Apparent Lack of MHC Restriction in Binding of Class I HLA Molecules to Solid-Phase Peptides

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Summary

The specificity of binding of solubilized, purified HLA-A,B molecules to solid-phase peptides has been examined using the assay described by Bouillet et al. [1989. *Nature (Lond.).* 339:473.] 64 peptides derived from the sequences of viral antigens, HLA-A,B,C heavy chains, and clathrin light chains were tested for binding to HLA-A2.1, Aw68.1, Aw69, B44, and B5, molecules that differ by 5–17 residues of the peptide binding groove. 15 of the peptides, including those known to be T cell epitopes, gave significant binding. The pattern of peptide binding for each of the five HLA-A,B molecules was not significantly different. Binding was demonstrated to be a property of native β_2m-associated HLA-A,B molecules that preserved conformational antigenic determinants after binding to peptide. In comparison to our previous results from solution-based assays the proportion of HLA-A,B molecules that can bind solid-phase peptides is very high. This accessibility of solid-phase peptides to the binding site of MHC molecules may be directly related to the observed absence of MHC specificity in the binding.

Study of T cells has shown that their antigen receptors (TCR) recognize a specific combination of MHC molecule and bound peptide (1–3). Crystallographic structures for HLA-A2.1 and HLA-Aw68.1 revealed the antigen recognition site (4, 5) and sequence comparisons showed that residues lining this groove are the foci for diversity in MHC molecules (6). Moreover, the substitutions at these positions are clearly the result of positive evolutionary selection (7–9). In general it has been reasoned that substitutions within the groove serve to affect the peptides bound by MHC molecules, a concept supported by the observed differences in the pockets of the peptide binding grooves of HLA-A2.1 and HLA-Aw68.1 and in the shape of the "extra" material they contain (4, 5, 10). That each individual uses a small number (3–6) of class I molecules to present numerous peptides to a diversity of TCR necessitates that each MHC molecule binds many peptides and engages many TCR. Thus, there are arguments supporting both specificity and degeneracy in MHC-peptide interactions. For class I MHC molecules experimental definition of the specificity of the binding site has proved difficult. This is due to the presence of tightly bound peptides in isolated class I molecules and uncertainty as to the relevance of experimental assays to the mechanism of peptide binding in vivo.

Previously we demonstrated binding of radio-iodinated influenza peptides to solubilized class I HLA molecules in a manner that correlated with the pattern of MHC restriction (11). We also obtained some evidence for the binding of peptides to nonpresenting MHC molecules. In these solution-based assays the proportion of class I molecules with accessible peptide binding sites was exceedingly low (<1 in 300), a property that significantly limits their applications. Bouillot and colleagues have developed a different assay, in which the binding of radio-iodinated class I HLA molecules to solid-phase peptides is measured (12). Binding was more readily detected than in the solution assay and though discerning quantitative differences in binding, Bouillot et al. (12) concluded that "Peptides which are recognized by cytotoxic T lymphocytes bind not only to the restricting MHC class I molecule but also to other class I molecules." A striking difference in results obtained with the solid-phase assay compared with those obtained with the solution assay was that up to ~25% of the radio-iodinated class I molecules were capable of binding to experimentally offered peptides. This property and the capacity to rapidly screen large numbers of peptides give the solid-phase assay potential for wide applications in the analysis of peptide binding to class I MHC molecules. Here we compare the binding of 5 class I HLA molecules to 64 peptides.

Materials and Methods

Peptides. Peptides were synthesized by a solid-phase method using FMOC or tBOC chemistry. Homogeneity of the peptides was indicated by reverse-phase HPLC.

Isolation of HLA Molecules. HLA molecules were purified from detergent solubilized cell lysates by immunoaffinity chromatography as previously described (13). HLA-A2.1, Aw69, and Aw68.1 molecules were purified from cell lysates of human lymphoblastoid

