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Immunology

Allele-specific B pocket transplant in class I major histocompatibility complex protein changes requirement for anchor residue at P2 of peptide

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ABSTRACT **To investigate the role of an anchoring pocket in allele-specific peptide presentation by a major histocompatibility complex class I molecule, we "transplanted" a B pocket from HLA-A*0201 into HLA-B*2705 by site-directed mutagenesis. The resulting protein, designated B27.A2B, binds a different set of endogenous peptides than B*2705 as evidenced by complete loss of allorecognition as well as restored expression in the antigen processing-defective mutant cell line T2. B27.A2B also fails to present an HLA-B27-restricted influenza virus peptide [nucleoprotein (383–391)] to cytotoxic T lymphocytes (CTLs). However, substitution of leucine, the predominant P2 anchor residue in A*0201-restricted peptides, for arginine, the P2 anchor in nucleoprotein-(383–391) and other B*2705-restricted peptides, restores recognition of B27.A2B by the same B*2705-restricted peptide-specific CTLs. These results demonstrate that a dominant polymorphic pocket in a class I molecule, through interaction with the anchor residue of an antigenic peptide, can distinguish among peptides differing by only a single amino acid and thus determine the allelic specificity of peptide presentation.**

Class I major histocompatibility complex molecules bind and present peptide antigens to T-cell receptors (TCRs) on cytotoxic T lymphocytes (CTLs). For the immune system to discriminate between self and nonself, and respond to a wide variety of invading pathogens, class I proteins must bind peptides of diverse amino acid sequences and yet maintain allelic specificity. With the combination of structural and functional studies, many of the principles that govern this process are beginning to emerge.

The class I antigen binding groove consists of six pockets (A–F) that vary in shape, depth, and chemical composition and appear to accommodate different structural features of bound peptides (1–10). Pockets A and F, located at either end of the groove, are composed mainly of conserved residues that form hydrogen bonds with the N and C termini of peptide, respectively (6–10). These hydrogen bonds in addition to interactions between other conserved class I residues and the peptide backbone, as opposed to interactions with peptide side chains, are the main determinants of peptide binding affinity (6–10).

In contrast to pockets A and F, pockets B–E contain many polymorphic residues and appear to influence what peptides will bind to a class I molecule. For example, HLA-B*2705, which contains a deep B pocket with a negatively charged glutamic acid at position 45, and other polar residues at positions 9 (histidine) and 24 (threonine), binds peptides with arginine at position 2 (Arg^{P2}) (11), while HLA-A*0201 has hydrophobic residues in these positions (Met^{45}, Phe^{9}, Ala^{24}) and binds peptides with predominantly Leu^{P2} (with some Met^{P2} and Ile^{P2}) (12). It has been hypothesized that these allele-specific peptide residues or anchors interact specifically with dominant pockets in the class I molecule and that shallow pockets would have less-stringent requirements and accommodate many different side chains (13). It should be noted that the B pocket is not always dominant and in some alleles may be quite small (e.g., H-$2K^b$) (6, 8) or even obstructed by large bulky residues (e.g., HLA-B*0801) (14) so that it will not accommodate a peptide side chain. In these instances, other pockets appear to be dominant and contain anchor residues (6, 8).

The B pocket has also been implicated as an important structural component in the association between HLA-B27 and susceptibility to spondyloarthropathies, since it is conserved in all disease-associated B27 subtypes (reviewed in ref. 15). While the mechanism(s) responsible for this association remains unclear, it has been hypothesized that "arthritogenic" peptides displaying the Arg^{P2} motif like other B27-associated peptides may be important in disease pathogenesis (16). A better understanding of the role the B pocket plays in peptide selection may help to define disease-associated peptides and aid in designing strategies to block their interaction with B27. To investigate the role of the B pocket in determining allele-specific peptide presentation, we have transplanted a B pocket from HLA-A*0201 into HLA-B*2705.

