Analysis of Physical Interactions between Peptides and HLA Molecules and Application to the Detection of Human Immunodeficiency Virus 1 Antigenic Peptides

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Summary

The physical association of 40 antigenic peptides and purified HLA class I and class II molecules was monitored using a direct peptide binding assay (PBA) in solid phase and an inhibition peptide binding assay (IPBA) in which the competing peptide was present in a soluble phase. We also examined the ability of different peptides to inhibit the lytic activity of human antiviral cytolytic T cells towards cells incubated with the corresponding target peptide. Our results showed that: (*a*) Binding of a given human T cell–recognized peptide to several HLA class I and class II molecules occurred frequently. Nevertheless, preferential binding of peptides to their respective restriction molecules was also observed. (*b*) Binding of HLA molecules to peptides recognized by murine T cells occurred less frequently. (*c*) 11 of 24 (46%) randomly selected HIV-1 peptides contained agretopic residues allowing their binding to HLA molecules. (*d*) The kinetics of HLA/peptide association depended on the peptide tested and were faster than or similar to those reported for Ia molecules. Dissociation of these complexes was very low. (*e*) Peptide/HLA molecule binding was dependent on length, number of positive charges, and presence of hydrophobic residue in the peptide. (*f*) A correlation was demonstrated between a peptide inhibitory effect in the IPBA and its blocking effect in the cytolytic test. Our data indicated that the restriction phenomenon observed in T cell responses was not strictly related to either an elective HLA/peptide association, or a high binding capacity of a peptide to HLA molecules. These data also showed that the PBA and IPBA are appropriate for the detection of agretopic residues within HIV-1 proteins.

T cells recognize antigens as small peptides bound to class I or class II MHC molecules (1–3). In most cases, T cell epitopes are recognized in association with only a few, if not a single, MHC restriction elements (reviewed in reference 4). In contrast, some peptides can be recognized in association with several Ia (5) or HLA-DR molecules (6, 7). Furthermore, a single peptide can be recognized by both MHC class I– and class II–restricted T cells (8, 9). Since class I and class II molecules have a similar predicted tertiary structure, including a single antigen binding site (10–12), interactions between peptides and these two types of molecules are also likely to be similar. The binding site is represented as a platform of eight antiparallel β-strands topped by α-helices. The β-pleated sheets contain a large number of conserved hydrophobic residues, whereas the α-helices contain more polymorphic residues (10, 11). Garrett et al. (13) have recently described several pockets apparently positioned to bind peptide side chains. The variable shapes and charges of these pockets might explain their different reactivities with multiple peptides. However, the molecular mechanism of peptide/MHC molecule association remains unclear: the conformations, orientations, and numbers of peptides interacting with a binding site are still hypothetical.

The formation of complexes between antigenic peptides and MHC class II molecules has been directly demonstrated in binding experiments involving labeled peptides and purified murine class II molecules either in soluble phase (14, 15), or in planar membranes (16). Although a physical association between HLA class I molecules and immunogenic peptides has been recently reported (17, 18), very little is known on peptide binding of class I molecules. We have previously described an assay to measure both the binding of radiolabeled HLA class I molecules to peptides attached to a support and the inhibition of this binding by soluble peptides (18). By using this assay, we showed that class I molecules bound to peptides described to be targets of class I–restricted

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CTL. Since we demonstrated that a single peptide was able to bind different HLA class I molecules, this indicated that interaction with MHC molecules was necessary but not sufficient for T cell recognition. In this paper, using both the direct peptide binding assay (PBA)[1] and the inhibition of peptide binding assay (IPBA), we analyzed the physical interactions between HLA molecules and 40 synthetic peptides. The peptides studied included specific targets for either $CD4^+$ or $CD8^+$ T cells, as well as a large number of peptides derived from HIV proteins and not yet described to bear T cell–reacting epitopes.

Materials and Methods

Purified HLA Preparations. Purified HLA preparations were obtained from three human lymphoblastoid cell lines transformed by EBV: JES THOM (HLA-A2/2, B27/27, DR1/1), HHKB (HLA-A3/3, B7/7, DRW 13/13), and KAS-011 (HLA-A1/1, B37/37, DR 2/2). Cells were lysed in 10 mM Tris-buffered saline (TBS), pH 7.5, containing 1% NP-40 (Sigma Chemical Co., St. Louis, MO) in the presence of protease inhibitors: 1 mM *p*-amidinophenyl-methyl-sulfonyl-fluoride, 10 μg/ml trypsin inhibitor, 10^{-5} M pepstatin A, 10^{-5} M leupeptin, and 10^{-4} M iodoacetamide (Sigma Chemical Co.). After centrifugation, supernatants were run through different columns as described by Walker and Reisfeld (19). Briefly, the columns, equilibrated in TBS containing 0.1% NP-40 and protease inhibitors, consisted of the succession of: (*a*) a CNBr-activated–Sepharose 4B column (Pharmacia France, Saint Quentin Yvelines), (*b*) a CNBr–sepharose 4B column coupled to normal BALB/c immunoglobulins, and (*c*) a CNBr–Sepharose 4B column coupled to anti-HLA mAbs. Purified immunoglobulins BB7.2 (20) and B27M2 (21) were used for HLA-A2 and -B27, respectively. Enriched HLA-B27 or -B37 preparations were also obtained by using B1.23.2, which reacts with most HLA-B molecules (22). HLA-DR preparations were obtained by using L243 (23). After three successive washes with TBS containing 1% NP-40, then TBS with 0.5 M NaCl and 0.1% NP-40, and finally PBS containing 0.1% *N*-octylglucopyrannoside (Sigma Chemical), HLA molecules were eluted from the columns with 0.05 M diethylamine, pH 11.5. Eluted fractions were immediately neutralized with 1 M Tris-HCl, pH 8.0. After an overnight dialysis at 4°C in PBS containing 0.1% NP-40, samples were aliquoted and frozen at −80°C. Purity of fractions was assessed by SDS-PAGE and IEF-PAGE after iodination of the HLA samples. Protein concentration was determined by using the BCA reagent (Pierce Chemical Co., Rockford, IL). Iodination of HLA molecules was performed by using the chloramine T method with 10 μg of purified HLA and 18.5 MBq ^{125}iodine.

