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HLA-B37 and HLA-A2.1 molecules bind largely nonoverlapping sets of peptides

(class I major histocompatibility molecules/antigen presentation/cytotoxic T cells)

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ABSTRACT **T-cell recognition of peptides that are bound and presented by class I major histocompatibility complex molecules is highly specific. At present it is unclear what role class I peptide binding plays relative to T-cell receptor specificity in determination of immune recognition. A previous study from our group demonstrated that the HLA-A2.1 molecule could bind to 25% of the members of a panel of unrelated synthetic peptides as assessed by a functional peptide competition assay. To determine the peptide-binding specificity of another HLA class I molecule, we have examined the capacity of this panel of peptides to compete for the presentation of influenza virus nucleoprotein peptide NP-(335–350) by HLA-B37 to NP-peptide-specific HLA-B37-restricted cytotoxic T-lymphocyte lines. Forty-two percent of peptides tested were capable of inhibiting NP-(335–350) presentation by HLA-B37. Remarkably, none of these HLA-B37-binding peptides belong to the subset that was previously shown to bind to the HLA-A2.1 molecule. Only the NP-(335–350) peptide was capable of binding to both HLA-A2.1 and HLA-B37. These findings demonstrate that the peptide-binding specificities of HLA-B37 and HLA-A2.1 are largely nonoverlapping and suggest that, from the universe of peptides, individual HLA class I molecules can bind to clearly distinct subsets of these peptides.**

The primary immunological function of HLA class I molecules appears to be the binding and presentation of peptides to T-cell receptors. Soluble class I molecules, like class II major histocompatibility complex (MHC) molecules (1–5), have been shown to be capable of directly binding synthetic peptides (6–8). The putative peptide binding site of class I molecules is bounded by two α-helices (one from the $\alpha 1$ and one from the $\alpha 2$ domain) with a β-pleated sheet floor (9, 10). The proper folding and assembly of class I heavy chains with β_2-microglobulin may actually require the presence of a bound peptide in this site (11).

Although it seems reasonable to assume that class I molecules must be able to bind a wide array of peptides to cover the universe of antigens which they must present to T-cell receptors, there is currently very little information on the size of the peptide repertoire that any particular class I molecule can bind, and the number of HLA alleles to which any given peptide can bind. In a previous study (12), we assessed the specificity of peptide binding by the HLA-A2.1 molecule by analyzing the ability of a panel of synthetic peptides derived from the sequences of a diverse group of proteins to functionally compete for presentation of the influenza virus matrix protein (M1) peptide 55–73 to peptide-specific cytotoxic T lymphocyte (CTL) lines. The results demonstrated that 25% of these unrelated peptides could compete for M1-(55–73) presentation, indicating that the HLA-A2.1 molecule can bind a clearly limited, but broad spectrum of peptides.

To determine the extent to which these findings with the HLA-A2.1 molecule might be generalizable, we have performed a similar analysis on the specificity of peptide binding by the HLA-B37 molecule. McMichael *et al.* (13) have previously shown that the HLA-B37 molecule can present an influenza virus nucleoprotein (NP) peptide 335–350 to virus-specific CTL. We have generated HLA-B37-restricted CTL that are specific for this NP peptide and have analyzed most of the same unrelated peptides utilized in the HLA-A2.1 study in a functional competition assay for HLA-B37 presentation of the NP-(335–350) peptide. These experiments were designed to answer two questions: (*i*) Would the HLA-B37 molecule bind a percentage of unrelated peptides similar to that bound by the HLA-A2.1 molecule? (*ii*) Would the HLA-B37 and HLA-A2.1 molecules bind the same, overlapping, or completely different set of these peptides?

MATERIALS AND METHODS

Cells. Peripheral blood lymphocytes (PBL) were obtained by batch leukapheresis of normal adult volunteers (14). HLA serotyping of PBL was kindly performed by the Human Antigen Typing Laboratory, Department of Transfusion Medicine, National Institutes of Health. JY, 2148, B75, DB1, F11B, and F2B are human B-lymphoblastoid cells lines.