MATERIALS AND METHODS

HLA-B*2705 Mutant Genes, Cell Transfections, and Flow Cytometry. Four to six amino acids in the B pocket of HLA-B*2705 were changed to the corresponding HLA-A*0201 residues by site-directed mutagenesis (Altered Sites; Promega) and confirmed by DNA sequencing. A list of these mutants and the amino acids that compose their B pockets compared with HLA-B*2705 and -A*0201 is shown in Table 1. Mutant B*2705 genes and pSV2neo were linearized with *Hin*dIII and *Bam*HI, respectively, and cotransfected into the HLA-A negative and -B35 low (17) cell line HMy2.C1R (C1R) (18) or the antigen processing-defective line 0.174 × CEM^R (T2) (19) by electroporation (Gene Pulser; Bio-Rad). Positive transfectants were selected with Geneticin (G418) at 1.8 mg/ml (1.2 mg/ml for T2 cells) and then maintained at 0.4 mg/ml. Cell-surface expression of the products of transfected or endogenous class I genes was determined by indirect immunofluorescence with ME.1 (anti-HLA-B27, -B7, -B42) (20) or MA2.1 (anti-HLA-A2) (21) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG followed by analytical flow cytometry (Epics 7-52). Cells expressing mutant B27 molecules at levels equivalent to B*2705 (in C1R)

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Abbreviations: CTL, cytotoxic T lymphocyte; TCR, T-cell receptor; NP, nucleoprotein; FITC, fluorescein isothiocyanate.

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Table 1. HLA-B*2705 B pocket mutants

Name	Amino acid residue					
	9	24	45	66	67	70
B*2705	H	T	E	I	C	K
B27.A24	H	A	E	K	V	H
B27.A25	H	A	M	K	V	H
B27.A2B	F	A	M	K	V	H
A*0201	F	A	M	K	V	H

Bottom and inner walls of the B pocket are formed by His^{9}, Thr^{24}, Val^{34}, Glu^{45}, and Cys^{67}, while Tyr^{7}, Glu^{63}, Lys^{70}, and Tyr^{99} surround the rim (4). Using site-directed mutagenesis, changes were made at several codons in the B*2705 gene to encode HLA-A*0201 residues at four, five, or six of the B pocket positions that differ between these two molecules. The B27.A2B gene encodes a B*2705 molecule whose B pocket is identical to that of HLA-A*0201.

were isolated by fluorescence-activated cell sorting and used for these studies.

The raw data for the histograms shown in Fig. 2 are obtained as cell number (y axis) in each of 256 channels that span 3 orders of magnitude of fluorescence intensity (arbitrary units; x axis). To reflect the logarithmic nature of this scale, where small peak shifts represent large changes in surface expression, these channels are expressed as 3 log units (85 channels per unit).

Peptides. The following peptides were used: nucleoprotein (NP)-(383–391), SRYWAIRTR derived from the influenza A X/31 NP; NP-(383–391;L384), SLYWAIRTR; NP-(383–391;K384), SKYWAIRTR; NP-(383–391;K389), SRYWAIKTR; NP-(383–391;K391), SRYWAIRTK. Peptides were synthesized on a Biosearch 9500 automated synthesizer by *tert*-butoxycarbonyl chemistry, or with a multipeptide synthesis block using 9-fluorenylmethoxycarbonyl chemistry, and HPLC purified prior to use.

CTL Lines and Clones. Alloreactive CTL lines specific for HLA-B27 were grown from peripheral blood mononuclear cells obtained from a healthy donor (DQ; HLA-A1, -A33, -B8, -Bw6, -Cw6) and stimulated weekly with mitomycin C-treated C1R expressing B*2705. Autologous HLA-B*2705-restricted CTL lines HF (HLA-A2, -A3, -B7, -B*2705) and SD (HLA-A2, -A32, -B*2705, -B49, Bw4) were grown from peripheral blood mononuclear cells that had been preincubated for 1 h with influenza A X/31 virus in the absence of serum and then for 7 d in RPMI 1640 medium with 10% fetal calf serum (22, 23). Cells from these bulk cultures were mixed with autologous B-lymphoblastoid cells preincubated for 1 h with influenza A NP-(383–391) at 50 μg/ml and irradiated with 3000 rad (1 rad = 0.01 Gy) and grown in medium containing recombinant interleukin 2 (10 units/ml) (Cetus). CTL lines were maintained in culture by restimulation every 10–14 d with autologous irradiated B-cell lines, which had been pulsed with peptide for 1 h. These lines were shown to be specific for the stimulating peptide presented by HLA-B27 (unpublished data). CTL clones specific for the NP-(383–391) peptide on HLA-B*2705 were obtained by limiting dilution (0.3 cell per well) of SD CTLs.