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1.	A1 60-84	WDQETRDMKAHSQTDRANLGTLRGY
2.	A2 98-113	MYGCDVGSDWRFLRGY
3.	A2 56-69	GPEYWDGETRKVKA
4.	A2 57-69	-PEYWDGETRKVKA
5.	A2 58-69	--EYWDGETRKVKA
6.	A2 59-69	---YWDGETRKVKA
7.	A2 60-69	----WDGETRKVKA
8.	A2 61-69	-----DGETRKVKA
9.	A2 62-69	------GETRKVKA
10.	A2 60-84	WDGETRKVKAHSQTHRVDLGTLRGY
11.	A2 70-85	HSQTHRVDLGTLRGYY
12.	A2 87-102	QSEAGSHTVQRMYGCD
13.	A2 112-129	KDYIALKEDLRSWTA
14.	A2 141-160	QTTKHKWEAAHVAEQLRAYL
15.	A2 161-171	GTCVEWLRRY
16.	A2 146-161	KWEAAHVAEQLRAYLE
17.	A2 137-148	DMAAQTTKHKWE
18.	A2 266-282	LPKPLTLRWEPSSQPTI
19.	Aw24 60-84	WDEETGKVKAHSQTDRENLRIALRY
20.	A32 60-84	WDQETRNVKAHSQTDRESLRIALRY
21.	Aw68.1 141-160	QTTKHKWEAAHVAEQWRAYL
22.	Aw68.1 98-113	MYGCDVGSDGRFLRG
23.	Aw68.1 60-84	WDRNTRNVKAQSQTDRVDLGTLRGY
24.	Aw68.1 56-69	GPEYWDRNTRNVKA
25.	Aw68.1 70-85	QSQTDRVDLGTLRGYY
26.	Aw68.1 87-102	QSEAGSHTIQRMYGCD
27.	Bw58 56-69	GPEYWDGETRNMKA
28.	Bw58 60-84	WDGETRNMKASAQTYRENLRIALRY
29.	B7 60-84	WDRNTQIYKAQAQTDRESLRNLRGY
30.	B27.1 60-84	WDRETQICKAKAQTDREDLRTLLRY
31.	B27.2 60-84	WDRETQICKAKAQTDRENLRIALRY
32.	B27.3 60-84	WDRETQICKAKAQTDRESLRTLLRY
33.	B13 60-84	WDRETQISKTNTQTYRENLRTALRY
34.	B14 60-84	WDRNTQICKTNTQTDRETLRNLRGY
35.	B41 60-84	WDRETQISKTNTQTYRESLRNLRGY
36.	B42 60-84	WDRNTQIYKAQAQTDRESLRNLRGY
37.	B44.1 60-84	WDRETQISKTNTQTYRENLRTAARY
38.	B44.1 141-160	QITQRKWEAARVAEQDRAYL
39.	B47 60-84	WDRETQISKTNTQTYREDLRTLLRY
40.	Cw1 60-84	WDRETQKYKRQAQTDRVSLRNLRGY
41.	Cw2.1 60-84	WDRETQKYKRQAQTDRVNLRKLRGY
42.	Cw3 60-84	WDRETQKYKPQAQTDRVSLRNLRGY
43.	β_2m 1-16	IQRTPKIQVYSRHPAE
44.	β_2m 8-25	QVYSRHPAENGKSNFLNC
45.	β_2m 25-41	CYVSGFHPSDIEVDLLK
46.	β_2m 38-54	DLLKNGERIEKVEHSD
47.	β_2m 51-66	HSDLSFSKDWSFYLLY
48.	β_2m 67-79	YTEFTPTEKDEYA
49.	β_2m 80-94	CRVNHVTLSQPKIVK
50.	β_2m 83-99	NHVTLSQPKIVKWDRDM
51.	FLU M57-68	KGILGFVFTLTV
52.	LMP	VMSDWTGGALLC
53.	HIV.gag 265-279	KRWILGLNKIVRMYC
54.	FLU.NP335-349	SAAFEDLRVLSFIRG
55.	LCa 47-71	FAILDGGAPGPQPHGEPPGGPDAVD
56.	LCa 113-137	MERLEALDANSRKQEAEWKEKAIKE
57.	LCa 148-164	QLQKTKANNRAAEEAFV
58.	LCa 188-208	AAEEAFVNDIEESSPGTEWER
59.	LCa 204-226	TEWERVARLCDFNPKSSKQAKDV
60.	LCb 45-72	EGFGAPAGSHAAPAQPGPTSGAGSEDMG
61.	LCb 113-135	RKRLQELDAASKVTEQEWAEKAK
62.	LCb 148-164	QVEKNKINNRASEEAFV
63.	LCb 188-208	ASEEAFVKESKEETPGTEWEK
64.	LCb 208-229	KVAQLCDFNPKSSKQCKDVSRL

Figure 1. Primary amino acid sequences of peptides. Peptides were synthesized on a Milligen/Biosearch 9600 automatic peptide synthesizer using Fmoc chemistry. β_2m = β_2 microglobulin; Flu = influenza; M = matrix protein; NP = nucleoprotein; HIV = human immunodeficiency virus; LMP = latent membrane protein of Epstein-Barr virus; LCa, LCb = light chain a and b of clathrin proteins.