Synthetic Peptides. Peptides prepared by solid-phase synthesis were obtained from Neosystem (Strasbourg, France). Sequences are given

[1] *Abbreviations used in this paper:* IPBA, inhibition of peptide binding assay; PBA, peptide binding assay.

Table 1. *Synthetic Peptides Tested for Binding to HLA Molecules*

Peptide	Sequence	Restricting element	Reference
Influenza A virus			
Matrix			
M.57-68	K G I L G F V F T L T V	HLA-A2	(24)
M.Y$^+$57-68	Y K G I L G F V F T L T V	HLA-A2	(18)
Nucleoprotein			
N.147-158R$^-$	T Y Q R T R A L V T G	H-2 K^d	(25)
N.335-349Y$^+$	S A A F E D L R V L S F I R G Y	HLA-B37	(3)
Haemagglutinin			
H.130-142	H N T N G V T A A C S H E	Ia^d	(26)
H.305-329	C P K Y V K Q N T L K L A T G M R N V P E K Q T R	HLA-DR	(1)
Lysosyme: Lys.46-61	N T D G S T D Y G I L Q I N S R	Ia^k	(14)
λ *repressor*: λR.12-26	L E D A R R L K A I Y E K K K	Ia^d	(27)
HLA-A2: A2.170-185	R Y L E N G K E T L Q R T D A P	H-2 K^d	(28, 29)
HIV 1			
Gag. 51-65	L E T S E G C R Q I L G Q L Q		
205-219	E T I N E E A A E W D R V H P	–	
219-233	H A G P I A P G Q M R E P R G	–	
265-279	K R W I I L G L N K I V R M Y	HLA-B27	(30)
378-391	M Q R G N F R N Q R K I V K	–	
418-433	K E G H Q M K D C T E R Q A N F	HLA-A2	(31)

continued

in Table 1. They were derived from influenza A virus proteins (Matrix, nucleoprotein, or hemagglutinin), hen egg lysozyme, λ repressor, HLA-A2 molecule, and HIV-1 proteins (Gag, Env, Nef, Vif, Rev, and Vpr) of the BRU isolate (36). Peptides Gag. 205–219 and Gag. 265–279 were derived from the HIV-1 SF2 isolate (30).

Direct Peptide Binding Assay. Wells of microtiter plates were pretreated with 100 μl 2.5% glutaraldehyde in distilled water for 2 h at 20°C, washed with distilled water, and coated for 16 h at 4°C with 100 μl of peptide diluted at 5 μg/ml either in carbonate-bicarbonate buffer, pH 9.6, or in PBS, pH 7.4 or pH 5.0. Remaining free sites were blocked by incubation for 2 h at 20°C with BSA diluted 1% in PBS containing 0.05% Tween 20 (Tw) and 0.02% sodium azoture. After washing, purified ^{125}I-HLA molecules (100 μl containing 1.5 × 10^5 cpm, 10^{-9} M) diluted in PBS containing 1% BSA, 0.05% Tw, 0.02% sodium azoture, 1 mM PMSF, and 10 μg/ml trypsin inhibitor were added and incubated for 20 h at 20°C. After extensive washing, the radioactivity of each well was counted. Each peptide was tested in triplicate and the average of three independent experiments was calculated.

Inhibition Peptide Binding Assay. Concentrations of 0.1–100 μM of competitor peptides were incubated in tubes for 0–3 h at 20°C with ^{125}I-HLA (10^{-9} M) diluted in PBS-BSA-Tw containing the protease inhibitors as mentioned above. Then the mixture was added to microtiter plate wells coated with a peptide that showed significant binding to HLA molecules in the direct test and incubated 20 h at 20°C. After extensive washing, the radioactivity in each well was counted and the percent of inhibition was calculated. Each peptide was tested in triplicate and in three independent experiments.