Cytotoxicity Assay. Alloreactive and influenza-specific CTL lines and clones were tested for recognition of mutant B*2705 molecules in a standard 4-h ^{51}Cr-release cytotoxicity assay (24). Specific lysis was quantitated as follows:

$$\% \text{ specific lysis} = [(cpm_{sample} - cpm_{spont})/(cpm_{max} - cpm_{spont})] \times 100.$$

Spontaneous release (cpm_{spont}) is determined from targets incubated with medium alone and maximal release (cpm_{max}) is determined from targets incubated with 5% Triton X-100. Spontaneous and maximal release for each condition are performed in quadruplicate, while experimental points represent triplicate or duplicate determinations. Positive controls for all CTL assays are represented by C1R transfected with wild-type B*2705 (and pSV2neo), and negative controls are C1R transfected with pSV2neo alone. All CTL assays have been performed at least twice with the same results.

RESULTS

Alloreactive CTL Recognition of B*2705 B Pocket Mutants. Using the HLA-B*2705 gene as a starting point, we constructed three mutants encoding B*2705 with four (B27.A24), five (B27.A25), or six (B27.A2B) substitutions to HLA-A*0201 residues in the B pocket. In B27.A2B, the B pocket is identical to HLA-A*0201, while the two other molecules contain B27 residues at positions 9 (histidine) and 9 (histidine) and 45 (glutamic acid) (Table 1). When transfected into C1R cells, these mutants are expressed on the cell surface at levels comparable to B*2705 as judged by cell-surface staining with the monoclonal antibody ME.1 and flow cytometry (unpublished data). However, these mutants are not recognized by B*2705-specific alloreactive CTLs (Fig. 1). A second alloreactive CTL line from a different individual yields the same results (unpublished data). Since B pocket residues are not accessible to the TCR (4, 7) and thus should not directly affect CTL recognition, it appears likely that the self peptides presented by these mutants are different.

Expression of B27.A2B in T2 Cells. Further evidence for altered peptide binding specificity of the B27 molecule containing an A2-like B pocket was demonstrated by transfecting B27.A2B into the antigen processing-defective cell line T2. These cells synthesize class I heavy chains (HLA-B5 and -A2) and β_2-microglobulin but, because of a large deletion in the class II region of chromosome 6, do not transport cytoplasmic peptides into the endoplasmic reticulum and therefore do not express stable HLA-B5 on the surface (19, 25–27). HLA-A2 is expressed, but only at 20–30% of control levels (19), which appears to be due to the binding of peptides derived from signal sequences (28, 29). T2 cells transfected with HLA-B*2705 express this molecule at only 10–20% of control levels (compared with B*2705 in C1R) but display a 3- to 4-fold increase in surface expression when incubated with the B27-restricted peptide NP-(383–391) (Fig. 2*C*). T2 cells transfected with B27.A2B express this molecule at levels 3- to 4-fold greater than B*2705 in T2 (Fig. 2*F*) and at 70–80% the level of B*2705 in C1R (data not shown). While it is possible that the T2 B27.A2B cells could be synthesizing

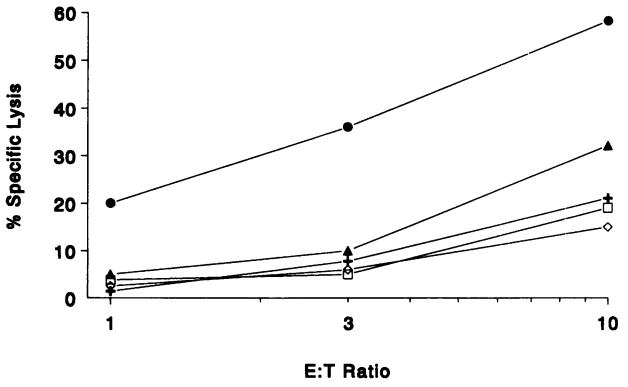

FIG. 1. Lack of recognition of B*2705 B pocket mutants by alloreactive CTLs. A DQ CTL line that recognizes B*2705 was tested for its ability to recognize and lyse C1R cells transfected with the B pocket mutant B27.A24 (◇), B27.A25 (+), or B27.A2B (□) in a standard 4-h ^{51}Cr-release assay. Positive and negative controls are represented by C1R-B*2705 (●) and C1R-Neo (▲), respectively. E:T, effector (CTL)/target cell ratio.