B cell lines 721.53 (A2), IDF (Aw69, A26, B18, Bw38), and C1R.Aw68.1 (a HLA-A,B-negative cell line transfected with the Aw68.1 gene), respectively, using a monoclonal anti-A2, Aw69, Aw68 (CR11.351) antibody column. HLA-B5 and B44 molecules were purified from cell lysates of B-lymphoblastoid cell line 721.144 (B5) and C1R.B44 (a B44 transfectant), respectively, using a monomorphic anti-HLA-A,B,C (W6/32) monoclonal antibody column.

Peptide Binding Assay. Direct binding of radiolabeled HLA molecules to peptides precoated onto polystyrene microtiter wells was as described by Bouillot et al. (12). Briefly, HLA molecules were labeled with 1 mCi of ^{125}I (Amersham Corp., Arlington Heights, IL) by the chloramine T method and fractions containing radiolabeled HLA molecules were collected from a Sephadex G50 column. The HLA molecules were diluted in PBS containing 0.5% (NP-40), 1% BSA, 0.02% NaN_3, and 1 mM PMSF. Peptides at 1 mg/ml were plated in duplicates in polystyrene microtiter plates (Dynatech, Alexandria, VA). Plates containing 10 μl peptides per well were incubated for 16 h at room temperature and washed twice with 0.1% BSA in PBS. Unoccupied sites were saturated with 1% BSA in PBS for 1 h. The plates were washed three times and radiolabeled HLA molecules were added and placed at room temperature for 18 h. The plates were then washed three times and radioactivity bound per well was measured by direct counting in a gamma counter.

Detection of HLA by mAbs. HLA molecules bound on peptide-precoated plates were detected by anti-HLA mAb and radiolabeled rabbit anti-mouse antibodies as secondary and tertiary reagents. HLA molecules were added to peptide precoated plates for 18 h at room temperature then washed three times with 0.1% BSA in PBS followed by incubation with 1% ovalbumin in PBS for 30 min

