells expressing the 45M mutant in a NP1-peptide independent fashion (Fig. 5 *b*). As the amino acid at position 45 is not assigned to interact directly with the CTL TCR, the implication of this observation is that the mutation alters the molecule in such a manner that an endogenous peptide bound to the 45M mutant or empty 45M molecules, resembles the complex of wild-type HLA-B*2705 and NP1 peptide (9).

Point mutations not in the B pocket also had diverse effects upon CTL recognition. The substitution of lysine at position 227 in the α_3 domain prevents CD8 binding (18, 31). Cells expressing the 227K mutant were not lysed by either CTL line, demonstrating their CD8 dependence. The 71T mutation, on the other hand, abrogated recognition by the DB CTL line and decreased that of the RB CTL line by 60%, implying residues with side chains not assigned to interact with either peptide or TCR can affect both peptide binding and CTL recognition. The 77S mutation, like the 70Q mutant, did not affect the amount of peptide binding but did reduce CTL recognition by about 50%, and the 97R mutant neither bound significant amounts of peptide, nor was lysed by the CTL lines.

In conclusion, these results indicate the 67A, 67S, and 97R mutations reduce CTL recognition by abrogating NP1 peptide binding. The 9Y, 45M, and 71T mutations reduce the extent of peptide binding and have variable effects on T cell

Table 2. *Polymorphisms of the B Pocket*

	9	24	34	45	67	**7**	**63**	**66**	**70**	**99**
B*2701,02,03,04,05,06,"HS"	H	T	V	E	C	Y	E	I	K	Y
B*3801,B*3901,B*1401,02	Y	S	–	–	–	–	N	–	N	–
B*0801	D	S	–	–	F	–	N	–	N	–
B*4201,B*0701	Y	S	–	–	Y	–	N	–	Q	–
B*4401,02	Y	–	–	K	S	–	–	–	N	–
B72	Y	S	–	K	S	–	–	–	N	–
B*4701	–	–	–	K	S	–	–	–	N	–
B*4901,B*4001,02,B*4101	–	–	–	K	S	–	–	–	N	F
B53,B*3501,B78,B*5101	Y	A	–	T	F	–	N	–	N	–
B*5201	Y	A	–	T	S	–	–	–	N	–
B*5801	Y	A	–	T	M	–	–	N	S	–
B*5701	Y	A	–	M	M	–	–	N	S	–
B*3701	–	S	–	T	S	–	–	–	N	S
B*1801	–	S	–	T	S	–	N	–	N	–
B*1301,02	Y	–	–	M	S	–	–	–	N	–
B*4601	Y	A	–	M	Y	–	–	K	Q	–
A*0201,02,03	F	A	–	M	V	–	–	K	H	–
A*0207	F	A	–	M	V	–	–	K	H	C
A*0101,A*3201	F	A	–	M	V	–	–	N	H	–
A*0301,02,A*3001	F	A	–	M	V	–	–	N	Q	–
A*0206,05	Y	A	–	M	V	–	–	K	H	–
A*0210	Y	A	–	M	V	–	–	K	H	F
A*0208	Y	A	–	M	V	–	–	N	H	–
A*6901,A*6801,02	Y	A	–	M	V	–	N	N	Q	–
A*2401	S	A	–	M	V	–	–	K	H	F
A*0101	F	A	–	M	M	–	–	N	H	–
Cw*0101	F	S	–	G	Y	–	–	K	Q	C
Cw*0601	D	S	–	G	Y	–	–	K	Q	–
Cw*0201,Cw*0501,Cw*1101	Y	A	–	G	Y	–	–	K	Q	–
Cw*0301	C	A	–	G	Y	–	–	K	Q	–
HLA-E	–	S	–	M	A	–	–	S	T	H
HLA-F	S	A	L	M	A	E	T	Y	N	N
HLA-G	S	A	–	M	T	–	–	N	H	I

The table shows the 10 amino acid residues that influence the nature of the B pocket in HLA-A2 and how they vary among 61 HLA class I sequences. Residues in the vicinity of the rim of the pocket are shown in bold print. All of the HLA sequences are from Zemmour and Parham (26), except for the HLA-B27 subtypes (12–14, 27, 28).

recognition. The 70Q, 77S, and 227K mutations do not alter peptide binding, but do affect recognition by T cells.