Competition between Peptides in a Lytic Assay. Human antipeptide CTL were generated as described by Carbone et al. (37). Briefly, 6–8 × 10^7 PBMC were stimulated with 100 μg of a synthetic peptide in 10 ml culture medium (RPMI 1640 supplemented with 100 μ/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamin, 2 mM nonessential amino acids, 1 mM sodium pyruvate, 10 mM Hepes, and 10% pooled heat-inactivated human AB serum). After a 7-d incubation, a secondary in vitro stimulation was performed by mixing 5–7 × 10^6 effector cells with 2–3 × 10^7 irradiated (4,000 rad) autologous PBMC in 10 ml culture medium containing 50–100 μg of peptide.

Weekly restimulations were similarly performed in culture medium supplemented with 5% of IL-2-containing supernatant prepared as described by Healy et al. (38). CTL from an HLA A2/3, B7/60, DR4/8 donor recognizing the peptides M.57-68 and

Table 1. *(continued)*

Peptide	Sequence	Restricting element	Reference
Env. 105-117	H E D I I S L W D Q S L K	Ia*	(32)
312-327	I R I Q R G P G R A F V T I G K	H-2 D^d	(33)
428-445	F I N M W Q E V G K A M Y A P P I S	Ia*	(32)
474-489	R P G G G D M R D N W R S E L Y	–	
510-521	V V Q R E K R A V G I G	–	
584-604	R I L A V E R Y L K D Q Q L L G I W G C S	HLA, Cl II*	(34)
827-843	Y V A E G T D R V I E V V Q G A C R	–	
846-860	R H I P R R I R Q G L E R I L	–	
Nef. 66-80	V G F P V T P Q V P L R P M T	–	
79-94	M T Y K A A V D L S H F L K E K	–	
113-128	W I Y H T Q G Y F P D W Q N Y T	HLA-B17.37	(35)
132-147	G V R Y P L T F G W C Y K L V P	HLA-B18	Our lab‡
137-145	L T F G W C Y K L	–	
160-174	E N T S L L H P V S L H G M D	–	
Vif. 1-15	M E N R W Q V M I V W Q V D R	–	
25-40	V K H H M Y V S G K A R G W F Y	–	
46-60	S P H P R I S S E V H I P L G	–	
60-72	G D A R L V I T T Y W G L	–	
71-85	G L H T G E R D W H L G Q G V	–	
Rev. 1-16	M A G R S G D S D E D L L K A V	–	
18-30	L I K F L Y Q S N P P P N	–	
37-50	A R R N R R R R W R E R Q R	–	
Vpr. 1-14	M E Q A P E D Q G P Q R E P	–	
55-68	A G V E A I I R I L Q Q L L	–	
68-80	L F I H F R I G C R H S R	–	

* Non precisely identified restricting element.

‡ Culmann B., E. Gomaud, M.-P. Kieny, B. Guy, F. Dreyfus, A.-G. Saimot, D. Sereni, D. Sicard and J.-P. Levy, manuscript submitted for publication.

M.Y$^+$57-68 in association with the HLA-A2 molecule and CTL from an HLA-A2/11, B5/37, DR2/3 donor recognizing the peptide N.335-349Y$^+$ in association with the HLA-B37 molecule were used in the lytic assay. The target cells were ^{51}Cr-labeled autologous EBV-transformed B lymphoblastoid cell lines. Blocking assays were performed by preincubating ^{51}Cr-labeled target cells for 1 h at 37°C with competitor peptides at concentrations ranging from 100 to 10 μM. The target peptide was then added at a final concentration of 0.4 μM (M.Y$^+$ 57-68) or 2 μM (N.335-349Y$^+$) with the effectors at an E/T ratio of 4:1. Chromium release into the supernatant was measured in a gamma counter. Specific lysis was calculated as: 100 ×[(experimental-spontaneous release)/(total ^{51}Cr incorporated-spontaneous release)]. Maximal specific lysis was obtained in the absence of competitor peptide. The results of duplicates were expressed as: percent inhibition of maximal specific lysis = [1 − (experimental specific lysis/maximal specific lysis)] × 100.

Results

Binding of HLA Class I Molecules to Peptides Recognized by Either CD8$^+$ or CD4$^+$ T Cells. Using the PBA, we tested nine peptides described to be targets for class I–restricted CTL. Three peptides corresponded to the influenza A virus matrix (M.57-68 and M.Y$^+$57-68) and nucleoprotein (N.335-349Y$^+$) protein; their recognition by human CTL was restricted by HLA-A2 (24) and HLA-B37 (3), respectively. Three other peptides were derived from HIV-1 Gag (Gag.265-279 and Gag.418–433), and Nef (Nef.113-128) proteins; the restriction molecules for these peptides were HLA-B27 (30), HLA-A2 (31) and HLA-B17 + B37 (35), respectively. The last 3 peptides were H-2^d-restricted targets of murine CTL issued from the nucleoprotein of the Influenza A virus (N.147-158R$^-$) (25), the HIV-1 Env protein (Env.312-327) (33), the HLA-A2 molecule (A2.170-185) (28, 29), respectively.