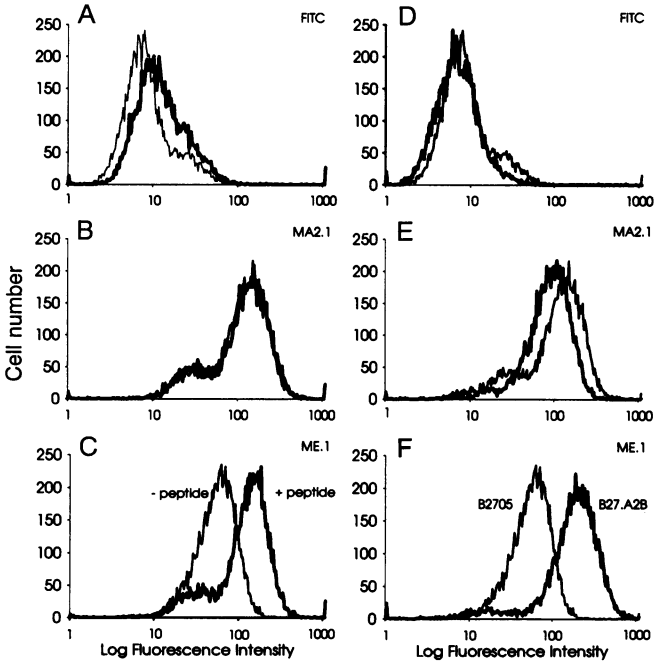

FIG. 2. Increased cell-surface expression of B*2705 in T2 B*2705 cells after incubation with NP-(383–391) peptide or after B pocket transplant. (*A–C*) Expression of HLA-A*0201 (*B*; MA2.1) and -B*2705 (*C*; ME.1) in T2 B*2705 cells incubated with (thick lines; + peptide) or without (− peptide) NP-(383–391) peptide (100 μM for 12 h) is shown. (*D–F*) Cell-surface expression of HLA-A*0201 (*E*; MA2.1) and -B*2705 or B27.A2B (*F*; ME.1) in T2-B*2705 and T2 B27.A2B (thick lines) cells. FITC staining alone (without first antibody) is shown in *A* and *D*. Histograms show cell number in each of 256 channels spanning 3 logs of fluorescence intensity and are overlaid for ease of comparison.