Synthetic Peptides. Synthetic peptides were synthesized as described previously (15) or by the RAMPS technique (DuPont Biotechnology) as described by the manufacturer. Peptides used in this report are shown in Table 1.

Generation and Assay of CTL. Generation and assay of HLA-B37-restricted influenza A/NT/60 NP-(335–350)-peptide-specific CTL lines were performed exactly as described for HLA-A2.1-restricted influenza M1-(55–73)-peptide-specific CTL lines (12, 18–21) except that Iscove's modified Dulbecco's medium (GIBCO) was used. PBL from HLA-B37$^+$ individuals were cultured *in vitro* with NP-(335–350) at 10 μg/ml (4 μM) for 8 days with recombinant interleukin 2 (rIL-2; Cellular Products) added at 5 units/ml on day 3. Primed responder cells were repeatedly restimulated by incubation with irradiated [2000 roentgens (500 mC/kg)] HLA-B37$^+$ stimulator PBL [that had been incubated with NP-(335–350) at 10 μg/ml for 3 hr at 37°C] plus rIL-2 added at 5 units/ml 24 hr later. The HLA-A2.1-restricted M1-(55–73)-peptide-specific CTL line 17B5.C2 was described previously (21). CTL activity was measured in a standard 4- to 5-hr ^{51}Cr-release assay as described previously (12), using B-cell targets that were either infected with influenza virus or incubated with synthetic peptides. Competitive peptide inhibition assays were performed as described (12) by incubat-

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Abbreviations: CTL, cytotoxic T lymphocytes; NP, nucleoprotein; M1, matrix protein; MHC, major histocompatibility complex; PBL, peripheral blood lymphocytes.

Table 1. Peptides utilized for CTL recognition

No.	Name*	Sequence	Known restriction element
1	FLU NP-(335–350)	SAAFEDLRVLSFIRGY	B37
2	PY CS 6-repeat	QGPGAPQGPGAPQGPGAPQGPGAP	
3	FLU NP-(50–63)	SDYEGRLIQNSLTI	K^k
4	TRY repeat	CDKKESGDSEDKKESGDSE	
5	PF HRP-II-(1–15)	AHHAHHAADAHHAAD	
6	RNAP-(1557–1577)	SPTSPSYSPTSPSYSPTSPSY	
7	FLU PB1-(537–548)	NDLGPATAQMAL	
8	FLU M1-(17–31)	SGPLKAEIAQRLEDV	DR1
9	PY CS 4-repeat	YPPQQPPQQPPQQPPQQ	
10	FLU B/Lee/40 MI-(57–68)	KALIGASICFLK	
11	FLU HA-(127–140)	NTTKGVTAACSHAG	IA^d
12	FLU HA-(518–528)	IYSTVASSLVL	K^d
13	RV VP4-(86–100)	CPTNQQVVLEGTNKTD	
14	FLU PB1-(246–257)	MQIRGFVYFVET	
15	FLU HA-(307–318)	PRYVRQNTLRLA	DR1
16	FLU NP-(365–380)	IASNENMDAMESSTLE	D^b
17	FLU HA-(111–120)	FERFEIFPKE	IE^d
18	SM 97.3-(347–364)	GDIGVMQADMDDAINAKQ	
19	DV NS4a-(102–119)	CMVLLIPEPEKQRTPQDNQ	
20	FLU PB2-(187–195)	ERELVRKTR	
21	FLU PB2-(303–313)	SFGGFTFKRTS	
22	HIV gp120 des-R	IQGPGAFVTIGK	
23	LCMV GP-(272–293)	LSDSSGVENPGGYCLTKWMILA	D^b
24	HAV VP4-(1–23)	MNMSKQGIFQTVGSGLDHILSLAC	
25	AT III-(281–298)	CMVLILPKPEKSLAKVEKE	
26	FLU PB1-(403–414)	LSPGMMMGMFNM	
27	FLU M1-(55–73)	LTKGILGFVFTLTVPSERG	A2