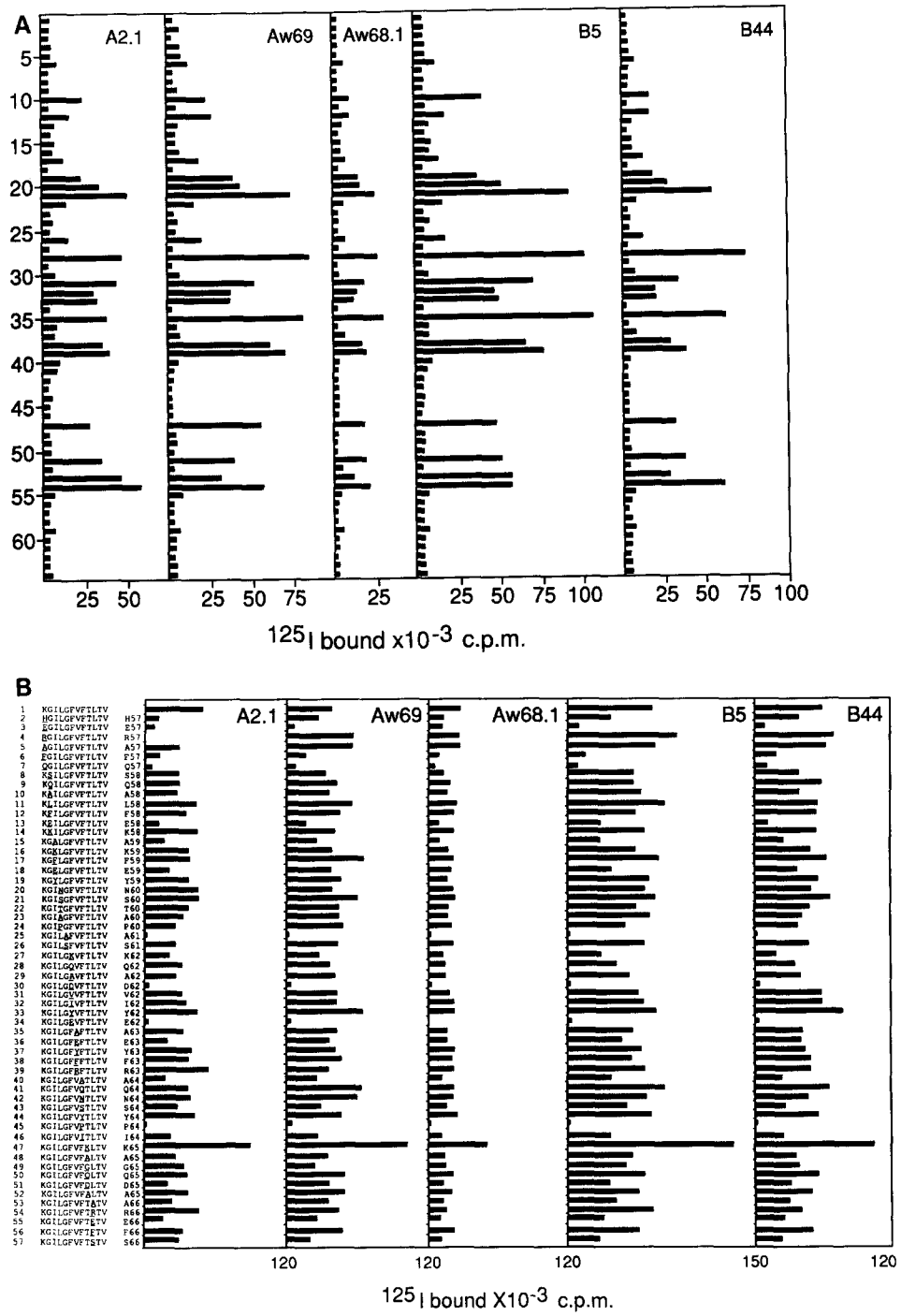

Figure 2. Direct binding of HLA molecules to peptides. Peptides were coated onto polystyrene microwells in duplicate at 10 μg/well and were placed at room temperature for 16 h before saturating the unbound sites with 1% BSA in PBS. HLA molecules radioiodinated by the chloramine T method were diluted in PBS containing 0.5% NP-40, 1% BSA, 0.02% NaN_3, and 1 mM PMSF and 10 ng was added to each microwell. The reaction plates were placed at room temperature for 18 h then washed three times with 0.1% BSA and 0.5% NP-40 in PBS, air dried, and radioactivity bound per well was counted. (*A*) Peptides of viral, HLA, and clathrin proteins. (*B*) Analogues of influenza matrix peptide Flu.M57-68.

at 4°C. After incubation, the microwells were washed twice and anti-HLA mAbs (250 μg/ml) were added to each well and placed at 4°C for 45 min. At that time, the microwells were then washed three times with 0.1% BSA in PBS and radioiodinated rabbit anti-mouse $F(ab')_2$ were added to each well at 600,000 cpm/well. The plates were placed at 4°C for 45 min, then washed three times and radioactivity bound per well was counted.

Results

Comparison of the crystallographic structures of HLA-A2.1 and HLA-Aw68.1 revealed differences in the detailed architecture of the peptide binding groove, indicating that these molecules would exhibit distinctive peptide binding specificity (5). In an attempt to identify discriminating peptides we tested 64 peptides for binding to HLA-A2.1 and HLA-Aw68.1 using the solid-phase assay of Bouillot et al. (12). Binding to HLA-Aw69, which has a hybrid peptide binding groove (α_1 derived from HLA-Aw68.1 and α_2 derived from HLA-A2.1) and to HLA-B44 and HLA-B5 was also assessed. The peptides tested had sequences derived from various viral antigens, HLA-A,B,C heavy and light chains and clathrin light chains (Fig. 1).