*Alloreactive CTL Recognition of HLA-B*2705 Point Mutations.* To see how point mutations affected recognition by CTL of different specificity, we analyzed the lysis of targets expressing the HLA-B*2705 mutants by an anti–HLA-B*2705 alloreactive CTL line, and by five clones derived from it by limiting dilution. Lysis by the CTL line and one clone was unaffected by any of the mutations. Four of five clones failed to lyse the 45M mutant, and one clone failed to lyse the 9Y mutant and another the 77S mutant. Thus, with the notable exception of the methionine mutation at position 45, alloreactive CTL are relatively insensitive to the point mutations we derived.

Table 3. *List of Mutations and Their Expression on Transfected C1R Cells*

Name	Wildtype	Mutant	Expression			
			W6/32	ME1	B27M1	Saline
C1R			174	5	4	4
B27C1R			1,456	1,234	276	4
9Y	His	Tyr	833	642	127	4
45M	Glu	Met	1,443	1,195	256	4
45K	**Glu**	**Lys**	**278**	**94**	**4**	**4**
67Y	**Cys**	**Tyr**	**201**	**12**	**4**	**4**
67A	Cys	Ala	1,284	998	252	4
67S	Cys	Ser	1,235	1,056	246	4
70Q	Lys	Gln	1,430	1,132	156	4
71T	Ala	Thr	1,380	653	54	4
77S	Asp	Ser	1,355	1,152	339	4
97R	Asn	Arg	1,216	1,034	90	4
227K	Asp	Lys	1,132	1,132	196	4

The table lists the 11 HLA-B*2705 mutations studied, and the nature of the mutation made. Expression was assessed by FACScan® analysis using the antibodies W6/32 (monomorphic anti-HLA class I); ME1 (anti–HLA-B27, B7, and B42); and B27M1 (anti–HLA-B27) or saline. Data are shown as the mean fluorescence channel for each sample. Results obtained for the poorly expressed 67Y and 45K mutants are shown in bold print.

Discussion

These experiments reveal a major role for the residue at position 45, and the B pocket in general, in HLA-B27 function (Table 4). Initially we observed that replacing the negatively charged glutamic acid residue at position 45 with positively charged lysine (as found in HLA-B*4001, B*4002, B*4101, B*4401, B*4701, and B*4901) resulted in a 12–15-fold decrease in cell surface expression, and the more conservative change to methionine (as found in HLA-A locus products) was expressed well. However, the 45M mutation greatly reduced the binding affinity for a known HLA-B*2705 binding peptide (NP1), although not to the point where CTL recognition was eliminated. Two further observations suggested that the 45M mutation also affected presentation of endogenously processed peptides. First, we found that the 45M mutant is recognized by the NP1-peptide–specific RB CTL line in a peptide-independent fashion, and second, it abrogated recognition by four of five alloreactive clones, and no other mutation affected recognition by more than a single clone. As the residue at position 45 is not assigned to interact with the TCR (9–11), and since alloreactive clones are often dependent on the nature of the endogenously bound peptide (32), these findings are best explained by an indirect effect of the 45M mutation on the nature or conformation of the endogenous peptide, such that it mimics the HLA-B*2705 molecule complexed to NP1 peptide, and is no longer recognized by the majority of alloreactive clones.

The importance of the peptide/B pocket interaction is further substantiated by the finding that mutations of other residues in the B pocket also markedly affect HLA-B27 function. Relatively conservative changes at positions 67 and 9 (67S, 67A, and 9Y) decreased NP1 peptide binding by >90%. In the case of the mutations at position 67, there was a concomitant loss of NP1-specific CTL recognition of the mutant HLA-B*2705 molecules, suggesting an absolute loss of peptide binding, and the mutation at position 9 only decreased the affinity of binding as NP1-specific CTL, which still recognized the mutant in a peptide dependent fashion.

