An HLA Class I Peptide-Binding Assay Based on Competition for Binding to Class I Molecules on Intact Human B Cells Identification of Conserved HIV-1 Polymerase Peptides Binding to HLA-A*0301

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ABSTRACT: A peptide-binding assay employing the HLA class I molecules on intact human B cells is described. The peptide antigens are stripped from the HLA class I molecules by mild acid treatment, after which the cells are incubated with a FL-labeled reference peptide together with different concentrations of the peptide of interest. The effectiveness by which the latter peptide competes for binding to the HLA class I molecules is assayed by measuring the amount of HLA-bound FL-labeled reference peptide with FACscan analysis. The assay is easy to perform because there is no need to purify HLA class I molecules, or to transfect cells with HLA class I molecules, and no radioactive label is used. Moreover, large panels of HLA-typed human B-cell lines are available as tools for peptide binding to a vast array of HLA molecules.

The binding assay was optimized and validated with peptides of known binding capacity to either HLA-A*0201 or HLA-A*0301. The kinetics of peptide binding in this assay were shown to be comparable to that in assays employing soluble HLA class I molecules. Application of the assay in the search for potential HLA-A*0301 restricted CTL epitopes, derived from HIV-1 polymerase, resulted in the identification of five high-affinity binding peptides. *Human Immunology 44, 189–198 (1995)*

ABBREVIATIONS

B-LCL	B-lymphoblastoid cell line
BSA	bovine serum albumin
CTL	cytotoxic T lymphocyte
ER	endoplasmic reticuium
FI	fluorescence index
FL	4-(iodoacetamido)fluorescein
HIV	human-immunodeficiency virus
HPV	human papiiloma virus
IC_{50}	concentration of peptide needed to inhibit binding of the FL-labeled peptide to 50%
IMDM	ISCOVE's modified Dulbecco's medium
MF	mean fluorescence
MHC	major histocompatibility complex
PBA196	PBS + BSA 1%
PBS	phosphate-buffered saline

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INTRODUCTION

Proteins in the cytosoi are processed into peptides and then transported to the endoplasmic reticuium (ER) where they bind to major histocompatibility complex (MHC) class I molecules. The peptide–class I complexes assembled in the ER are then transported to the cell

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surface [1], where they are presented to CD8 + cytotoxic T lymphocytes (CTLs) [2–4].

Several features of MHC class I presentation have now been elucidated. Allele-specific motifs predictive of peptide binding to MHC class I, based on the presence of amino acids at anchor positions in the peptide, were defined by the analysis of naturally processed peptide pools eluted from class I molecules [reviewed in 5, 6] and peptide-binding studies [7, 8]. The structures of several MHC class 1 molecules were studied by x-ray crystallography which revealed that these anchor amino acids engage specific pockets in the MHC class I molecule [reviewed in 9]. The elucidation of these allele-specific MHC class I peptide-binding motifs and the definition of size requirement for peptide binding [10–12] facilitate the identification of CTL epitopes in proteins of interest [13, 14]. The approach is based on scanning the amino acid sequences of virus-derived proteins or tumor-specific proteins for the presence of potential class I binding peptides [15].

Peptide-binding assays employ either cell-bound MHC class I molecules [16–21] or purified "cell-free" MHC class I molecules [22–25]. Assays relying on cell-bound MHC class I molecules are based on upregulation [17, 18, 21] or reconstitution of MHC class I molecules [19, 20] as detected by MHC class I conformation-specific antibodies. Cell-free systems are quantitative and make use of purified MHC molecules, to which labeled reference peptides are bound in a competition set-up [25]. Purification of MHC class I molecules, however, is laborious and conformational changes may occur during purification and/or storage.

In the present study we report the utilization of fluorescein (FL)-labeled reference peptides that bind to HLA class I molecules on HLA-homozygous B cell lines, of which the bound peptides have been removed by mild acid treatment. We show that the binding of FL-labeled peptides to these peptide-stripped HLA class I molecules is specific and allows the semiquantitative determination of the binding capacity of peptides. The kinetics of peptide binding to these peptide-stripped HLA class I molecules is comparable to that of soluble HLA class I molecules and independent of biosynthesis of new HLA class I molecules. We applied this new assay to identify potential HLA-A*0301 restricted conserved CTL epitopes derived from human immunodeficiency virus (HIV)-1 polymerase.