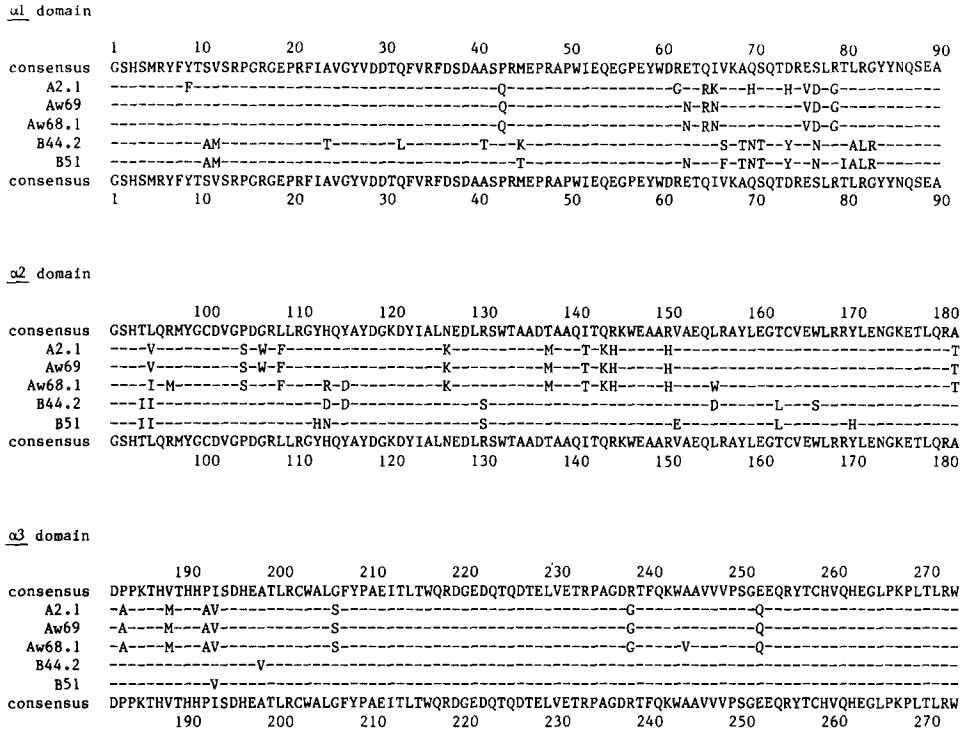

Figure 3. The amino acid sequences of the α_1, α_2, and α_3 domains of the five HLA-A and -B molecules studied here. The standard one-letter amino acid code is used. A dash indicates that a residue is identical to that found in the consensus sequence.

Fifteen of the 64 peptides bound to HLA-A2.1 at levels >10-fold of background, 15 peptides gave binding twofold above background levels and 34 were negative (Fig. 2 *A*). Positively binding peptides were found among the HLA peptides and the viral peptides, but not among the clathrin peptides. The lack of HLA binding to the clathrin peptides was not due to poor absorption of peptides to the microtiter plates as many of these peptides bind to specific anti-clathrin monoclonal and polyclonal antibodies in similarly configured assays (14). Thus, there is, as previously demonstrated (12), discrimination between peptides in this assay. Calculation also shows that up to 30% of the total input of radio-iodinated HLA-A2.1 molecules can bind to solid-phase peptide. Among the strongly binding peptides is that corresponding to residues 57–68 of the influenza matrix peptide (Flu.M57-68) and the binding of HLA-A2.1 to this peptide we observed is comparable to that reported by Bouillot et al. (12). This peptide has repeatedly been shown to be presented to influenza specific CTL by HLA-A2.1 and HLA-Aw69 (15). In addition, Gotch et al. (16) have tested peptide analogues for their capacity to substitute for Flu.M57-68 and have identified positions at which substitution prevents presentation to specific CTL. The pattern of binding of HLA-A2.1 to these analogue peptides observed in the solid-phase assay closely correlates with the pattern of sensitization observed by Gotch and colleagues (Gotch, F., and McMichael, A. J., unpublished data, personal communication), showing that among this highly related set of peptides, binding correlates with presentation (Fig. 2 *B*) with the exception that peptides E63, Y63, and F63 bound in the solid-phase assay and were not recognized by CTL and did not inhibit presentation of the matrix peptide by HLA-A2.

It was therefore surprising that no significant discrimination between five different MHC molecules was observed in this binding assay; the pattern of binding of HLA-A2.1 to the 64 peptides was mimicked by HLA-Aw68.1, Aw69, B5, and B44 (Fig. 2). Thus every peptide that bound to one HLA molecule bound to the other four; peptides that failed to bind to one HLA molecule did not bind to the other four. Even when the levels of binding of different peptides to the five HLA molecules were compared, there were no significant differences that could be ascribed to "HLA type."