Furthermore, we found that changing cysteine at position 67 to tyrosine caused a loss of HLA-B*2705 expression in C1R cells. Tyrosine at this position is found in HLA-B*0701, B*4201, and B*4601, and is predicted to block access to the B pocket (10). That two mutations (45K and 67Y) which radically alter the B pocket lead to a decrease in cell surface expression is remarkable. We favor the explanation that there is a relative lack of endogenous peptides able to bind these mutant molecules with sufficient affinity to stabilize their cell surface expression, although other possibilities cannot be formally excluded. It is interesting that El-Zaatari et al. (33) showed that the 67Y mutant molecule is expressed in mouse L cells, albeit with a loss of the overlying ME1 antibody epitope. We confirmed these results on transfecting our 67Y mutant construct into human-β_2m expressing L cells (data not shown). These findings may be explained by the presence of a endogenous peptide in mouse, and not human

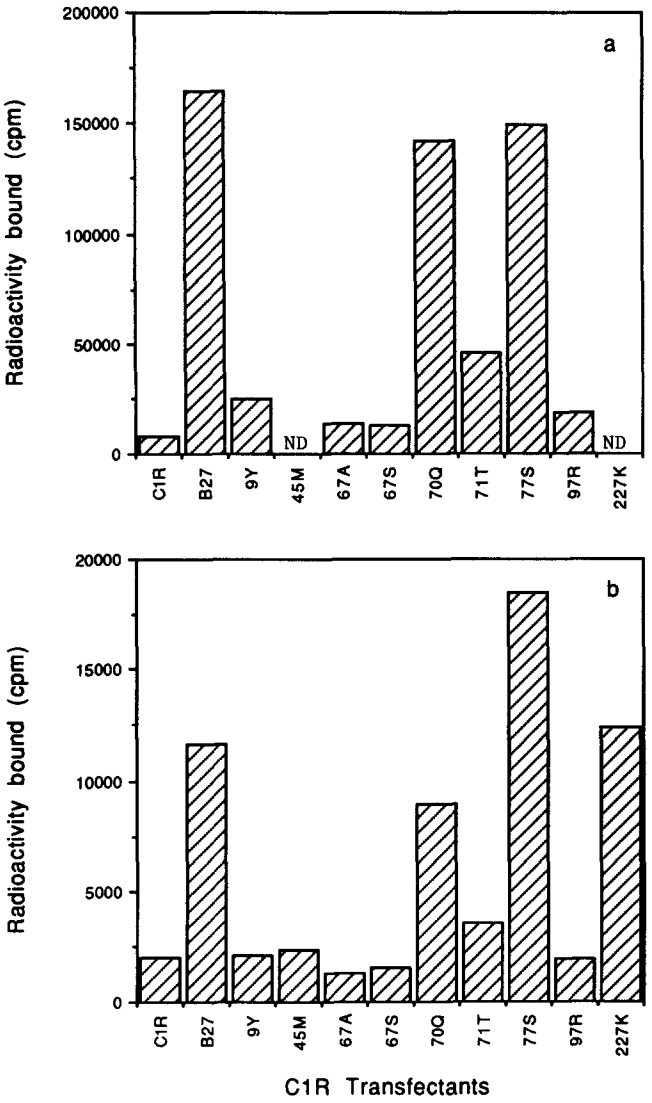

Figure 2. ^{125}I-NP1 binding to HLA-B*2705 mutant C1R cells. Peptide binding to C1R, B27C1R, and HLA-B*2705 mutant molecules after culture of C1R transfectants expressing these molecules in the presence of either (*a*) 50 μg/ml or (*b*) 5 μg/ml of ^{125}I-NP1.

cells, that is able to bind and stabilize the 45M mutant molecule.

