Immunogenicity of Peptides Bound to MHC Class I Molecules Depends on the MHC-Peptide Complex Stability[1]

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The impact of the MHC class I peptide binding stability on the immunogenicity of particular peptide Ags in class I-restricted cytotoxic T lymphocyte responses is not clearly established. Therefore, we have determined the dissociation rate of each peptide from MHC class I at 37°C and compared this to that of a consensus CTL epitope. Newly defined immunogenic peptides formed relatively stable MHC-peptide complexes as shown by their low dissociation rates, whereas nonimmunogenic peptides displayed high dissociation rates. In addition virtually all previously described HLA-A*0201-restricted T cell epitopes showed low dissociation rates. Furthermore, we show that the immunogenicity of HIV-1-derived peptides can be predicted more accurately by their dissociation rate than by the MHC class I binding affinity. Selection of peptides based on affinity and their dissociation rate leads to a more precise identification of candidate CTL epitopes than selection based on affinity alone. These results help to understand why some peptides are recognized by CTL and, along with detailed knowledge of protein processing rules, therefore have important implications for the selection of peptides in peptide-based vaccines. *The Journal of Immunology,* **1996, 156: 3308–3314.**

The protective role of CTL against tumors and viruses has recently been reviewed (1–3). CTL recognize peptide fragments of cellular or viral proteins bound in the antigen-presenting groove of MHC class I molecules (4–8). In several animal models MHC class I binding peptides can induce protective CTL-mediated immunity against certain viruses (reviewed in Ref. 1). Therefore prediction of potential CTL epitopes has important implications for preventive and/or therapeutic applications in human diseases.

Although many factors influencing MHC class I-restricted CTL responses to peptides have been elucidated, it is still not clear why only a few peptides are selected for recognition by CTL despite the presence of other putative antigenic peptides within the same Ag. One important parameter appears to be the binding affinity of the peptide. In a large group of peptides binding with high and intermediate affinity to MHC class I molecules in mouse (9) and human (10, 11) most, but not all, peptides were immunogenic. Peptides binding with lower affinity to MHC class I may form less stable MHC-peptide complexes leading to decreased immunogenicity (10). Indeed, in the MHC class I-restricted response to OVA, the dominance and subdominance of two epitopes was not determined by a difference in T cell precursor frequency but was due to a difference in binding affinity and dissociation rate between both epitopes (12). Likewise the immunogenicity of at least some peptides binding to MHC-class II was determined by their ability to stabilize the class II molecule in SDS-containing solutions, which correlated with the dissociation rate of the peptide (13).

The binding affinity of peptides to MHC molecules at equilibrium is the result of the continued association and dissociation of the trimolecular complex of peptide, MHC class I molecule, and β_2m.[3] The dissociation rate of peptides bound to MHC class I is influenced neither by the presence of competing peptides (14) nor by the concentration of the competing peptides (15). On the other hand, the amount of free MHC peptide binding sites is influenced and limited by the dissociation rate of previously bound peptide (15). Thus a peptide with a low dissociation rate will, once bound, probably form a stable MHC-peptide complex in the endoplasmic reticulum, be transported to the cell surface, and persist there for a time sufficient to allow T cell recognition.

To support this hypothesis we have determined the dissociation rate of a group of MHC class I-binding peptides, focusing on the correlation between peptide binding affinity and immunogenicity on the one hand and between the dissociation rate of peptide from MHC class I molecules on the other hand. Immunogenicity appears to correlate better with the dissociation rate than with peptide binding affinity.

Materials and Methods

Cell lines

The EBV-transformed B cell line JY (HLA type: A*0201, B7, Cw7, DR4, DRw6, DPw2) was cultured in complete culture medium consisting of RPMI 1640 Dutch modification (Life Technologies, Paisley, Scotland) supplemented with 10% FCS, antibiotics (100 IU/ml penicillin (Brocades Pharma, Leiderdorp, The Netherlands) and 100 μg/ml kanamycin (Sigma

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[3] Abbreviations used in this paper: HBV, hepatitis B virus; HCV, hepatitis C virus; HPV16, human papillomavirus type 16; HPV11, human papillomavirus type 11; HTLV1, human T cell lymphotropic virus type 1; E6 and E7, early region 6- and 7-encoded proteins; pol, polymerase; NP, nucleoprotein; NS3 and NS4, nonstructural region 3 and 4; LMP2, latent membrane protein 2; β_2m, human β_2-microglobulin; IC_{50}, 50% inhibitory concentration; DT_{50}, time required for 50% of the molecules to decay; HBVc18–27, hepatitis B virus core peptide amino acids 18 to 27; FL, fluorescein; MF, mean fluorescence.