These results clearly challenge the hypothesis that variation between class I MHC molecules, which is mainly found in the peptide binding groove, acts significantly to alter the peptide binding specificity. The five HLA-A,B molecules studied here show considerable variation in the residues that point into the groove, between 5 and 17 amino acid differences (Fig. 3), and yet their pattern of peptide binding appears identical.

This raised the possibility that it was denatured HLA molecules, or an iodinated contaminant in the HLA preparations, that was binding the peptides. To address this question we separated radio-iodinated HLA-A2 on a column of a W6/32, a mAb that only binds to native β_2m-associated molecules, and measured the binding to peptides of the bound and the unbound radioactivity. All peptide binding activity of the ^{125}I-HLA-A2 preparation was removed by passage over the W6/32 column, showing that the observed peptide binding was due to β_2m-associated HLA-A2 heavy chains and not to free heavy chains or a contaminant. This conclusion was confirmed by the recovery of peptide binding activity by elution of material bound to the W6/32 column. Moreover,

Table 1. *Binding of Peptides by Radioiodinated HLA-A2.1 Molecules Reisolated by Affinity Chromatography*

	Binding by fractions of HLA-A2.1 preparation			
	Pre W6/32 column		Eluate	Run through
Peptides	600,000*	50,000	50,000	50,000
FLU.M57–68	7,640‡	1,234	6,585	638
Bw58.60–84	11,347	1,876	13,856	1,214
B41.60–84	15,867	2,413	15,888	1,070
None	1,812	1,180	1,593	440

* Amount of radioactivity added to each well.
‡ Amount of radioactivity bound to each well after three washes to remove excess radioactivity.

only a fraction (1/10) of the radioactivity was needed to achieve binding comparable to the level obtained with unfractionated radiolabeled HLA-A2 (Table 1).

A further source of potential artefact is modification of the HLA molecules by the iodination reaction. The peptide binding groove of MHC molecules is characterized by a number of strategically placed tyrosine residues (4) and it is possible that their iodination could alter the combining site specificity to give a degenerate pattern of peptide binding. To circumvent this issue we developed a modified assay in which the binding of noniodinated HLA molecules to plate-bound peptides was measured using specific anti-HLA-A,B mouse mAbs and radioiodinated anti-mouse Ig (Fig. 4). This indirect assay gave similar patterns of peptide binding to those seen in the direct assay and equivalent results were obtained with six mAbs directed against different epitopes of HLA-A,B molecules; results obtained with W6/32 and A2.1 mAb are shown (Fig. 4 *A*). Protein modification due to radio-iodination is therefore not the source of the degenerate binding.

Discussion

Bouillet et al. (12) previously analyzed the binding of radio-iodinated HLA-A2.1, HLA-B37, and HLA-B27 to solid-phase peptides. Peptides that were known to be antigenic for T cells bound to their appropriate restriction element, but although some quantitative differences were observed, these authors found that peptides binding to one class I molecule tended to bind to others. In three cases where direct comparison can be made, the binding of HLA-A2.1 to influenza nuclear protein peptide FLU.NP335-349, influenza matrix protein peptide FLU.M57-68, and HIV gag protein peptide HIV.gag.265-279, we obtain comparable results to Bouillot et al. Furthermore we find the assay robust, highly reproducible, and suitable for screening large numbers of samples.

Comparison of the binding of the same MHC molecule to different peptides showed specificity that correlated with