As illustrated in Table 2, in all cases, a significant reactivity of each peptide with its restriction molecule was observed. Also, and according to our previous report (18), HLA-A2 bound more efficiently peptide M.Y$^+$57-68 than the natural peptide M57-68. We verified that all the material retained

Table 2. *Direct Binding of HLA Class I Molecules to Peptides Described as Target for MHC Class I–restricted CTL*

Peptide	Restricting element	HLA bound (cpm) A2	B27	B37
M.57-68	HLA-A2	926	677	924
M.Y$^+$57-68	HLA-A2	9,150	2,073	2,908
N.335-349Y$^+$	HLA-B37	5,200	658	2,175
Gag.265-279	HLA-B27	24,010	37,590	23,000
Gag.418-433	HLA-A2	633	0	294
Nef.113-128	HLA-B17,-B37	0	0	701
N.147-158R$^-$	H-2 K^d	0	0	0
Env.312-327	H-2 D^d	22,800	23,241	21,066
A2.170-185	H-2 K^d	0	0	0

Table 3. *Direct Binding of HLA Class I Molecules to Peptides Bearing MHC Class II–associated Epitopes*

Peptide	Restricting element	HLA bound (cpm) A2	B27	B37
Env.584-604	HLA.II*	1,200	1,081	2,206
H.305-329	HLA.DR*	15,898	3,915	3,050
H.130-142	Ia^d	0	0	0
λR.12-26	Ia^d	6,807	354	943
Env.105-117	Ia*	0	0	0
Env.428-445	Ia*	2,600	0	0
Lys.46-61	Ia^k	2,709	0	516

Wells of microtiter plates were coated with 5 μg/ml of peptide and 1.5 × 10^5 cpm of iodinated HLA molecules were added. Each value represents the average of three independent experiments.
* Nonprecisely identified restricting element. The results corresponding to the restricting HLA molecule are underlined in Table 2.

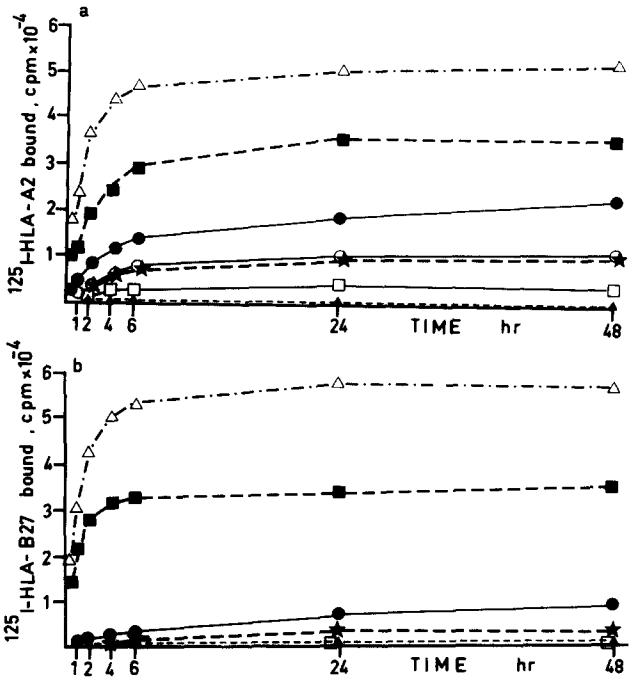

Figure 1. Time course of peptide/HLA association. Synthetic peptides (10 μg/ml) were immobilized onto microtiter plate wells as indicated in Materials and Methods. The buffers used were respectively: PBS, pH 7.4, for peptides M.Y$^+$57-68 (●) and M.57-68 (○), PBS, pH 5.0, for peptides N.335-349Y$^+$ (★). N.147-158R$^-$ (▲), and Gag.418-433 (□) and carbonate-bicarbonate buffer, pH 9.6, for peptides Gag.265-279 (△) and Env.312-327 (■). Binding of iodinated HLA-A2 (*a*) -B27 (*b*) molecules (2 × 10^5 cpm) was determined by radioactivity counting.

Table 4. *Concentration of Soluble Peptide Required for 50% Inhibition of HLA/Peptide Association*

Competitor peptide	Restricting element	HLA/Peptide association		
		HLA-A2 + M.Y⁺57-68	HLA-B27 + Gag.265-279	HLA-B37 + N.335-349Y⁺
		μM	*μM*	*μM*
M.57-68	HLA-A2	10	30	10
M.Y⁺57-68	HLA-A2	0.1	3	5
N.335-349Y⁺	HLA-B37	0.3	3	10
Gag.265-279	HLA-B27	30	10	E
Gag.418-433	HLA-A2	30	>100	E
Nef.113-128	HLA-B17-37	30	30	>100
N.147-158R⁻	H-2 K^d	N	N	N
Env.312-327	H-2 D^d	>100	N	N
A2.170-185	H-2 K^d	N	N	N
Env.584-604	HLA-Cl II*	30	30	100
H.305-329	HLA-Cl DR*	>100	N	N
H.130-142	Ia^d	N	N	N
λR.12-26	Ia^d	N	N	N
Lys.46-61	Ia^k	100	100	100
Env.105-117	Ia*	3	100	10
Env.428-445	Ia*	10	30	100