That the residue at position 45 plays a major role in HLA-B*2705 function can be explained in terms of the structure of HLA-B*2705, modeled on the known structures of HLA-A*0201 and HLA-A*6801 (9–11). In these molecules, the core of the B pocket is formed by hydrophobic residues (9F or Y; 24A; 45M; 67V and 34V) and the pocket is thought to bind either a leucine or isoleucine side chain. Site-directed mutagenesis studies of HLA-A*0201 reveals that mutations in the B pocket also markedly affect peptide-specific and alloreactive CTL recognition (34). In comparison, most of the residues lining the core of the B pocket in HLA-B27 are hydrophilic (9H, 24T, and 67C) with the negatively charged 45E side chain situated at the apex of the pocket. This has led to the suggestion that the B pocket may bind a positively charged side-chain (e.g., histidine, arginine, or lysine) that forms a salt bridge with the 45 glutamate side chain (10). Such a salt bridge may drastically increase the binding affinity of the side chain in the pocket, and effectively anchor the peptide in the groove. Mutations at position 45 would prevent the formation of the salt bridge and decrease the affinity of peptide binding without affecting the conformation of the bound peptide. Our finding that NP1-peptide binding to the 45M mutant could not be detected in the binding assay, yet was detected by NP1-peptide specific CTL, supports this model. Mutations that dramatically affect peptide binding affinity would also affect endogenous peptide selection by HLA-B*2705. Again, this is supported by our finding that the 45M mutant had a major effect on alloreactive CTL recognition.

Mutations outside of the B pocket also had diverse effects on HLA-B27 function and clearly showed that the B pocket is not the only feature of the HLA-B27 groove that determines binding and presentation of the NP1 peptide. Indeed, even changing a residue that is not assigned to interact with either the TCR or the peptide (threonine for the natural alanine at position 71) reduced peptide binding and abrogated recognition by the DB NP1-specific CTL line. This residue is polymorphic in the products of HLA-B locus genes and clearly must have an indirect effect on peptide binding and presentation, probably by affecting the side chain conformation of adjacent amino acid residues that impact on the binding groove. Next, we found that changing the asparagine residue for aspartate at position 97 both abrogated NP1 binding and NP1-specific CTL recognition, suggesting an absolute loss of binding. Asparagine at position 97 forms part of the core of both the C and E pockets, and our results suggest that interactions with these pockets may also critically affect peptide binding. Mutations at the rim of the B pocket (70Q) and the side wall of the F pocket (77S), on the other hand, had no affect on NP1 peptide binding but did reduce NP1-specific CTL recognition. This implies that these residues may not be involved in determining which peptides bind to HLA-B*2705, but do affect T cell recognition, probably by changing the conformation of the peptide in the groove.

In these studies, widely different results were obtained with the various functional assays used to analyze the point mutants of HLA-B*2705. The NP1-peptide binding assay was very sensitive to changes in the amount and affinity of peptide binding to HLA-B*2705, but was a poor indicator of low-level binding. Peptide-specific CTL detected weak peptide binding but gave no indication of subtle changes in peptide binding unless careful titrations of limiting amounts of peptide were performed. Thus the two assays complement each other by their differing ranges of sensitivity. In contrast, alloreactive CTL were generally insensitive to the point mutations we derived, with the marked exception of the 45M mutant. These findings may be explained in terms of the different reactivities of peptide-specific and alloreactive CTL. Epitopes on both the presenting class I molecule and endogenous peptide(s) contribute to the alloantigen, and recognition by peptide-specific CTL depends on bound peptide.