*Abbreviated peptide names refer to the following: FLU NP is influenza A/NT/60/68 nucleoprotein; PY CS is *Plasmodium yoelii* circumsporozoite protein; TRY repeat is the *Trypanosoma cruzi* 85-kDa trypomastigote surface antigen nonapeptide repeat unit; PF HRP-II is the histidine-rich protein II of *Plasmodium falciparum*; RNAP is yeast RNA polymerase; FLU PB1 is influenza A/NT/60/68 basic polymerase 1; FLU M1 is influenza type A matrix protein; FLU B/Lee/40 M1 is influenza type B matrix protein; FLU HA is influenza A/Puerto Rico/8/38 hemagglutinin; RV VP4 is rotavirus VP-4 protein; SM 97.3 is the *Schistosoma mansoni* 97-kDa protein; DV NS4a is Dengue type 4 virus nonstructural protein 4a; FLU PB2 is influenza virus A/NT/60/68 basic polymerase 2; HIV gp120 des-R is the human immunodeficiency virus type 1 glycoprotein 120 CTL epitope without the arginine residues; LCMV GP is lymphocytic choriomeningitis virus glycoprotein; HAV VP4 is hepatitis A virus VP-4; and AT III is human anti-thrombin III. For individual references for these peptides sequences, see ref. 12. The reference for peptide 3 is ref. 16 and that for peptide 10 is ref. 17.

ing target cells with one peptide (at 0.2 μM) in combination with one other competitor peptide (at 90 μM) at 37°C for 1 hr, washing two times, and assaying with the standard ^{51}Cr-release assay.

RESULTS

Specificity of NP-(335–350)-Peptide-Specific CTL. Generation of CTL lines from the PBL of HLA-B37$^+$ individuals by repeated stimulation with a synthetic peptide corresponding to residues 335–350 of NP of influenza virus A/NT/60 resulted in the generation of CTL that could specifically lyse HLA-B37-matched targets that were preincubated with the NP-(335–350) peptide (Fig. 1*A*), but not other peptides, as exemplified by PY CS 6-repeat. Likewise, A/NT/60 virus-infected cells were lysed but not influenza virus B/Ann Arbor-infected cells (Fig. 1*A*). The NP-(335–350)-peptide-specific CTL could lyse only peptide-exposed HLA-B37-matched targets and not targets matched for other HLA specificities (Fig. 1*B*). These results confirm the ability of HLA-B37 to present the NP-(335–350) peptide (13) and demonstrate that this peptide can induce CTL that can recognize A/NT/60 virus-infected target cells.

Competitive Inhibition of NP-(335–350) Presentation by HLA-B37 by a Panel of Unrelated Synthetic Peptides. The specificity of peptide binding by the HLA-B37 molecule was assessed by a functional assay of peptide competition at the target cell level. Exposure of HLA-B37$^+$ target cells to 0.2 μM antigenic peptide NP-(335–350) plus a known positive competitor peptide, PY CS 6-repeat (peptide 2 in Table 1), followed by washing of the targets and assay with NP-(335–350)-peptide-specific CTL, results in inhibition of lysis of these targets relative to targets incubated with 0.2 μM NP-(335–350) peptide alone (Fig. 1*C*). Increasing the concentration of the antigenic NP-(335–350) peptide results in quantitatively decreased inhibition of lysis caused by 90 μM competitor peptide (Fig. 1*C*), thus demonstrating that there is dose-dependent competition for binding of the NP-(335–350) peptide to the HLA-B37 molecule. All competition experiments were performed with 90 μM competitor peptides because this was the highest peptide concentration that reproducibly did not cause toxicity to target cells.