Results are expressed as μM of competitor peptide giving 50% inhibition of binding. Wells were coated with 5 μg/ml M.Y⁺57-68, in PBS, pH 7.4, 5 μg/ml Gag.265-279 in buffer pH 9.6, and 10 μg/ml N.335-349Y⁺ in PBS pH 5. Maximum response obtained for HLA-A2/M.Y⁺57-68 was 8,000-12,000 cpm., for HLA-B27/Gag.265-279, 30,000-40,000 cpm, and for HLA-B37/N.335-349Y⁺, 6,000-8,000 cpm. N, No competitor effect; E, enhancement of binding.
* Nonprecisely identified restriction molecule.

on peptides had the migration pattern of HLA class I molecules by using IEF electrophoresis (not shown). Peptides such as M.57-68, M.Y⁺57-68, N.335-349Y⁺, or Gag.265-279 had a high binding efficiency and in addition to their restriction molecule bound also other class I molecules. In contrast, less efficient peptides interacted only or preferentially with their restriction molecule. Interestingly, peptide N.335-349Y⁺, which was HLA-B37-restricted, interacted more efficiently with HLA-A2 than with HLA-B37 molecules, but still reacted very weakly with HLA-B27 molecules. Among the three peptides described as targets for murine CTL and restricted by H-2K^d or H-2D^d, only Env.312-327 bound very efficiently the three HLA class I molecules tested.

Table 3 shows typical results obtained with seven peptides described as targets for MHC class II–restricted T cells. Peptides H.305-329 and Env.584-604 corresponded to the hemagglutinin of influenza virus and the HIV-1 Env protein, respectively. These two peptides containing epitopes recognized by human CD4⁺ T cells bound efficiently the three HLA class I molecules tested. The five other peptides were recognized by murine T cells in the context of an Ia molecule (see Table 1) and three of them associated with HLA molecules.

Altogether, these experiments showed, on the one hand, that the ability for a given peptide to bind several HLA molecules is a frequent event, and, on the other hand, that the peptides known as targets for murine T cells reacted less efficiently with HLA molecules than peptides bearing human T cell–reactive epitopes.

Time Course of Antigenic Peptide/HLA Complex Formation. To study the kinetics of formation of peptide/HLA complex in solid phase, iodinated HLA-A2 and -B27 molecules were tested for their ability to interact with immobilized peptides between 30 min and 48 h of incubation at 20°C. Fig. 1 shows that most of the binding occurred at 6 h, although a plateau was not reached before 24 h in most cases. The equilibrium level varied considerably depending on the peptide, and to a lesser extent, on the HLA class I molecule (Fig. 1). In these solid phase conditions, the dissociation rate of peptide/HLA complexes was very low for all the molecules tested. This rate did not exceed 20% after 24 h of incu-

bation either in the presence of the relevant solubilized peptide or without addition of peptide (not shown).

Inhibition by Antigenic Peptides of Different Peptide/HLA Class I Molecule Associations. Since the PBA might be influenced by altered peptide conformation after immobilization on glutaraldehyde-treated microtiter plate wells, false-negative results might be due to this alteration. For this reason, quantitative IPBA was performed systematically to compare the ability of peptides to inhibit three different HLA/peptide associations. We tested the HLA/peptide associations of HLA-A2/M.Y$^+$57-68, HLA-B27/Gag.265-269, and HLA-B37/N.335-349 Y$^+$.

All peptides described as targets for HLA class I–restricted CTL had an efficient competitive activity in at least two of the three HLA/peptide systems tested. The HLA-A2-restricted peptides M.57-68 and M.Y$^+$57-68 and the HLA-B37-restricted peptide N.335-349Y$^+$ were particularly efficient competitors in all three systems (Table 4). Some peptides had variable competitive activities: also, we observed that the HLA-B27-restricted peptide Gag.265-279 and the HLA-A2-restricted peptide Gag.418-433 did not inhibit the HLA-B37/N.335-349 Y$^+$ association, but gave strong enhancements of binding at concentrations varying from 0.3 to 10 μM. Interestingly, the HLA-B17 and B37-restricted peptide, Nef.113-128, competed more efficiently in systems involving HLA-A2 or B27 molecules than in the HLA-B37/N.335-349Y$^+$ system. The three murine class I–restricted peptides did not inhibit the HLA/peptide interactions with the exception of a weak inhibition ($\sim$30%) obtained with 100 μM of Env.312-327 on the HLA-A2/M.Y$^+$57-68 association. Finally, among the seven class II–restricted peptides tested, two did not compete, two competed weakly, and three peptides derived from the HIV-1 Env protein sequence competed in the three HLA/peptide associations with different efficiencies.