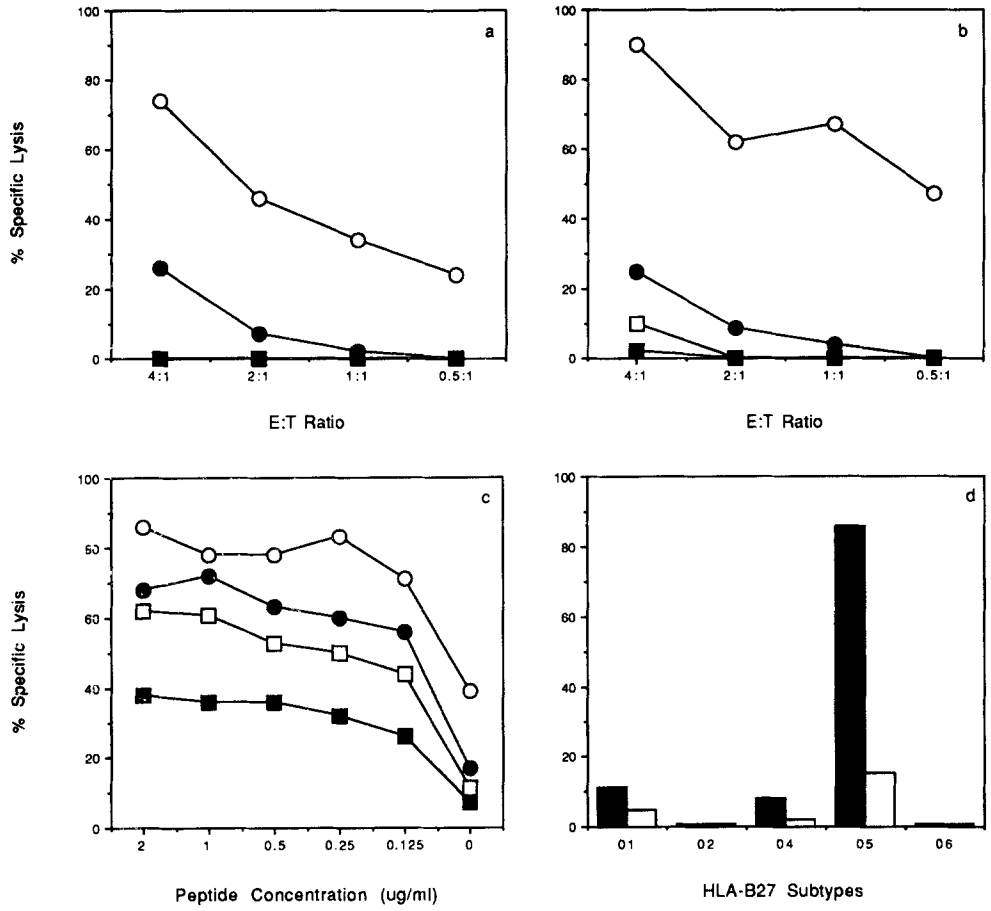

Figure 3. CTL lysis of HLA-B*2705 cells is peptide and allele specific. (*a* and *b*) Lysis of either C1R (*squares*) or B27C1R (*circles*) cells in the presence (*open symbols*) or absence (*solid symbols*) of 1 μg/ml NP1 peptide by the CTL lines (*a*) DB or (*b*) RB at various E/T ratios. (*c*) Lysis of B27C1R cells by RBCTL in the presence of various concentrations of NP1 peptide at E/T ratios of 10:1 (*open circles*), 5:1 (*solid circles*), 2:1 (*open squares*), and 1:1 (*solid squares*). (*d*) Lysis of naturally occurring HLA-B27 subtypes by RBCTL in the presence of 1 μg/ml NP1 peptide (*solid columns*) and in the absence of peptide (*open columns*) at an E/T of 5:1. Similar results were obtained at E/T ratios of 10:1, 2:1, and 1:1. Full tissue types of HLA-B27 subtype lines are given in Materials and Methods.

Table 4. *Summary of Functional Analyses of Native and Mutant HLA-B*2705-transfected C1R Cells*

Name	Expression	NP1 binding	RBCTL		DBCTL		Allo-CTL clones
			+NP1	−NP1	+NP1	−NP1	
C1R	−	−	−	−	−	−	0/5
B27C1R	+ + +	+ + +	+ + +	−	+ + +	−	5/5
9Y	+ + +	±	+ +	−	+ +	−	4/5
45M	+ + +	−	+ + +	+ +	+ +	−	1/5
45K	+	ND	ND	ND	ND	ND	ND
67Y	−	ND	ND	ND	ND	ND	ND
67A	+ + +	−	−	−	−	−	5/5
67S	+ + +	−	−	−	−	−	5/5
70Q	+ + +	+ + +	+ +	−	+ +	−	5/5
71T	+ + +	+	+	−	−	−	5/5
77S	+ + +	+ + +	+ +	−	+ + +	−	4/5
97R	+ + +	−	−	−	−	−	5/5
227K	+ + +	+ + +	−	−	−	−	4/5

The table summarizes the data shown in Table 3 and Figs. 2, 4, and 6. (+ + +) A result >70% of the wild-type, (+ +) 30–70%, and (+) <30% of the result with wild-type B27C1R cells. The last column shows the number (out of five) of alloreactive CTL clones that lysed the given HLA-B*2705 mutant at levels >10% of the lysis of wild-type B27C1R cells.