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Chemical Co., St. Louis, MO)), and 20 μM 2-ME (Merck, Darmstadt, Germany) at 37°C in humidified air containing 5% CO_2.

Jurkat A*0201K^b cells are stable transfectants of the human T cell leukemia line, Jurkat, which express the product of the HLA-A*0201K^b chimeric gene (16). They are cultured in complete culture medium in the presence of 200 μg/ml G418 sulfate.

Peptides

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 of DMSO, diluted in 0.9% NaCl to a peptide concentration of 5 mg/ml, and stored at −20°C before usage.

Fluorescein (FL)-labeled peptides as used in the competition-based HLA class I binding assay were synthesized, labeled, and characterized as described earlier (17). The sequence of the reference peptide used for HLA-A*0201 was FLPSDYFPSV (18, 19) wherein we substituted the tyrosine with a cysteine to tag a fluorescein group to the peptide: FLPSDC(FL) FPSV (17).

Transgenic mice

HLA-A*0201K^b transgenic mice were kindly provided by Dr. L. Sherman (Scripps Laboratories, San Diego, CA, through animal distributor Harlan Sprague-Dawley, Inc., Indianapolis, IN). Mice were held under clean conventional conditions. The transgenic mice express the product of the HLA-A*0201K^b chimeric gene in which the α3 domain of the heavy chain is replaced by the corresponding murine H-2 K^b domain while leaving the HLA-A*0201 α1 and α2 domains unaffected (16). This allows the murine CD8 molecule on the murine $CD8^+$ T cells to interact with the syngeneic α3 domain of the hybrid MHC class I molecule.

In vivo immunizations and murine T cell cultures

Groups of three to six HLA-A*0201K^b transgenic mice were injected s.c. in the base of the tail with 100 μg of peptide emulsified in IFA in the presence of 140 μg of the H-2 I-A^b-restricted HBV core antigen-derived T helper epitope (128–140; sequence TPPAYRPPNAPIL) (20). After 11 days, mice were killed and spleen cells (30×10^6 cells in 10 ml) were restimulated in vitro with syngeneic-irradiated LPS-stimulated B cell lymphoblasts (ratio 3:1), and 1 μg/ml peptide in complete culture medium in T25 flasks (Falcon; Becton Dickinson, Franklin Lakes, NJ). At day 6 of culture, the cytotoxicity of these bulks was tested in a standard 5-h ^{51}Cr release assay.

^{51}Cr cytotoxicity assay

CTL activity was measured in a standard chromium release assay as described previously (21). Target cells were sensitized with 10 μg/ml peptide for 30 min at 37°C. Target cells were added to various numbers of effector cells in a final volume of 100 μl of complete culture medium in 96-well U-bottom microtiter plates. After 5 h of incubation at 37°C, supernatants were harvested. The mean percentage specific lysis of triplicate wells was calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100$$

The percentage of specific lysis is expressed in $LU_{30}/10^6$ cells, in which 1 LU_{30} corresponds to the number of effector cells required to induce 30% ^{51}Cr release from 2000 Jurkat A *0201/K^b target cells during a 5-h assay.

Peptide "stripping" by mild acid treatment

JY cells were washed twice with PBS and then put to rest on ice for 5 min. Ten million cells were then treated for 90 s with 2 ml ice-cold citric acid-Na_2HPO_4 buffer (a mixture of an equal volume of 0.263 M citric acid and 0.123 M Na_2HPO_4), pH 3.2 (17, 22). Immediately thereafter the eluted cells were buffered with cold Iscove's modified Dulbecco's medium (IMDM), washed with IMDM, and resuspended at 10^6 cells in IMDM with 1 μg/ml β_2m (Sigma Chemical).

Competition-based HLA class I peptide-binding assay

Peptides were tested for their binding affinity using the previously described peptide-binding assay (17). Briefly, cells were stripped (see above) but resuspended at 7×10^5 cells/ml in IMDM + 1.5 μg/ml β_2m. A FL-labeled reference peptide, 25 μl (end concentration, 150 nM), was incubated with 25 μl of competitor peptide (different end concentrations) in a 96-well V-bottom plate (Costar, Cambridge, MA). One hundred microliters of mild acid-treated JY cells were added to these wells. The mixture was incubated for 24 h at 4°C, washed twice with PBS containing 1% BSA (PBA1%), resuspended in PBA1% containing 0.5% paraformaldehyde, and analyzed by FACScan (Becton Dickinson, Etten-Leur, The Netherlands).