A panel of unrelated peptides (Table 1) was chosen at random from a collection of peptides that had been made for studies unrelated to the present investigation and that was previously used to study the specificity of peptide binding by HLA-A2.1 (12). Ten of these peptides had been previously shown to be recognized in association with MHC molecules (Table 1). A representative experiment that utilizes 8 of the 26 synthetic peptides in Table 1 for competition of presentation of NP-(335–350) by HLA-B37 is shown in Fig. 2. The results in Fig. 2*A* demonstrate that exposure of target cells to each of these 8 competitor peptides alone at 90 μM is incapable of sensitizing the HLA-B37$^+$ targets for significant lysis by peptide-specific CTL relative to the lysis obtained by sensitization of targets with 0.2 μM NP-(335–350). The results in Fig. 2*B* show that 90 μM peptides 2, 3, 4, 9, 10, and 11 produce greater than 40% percent inhibition of NP-

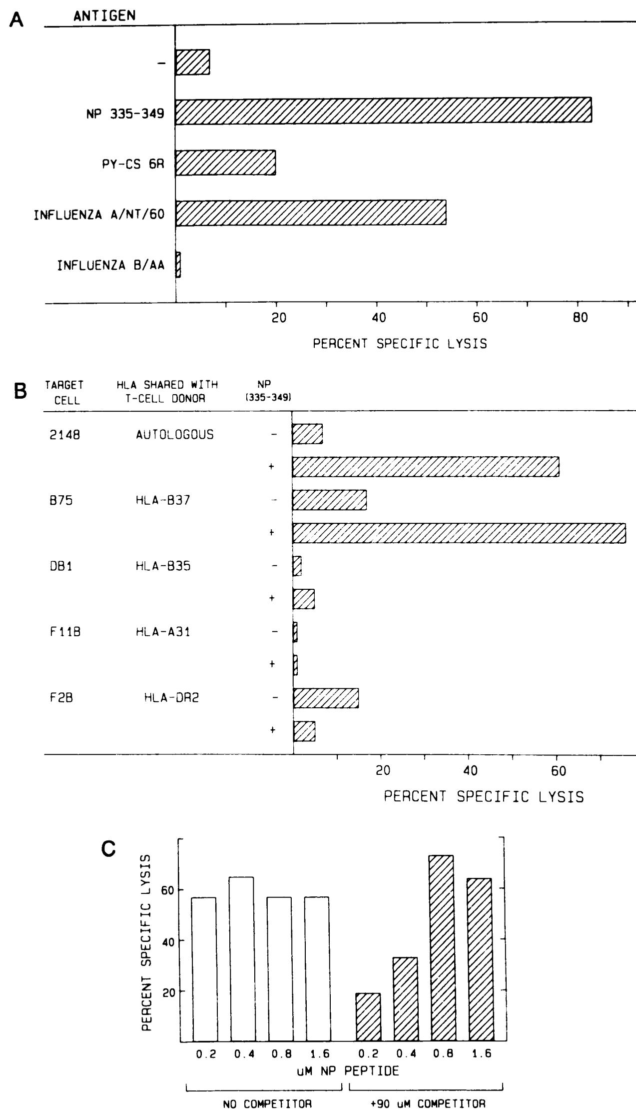

FIG. 1. Specific interactions of NP-(335–350)-peptide-specific HLA-B37-restricted CTL lines. (*A*) Antigen specificity. CTL line 2148.1 was assayed on an autologous B-cell line that was either incubated with 4 μM peptide [NP-(335–350) or an unrelated peptide, PY CS 6-repeat (Table 1)], or infected with A/NT/60 or B/Ann Arbor influenza virus. (*B*) HLA restriction. A panel of B-cell lines, matched for one HLA antigen with the T-cell donor, were pulsed with 4 μM NP-(335–350) peptide for 1 hr at 37°C, washed twice, and tested for their ability to be lysed by CTL line 2148.1. HLA typing of the cell panel is as follows: 2148 (autologous cell), HLA-A31,-B37,53,-C2,4,-DR2,8; B75, HLA-A2,-B27,37,-C2,-DR6,10; DB1, HLA-A2,32,-B44,53,-C-,-DR3; F11B, HLA-A25,31,-B15,40, -C3,-DR4; F2B, HLA-A1,2,-B7,8,-C7,-DR2,4. HLA antigens shared with the T-cell donor are underlined. (*C*) Peptide competition assay for presentation of NP-(335–350) by HLA-B37. HLA-B37$^+$ target cells (2148) were exposed to increasing concentrations of NP-(335–350) (0.2–1.6 μM) in the absence (open bars) or presence (hatched bars) of 90 μM positive competitor peptide (PY CS 6-repeat). After incubation for 1 hr at 37°C, cells were washed twice and tested for their ability to be lysed by NP-(335–350) peptide-specific CTL line M30.