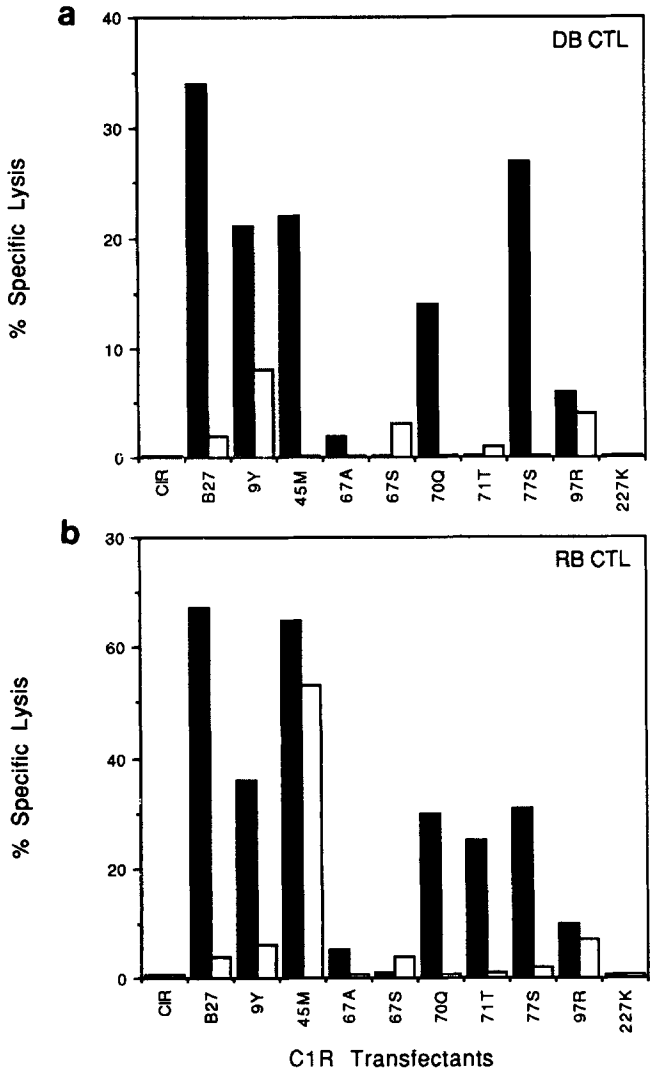

Figure 4. NP1-specific CTL lysis of HLA-B*2705 wild-type or mutant HLA-B*2705 transfected C1R cells by the DB (*a*) or RB (*b*) CTL lines at an E/T of 2:1 in the presence (*solid columns*) or absence (*open columns*) of 1 μg/ml NP1 peptide. Similar results were found at E/T ratios of 4:1, 1:1, and 0.5:1.