The mean fluorescence (MF) value obtained in the wells without competitor peptide was regarded as maximal binding and equated to 0% inhibition; the MF obtained from the wells without reference peptide was equated to 100% inhibition. Percentage 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 peptide − MF no reference peptide)) × 100%

The relative binding affinity of the peptide is expressed as peptide concentration needed to inhibit 50% of the binding of the reference peptide (IC_{50}). Affinity is categorized as follows: high $IC_{50} < 5$ μM, intermediate >5 μM $IC_{50} < 15$ μM, or low $IC_{50} > 15$ μM. Peptide HIV-1*pol*-468 bound not with high (17) but with intermediate binding affinity.

Measurement of MHC-peptide complex stability at 37°C

JY cells at a concentration of 1 to 2 million cells/ml were incubated with 10^{-4} M emetine (Sigma Chemical) for 1 h at 37°C to stop protein synthesis and thus the emergence of de novo synthesized class I molecules at the cell surface (22). Cells were washed twice with PBS and peptide stripped (see above). One million cells were added to 200 μg of peptide in 1 ml and incubated for 1 h at room temperature. Cells were washed twice with ice-cold IMDM and resuspended in 1 ml IMDM. Subsequently, the cells were incubated for 0, 2, 4, and 6 h at 37°C and thereafter stained with BB7.2, an HLA-A2 conformation-specific mAb (23), and GaM/FITC. Thereafter the cells were fixed by resuspension in PBA1% containing 0.5% paraformaldehyde. Cells were analyzed by FACScan. The fluorescence index (FI) was calculated as FI= (mean fluorescence sample − mean fluorescence background) ÷ mean fluorescence background (without peptide). Samples were tested in duplicate and the variation between both samples was always less that 10%.

The percentage of residual HLA-A2 molecules was calculated by equating for each peptide, the FI of $t = 0$ to 100% and then using the formula: % remaining = ($FI_{t=n} \div FI_{t=0}$) × 100%. Because the dissociation of peptides from MHC is a linear process, the stability of the peptide-MHC complexes was measured as the time required for 50% of the molecules to decay (DT_{50}). We have used $t = 2$ h at 37°C as starting point for the reason that from this time point only the DT_{50} are determined from peptides that are able to form stable peptide-MHC complexes.

Statistics

Using Fisher's test for 2 by 2 tables (Fisher's exact two-tailed test), the dissociation rate (DT_{50}) of peptides at 37°C was correlated to the immunogenicity of the peptides. Binding affinity could not be correlated to immunogenicity using a χ^2 test due to the relatively small number of peptides. Therefore we compared high affinity binding peptides with low affinity binding peptides to establish the strongest correlation between affinity and immunogenicity using Fisher's test for 2 by 2 tables.

Results

*Stability of MHC class I molecules complexed with HBV- or HPV16-derived peptides of known binding affinity and immunogenicity in HLA-A*0201/K^b transgenic mice*

To study the relation between dissociation of peptides bound to MHC class I molecules and their ability to induce a CTL response, we used nine peptides derived from HBV polymerase (pol) and eight peptides of HPV16 of which the relative binding affinity and immunogenicity in HLA-A*0201/K^b transgenic mice was reported previously (10, 11, 24). To show that all 17 peptides indeed bound to HLA-A*0201, we tested their affinity in a previously described competition-based HLA-class I binding assay (17). HBV*pol*-635, HPV16*E7*-11, and HPV16*E7*-86 bound with relatively high affinity (<5 μM). Fourteen peptides bound with intermediate (between 5 and 15 μM) or low affinity (> 15 μM (Table I). Peptide binding affinities measured and classification of the peptide binding affinity into high, intermediate, and low are comparable to the affinities and classifications of Sette et al. (10) and Kast et al. (24).

Table I. *Comparison of the immunogenicity of HBV- or HPV16-derived peptides to the dissociation rate*

Peptide	Amino Acid Position	Sequence	Affinity		Immunogenicity	Stability (DT_{50})
			IC_{50} (nM)	IC_{50} (μM)		
HBV Pol[a]	635–643	GLYSSTVPV	33	4.5[b]	+[c]	>4 h[d]
HBV Pol	1076–1084	HLYSHPIIL	38	8.0	+	>4 h
HBV Pol	1344–1352	WILRGTSFV	278	11.0	−	1 h
HBV Pol	996–1004	NLSWLSLDV	385	6.0	+	3 h
HBV Pol	992–1000	LLSSNLSWL	1,087	19.5	−	1 h
HBV Pol	985–993	NLQSLTNLL	2,000	22.0	−	NS
HBV Pol	43–51	HLLVGSSGL	2,778	24.0	−	<1 h
HBV Pol	28–36	LLDDEAGPL	>25,000	69.0	−	NS
HBV Pol	594–602	PLEEELPRL	>25,000	>100	−	NS
HPV16 E7	86–93	TLGIVCPI	7	0.7	+	>4 h
HPV16 E7	11–20	YMLDLQPETT	46	0.7	+	>4 h
HPV16 E6	52–60	FAFRDLCIV	130	9.0	−	2 h
HPV16 E7	7–15	TLHEYMLDL	188	5.0	−	2 h
HPV16 E7	82–90	LLMGTLGIV	208	5.0	+	>4 h
HPV16 E6	18–26	KLPQLCTEL	328	8.5	−	2 h
HPV16 E6	7–15	AMFQDPQER	1,818	17.5	−	NS
HPV16 E6	26–34	LQTTIHDII	3,157	20.5	−	NS