Association of Antigenic Peptides with HLA Class II Molecules. Since peptides described as targets for class II–restricted CD4$^+$ T cells bound HLA class I molecules, we examined whether peptides described to be class I–restricted CTL targets could also bind HLA class II molecules. 14 antigenic peptides were tested in the PBA, and in the IPBA using the HLA-DRw13/H.305-329 association as reference system (Table 5). Peptides that had a high ability to bind HLA class I molecules in the previous experiments (H.305-329, Env.312-327, M.Y$^+$57-68, or Gag.265-279) also bound efficiently class II molecules (Table 4). Peptides that had a low interaction with class I molecules (Env.428-445 or λR.12-26) also had low interaction with class II molecules. The two peptides shown to be HLA-DR-restricted, H.305-329 and Env.584-604, bound the two HLA-DR molecules tested in the PBA, whereas Ia-restricted peptides either were negative or reacted only with HLA-DR2. Using the IPBA, we found that the antigenic peptides acting as efficient competitors on HLA class I/pep-

Table 5. *Interaction of HLA-Class II Molecules with Peptides Described as Targets for CD4$^+$ or CD8$^+$ T Cells*

Peptide	Restricting element	PBA HLA bound (cpm) DR2	PBA HLA bound (cpm) DRW13	IPBA HLA-DRw13/H.305-329
				μM
H.305-329	HLA-DR	3,900	3,147	50
Env.584-604	HLA-Cl II	600	1,308	10
Env.105-117	Ia	0	0	50
Env.428-445	Ia	806	0	>100
Lys.46-61	Ia^k	0	0	30
λR.12-26	Ia^d	1,200	0	N
H.130-142	Ia^d	0	0	N
Env.312-327	H-2 D^d	4,806	11,911	N
A2.170-185	H-2 K^d	0	0	N
M.Y$^+$57-68	HLA-A2	4,162	4,943	0.1
N.335-349 Y$^+$	HLA-B37	390	897	1
Gag.265-279	HLA-B27	12,300	22,580	10
Gag.418-433	HLA-A2	0	0	100
Nef.113-128	HLA-B17 + B37	0	0	3

IPBA, Results are expressed as concentration (μM) of peptide giving 50% inhibition of binding on the HLA-DRw13/H.305-329 association. The maximum response was 4,500 cpm.
N, No competitor effect.

tide associations were also efficient competitors on the HLA-DRw13/H.305-329 association.

Systematic Attempts to Detect HLA Class I Binding to HIV Peptides. All the peptides used above have been shown previously to serve as targets in either class I- or class II-restricted T cell responses. We also tested a more important panel of HIV peptides regardless of their ability to serve as T cell targets. 24 HIV peptides not yet described to be T cell antigens were randomly chosen. Using the PBA, 14 of these peptides (58%) did not bind the HLA molecules tested (Table 6). Peptide Vpr.55-68 reacted weakly with both HLA-B37 and DRw13. The nine remaining peptides reacted with all three HLA molecules tested. Using the IPBA, six peptides had competitor activity in the three HLA/peptide associations tested: Nef.137-145, Vif.1-15, Vif.60-72, Rev.18-30, Vpr.55-68, and Vpr.68-80. Peptides Vif.25-40 and Nef.132-

Table 6. *Binding of HLA Class I or Class II Molecules to HIV Peptides not Described as T Cell Targets*

HIV Peptide	PBA: HLA bound A2	PBA: HLA bound B37	PBA: HLA bound DRw13	IPBA: HLA-A2 + M.Y$^+$ 57-68	IPBA: HLA-B37 + N.335-349Y$^+$	IPBA: HLA-DRw13 + H.305-329
	cpm			*μM*	*μM*	*μM*
Nef						
66-80	0	0	0	N	N	N
79-94	0	0	0	N	N	N
132-147	18,504	31,882	29,883	*	E	10
137-145	6,638	5,718	2,515	10	3	0.1
160-174	0	0	0	N	N	N
Vif						
1-15	3,100	3,714	3,468	3	10	0.1
25-40	9,368	31,500	27,595	30	E	1
46-60	0	0	0	N	N	N
60-72	350	3,588	2,326	30	100	30
71-85	0	0	0	N	N	N
Rev						
1-16	0	0	0	N	N	N
18-30	0	0	0	100	100	10
37-50	6,150	10,262	13,729	>100	E	>100
Vpr						
1-14	0	0	0	N	N	N
55-68	0	722	437	3	10	100
68-80	40,768	34,031	24,356	1	10	0.1
Gag						
50-65	0	0	0	N	N	N
205-219	0	0	0	N	N	N
219-233	0	0	0	N	N	N
378-391	30,874	15,596	34,278	N	N	N
Env						
474-489	0	0	0	N	N	N
510-521	0	0	0	N	N	N
827-843	0	0	0	N	N	N
846-860	5,400	2,402	944	>100	N	N

The IPBA results are expressed as concentration (μM) of peptide giving 50% inhibition of HLA/reference peptide binding. Maximum response for HLA-A2/M.Y$^+$57-68 was 8,000–12,000 cpm, for HLA-B37/N.335-349Y$^+$, 6,000–8,000 cpm and for HLA-DRw13/H.305-329, 4,000–5,000 cpm. N, No competitor effect; E, enhancement of binding.