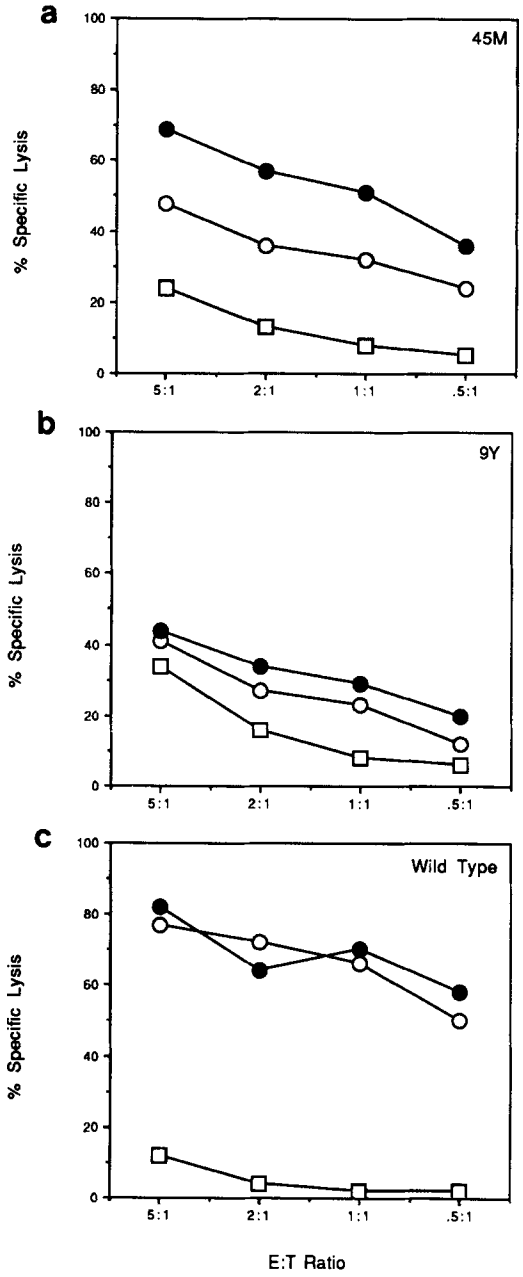

Figure 5. 45M and 67Y mutants cells require the presence of NP1 peptide for optimal lysis. 45M (*a*), 9Y (*b*), and control B27-C1R (*c*) cells were incubated for 14 h in the presence of 1 μg/ml NP1 and then washed three times. These target cells were then cultured with RB CTL at various E/T ratios in the presence (*solid circles*) or absence (*open circles*) of 1 μg/ml NP1 for the standard 4-h CTL assay. Negative control cells (*open squares*) were cultured without peptide during both the preincubation and the CTL assay.

Alloreactive CTL may therefore be less sensitive than peptide-specific CTL to subtle changes in the conformation of the bound peptide induced by point mutations within the groove. Indeed, Calvo et al. (35) reported similar results from their analysis of a different set of HLA-B*2705 point mutants with 20 alloreactive CTL clones. They found that only one mutant, in their case, that at position 152, affected most of the clones. These results suggest that certain residues within the peptide binding groove, such as those at position 45 and 152, play a dominant role in determining allorecognition.

Although some point mutations (45K and 67Y) appear to alter the class I molecule so much as to prevent expression altogether, the NP1-specific CTL data suggests that most point mutations compatible with cell surface expression alter recognition by directly affecting bound peptide. Previous results indicated that the mechanism of the binding assay involves the interaction of the radiolabeled peptide with an apparently large number of "empty" HLA molecules on the cell surface. The CTL data here complements these results and suggests that most point mutations do not alter the stability of the empty molecules, or the proportion of molecules that arrive at the cell surface empty.