[a] Peptide origin, position of first and last amino acid, and the amino acid sequence and binding affinity as described previously (10, 24)
[b] Affinity was measured as described recently (17). IC_{50} represents the amount of peptide required for 50% inhibition of binding of the fluorescein-labeled reference peptide to HLA-A*0201.
[c] Immunogenicity of the peptide was determined by injection of peptide doses of 10- to 100-fold in excess of the amount required to elicit optimal CTL responses emulsified in IFA together with an equimolar amount of I-A^b T-helper epitope (10, 11): −, nonimmunogenic; +, immunogenic.
[d] DT_{50} is given starting from t = 2 h at 37°C. NS = nonstable: <10% of HLA molecules were detectable after a 2-h incubation at 37°C.

Table II. *Comparison of peptide binding affinity, dissociation rate, and immunogenicity of HBV- and HPV16-derived peptides*

Peptide Binding Affinity	Dissociation Rate (DT_{50})		Immunogenicity
	≥3 h	<3 h	
High	3	0	Immunogenic
	0	0	Nonimmunogenic
Intermediate	3	0	Immunogenic
	0	4	Nonimmunogenic
Low	0	0	Immunogenic
	0	7	Nonimmunogenic

Subsequently with the use of a conformation-specific anti-HLA-A2 Ab, the amount of residual HLA-A*0201 peptide complexes was monitored in time. The loss of peptide-stabilized HLA-A*0201 molecules at the cell surface represents the dissociation of the peptide from the class I molecule to which the peptide is bound. The stability is then presented by the time required for 50% of the molecules to decay (DT_{50}). All three high affinity binding peptides and three of the intermediate affinity binding peptides, HBV*pol*-996, HBV*pol*-1076, and HPV16*E7*-82 showed a $DT_{50} \geq 3$ h (Table I). The four other peptides of intermediate affinity, HBV*pol*-1344, HPV16*E6*-18, HPV16*E6*-52, and HPV16*E7*-7 showed a DT_{50} between 1 and 2 h (Table I). The low affinity binding peptides showed a DT_{50} of 1 h or less. In Table II we show a comparison between the dissociation rate, binding affinity, and immunogenicity of these peptides. All high affinity binding peptides form stable MHC-peptide complexes and are immunogenic, whereas the group of peptides of intermediate affinity contains either peptides that are immunogenic and form stable MHC-peptide complexes or are nonimmunogenic and do not form stable MHC-peptide complexes as shown by their high dissociation rates (Table II).

Stability of MHC class I molecules complexed with known human CTL epitopes

Seventeen HLA-A*0201-binding peptides earlier reported to be immunogenic (e.g. found as CTL epitope or capable of inducing a primary response) (20, 24–36) were tested for their binding affinity to HLA-A*0201. Eight peptides bound with high affinity, seven peptides bound with intermediate affinity, and two peptides bound with low affinity (Table III). The dissociation rates were determined and virtually all peptides showed a $DT_{50} \geq 4$ h, except for the peptides HPV11*E7*-4 and HIV-1*pol*-267. The HPV11*E7*-4 and HIV-1*pol*-267 CTL epitopes, both found by primary CTL induction using synthetic peptide or cells expressing extremely high amounts of Ag, dissociated faster ($DT_{50} \leq 2$ h (Table III). Interestingly, the sequence of the HCV1core-131 peptide [ADLMGYI PLV] does not correspond precisely to the HLA-A*0201 motif. The HCVcore-132 peptide that lacks the N-terminal alanine (DLMGYIPLV) fits better to the HLA-A*0201 motif. This is also reflected in the higher affinity of this shorter peptide (IC_{50} = 5.0 μM), but the peptide dissociates more quickly (Fig. 1) than the HCVcore-131 peptide.

Immunogenicity is correlated with the dissociation rate

A significant correlation exists between the immunogenicity of a peptide and the dissociation rate. Of the investigated known HLA-A*0201-restricted immunogenic peptides, 21 of 23 showed a $DT_{50} \geq 3$ h, while none of the 11 nonimmunogenic peptides showed a $DT_{50} \geq 3$ h (p = 0.0000003, Table IV). This correlation is closer than that between peptide binding affinity and immunogenicity (p = 0.0005, Table IV) and confirms the trend visible in Table II. When the correlation between immunogenicity and dissociation rate was investigated for peptides binding with intermediate or low affinity, this was still better correlated (p = 0.00007, Table V) to immunogenicity than affinity (p = 0.04). This implies that peptides that are processed, transported to the endoplasmic reticulum, and are able to form stable MHC-peptide complexes are likely to be CTL epitopes.