MATERIAL AND METHODS

Cell lines. The Epstein-Barr Virus-transformed B-lymphoblastoid cell lines (B-LCLs) used for the competition assays are JY (HLA type: A*0201, B7, Cw7, DR4, DRw6, DPw2) and EKR (HLA type: A3, B7, DR7, DQw2).

The B-LCLs used to confirm specific binding of reference peptides are B109, BRM, D100, D110, K97, ML, NL, P98, S59, and S99. The HLA type of these cell lines is given in Fig. 1.

Peptides. FL-labeled reference peptides were synthesized as Cys-derivative. Labeling was performed with 4-(iodoacetamido)fluorescein (Fluka Chemie AG, Buchs, Switzerland) at pH 7.5 (Na-phospate in water/acetonitrile 1:1). The labeled peptides were desalted over Sephadex G-10 and further purified by C18 RP-HPLC. Labeled peptides were characterized by MALDI-MS (Lasermat, Finnigan, UK). The reference peptide used for HLA-A*0301 binding was KVFPC(FL)ALINK (MH^+_{calc} = 1521.8, MH^+_{meas} = 1521.4); the reference peptide for HLA-A*0201 was FLPSDC(FL)FPSV (MH^+_{calc} = 1500.6, MH^+_{meas} = 1500.1).

The reference peptides used for binding to HLA-A*0301 or HLA-A*0201 were published by Sette et al. [25]. In both peptides these investigators introduced a tyrosine which they used to tag a radioactive label to the peptide. We have substituted this tyrosine for a cysteine. The cysteine allowed the conjugation of 4-(iodoacetamido)fluorescein. The polymerase amino acid sequences of 14 different full-length sequenced HIV-1 virus strains, LAI, MN, NL43, OY1, SF2, RF, MAL, D31, CAM1, HAN, ELI, NDK, JRCSF, and JRFL [26], were screened for possible HLA-A*0301 restricted CTL epitopes using a scoring system [15]. The HLA-A*0301 motif used was based on the studies of Kubo et al. [8] and Engelhard [5]. At the anchor at position 2 a L, I, V, or M was preferred and at the C-terminal anchor a K, R, or Y was preferred. Peptides were synthesized that contained the mentioned residues at both anchor positions and were completely conserved among all 14 HIV-1 strains.

Peptides were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422, Langenfeld, Germany) using Fmoc-chemistry. Peptides were analyzed by reverse-phase HPLC, dissolved in 20 µl dimethyl sulfoxide (DMSO), diluted in 0.9% NaCl to a peptide concentration of 5 mg/ml, and stored at —20°C before usage.

Mild acid treatment of B-LCLs. Mild acid treatment of HLA-A2 or HLA-A3 on B-LCLs was performed according to Bremers modification [manuscript submitted] of the procedure of Storkus et al. [20]. Briefly, cells were washed twice with phosphate-buffered saline (PBS) and then put to rest on ice for 5 minutes. The cells were then treated 90 seconds with ice-cold citric acid Na_2HPO_4 buffer (mixture of an equal volume of 0.263 M citric acid and 0.123 M Na_2HPO_4) [27]. For HLA-A3 the buffer was adjusted to pH = 2.9 and to pH = 3.2 for HLA-