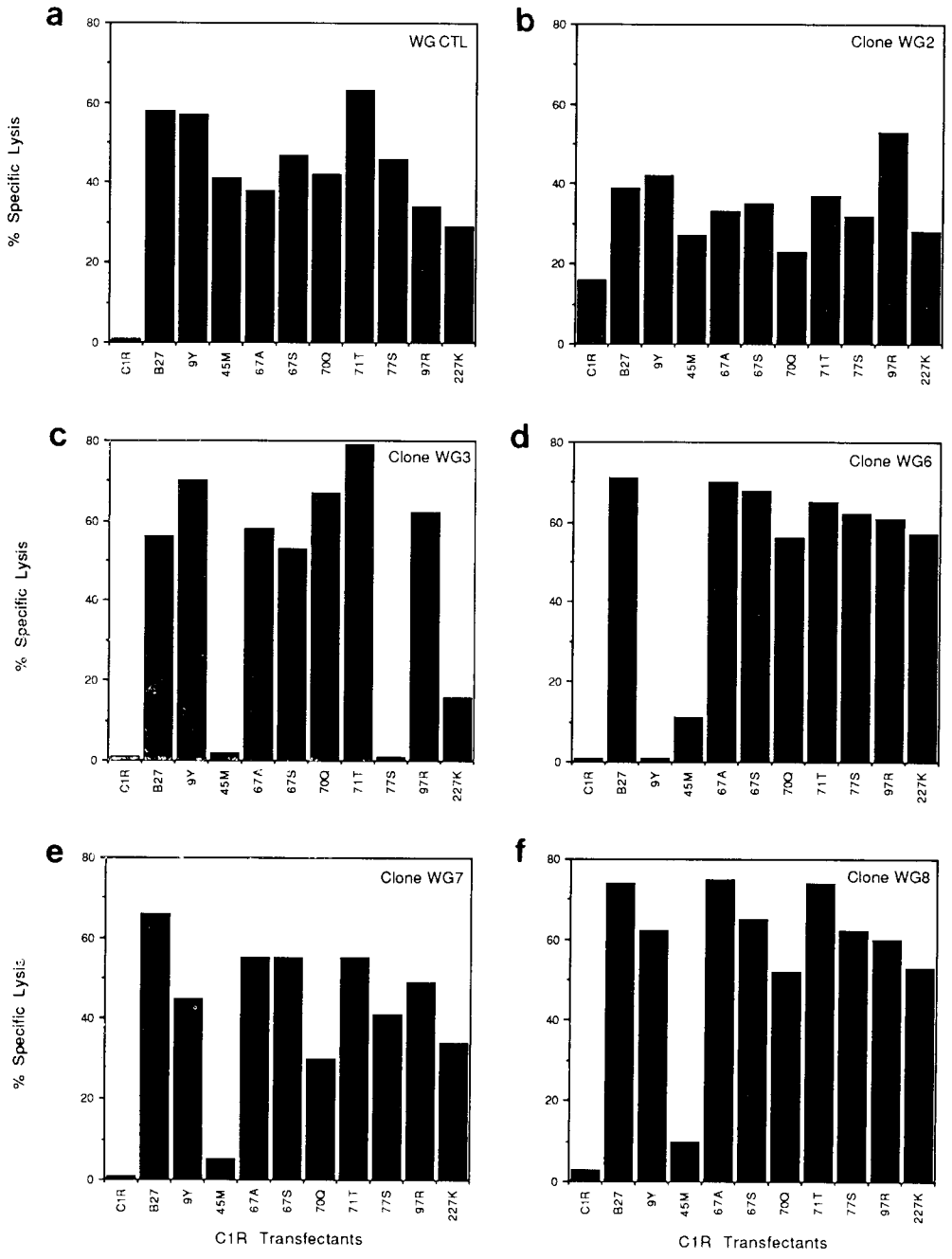

Figure 6. Alloreactive CTL lysis of HLA-B*2705 wild-type and mutant-transfected C1R cells by the WG CTL line (*a*) and 5 WG clones, WG2 (*b*), WG3 (*c*), WG6 (*d*), WG7 (*e*), and WG8 (*f*) at an E/T of 5:1. Similar results were obtained at E/T ratios of 10:1, 2:1, and 1:1.

In conclusion, we have shown that B pocket is highly conserved among the HLA-B27 subtypes, and that it plays an important role in the expression of HLA-B*2705 and in the presentation of both endogenous and exogenous peptides. In light of these findings, we predict that the B pocket anchors an antigenic peptide amino acid side chain that will be found in a similar position in most endogenously-processed peptides bound by HLA-B*2705. Furthermore, the conservation of the B pocket among HLA-B27 subtypes suggests that peptides bound to all of these will carry the same motif. Wiley and colleagues have recently solved the HLA-B27 x-ray crystallographic structure, emphasizing the importance of the B pocket (35a). Furthermore, they have sequenced 11 naturally processed peptides isolated from HLA-B27, and found that all of these have an arginine residue at position 2 (35b). Together, these observations have implications for the association of HLA-B27 with Ankylosing Spondylitis, a disease which is thought to be mediated by CTL that recognize a self-peptide presented by HLA-B27 (36). One argument against this "arthritogenic peptide" hypothesis is the finding that multiple subtypes (HLA-B*2702; 04, 05, and 06) are disease associated, and differ greatly in peptide binding, alloreactive-CTL and peptide-specific CTL recognition (22, 25, 37). Our findings suggest that these subtypes all bind peptides with at least one common anchor motif, rendering it more likely that a single arthritogenic peptide may be presented to CTL by multiple HLA-B27 subtypes, thereby explaining the shared disease association.

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