*Immunogenicity in HLA-A*0201/K^b transgenic mice of HIV-1-derived peptides with known affinity and dissociation rate*

To assess the in vivo immunogenicity of peptides of which the binding affinity and the dissociation rate was measured, HLA-A*0201/K^b transgenic mice were vaccinated with two control

Table III. *Stability of HLA-A*0201 complexed with known CTL epitopes*

Peptide	First aa Position	Sequence	Affinity (IC_{50} (μM))	Stability (DT_{50})	Immunogenicity
HCV1 core	131	ADLMGYIPLV[a]	50.0[b]	>4 h[c]	RC[d]
HCV1 core	178	LLALLSCLTV	7.5	>4 h	RC
HCV1 NS3	1406	KLVALGINAV	5.0	4 h	RC
HCV1 NS4	1789	SLMAFTAAV	1.5	>4 h	RC
HBV surface	335	WLSLLVPFV	1.0	>4 h	RC
HBV surface	348	GLSPTVWLSV	2.0	>4 h	RC
EBV LMP2	426	CLGGLLTMV	2.5	4 h	PR1
HTLV1 tax	11	LLFGYPVYV	0.8	>4 h	RC
HPV11 E7	4	RLVTLKDIV	52.0	2 h	PR2
INF B NP	85	KLGEFYNQMM	5.5	>4 h	CTL
INF A matrix	58	GILGFVFTL	0.6	>4 h	CTL
HIV-1 Gag	76	SLYNTVATL	1.5	>4 h	CTL
HIV-1 Pol	267	VLDVGDAYFSV	7.0	NS	PR3
HIV-1 Pol	468	ILKEPVHGV	8.0	>4 h	CTL
pmel17/gp100	[1]	YLEPGPVTA	8.5	4 h	CTL
pmel17/gp100	[2]	LLDGTATLRL	5.5	4 h	CTL
Tyrosinase	369	YMNGTMSQV	4.5	>4 h	CTL

[a] Peptide origin, position of first amino acid, and the amino acid sequence of the different HLA-A*0201-restricted CTL epitopes are given (20, 25–36).
[b] Binding affinity was measured as described recently (17). IC_{50} represents the amount of peptide required for 50% inhibition of binding of the fluorescein-labeled reference peptide to HLA-A*0201.
[c] DT_{50} is given starting from t = 2 h at 37°C. NS = nonstable, <10% of HLA molecules were detectable after a 2-h incubation at 37°C.
[d] RC, recall experiment wherein CTL already primed by viral infection of the patient in vivo were boosted in vitro with peptide to detect the precise epitope. All authors used similar protocols. CTL, peptides were used to identify the epitopes recognized by CTL that were obtained from patients. PR1, CTL were primed in vitro with an autologous EBV-transformed B cell line and then cloned, peptides were used to map the epitope recognized. PR2, CTL were induced in vitro using repeated stimulation with recombinant vaccinia virus-HPV11 E7-infected B cells. PR3, CTL were induced in vitro using repetitive stimulation with peptide pulsed APCs.

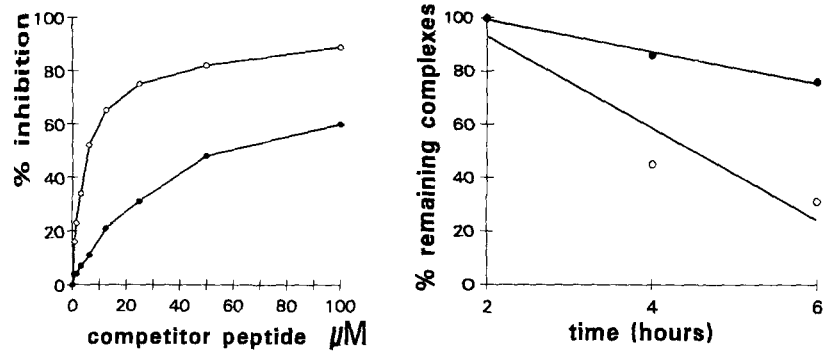

FIGURE 1. Binding affinity and dissociation rate of the HCV1core-131 peptide and the shorter variant without the N-terminal alanine. The binding affinity (*left*) and the dissociation rate (*right*) of the HLA-A*0201-restricted CTL epitope HCV1core-131 (●; ADLMGYIPLV) (25) and shorter variant (○; DLMGYIPLV), which corresponds more precisely to the HLA-A*0201 motif, was tested (see *Materials and Methods*). The mean inhibition of the reference peptide at each concentration of competitor peptide, obtained in two independent experiments, is shown at the left. The right figure shows the percentage of residual peptide-MHC molecules for both peptides at each time point (mean of two independent experiments). The percentage of molecules present at t = 2 h was set to 100%. The lines are the result of linear regression analysis.