(335–350) presentation at an effector-to-target ratio of 1.25:1, whereas peptides 16 and 17 show little or no inhibitory capacity.

A summary of all competitive inhibition experiments with the panel of peptides shown in Table 1 is presented in Fig. 3*A*. Competitor peptides that are scored as positive for inhibition of presentation of NP-(335–350) by HLA-B37 produced greater than 40% inhibition in all experiments in which they

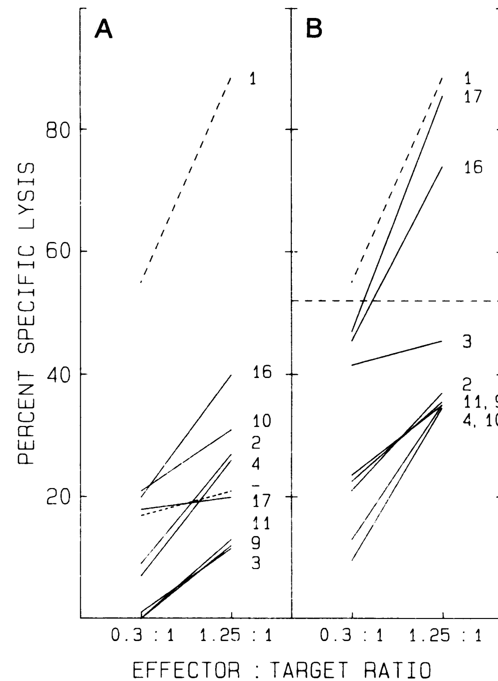

FIG. 2. Competitive inhibition by unrelated peptides of NP-(335–350) peptide presentation by HLA-B37. The results of a representative experiment using 8 of the 26 peptides (see Table 1) tested are shown. (*A*) HLA-B37$^+$ 2148 target cells were incubated for 1 hr at 37°C with no peptide (-------), 0.2 μM NP peptide (no. 1, – – –), or 90 μM indicated peptides (numbers correspond to peptide numbers in Table 1, ——), washed twice, and assayed for their ability to be lysed by HLA-B37-restricted NP-(335–350)-peptide-specific CTL lines. (*B*) The same target cells were incubated 1 hr at 37°C with 0.2 μM NP-(335–350) plus the indicated competitor peptide (——) at 90 μM and assayed as described above. The horizontal dashed line represents the lysis of 40% inhibition at an effector-to-target ratio of 1.25:1.

were assayed, and peptides that produced less than 40% inhibition were scored as negative [a frequency analysis of mean percent inhibition values shows a bimodal distribution on either side of 40% (data not shown)]. The results demonstrate that 11 of the 26 peptides (nos. 2–12) were reproducibly scored as positive competitors. No structural similarities are readily apparent from examination of the primary sequences of these 11 competitor peptides, although one remarkable feature is that 5 of the 11 peptides contain repetitive sequences. The other 6 inhibitory peptides are all derived from influenza viral molecules. Thus, the HLA-B37 molecule can apparently bind peptides with no apparent common primary structural motif.

Comparison of HLA-B37- and HLA-A2.1-Binding Peptides. Our previous study (12) demonstrated that six of the peptides in Table 1 could bind to HLA-A2.1 as determined by target cell sensitization and recognition by CTL (nos. 13, 27) or by inhibition of M1 peptide presentation by HLA-A2.1 (nos. 14, 23, 24, 25, and 26). None of these six HLA-A2.1-binding peptides were scored as positive for inhibition of HLA-B37 presentation (Fig. 3*B*). Similarly, none of the peptides that were scored as positive for inhibition of HLA-B37 presentation (nos. 2–12) were scored as positive for inhibition of HLA-A2.1 presentation (Fig. 3*B*). Thus, HLA-B37 and HLA-A2.1 apparently each bind a subset of the peptides in this panel, and these subsets are nonoverlapping.