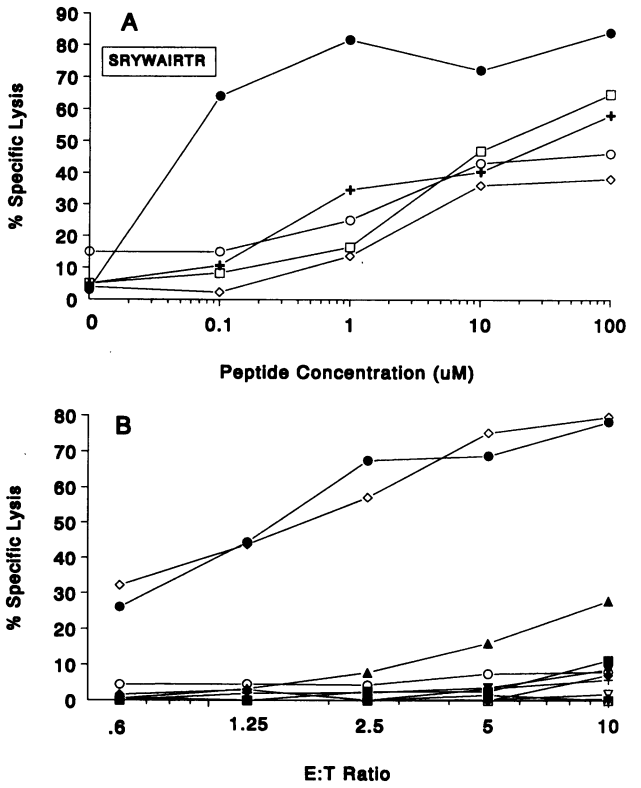

FIG. 3. Lack of recognition by influenza A NP-(383–391)-specific CTLs of B pocket mutants incubated with NP-(383–391) peptide or infected with influenza A X/31 virus. (*A*) Target cells were incubated with effectors for 4 h at an effector/target cell ratio of 5:1 at the indicated peptide concentrations. ●, B*2705; ○, Neo; □, B27.A2B; ◇, B27.A24; +, B27.A25. (*B*) Target cells were labeled with $Na^{51}Cr$ for 1 h, washed, divided into three aliquots, and then further incubated for 1 h with influenza A X/31 virus at a final hemagglutinating titer of 1:500 in the absence of serum (flu), medium alone (− peptide), or NP-(383–391) at a final concentration of 10 μM (+ peptide). At the end of 1 h, all targets were diluted 1:75 and flu-infected targets were then incubated for another 3 h. A CTL assay was then performed, with targets and effectors plated at the indicated effector/target cell (E:T) ratios, and percentage specific lysis was determined after the standard 4-h incubation. ○, B2705 − peptide; ●, B2705 + peptide; ◇, B2705 + flu; △, B27.A2B − peptide; ▲, B27.A2B + peptide; +, B27.A2B + flu; ▽, B27.A25 − peptide; ▼, B27.A25 + peptide; +, B27.A25 + flu; □, B27.A24 − peptide; ■, B27.A24 + peptide; ◆, B27.A24 + flu.

more heavy chain than T2 B*2705, this would be unlikely to account for the increased surface expression of B27.A2B, since peptide supply is still the limiting factor (see Fig. 2*C*; see also refs. 26 and 27). Thus, expression of B27.A2B in T2 is comparable to HLA-A*0201 or -B*2705 stabilized with exogenous peptide, suggesting that this B27 molecule with an A2-like B pocket may be binding signal sequence-derived peptides similar to those bound to A*0201 or perhaps other as yet unidentified sequences. The small decrease in expression of A*0201 seen in T2 cells transfected with B27.A2B (Fig. 2*E*) is consistent with the idea that B27.A2B is competing for a limiting number of signal sequence peptides that would otherwise bind to A*0201. It is worth noting that T2 transfected with B27.A24 showed no surface expression of this molecule (unpublished data), suggesting that an A2-like B pocket with B27 residues at positions 9 (histidine) and 45 (glutamic acid) confers different peptide binding specificity than either an A2 or B27-like B pocket. Our attempts at transfecting B27.A25 into T2 have so far been unsuccessful.

Recognition of B*2705 B Pocket Mutants by NP-(383–391) Peptide-Specific CTLs. To examine the presentation of a single peptide by these B pocket mutants, we chose an HLA-B27-restricted influenza A NP epitope, NP-(383–391) (22, 23, 30). All three mutants fail to present this peptide to a B27-restricted peptide-specific CTL line (HF). The same result is seen with virus infection or incubation of target cells with the optimal nonamer peptide NP-(383–391) (SRYWAIRTR) (Fig. 3), which contains an Arg^{P2} residue similar to other B27-restricted peptides (11, 22). If the B pocket is binding the side chain of P2, then transforming the B pocket of B27 into an A2-like structure should change the specificity of this interaction and favor binding of a peptide with Leu^{P2} and not Arg^{P2}. To test this directly, we synthesized the leucine-containing NP-(383–391) peptide SLYWAIRTR. The B27.A2B mutant presents this peptide to the same HF CTLs that recognize NP-(383–391) on B*2705, while presentation by B*2705 and B27.A25 is 100-fold less (Fig. 4). B27.A24 is not lysed any better than the C1R-Neo negative control. The small increase in lysis observed for B*2705 and B27.A25, compared to Neo and B27.A24, is unlikely to be physiologically relevant since the peptide concentrations required for this effect are in the 10–100 μM range (i.e., 100- to 1000-fold greater than for the B27.A2B molecule). A similar result showing pocket specificity for the P2 side chain is obtained with a CTL clone derived from a different individual (SD) (Fig. 5).

Specificity of the B Pocket for Arginine. To investigate the specificity of the B*2705 B pocket for arginine (7), lysine, which is also positively charged and approximately the same length as arginine, was substituted for arginine at P2 in the NP-(383–391) peptide (SKYWAIRTR). This conservative substitution nearly completely abolishes peptide presentation, while an arginine to lysine substitution at P9, an area that binds in the F pocket (31), has no deleterious effect (Fig. 6). An arginine to lysine substitution at P7, another peptide

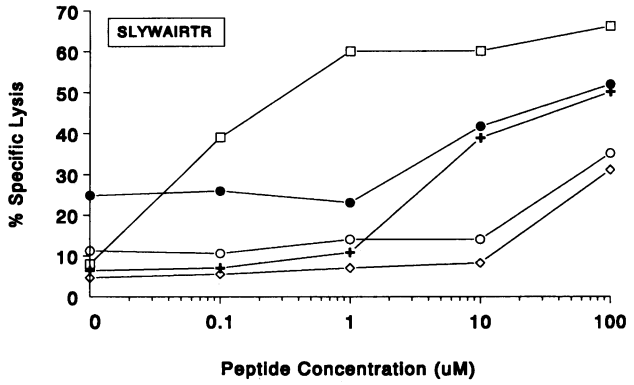

FIG. 4. Restored recognition by influenza A NP-(383–391)-specific CTLs of the B27.A2B mutant by substituting a leucine at P2 in the NP-(383–391) peptide. Experiment was performed exactly as described in Fig. 3*A*, except Leu^{P2}-substituted NP-(383–391) (SLYWAIRTR) was used. ●, B*2705; ○, Neo; □, B27.A2B; ◇, B27.A24; +, B27.A25.