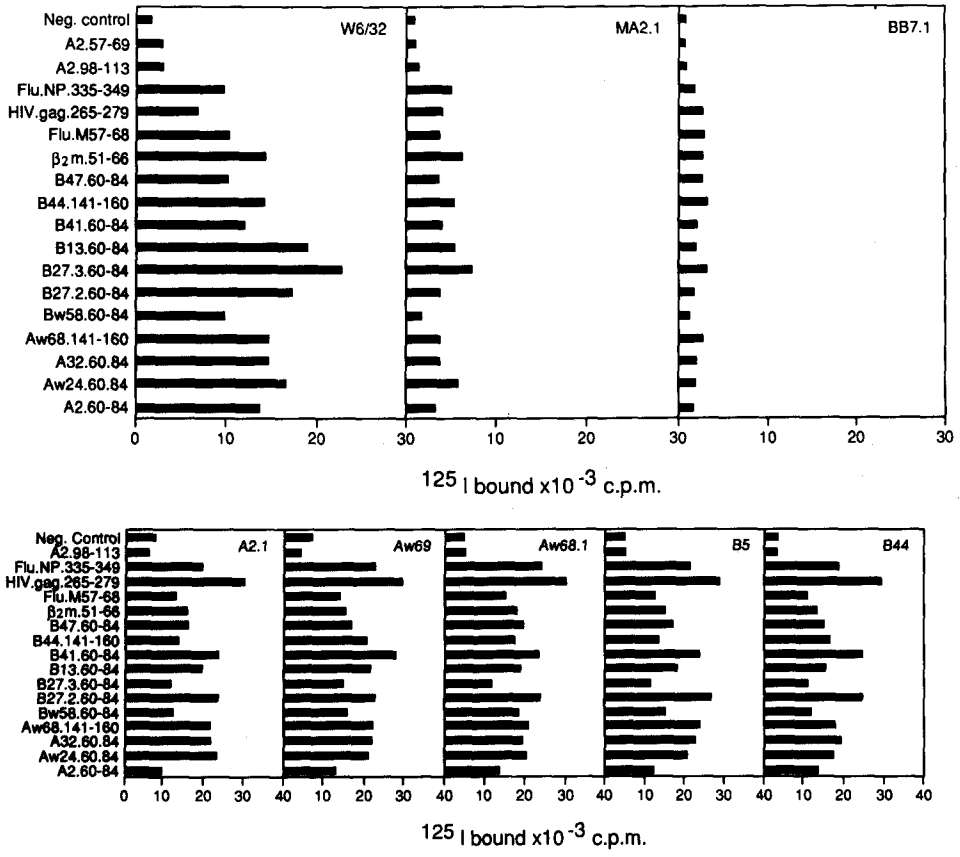

Figure 4. Binding of HLA molecules to peptides detected by an indirect binding assay. HLA preparations were added to microwells precoated with peptides and presaturated with 1% BSA. The reaction plates were placed at room temperature for 18 h then washed three times, and unbound sites were saturated with 1% ovalbumin in PBS. Monomorphic anti-HLA antibody W6/32 at 250 μg/ml was added to each well in 50 μl and incubated at 4°C for 45 min. The microwells were washed three times and ^{125}I-labeled rabbit anti-mouse F(ab')$_2$ were added to each well for an additional 45 min. Radioactivity bound per well was measured by direct counting of well washed to remove excess radioactivity.

presentation to T cells. In contrast we found no significant differences in the patterns of peptide binding of five different class I molecules. Bouillot et al. were able to discern some quantitative differences in titration experiments. In this study peptides were plated at 1 mg/ml, a concentration that for known antigens gave maximal binding, and this protocol may have served to reduce the discriminating capacity of the assay. Nevertheless it is clear from our results and those of Frelinger et al. (17) with this assay that it is hard to argue for a profound effect of MHC polymorphism on the binding of peptides to HLA-A,B molecules. The critical issue is whether this is a true reflection of the specificity of peptide binding by class I molecules or an artefact of the assay.

Trivial artefacts have been ruled out. The binding is clearly the product of β_2m-associated HLA-A,B heavy chains and is not dependent upon radioiodination. Conformational antigenic determinants of the HLA-A,B molecules are preserved on binding to solid-phase peptides. In addition we know that binding does not require the presence of detergent or the hydrophobic transmembrane region of the HLA-A,B molecule. Papain solubilized and genetically engineered, secreted HLA molecules give similar binding to the detergent solubilized product.

These results combined with the peptide specificity and its correlation with T cell reactivity argue for binding being within the peptide binding groove. That the binding grooves of a much greater proportion of HLA-A,B molecules are accessible to solid-phase peptides than to peptides in solution suggests that interactions at the solid phase facilitate either displacement of the endogenous peptide or the simultaneous binding of the endogenous peptide and the experimentally offered peptide. This might involve nonphysiological interactions between the class I molecules and plastic that loosen up the structure and thereby reduce the specificity of the combining site. Alternatively the plastic surface might mimic conditions that MHC molecules encounter *in vivo* and which contribute to their function.

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