The NP-(335–350) Peptide Can Bind to Both HLA-B37 and HLA-A2.1. To determine whether there was any overlap between peptides that could bind to HLA-B37 and HLA-A2.1, we determined the capacity of peptide NP-(335–350) to bind to HLA-A2.1 as assessed by its ability to inhibit pre-

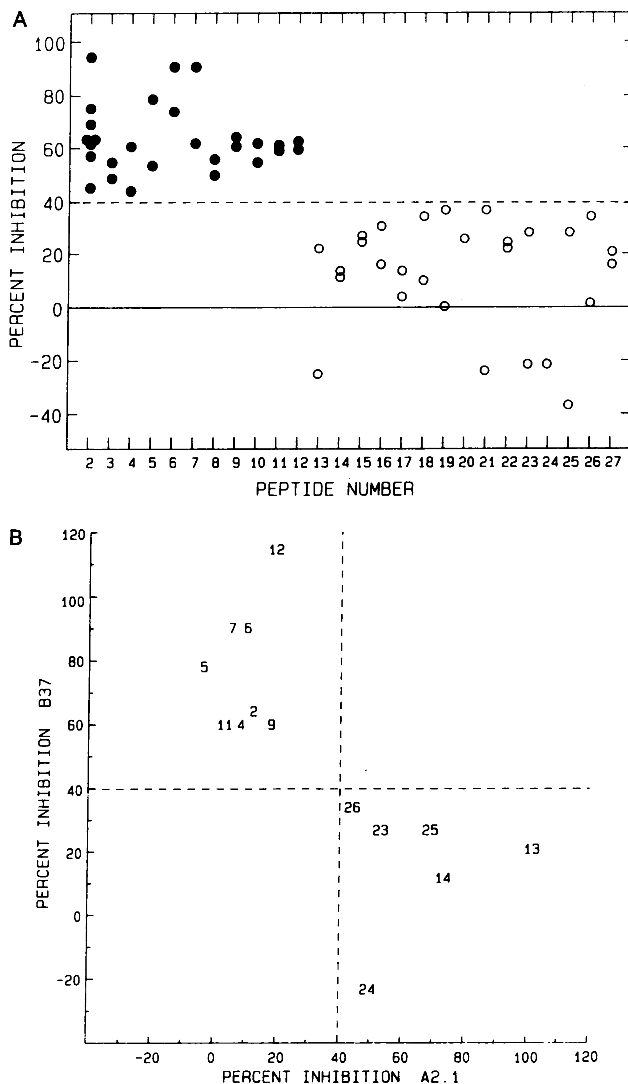

FIG. 3. (*A*) Summary of peptide competition for presentation of NP-(335–350) by HLA-B37. Results of the experiment shown in Fig. 2 and data not shown are expressed as percent inhibition and are calculated by the formula: percent inhibition = [(lysis in the absence of competitor peptide − lysis in the presence of competitor peptide)/lysis in the absence of competitor peptide] × 100, in which lysis values are those at the highest effector-to-target ratio used. Positive inhibition values were scored as those equal to or greater than 40% inhibition. Each symbol represents results obtained either in separate experiments or with individual CTL lines. •, Peptides scored as positive competitors; ○, negative competitors. Peptides are numbered according to Table 1. (*B*) HLA class I allele specificity of peptide binding. Of the 26 peptides tested for HLA-B37 binding, 21 have been previously tested for their capacity to bind to HLA-A2.1 (12). The percent inhibition values obtained for HLA-B37-binding peptides (nos. 2, 4, 5, 6, 7, 9, 11, 12) and HLA-A2.1-binding peptides (nos. 13, 14, 23, 24, 25, 26) in the HLA-B37-restricted NP-(335-350)-specific and HLA-A2.1-restricted and M1-(55–73)-specific systems are compared.