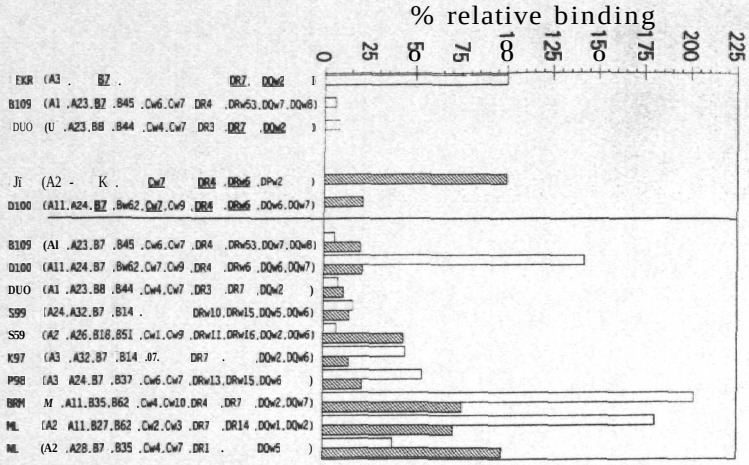

FIGURE 1 Specificity of FL-labeled reference peptides. Reference cell line EKR (HLA-A*0301) was mild acid treated at pH = 2.9. The reference cell line JY (HLA-A*0201) was mild acid treated at pH = 3.2, and the 10 different other B-LCL lines were mild acid treated at pH = 2.9, when subjected to incubation with the HLA-A*0301 FL-labeled reference peptide, or at pH = 3.2 when incubated with the HLA-A*0201 FL-labeled reference peptide. EKR cells are incubated with 150 nM of the HLA-A*0301 FL-labeled reference peptide *(open ban)*, JY cells are incubated with 150 nM of the HLA-A*0201 FL-labeled reference peptide (*hatched ban)*, and the 10 different other B-LCL lines were incubated with 150 nM of either the HLA-A*0301 *(apen bars)* or HLA-A*0201 FL-labeled reference peptide (*hatched barf),* for 4 hours at 26°C. The FI was calculated for each cell line and the FI of FL-labeled reference peptide bound to EKR (for binding to HLA-A*0301) and the FI of FL-labeled reference peptide to JY (for binding to HLA-A*0201) was equated to 100% binding. By the formula: (FI cell line/FI reference cell line) X 100% the relative peptide-binding percentages of the 10 different B-LCL lines were calculated. The *upper left side* shows the full HLA-type of the reference cell lines together with the overlapping HLA-type of other cell lines. The *lower left side* shows all 10 B-LCL lines with their full HLA-type.

A2; these pH differences are essential for optimal elution of bound peptides and reconstitution of the MHC class I molecule with the exogenous added peptide [Bremers, manuscript submitted]. Immediately thereafter the eluted cells were buffered with cold ISCOVE's modified Dulbecco's medium (IMDM), washed with IMDM, and resuspended at 700.000 cells/ml in IMDM + 1.5 μg/ml β_2 microglobulin (Sigma, St. Louis, MO, USA).

Peptide competition assay. For competition assays, 25 μl FL-labeled reference peptide (end concentration: 150 nM in PBS) was incubated with 25 μl competitor peptide (different end concentrations in PBS) in a 96-well U-bottom plate (Costar, Cambridge, MA, USA). A total of 100 μl of the mild acid treated B-LCLs (A2:JY, A3:EKR) was added to these wells.

The mixture was incubated for 3 or 24 hours at 4°C or 26°C, washed twice with PBS containing 1% bovine serum albumin (BSA) (PBA 1%), resuspended in PBA 1% containing 0.5% paraformaldehyde, and analyzed at a FACscan (Becton-Dickinson, Etten-Leur, the Netherlands).

The mean fluorescence (MF) value obtained in the experiment without competitor peptide was regarded as maximal binding and equated to 0% inhibition; the MF obtained from the experiment without reference peptide was equated to 100% inhibition.

Percent inhibition of binding was calculated using the following formula:

[1 — (MF 150 nM reference and competitor peptide — MF no reference peptide) ÷ (MF 150 nM reference - MF no reference peptide)] X 100%

In experiments where no competitor peptide was added the fluorescence index (FI) was calculated to indicate how much fluorescence above the background (no reference peptide) was measured. The FI = (MF sample — MF background)/MF background.

To block protein synthesis in B-LCLs a final concentration of 100 μM emetine (Sigma) was used, as shown previously [27].

RESULTS

Sensitivity and specificity of FL-labeled reference peptides binding to HLA class I. The reference peptides binding to HLA-A*0201 and HLA-A*0301 were described and used in a molecular binding assay by Sette et al. [25] In both peptides a tyrosine was used to tag a radioactive label to the peptide. We substituted this tyrosine with cysteine, to which 4-(iodoacetamido)fluorescein was conjugated.

The amount of fluorescent peptide needed for the competition assay was established. For this purpose a peptide titration was performed. After incubation of 3 hours at 26°C the MF was measured. At concentrations from 2 nM to 100 nM for the HLA-A*0201 reference peptide and from 2 nM to 150 nM for the HLA-A*0301 reference peptide a sharp increase in MF was found (data not shown). Mild acid treatment of the B cells before incubation with FL-labeled reference peptide resulted in a higher fluorescence maximum and also sharper increase of the MF at low peptide concentrations (Fig. 2).