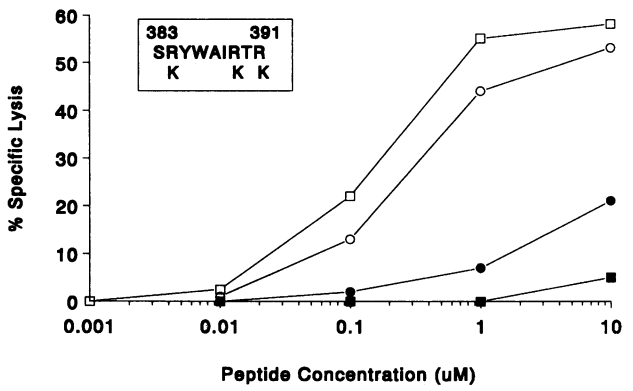

FIG. 6. Specificity of the B pocket in HLA-B27 for arginine at P2 of the influenza A NP-(383–391) peptide. CTL assay was performed in the presence of NP-(383–391) (SRYWAIRTR) (○) or with the following lysine-substituted peptides: K384, SKYWAIRTR (■); K389, SRYWAIKTR (●); K391, SRYWAIRTK (□) at an effector/target cell ratio of 5:1.

side chain that interacts with the B27 molecule (5), also significantly reduces presentation. In addition, none of the mutant molecules (B27.A2B, B27.A25, or B27.A24) is recognized with the Lys^{P2} peptide (unpublished data), consistent with the observed specificity of an A2-like B pocket for hydrophobic side chains (leucine, methionine, or isoleucine) (12).

DISCUSSION

Previous studies have shown that single B pocket mutations in HLA-B*2705 and -A*0201 alter their phenotypes (30, 32–34). Single substitutions at positions 9, 24, 45, and 67 can affect both self peptide and viral peptide presentation, but the effect depends on the peptide being presented (34), its mode of presentation (intra- vs. extracellular) (M. Matsui and J.A.F., unpublished work), and/or the particular CTL line or clone used. It is clear from these functional studies that individual residues in the B pocket can influence binding of some (but probably not all) self peptides and viral epitopes and therefore affect recognition by their cognate peptide-specific CTLs. We have addressed the function of the B pocket as an entire unit and show that by "transplanting" a B pocket from HLA-A*0201 into HLA-B*2705 we have changed the specificity of the class I molecule for peptides in an allele-specific fashion. Peptides with Arg^{P2} are no longer recognized on B*2705 with an A2-like B pocket, while the single substitution of leucine for arginine at P2 restores recognition. These results are consistent with the Arg^{P2} (but not Leu^{P2}) side chain binding in a B27-like B pocket and, conversely, the Leu^{P2} (but not Arg^{P2}) side chain binding in an A2-like B pocket. However, it should be noted that mutation of class I residues could abrogate CTL recognition independent of peptide binding. This is unlikely to be occurring here for two reasons; first, the mutated residues are in the B pocket and are not directly accessible to the TCR (4, 7); second, restored recognition with the Leu^{P2} peptide indicates that essential TCR class I contact residues remain intact. In addition, using the T2 assembly assay (35), we find that the Leu^{P2} NP-(383–391) peptide will not promote assembly of B27 molecules, even at concentrations up to 100 μM (unpublished data). This same result for B*2705 has recently been reported (36). As yet we have not been able to demonstrate assembly of B27.A2B with the Leu^{P2} peptide. This appears to result from an unusual stability of B27.A2B compared with HLA-A2, -B5, and -B27 in T2 cells (unpublished data), presumably because of the binding of peptides present in the endoplasmic reticulum (28, 29).