sentation of M1-(55–73) peptide to M1-peptide-specific CTL (12). The reciprocal experiment of using the M1-(55–73) peptide to inhibit presentation of NP-(335–350) by HLA-B37 was also performed. The results (Fig. 4) demonstrate that the NP-(335–350) peptide (no. 1) could inhibit presentation of the M1 peptide (no. 27) by HLA-A2.1 to an M1-peptide-specific CTL line to an extent greater than the inhibition produced by the positive control competitor peptide S61 (Fig. 4 *A* and *B*). On the other hand, the M1 peptide could not inhibit presentation of the NP-(335–350) peptide by HLA-B37 to a NP-peptide-specific CTL line (Fig. 4 *C* and *D*), although the positive control peptide 2 produced strong inhibition of NP-(335–350) presentation. These results demonstrate that at least one peptide [NP-(335–350)] can bind to both HLA-B37 and HLA-A2.1.

DISCUSSION

The results presented in this and a previous report (12) demonstrate that there are clear differences in the peptides that can compete for presentation of antigenic peptides by HLA-B37 and HLA-A2.1. The different patterns of peptide binding by these two class I molecules are apparently not due to differences in their capacities to bind and present the antigenic peptides, since in both systems the antigenic peptides were used at equal molar concentrations (0.2 μM). The observed differences in peptide competition between HLA-B37 and HLA-A2.1 most likely represent real differences in the specificity of their peptide binding sites (9, 10). While apparently not common, it is clearly possible for one peptide to bind to both HLA-A2.1 and HLA-B37, as exemplified by the NP-(335–350) peptide. The binding of NP-(335–350) to HLA-A2.1 and HLA-B37 has also been reported by Bouillot *et al.* (7), using a class I peptide binding assay.

The demonstration that HLA-A2.1 and HLA-B37 bind largely nonoverlapping sets of peptides strongly indicates that the existence of multiple functional class I genes is related to the capacity of their encoded molecules to present a wide variety of antigenic peptides to T cells (22, 23). A relatively high percentage of peptides bound to each of these class I molecules (42% to HLA-B37 and 25% to HLA-A2.1). It is currently unclear how these percentages reflect the number of naturally processed peptides that could be bound by class I molecules within a cell. However, these percentages might represent an underestimation of the peptide-binding capacities of HLA-B37 and HLA-A2.1 for at least two reasons: (*i*) peptides might bind but not compete due to a much lower binding affinity than the antigenic peptide, and (*ii*) two peptides might bind simultaneously to two different sites in the peptide-binding groove. If the finding that HLA-A2.1 and HLA-B37 bind largely nonoverlapping sets of peptides is generalizable to other HLA-A and -B allelic products (24), it may suggest that these two loci have evolved to provide different peptide-binding specificities. The existence of A-ness and B-ness in the nucleotide sequences of HLA-A and -B genes (25) is also indicative of a functional diversification of these loci.

The structural basis for the observed differences in peptide binding specificity of HLA-B37 and HLA-A2.1 can be analyzed by comparison of the amino acid sequences of HLA-A2.1 (26) and HLA-B37 (27). These two class I molecules differ in their $\alpha 1$ and $\alpha 2$ domains at 32 amino acid positions, 13 of which point into the peptide-binding site and are available for ligand binding (10). These 13 amino acids are located at positions 9, 24, 45, 62, 66, 67, 70, and 74 in the $\alpha 1$ domain and at positions 95, 99, 114, 116, and 156 in the $\alpha 2$ domain. Residues 9, 95, 99, 114, and 116 are located on the floor of the peptide-binding groove. Mutagenesis experiments have demonstrated that amino acid substitutions in each of these positions on the floor of the peptide-binding groove of the HLA-A2.1 molecule can alter the binding and/or presentation of influenza viral peptides to CTL (18–21, 28–30). Thus, these residues might also be involved in peptide selection by HLA-B37. In addition, HLA-A2.1 and HLA-B37 also differ at residues 24, 45 and 67, which are located in the "45 pocket" (31, 32), a cavity that extends from the peptide-binding groove and is postulated to be capable of influencing peptide binding (31, 32). This "45 pocket" is hydrophobic in the HLA-A2.1 molecule (composed of residues Ala-24, Met-45, Val-67) while it is hydrophilic in the HLA-B37 molecule (Ser-24, Thr-45, Ser-67). Construction of