In order to investigate if aspecific peptide binding to cell components, including other HLA class I alleles, at the surface of the cell line used occurred, 10 different B-LCL cell lines were incubated with 0 or 150 nM of FL-labeled reference (either HLA-A*0201 or HLA-A*0301) peptide. The FI for each cell line was calculated and the FIs obtained for reference cell lines JY (binding of peptide to HLA-A*0201) and EKR (binding of peptide to HLA-A*0301) were equated to 100% binding. To relate the binding of FL-labeled reference peptide to the 10 different cell lines with the binding of the FL-labeled reference peptide to JY or EKR, the relative peptide-binding percentages were determined. The relative peptide-binding percentages of the FL-labeled reference peptides to each cell line were calculated as: (FI cell line/ FI reference cell line) X 100%. For both FL-labeled reference peptides the nonspecific binding to other cell components, of the cell lines used in the competition assay, never exceeded 20% (Fig. 1). Because the peptide binding motif of HLA-A*0301 is very similar to the binding motif of HLA-A11 [8], binding of the HLA-A*0301 FL-labeled reference peptide to B-LCL cell lines expressing this allele was also observed (Fig. 1). The cell line NL binds the HLA-A*0301 FL-labeled reference peptide. It expresses the HLA-A28 allele of which two subtypes, HLA-Aw6801 and HLA-Aw6803, shire the peptide-binding motif with HLA-A*0301 [A. Sette, personal communication].

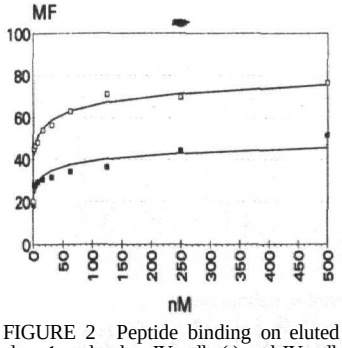

FIGURE 2 Peptide binding on eluted vs noteluted HLA class 1 molecules. JY cells (•) and JY cells with HLA class I molecules mild acid treated (D) were incubated with increasing amounts (nM) of the HLA-A*0201 FL-labeled peptide. Cells were incubated for 3 hours at 26°C, washed, and MF was measured at a FACScan. The lines shown are the result of logarithmic regression analysis of the concentration of FL-labeled reference peptide versus the MF.

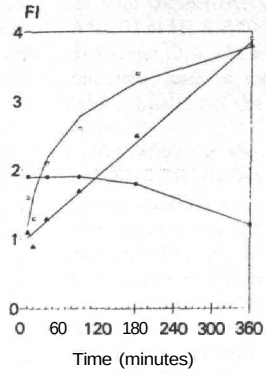

FIGURE 3 Kinetics of peptide binding to mild acid treated HLA class I molecules. EKR cells were mild acid treated and incubated with 150 nM of HLA-A*0301 FL-labeled reference peptide for different periods of time at 4°C (A), 26°C (D), or 37°C (•). At 10, 20, 40, 90, 180, and 360 minutes the binding of FI-labeled peptide was measured. Binding is given as the fluorescence index (FI). The lines shown for 4°C and 26°C are the result of linear or logarithmic regression analysis, respectively.

Kinetics of peptide binding to mild acid treated HLA class 1 molecules. To study the effect of peptide binding at different temperatures, EKR cells were eluted and incubated with FL-labeled peptide for different periods of time at 4°C, 26°C, or 37°C. At 4°C the peptide binds rapidly initially and then increases steadily in time (Fig. 3). Peptide binding at 26°C is faster (Fig. 3). The amount of peptide bound after 6 hours at 26°C did not differ from the amount of peptide bound at 4°C. Peptide binds fast at 37°C but no increase of bound peptide is found when incubated longer (Fig. 3). The tack of increase in bound peptide at 37°C is probably due to two

phenomena. The HLA class I molecules, present on the surface of the cell to which no peptide was bound, disintegrate at this temperature [21]. Second, the dissociation of peptides is dramatically faster at 37°C compared to the dissociation of peptides when incubated at 4°C [23].