We have demonstrated a striking specificity of the B27 B pocket for arginine by showing that the conservative substitution of lysine at P2 of the NP-(383–391) peptide abolishes CTL recognition. This specificity, as suggested by Madden *et al.* (7), may result from the planar configuration of hydrogen bonds between the P2 guanidinium group and the side chains of His^9, Thr^{24}, and Glu^{45}. Thus lysine, which has a tetrahedral amino group at P2, may not be able to form stable hydrogen bonds. The lack of presentation of the Arg^{P2} peptide by the mutants with B27 residues at His^9 (B27.A25) and His^9 and Glu^{45} (B27.A24) (Fig. 3*A*) demonstrates that while hydrogen bonding between these residues and Arg^{P2} may be an impor-

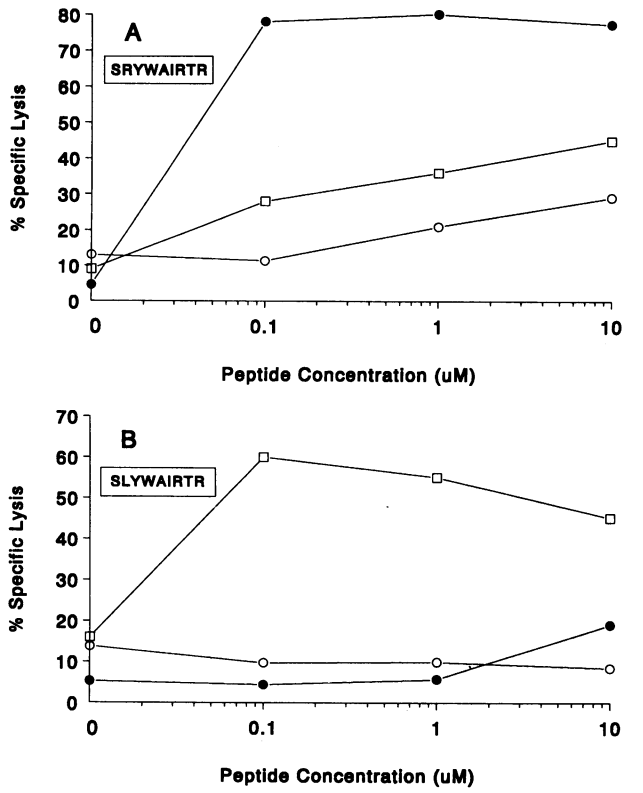

FIG. 5. Recognition of the NP-(383–391) (Arg^{P2})–B*2705 complex by the same clone that recognizes the NP-(383–391) Leu^{P2}–B27.A2B complex. CTL assay was done at various concentrations of SRYWAIRTR (*A*) or SLYWAIRTR (*B*). Clone was obtained by limiting dilution of bulk SD CTLs, and assay was performed using an effector/target cell ratio of 1:1. ●, B*2705; ○, Neo; □, B27.A2B.

tant contributor to the free energy of peptide binding, it is unlikely to be the only factor. Our studies also show that in an A2-like B pocket, a single change from phenylalanine to histidine at position 9 (B27.A25) is sufficient to greatly reduce recognition with the Leu^{P2} peptide. This finding is consistent with the results of Matsui *et al.* (ref. 32; M. Matsui and J.A.F., unpublished work), where other mutations at position 9 (phenylalanine to leucine or phenylalanine to tyrosine) in HLA-A*0201 affect recognition by many alloreactive A2-specific clones, as well as recognition of FMP-(58–66) by flu-specific CTLs. In addition, Rötzschke *et al.* (37) have found that the HLA-A*0205 subtype of A2, which contains tyrosine at position 9 (as well as three other non-B pocket substitutions), loses the dominant leucine anchor residue at P2 of eluted peptides, allowing polar residues such as glutamine to bind. While it may be that other amino acids with small side chains at P2, such as alanine or glycine, would be tolerated in either a B27- or A2-like B pocket, their absence from the pool of the most abundant self peptides (11, 12) suggests that they do not compete effectively for binding with endogenous peptides that contain arginine (for B27) or leucine (for A2) at this position.

By transplanting a B pocket from one class I molecule into another, we have demonstrated how class I molecules, through the interaction between a dominant pocket and a peptide anchor residue, can discriminate between nonamer peptides differing by only a single amino acid. These results, together with recent crystallographic analyses (6–10), provide direct evidence that while high-affinity peptide binding may be a function primarily of conserved class I residues, dominant pockets within the class I molecule can determine the allelic specificity of peptides that are presented to the TCR.

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