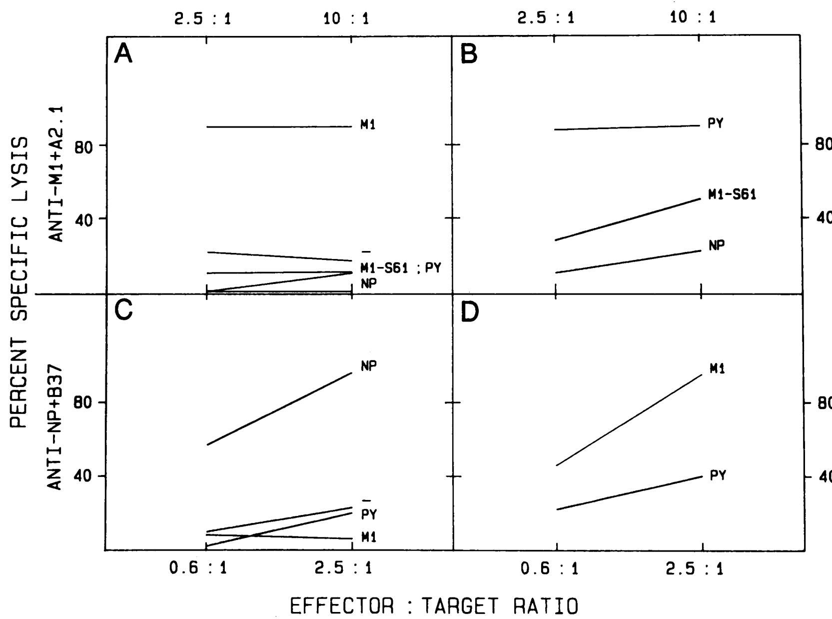

FIG. 4. The NP-(335–350) peptide can bind to both HLA-B37 and HLA-A2.1. Competitive inhibition of presentation of M1-(55–73) by HLA-A2.1 (*A*, *B*) and NP-(335–350) by HLA-B37 (*C*, *D*) was performed using CTL lines 17B5.C2 [anti-M1-(55–73) + A2.1] and B75.4 [anti-NP-(335–350) + B37], respectively. In conditions *A* and *C*, target cells were preincubated 1 hr at 37°C with 0.2 μM M1 peptide (no. 27 in Table 1), 0.2 μM NP peptide (no. 1), 90 μM PY CS 6-repeat (PY, no. 2), 90 μM M1-S61, or no peptide (−). M1-S61 is the same as M1-(55–73) but has a serine substituted for glycine at position 61. The target cells were washed twice and assayed for their ability to be lysed by the appropriate CTL lines. In conditions *B* and *D*, targets were incubated for 1 hr at 37°C with 0.2 μM M1 or NP peptide plus the indicated competitor peptides at 90 μM, washed twice, and assayed in the same way as above.

mutants in which one or more of these residues are exchanged between HLA-B37 and HLA-A2.1 should permit the determination of the role these residues play in peptide-binding specificity.

In a study somewhat comparable to the present report, Sette *et al.* (33, 34) have analyzed the peptide-binding specificity of class II MHC molecules. Fourteen overlapping peptides were derived from the staphylococcal nuclease protein sequence and were tested for binding to soluble IA^d, IA^k, and IE^d molecules. Forty-three percent of the peptides bound to IA^d, 14% bound to IA^k, and 36% bound to IE^d. Only 1 out of the 14 peptides bound to all three class II MHC molecules. These findings are similar to the present findings for HLA class I molecules in that comparable percentages of unrelated peptides bound to these murine class II molecules, and a remarkable degree of discrimination was observed in the peptide-binding capacities of products of different loci (i.e., IA vs. IE, HLA-A vs. HLA-B). It remains to be determined whether the peptide-binding patterns observed for HLA-B37 and HLA-A2.1 can be extrapolated to other HLA-A and HLA-B alleles as well as to HLA-DR and HLA-DQ molecules.

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