* Peptide 132-147 gave a maximal inhibition of 30% at 10 and 100 μM.

147 had either competitor or enhancer activity depending on the system tested. Peptide Rev.37-50, which had a high binding in the PBA, had either a very low competitor activity or an enhancing effect in the IPBA. Also, peptide Env.846-860, competed poorly in one association only. The other 14 peptides (58%) did not compete at all.

Using Table 7, one can figure the different possible patterns of reactivity obtained in the PBA and in the IPBA with this panel of HIV peptides in comparison with previously described antigenic peptides. All the peptides bearing human T cell epitopes interacted with HLA molecules, whereas 62% of the peptides bearing murine T cell epitopes and only 46% of randomly selected HIV-1 peptides did.

Correlation between the Competitor Activity of Peptides in IPBA and their Ability to Inhibit CTL-mediated Cytolysis. The results obtained in the IPBA strongly suggest that different peptides compete for the same binding site on HLA molecules. To determine the functional significance of these data, nine non-HIV antigenic peptides and eight HIV peptides not described as antigens were tested for their ability to inhibit the cytolytic interaction between anti-influenza A CTL and cells incubated with the relevant target peptide. As shown in Table 8, in 15 of these 17 peptides a good correlation was observed between the competitor activity detected in the IPBA and the blocking effect observed in the cytolytic test. Discrepancies were observed in only two cases: (*a*) Peptide Lys.46-61 partially inhibited the binding in the IPBA but did not block the CTL induced lysis; (*b*) peptide Gag.265-279 gave puzzling results: it enhanced the lysis by relevant CTL of HLA-B37$^+$ cells incubated with N.335-349Y$^+$, and in agreement with these data, this peptide provided enhancement in the IPBA. However, when tested on the HLA-A2/M.Y$^+$57-68 association, the same peptide enhanced the lysis, although it had either a low competitor activity or an enhancing effect in the IPBA.

Discussion

In the present study we identified general characteristics of the physical interactions between antigenic peptides and HLA class I molecules. Using a recently described method (18), we found that the kinetics of binding of HLA molecules varied with the peptide studied and were similar to or faster than those observed with murine class II molecules by using equilibrium dialysis (39). In addition, the resulting HLA class I/peptide complexes were extremely stable. We also found that the binding efficiency of HLA molecules was variable: <1% for most peptides, but reaching 6% in some cases, and even 15–25% in the cases of peptides Gag.265-279, Env.312-327, or Vpr.68-80. These numbers were unusually high when compared with bindings obtained with HLA class I molecules by using other methods (17). Nevertheless, our results were in agreement with the recently reported data of Demotz et al. (40) demonstrating that 10–40% of I-E^d molecules could be occupied by naturally processed peptides of hen egg lysozyme. Since, during our experimental conditions, purified HLA molecules were most likely occupied by endogenously bound peptides, as reported for murine class II molecules (41), such high levels of specific binding were surprising. This could be due either to the fact that 25% of HLA class I molecules might be naturally free of peptides, and/or to the fact that a proportion of endogenous peptides could be readily substituted. Another possibility would be that exogenous peptides bind HLA molecules already occupied by endogenous peptides, although this would be in opposition to the crystallography data of HLA-A2 molecule (10, 11) according to which there is only a single peptide binding site per molecule.

Our data obtained with peptides derived from the same region of a given protein and varying in length, indicated the importance of the size of the peptide in binding. Thus peptide Nef.132-147 bound very efficiently in the PBA, although this peptide poorly inhibited the HLA-A2/M.Y$^+$57-68 association, or did not at all inhibit the HLA-B37/N.335-349 Y$^+$ association. In contrast, the shorter analogue of this peptide, Nef.137-145, had a low level of binding in the PBA and a strong inhibitory activity in the IPBA. The immobilization of this latter nine residue long peptide onto microplates might have modified its structure and reactivity. Nevertheless, the inability of the larger peptide to compete in the IPBA remains to be elucidated.

The different patterns of HLA/peptide interaction observed in the PBA and IPBA showed that 9 of 24 (38%) HIV-1 peptides not yet described as antigens reacted in both assays, whereas 1 of 24 (4%) bound only in the PBA, and the same proportion reacted only in the IPBA. When testing a panel of 156 HIV-1 peptides, including a minority of previously

Table 7. *Patterns of Reactivity*

	Pattern 1	2	3	4	
Peptide	PBA + IPBA +	PBA + IPBA −	PBA − IPBA +	PBA − IPBA −	Reactive peptides (number tested)
Human CD4$^+$ or CD8$^+$ epitopes	8	0	0	0	8/8 (100%)
Murine CD4$^+$ or CD8$^+$ epitopes	2	2	1	3	5/8 (62%)
Randomly chosen HIV peptides	9	1	1	13	11/24 (46%)

described T cell epitopes, the numbers observed were 44 of 156 (28%), 17 of 156 (11%), and 12 of 156 (8%), respectively (Choppin, J., F. Nartinon, F. Connan, N. Pauchard, E. Gomard and J. P. Levy, manuscript submitted for publication). The differences observed between the numbers obtained with the two assays can be due to: (*a*) conformational alterations of peptides immobilized onto the microplate wells which might result in false-negative results in the PBA; (*b*) to fixation of the peptide on the HLA molecule at a position which is different of that of the reference peptide used in the IPBA and might result in non-inhibitory effect. This simultaneous fixation of two peptides might increase the capacity of interaction of the HLA molecules with the reference peptide by conformational effect and explain the enhancing effect observed with certain peptides as reported also by Bhayani and Paterson (42) in functional assays; (*c*) to a peptide/peptide interaction which might also allow the fixation of two peptides on a single HLA molecule and give false-negative results in the IPBA. We are currently investigating these possibilities.