Table IV. *Statistical analysis of the dissociation rate (DT_{50}) or binding affinity vs immunogenicity of HLA-A*0201-binding peptides*

	Immunogenic	Nonimmunogenic
$DT_{50} \geq 3$ h	21	0
$DT_{50} < 3$ h	2	11
		$p = 0.0000003$[a]
High affinity	11	0
Intermediate affinity	10	4
Low affinity	2	7
		$p = 0.0005$[b]

[a] Fisher's two-tailed exact test for 2 by 2 tables.
[b] The relation between binding affinity and immunogenicity was determined by comparison of the high affinity-binding peptides with the low affinity-binding peptides, using Fisher's two-tailed exact test for 2 by 2 tables.

Table V. *Statistical analysis of dissociation rate (DT_{50}) or binding affinity vs immunogenicity of peptides binding with intermediate or low affinity to HLA-A*0201*

	Immunogenic	Nonimmunogenic
$DT_{50} \geq 3$ h	10	0
$DT_{50} < 3$ h	2	11
		$p = 0.00007$[a]
Intermediate affinity	10	4
Low affinity	2	7
		$p = 0.04$[a]

[a] Fisher's two-tailed exact test for 2 by 2 tables.

peptides (HPV16*E7*-86 and HBV*core*-18; FLPSDDFPSV) and four HIV-1-derived peptides (Table VI). The derivation of these transgenic mice (16) and their use to analyze in vivo immunogenicity have been described previously (10, 11). The HIV-1*pol*-468; (ILKEPVHGV) is a CTL epitope and binds with intermediate affinity. The HIV-1*pol*-267;(VLDVGDAYFSV) peptide was found to be immunogenic in a human primary CTL induction after repetitive stimulations with relatively high doses of peptide (20). To test the predictive value of the in vitro-measured MHC-peptide complex stability, we determined the binding affinity and dissociation rate of the two other HIV-1*pol* peptides (HIV-1*pol*-343: YM DDLYVGSDL and HIV-1*pol*-576: LLWKGEGAV) (Table VI).

Table VI. *Immunogenicity of HIV-1-derived peptides with known dissociation rate tested in HLA-A*0201/Kb transgenic mice*

Sequence + Origin		Affinity (IC_{50} (μM))	Stability (DT_{50})	$LU_{30}/10^6$ Cells	CTL Response
FLPSDDFPSV	HBV*core*-18[a]	0.4[b]	>4 h[c]	53 (25–71)[d]	3/3[e]
TLGIVCPI	HPV16*E7*-86	0.7	>4 h	183 (70–400)	3/3
VLDVGDAYFSV	HIV-1*pol*-267	7.0	NS	<2	0/7
YMDDLYVGSDL	HIV-1*pol*-343	8.0	1 h	<2	0/3
LLWKGEGAV	HIV-1*pol*-576	6.0	>4 h	84 (67–100)	2/3
ILKEPVHGV	HIV-1*pol*-468	8.0	>4 h	56 (17–100)	5/6

[a] Peptide amino acid sequence, protein, and the position of the first amino acid of the different HLA-A*0201 binding potential CTL epitopes are given.
[b] Average binding affinity was measured as described recently (17). IC_{50} represents the amount of peptide required for 50% inhibition of binding of the fluorescein-labeled reference peptide to HLA-A*0201.
[c] DT_{50} is given starting from t = 2 h at 37°C. NS = nonstable: <10% of HLA molecules were detectable after a 2-h incubation at 37°C.
[d] Average of all mice and range of observed responses.
[e] Number of mice that mounted a peptide-specific CTL response per total mice vaccinated.