Binding to mild acid treated class I molecules is not dependent on de novo protein synthesis. To characterize the interaction of peptides with cell-associated mild acid treated HLA molecules, peptide-stripped EKR cells were incubated with FL-labeled peptide for different periods of time at 4°C or 26°C. As shown in Fig. 4, the fluorescent labeling at 4°C of the cells steadily increases in time. The use of 100 μM protein synthesis inhibitor emetine for 1 hour prior to elution decreased the amount of peptide bound at 26°C but not at 4°C (Fig. 4).

Thus, the binding of a peptide to mild acid treated HLA class I molecules at 4°C was unaffected by the use of a protein-synthesis-inhibiting drug. Because metabolic processes are reduced at 4°C, the binding of peptides to the eluted HLA class I molecules is only dependent on the availability of the HLA class I molecules already present at the outer surface of the cell.

Competition assay. Plotting MF against the concentration of FL-labeled reference peptides resulted in a log-shaped curve. We chose 150 nM of FL-labeled reference peptide as standard concentration in all competition experiments. The use of 150 nM FL-labeled reference peptide resulted in a MF of about 4 to 5 times the background (not shown). The nonlabeled reference peptide was titrated into 150 nM of FL-labeled reference peptide, the percentage inhibition was calculated and plotted against the concentration of the unlabeled peptide (Fig. 5). In a 24-hour competition assay at 4°C the nonlabeled HLA-A*0201 or HLA-A*0301 reference peptide needed about 3-5 times (0,4 μM and 0,7 μM, respectively) the concentration used of the FL-labeled reference peptide to inhibit binding of the FL-labeled peptide to 50% (IC_{50}) (Table 1).

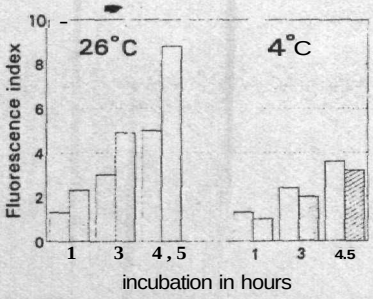

FIGURE 4 Binding of FL-labeled pepcide to protein-synthesis-inhibiting, drug-treated cells. EKR cells were treated with 10^{-4} M emetine (*open bars*) or not (*hatched bars*), for 1 hour prior to mild acid treatment [27]. Then, 150 nM of HLA-A*0301 FL-labeled reference peptide was added and binding was monitored at 1, 3, or 4.5 hours of incubation. Cells were incubated at 26°C or 4°C.

To determine the optimal experimental conditions and to validate the assay we tested peptides derived from human papilloma virus (HPV)16 E6 and E7 proteins with known binding properties to HLA-A*0201 or HLA-A*0301 [28, 29] at different concentrations, for 3 or 24 hours at 4°C or 26°C (Table 1). When the cells were incubated for 24 hours less peptide was needed (Table 1). The lowest amount of competitor peptide was needed when the cells were incubated for 24 hours at 4°C (Table 1). No difference was observed between an incubation time of 24 hours or 48 hours at 4°C (not shown). This implicates that the test is more sensitive when equilibrium is reached. Probably, due to a faster association of the FL-labeled reference peptide, more competitor peptide is needed to reach IC_{50} in short incubations. Ranking the peptides to their IC_{50} shows that when the cells are incubated at 4°C for 24 hours, their order is comparable to that found by Kast et al. [28] using the molecular binding assay (Table 1). All peptides that did not possess the described binding motif showed low binding affinity. Taken together these results and the results of peptide-binding to HLA class I molecules on emetine-treated cells, we conclude that the competition assay is best performed at 4°C with an incubation time of at least 24 hours.

Competition with known CTL epitopes. Five HLA-A*0201-restricted CTL epitopes, one HLA-A*0301-restricted CTL epitope, and two HLA-A*0301 peptides, identified via peptide pool-sequencing, were used to determine the IC_{50} values of high affinity binding peptides. The five peptides tested for binding to HLA-A*0201 all competed very well with an $IC_{50} \leq 1.7$ μM (Table 2). The known HLA-A*0301-restricted CTL epitope derived from HIV was tested. This peptide, derived from HIV-nef, bound with an IC_{50} of 0.5 μM. The two peptides, which were identified via peptide pool sequencing bound with an $IC_{50} \leq 15$ μM (Table 2). We therefore conclude that peptides competing with an $IC_{50} \leq 15$ μM must be considered potential CTL epitopes.