In our experiments, a high binding in the PBA (>6%) correlated to the presence of positive charges. Thus, peptides giving the highest bindings, such Gag.265-279, Env.312-327, Nef.132-147, Vif.25-40, Rev.37-50, Vpr.68-80, or Gag.378-391, have three to eight positive charges in their sequences. In contrast, a competitor activity in the IPBA did not correlate to the presence of positive charges, but rather was dependent on the presence of several hydrophobic residues. Bogen and Lambris (43) have indicated the importance of positive charges for the binding of peptides to I-E^d molecules and suggested the implication of such charged residues in salt bridges established with the negative charges of MHC molecules. Initial formation of such salt bridges would then be followed by formation of a stable binding between the hydrophobic residues of a peptide and the underlying β strands. In peptides comprising numerous positive charges, the initial interaction would be enhanced, whereas the second step would not take place and fixation would be unstable in soluble phase. Alternatively, such peptides might preferentially react with HLA molecules after immobilization onto microplates.

Among the 40 peptides tested eight of eight described as targets for human T cells (either $CD8^+$ or $CD4^+$), 5 of 8 described as targets for murine T cells and 11 of 24 of randomly chosen HIV peptides evidenced positive binding to HLA molecules. Using 36 peptides derived from HIV-1 nef, preliminary results showed that the binding was also correlated to the recognition of peptide by human T cells. Our results suggest a preferential association of human antigenic peptides with HLA molecules and they also agree with the well-known observation, that, in most cases, human and murine CTL do not recognize identical epitopes in a single viral

Table 8. *Relationships between Peptide Competitor Activity in IPBA and Inhibition of CTL-mediated Lysis*

	HLA-A2/M.Y$^+$ 57-68		HLA-B37/N.335-349Y$^+$	
Peptide	CTL blocking	IPBA	CTL blocking	IPBA
	μM		*μM*	
M.Y$^+$ 57-68	ND	0.1	3	3
Nef.137-145	3	10	1	3
Vpr.68-80	10	1	30	10
Vif.1-15	30	3	3	3
Vpr.55-68	30	3	50	10
N.335-349Y$^+$	50	0.3	ND*	10
Vif.60-72	50	30	100	100
Rev.18-30	100	100	100	100
Nef.113-128	>100	100	>100	>100
Env.312-327	>100	>100	N	N
Nef.79-84	N	N	N	N
N.47-158R$^-$	N	N	N	N
Env.827-843	N	N	N	N
A2.170-185	N	N	N	N
λR.12-26	N	N	N	N
Gag.265-279	E	30 or E	E	E
Lys.46-61	N	100	N	100

Results are expressed as concentration (μM) of competitor peptide giving 50% or inhibition in CTL lysis and IPBA. ND, Not done: this test could not be performed since the competitor peptide was similar to the sensitizing one. N, No effect; E, enhancement.

protein. Moreover, it must be noted that among the murine T cell target peptides, Env.312-327 which strongly bound HLA molecules, was also described as a human T cell target (44).

In most cases, peptides identified as targets for human antiviral CTL reacted preferentially with their restriction HLA molecule in the PBA. This preference was particularly obvious when using certain low binders, but was not frequently observed in the IPBA. The fact that antigenic peptides were either strong or weak binders might be due to different alignments within the binding site of HLA molecules as suggested by others (45, 46). Altogether, our results indicated that ability of a given peptide to bind HLA molecule was a prerequisite for antigenicity but this binding ability alone could not explain the restriction phenomenon. As already suggested (7, 17, 18), the restriction must be determined at multiple levels, and predominantly at the level of the T cell repertoire. Our results are also in agreement with several reports describing the binding of certain antigenic peptides to several murine or human MHC class II molecules (5–8, 17, 27, 42). It was likely that interactions between antigenic peptides and HLA molecules not described as restricting element were not artefactual since an excellent correlation was found between the inhibitory effect of a given peptide in the IPBA and its ability to block cytolytic responses. Moreover, such a correlation was also found between the enhancing effect of a peptide in the IPBA and the cytolytic assay.

The PBA and IPBA might then be useful to select potentially immunogenic peptides according to their ability to interact with HLA molecules. Now, it would be interesting to compare the results obtained in the PBA and IPBA with theoretical model predictions of peptide T immunogenicity previously described (4, 31, 33, 47, 48) for an accurate determination of antigenic peptides.

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