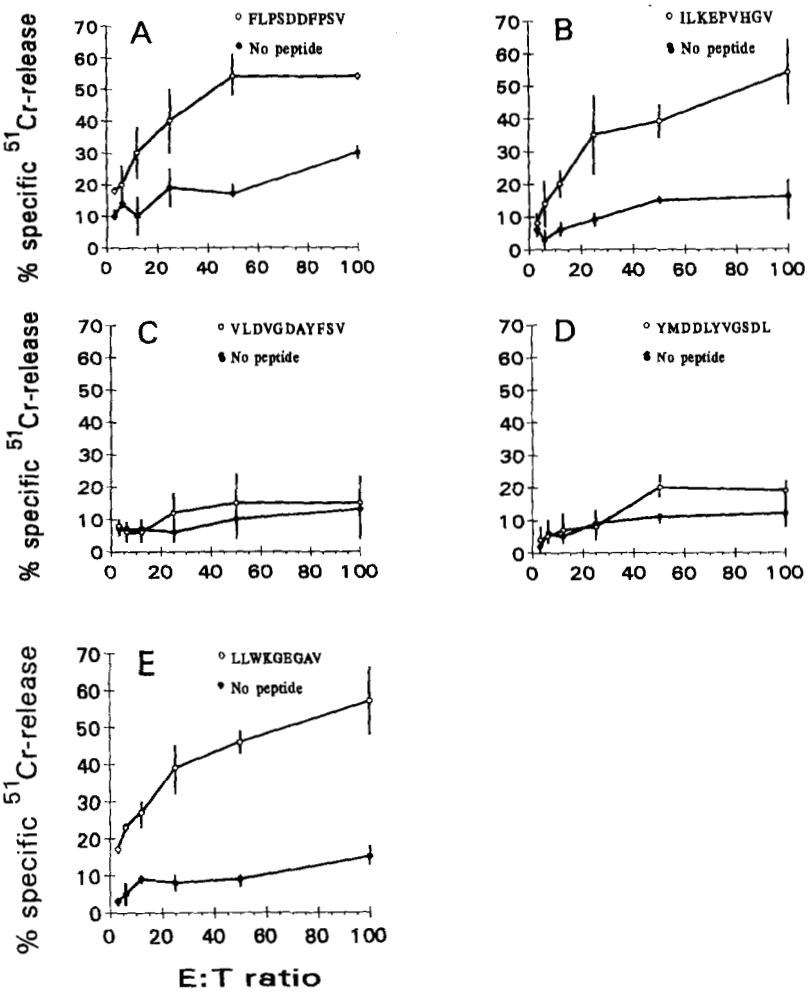

FIGURE 2. Peptide-specific cytotoxicity induced by vaccination of HLA-A*0201K^b transgenic mice. A representative experiment in which HLA-A*0201K^b transgenic mice were vaccinated with indicated peptide displaying a low dissociation rate (*A*, *B*, and *E*) or high dissociation rate (*C* and *D*) in combination with an HBV core-encoded T helper epitope in IFA (see *Materials and Methods*). Bulk CTL cultures derived from spleen cell of these mice were tested for peptide specificity in cytotoxicity assays on Jurkat A*0201K^b target cells pulsed with (○) or without (●) specific peptide. Shown is the mean specific lysis of bulk CTL from three to six animals with indicated standard deviation. Specific lysis is depicted at an E:T ratio varying from 1.5 to 100.

Both peptides were detected when the highly conserved regions of HIV-1*pol* were screened for amino acid sequences that contained two anchors for binding to HLA-A*0201, as described previously (20). We vaccinated groups of mice with all the peptides. Bulk CTL derived from mice vaccinated with the control peptides specifically lysed peptide-sensitized Jurkat A*0201/K^b cells (Fig. 2; Table VI). As expected, all peptides with a low dissociation rate mounted a CTL response (Fig. 2; Table VI), whereas the two peptides with high relative dissociation rates did not induce a CTL response (Fig. 2; Table VI). Thus, the immunogenicity of these peptides was perfectly predicted by their dissociation rates.

Discussion

The results of this study show that peptide-induced MHC stability and immunogenicity of the peptide are strongly correlated. We find a closer correlation between the dissociation rate of a peptide and immunogenicity (p = 0.0000003) than between binding affinity and immunogenicity (p = 0.0005). The better correlation is gained in the group of peptides that bind with intermediate or low affinity. Immunogenic peptides in this group are characterized by a low dissociation rate, meaning that they induce stable MHC-peptide complexes.

To study the impact of a stable MHC-peptide complex on its immunogenicity, we tested 34 peptides with known immunogenicity, determined either in HLA-A*0201/K^b transgenic mice and/or in humans, for their capacity to form stable MHC class I-peptide complexes at 37°C. All peptides with a high affinity for the MHC class I molecule have been reported to be immunogenic (10, 11) and the dissociation rates of these peptides were found to be low, as shown by a $DT_{50} \geq 4$ h. Furthermore, low or intermediate affinity MHC-binding peptides with a low dissociation rate were immunogenic, whereas those with a high dissociation rate were not. Moreover, selection of potential CTL epitopes based on binding affinity alone would have favored the HCV1core-132 (DLMGYIPLV) peptide, which corresponds more precisely to the HLA-A*0201 binding motif, over the HCV1core-131 (ADLM GYIPLV) peptide. The HCV1core-131 peptide, however, would be selected by its superior capacity to form stable MHC-peptide complexes, and this peptide was reported to be more immunogenic (25, 37) (A. Cerny, personal communication).