*Binding of conserved HIV-1 pal sequences to HLA-A*0301.* Twenty peptides 8–11 amino acids long were selected on the basis of the HLA-A*0301 binding motif and their conservation in the polymerase gene products of different HIV-1 strains. The peptides were tested in the competition assay for 24 hours at 4°C. Nine peptides

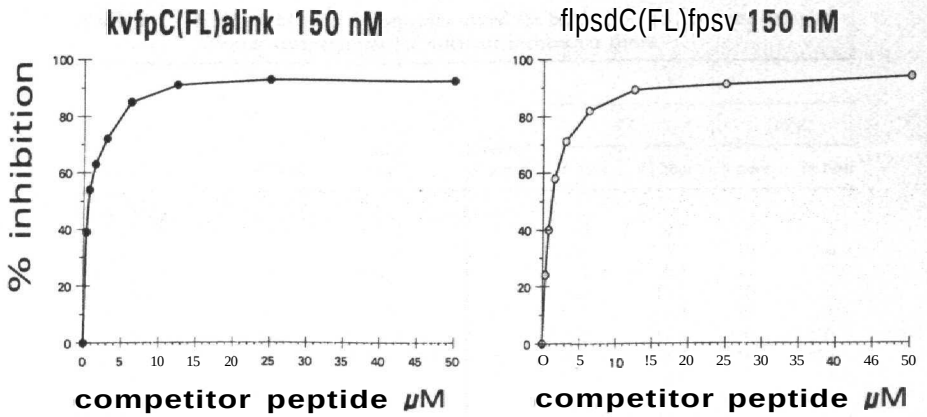

FIGURE 5 Competition of nonlabeled reference peptide with FL-labeled reference peptide. EKR cells (left) or JY cells (right) were incubated with 150 nM of FL-labeled reference peptide, kvfpC(FL)alink or flpsdC(FL)rpsv, respectively, and increasing amounts (μM) of nonlabeled reference peptide. Inhibition of binding was calculated and is shown in relation to the amount of nonlabeled reference peptide used.

were shown to bind to HLA-A*0301. Four peptides bind with intermediate affinity and competed with an $IC_{50} \leq 15$ μM (Table 3); the other five peptides (marked with an asterisk) bind with high affinity and competed with an $IC_{50} \leq 2.9$ μM. Considering the IC_{50} obtained with the known CTL epitopes, these five peptides may be candidate CTL epitopes.

DISCUSSION

In recent years various assays have been developed to identify potential CTL epitopes in proteins of cellular or viral origin [16–25, 37–40]. Peptides with a high capacity to bind to a MHC class I molecule may be immunogenic whereas low-affinity binding peptides are not [29, 41]. We report an assay that employs the HLA class 1 molecules present on human B cells and show that the kinetics of binding of peptides to these HLA class I molecules is comparable to the kinetics observed with soluble HLA class ! molecules [42].

The FL-labeled reference peptides specifically bound to their HLA molecules and not to other components at the surface of the cell lines used for the assay (Fig. 1).

The use of a protein synthesis inhibitor decreased the amount of peptide bound at 26°C but not at 4°C (Fig. 4). This indicates that during incubation at 26°C novel MHC class 1 molecules reach the cell surface [21], to which peptides might bind. Due to the limiting amount of FL-labeled peptide only the competitor peptide will bind to the de novo produced HLA class I molecules and therefore a higher amount of competitor peptide is needed to compete in the assay at 26°C. We conclude that, because peptides bind at 4°C and emetine has no effect at this temperature, the binding of peptide to eluted HLA class 1 molecules occurs at 4°C and is not determined by metabolic processes.

Luescher et al. [42] reported that binding of peptide to peptide-loaded HLA molecules at the cell surface did not take place at 4°C and that binding of peptides was dependent on de novo produced HLA class I molecules. However, when these peptide-loaded HLA molecules were stripped from their peptides by mild acid treatment the FL-labeled reference peptides did bind at 4°C as shown in our study (Fig. 3). We observed that the fate of peptide upon incubation at different temperatures shows comparable characteristics to those seen with peptide binding to soluble MHC molecules [42]. We therefore conclude that the kinetics of peptide binding to acid-eluted cell-bound MHC molecules are comparable to those of peptide binding to soluble MHC molecules.

Validation of the assay shows that the assay is most sensitive when performed at 4°C with an incubation time of at least 24 hours. The ranking of the HLA-A*0201 and HLA-A*0301 motif-bearing peptides, according to their IC_{50}, is comparable to that seen by Kast et ai. [28]. When the HLA-A*0201 motif-bearing peptides are ranked to the IC_{50} reported by Sette et al. [29], ranking of the motif-bearing peptides is similar in our assay. The four peptides also tested for binding to HLA-A*0301 in the cell-free assay, which uses FL-labeled reference peptide [Drijfhout, manuscript in preparation], competed to the same extent in the cellular binding assay.

When the competition assay is performed for 3 hours at 4°C, the HPV16 peptides LLMGTLGIV (E7 82-90, HLA-A*0201), KLPQLCTEL (E6 18-26, HLA-A*0201) which is naturally processed [43], and

TABLE 1 HPV 16 E6 and *E1* peptides tested for binding in competition assay at different temperatures for different incubation times

Sequence[a]	HPV 16	IC_{50}[b] (nM)	Presence[c] of motif	IC_{50}[d] (μM) 4°C 3 hour	4°C 24 hour	26°C 3 hour	26°C 24 hour
HLA-A*0201							
TLGIVCPI	E7	7	+	3.9	0.7	6.5	3.1
LLMGTLGIV	E7	8/208	+	20	5	10	6.9
YMLDLQPETT	E7	46/33	+	2.9	0.7	11	7.3
TLGIVCPIC	E7	153	−	>25	15.7	29	10
KLPQLCTEL	E6	328/5556	+	>25	8.3	36	34.5
AMFQDPQER	E6	1818/>25000	−	>25	17.5	>50	>50
LQTTIHDII	E6	3157/>25000	−	>25	20.3	>50	>50
FLPSDCFPSV[e]			+	0.8	0.4	1	1.1
HLA-A*0301							
IVYRDGNPY	E6	10/660	+	2.1	0.8	2.5	2.5
HLDKKKQRFH	E6	68	+	1.9	1.7	9	17
AMFQDPQER	E6	290	+	7.6	3.3	12.5	15
TTLEQQYNK	E6	384	+	16.5	4.2	41	38
IVCPICSQK	E7	1111	+	4.1	3.9	24	24
AMSAARSSR	E6	4285/6600	+	4.3	4	6	7
QQLLRREVY	E6	5000/>6600	−	17	>25	>50	>50
KVFPCALINK[f]		-/660	+	0.5	0.7	3	4

[a] Amino acid sequence of the HPV peptides.

[b] Binding capacity (IC_{50}) to the given HLA class I molecule as tested in the molecular binding assay [28]. For some peptides two IC_{50} values are given: HLA-A*0201, che value at the *right of the backslash*, was reported in a later publication [29]; HLA-A*0301, the value at the *right side*, was determined using a molecular binding assay which employs the same FL-labeled reference pepcide as used in the cellular binding assay [Drijfhout, manuscript in preparation].

[c] The presence of the HLA-A*0201 or HLA-A*0301 binding motif in the peptide.

[d] Binding capacity of the peptides is shown as the concentration of peptide needed to inhibit binding of che FL-labeled peptide EO 50% (IC_{50} in μM).

[e] The nonlabeled reference peptides; che dash means that their IC_{50} in the molecular binding assay is not known.

TABLE 2 Binding capacity of known processed and presented peptides

Sequence[a]	IC_{50}[b] (nM)	IC_{50}[c] (μM)	Origin	Reference
HLA-A*0201				
FLPSDFFPSV	2.8	0.4	Hepatitis B nucleocapsid	30
GILGFVFTL	6	0.4	Influenza A matrix	31
ILKEPVHGV	242	1.7	HIV-1 RT	32
SLYNTVATL	—	1.3	HIV-1 gag	33
YMNGTMSQV	—	1.7	Tyrosinase	34
HLA-A*0301				
QVPLRPMTYK	11	0.5	HIV-1 nef	35
KLFNIMVTY	—	15	Unknown	36
KLHKQRAKS	—	12	Unknown	36

[a] Amino acid sequence of the peptides.