Therefore the dissociation rate can be used to predict the immunogenicity of candidate CTL epitopes. As shown in Table V, the dissociation rate of peptides binding with intermediate affinity correlated well with their immunogenicity, indicating that this is an important parameter for the prediction of their immunogenicity. Indeed peptides that formed stable MHC-peptide complexes were immunogenic in HLA-A*0201/K^b mice (Table VI), whereas peptides displaying high dissociation rates were not, although immunizing doses were used that were 10- to 100-fold in excess of what is required to elicit optimal CTL responses in a system for evoking CTL, in which help is not limiting (10).

The influence of temperature on binding affinity (14, 38) might explain why some peptides binding with intermediate affinity are nonimmunogenic. For technical reasons binding affinity is measured at nonphysiologic temperatures ($\leq$ 23°C) when the class I molecule is probably in an "open" conformation. The peptide will interact with sites at the peptide binding groove resulting in a conformational change of the molecule and a tight fit for the anchor residues. Without this conformational change a peptide will dissociate at 37°C (14). Still a substantial group of immunogenic peptides exists that bind with intermediate or low affinity. These peptides can differ from the group of peptides that bind with high affinity by either a lower association rate or a higher dissociation rate. Peptides that display a higher dissociation rate are probably nonimmunogenic, whereas those peptides with lower dissociation rates will be immunogenic.

A $DT_{50} \geq 3$ h as a cutoff point is based on the results of the first two series of immunogenic and nonimmunogenic peptides tested (Table I). From all the known CTL epitopes (Table III), three epitopes were found by primary CTL induction in vitro. All the others were found by culturing CTL obtained from patients. Two of these three immunogenic peptides displayed a $DT_{50} < 3$ h and seem to form an exception. The HIV-1*pol*-267 and HPV11*E7*-4 peptides formed unstable MHC-peptide complexes but were found to be immunogenic in a primary CTL response. Although both primary responses were induced in different ways, they were the result of repetitive stimulation with either exogenously added peptide or recombinant vaccinia-infected cells overexpressing the epitope. Vaccinia virus shuts down host protein synthesis and replicates in the cytoplasm. Therefore the high level of protein expressed by the recombinant vaccinia construct leads to high levels of peptide derived from this protein that will be presented in the class I molecules. In the case of nonlytic viruses, on the other hand, a relatively low amount of virus proteins must compete with host proteins for generation and presentation of peptides; thus, only peptides with low dissociation rates will be presented at the cell surface. A good example for this is the third epitope found by in vitro primary induction, the EBV LMP2 epitope (27). CTL were obtained by culturing PBMC with autologous EBV-transformed B cells. In this case, the peptides generated from EBV proteins must compete with those from host-derived proteins and indeed the peptide found shows a low dissociation rate. The HPV16*E6*-18 peptide earlier reported to be processed (39) was eluted from recombinant vaccinia-infected cells. This peptide, however, is not immunogenic in the HLA-A*0201/K^b mice and showed a high dissociation rate. Furthermore, other HPV16-derived peptides that displayed higher dissociation rates and are nonimmunogenic in the HLA-A*0201/K^b mice, are occasionally able to induce primary T cell responses but only after repetitive stimulation with high doses of exogenously added peptide (11). Thus, it is possible for T cells to react to unstable MHC-peptide complexes in such a way that they recognize the endogenously processed peptide when this particular epitope is expressed at high levels (20, 29), but in the case of tumor Ags and nonlytic viruses the in vivo relevance of such peptides is uncertain on the basis of our results.

We show that the in vitro dissociation rate is related to the immunogenicity of a peptide. Apparently this predicts whether a peptide can persist at the cell surface for a time sufficient to allow the induction of a CTL response. Unfortunately, it does not predict whether a peptide will be endogenously processed and presented in the context of MHC class I. This is dependent on the presence of enzymatic cleavage sites in the flanking sequences of the peptide as shown by Ossendorp et al.[4] They found that a single amino acid difference within a viral epitope caused the destruction of the epitope derived from a related virus due to specific proteasome cleavage. Both peptides showed the same binding affinity and both were immunogenic when injected as a synthetic peptide. Also of influence is the transport of peptides to the endoplasmic reticulum by TAP although human TAP molecules are reported hardly to select for peptides (40). There might also be a relation between the amount of peptides created and immunogenicity; it is conceivable that due to a limited amount of protein in the cytoplasm, not enough peptides are presented at the cell surface to induce a CTL response. If the rules for processing of peptides are more precisely known we will be able to even more accurately predict which protein-derived peptides will be in vivo relevant CTL epitopes and might be selected for use in peptide-based immunotherapeutic vaccines.

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