[b] Binding capacity (IC_{50} in nM) to the given HLA class I molecule as tested in che molecular binding assay [29].

[c] Binding capacity of the peptides in the present study is shown as the concentration of pepcide need to inhibit binding of the FL-labeled pepcide to 50% (IC_{50} in μM).

TTLEQQYNK (E6 93-101, HLA-A*0301) are measured as low-affinity binding peptides. However, these peptides display *a* higher affinity in a 24-hour assay. Obviously, these peptides have a low association rate and only at equilibrium do they show high-affinity binding. This means that these peptides must have a low dissociation rate at 4°C. What this observation means, in terms of eliciting a CTL response by the peptide in vivo, is still speculative. It could be that the immunologie importance of a peptide is dependent mainly on the dissociation rate of the peptide.

Two HLA-A*0301-restricted peptides, which were identified via peptide-pool sequencing, competed with an $IC_{50} \leq 15$ μM. Therefore peptides binding to HLA class I molecules with an $IC_{50} \leq 15$ μMare considered candidate CTL epitopes. All six known CTL epitopes, however, competed with an $IC_{50} < 2$ μM. In the search of potential CTL epitopes it is, therefore, best to focus on peptides that display binding affinities of approximately 2 μM.

The screening of HIV polymerase protein sequence for conserved sequences bearing the HLA-A*0301 motif re-

TABLE 3 Binding of conserved HIV-1 pol sequences compliant with the HLA-A*0301 binding motif

Sequence*	Position[b]	IC_{50} (μM)[c]
PISPIETVPVK	160–170	>100
PIETVPVKLK	163–172	>100
PIETVPVK	163-170	>100
PLTEEKIK	184–191	>100
AIKKKDSTK	221–229	1.0–3.0*
GIPHPAGLK	252-260	0.3–0.5*
SVTVLDVGDAY	264–274	>100
TVLDVGDAY	266–274	>100
VLDVGDAY	267-274	>100
NVLPQGWK	306–313	30.0–40.0
WMGYELHPDK	388-397	14.0–20.0
ELELAENR	459–466	>100
ELAENREILK	461–470	14.0–20.0
QLDCTHLEGK	781-790	8.5-10.0
AVHVASGY	795–802	21.5-25.0
QVRDQAEHLK	883–892	2.9–3.0*
AVFIHNFKR	898–906	0.3–0.5*
GIGGYSAGER	909–918	6.5-10.0
KIQNFRVYY	938-946	1.8–2.5*
KIQNFRVY	938-945	70.0–90.0

[a] Amino acid sequence of conserved peptides derived from HIV-1.

* Position of first and last amino acid in HIV-1 polymerase derived from strain JR-CSF.

[c] Peptides were tested in the competition assay at 4°C with an incubation rime of 24 hours. The binding capacity of the peptides is shown as the range of the concentration of peptide needed to inhibit binding of the FL-labeled peptide to 50% (IC_{50} in μM). Peptides marked with a *asterisk* (*) are considered to be potential CTL epicopes.

suited in the selection of 20 peptides, five of which were shown to bind with a binding affinity comparable to that of already known epitopes. These peptides are considered potential CTL epitopes and their immunogenicity is currently under investigation.

The system developed has a number of advantages compared to previously described assays. Native HLA class I molecules present on the surface of B-LCLs are used and the binding of peptides is measured via FACS analysis. The FACscan system is very sensitive and allows the measurement of low amounts bound FL-labeled peptide (Fig. 2), also in short incubations. The system is relatively simple because there is no need to purify soluble HLA class I molecules or to transect these molecules in cells and no radioactive label is used. Although the data of the assay when it is compared to the molecular assay is compressed, and therefore discrimination between high-affinity and very high-affinity binding peptides is more difficult, peptides are categorized in the same ranking order. Furthermore, in contrast to other cellular peptide-binding assays, no monoclonal antibodies are needed to detect peptide binding to HLA class I. Moreover, large panels of HLA-typed B-cell lines are available for measurement of peptide binding to a vast array of HLA molecules. Presently the system is also used successfully for the HLA class 1 molecules HLA-A1 and HLA-B7

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