Journal of Immunotherapy
18(2):77–85 © 1995 Lippincott–Raven Publishers, Philadelphia

The Use of Epstein–Barr Virus-Transformed B Lymphocyte Cell Lines in a Peptide-Reconstitution Assay: Identification of CEA-Related HLA-A*0301–Restricted Potential Cytotoxic T-Lymphocyte Epitopes

*†‡André J. A. Bremers, *†Sjoerd H. van der Burg, *Peter J. K. Kuppen, †W. Martin Kast, *Cornelis J. H. van de Velde, and †Cornelis J. M. Melief

*Department of *Surgery and †Immunohematology and Blood Bank, University Hospital Leiden, Leiden: and ‡Department of Surgery, Leyenburg Municipal Hospital, The Hague, The Netherlands*

Summary: In the development of cytotoxic T lymphocyte (CTL)–mediated immunotherapy, the identification of CTL epitopes is of crucial importance. Binding of a peptide to major histocompatibility complex (MHC) class I molecules is one of the prerequisites for its function as a CTL epitope. We describe the technique, validation, and application of a simple cellular assay, intended for the screening of peptides for binding, that can be applied to any human leukocyte antigen (HLA) allele. Reconstitution of peptides in MHC class I molecules after elution by acid treatment was previously shown to be possible in specially engineered cell lines expressing only one type of MHC class 1, and was applied for the HLA-A*0201 allele. We now report the optimal conditions for application of this type of binding assay to the HLA-A*0301 allele. The adaptations that were necessary to make the technique operational for HLA-A*0301 are shown in detail. These consisted of lowering the pH during acid treatment to 2.9 and lengthening the duration of elution to 90 s. Furthermore, immediate aspiration of eluted peptides appeared to be essential for this allele. We found also that the use of Epstein-Barr virus (EBV)–transformed B cell lines (B-LCL) yields results similar to those of the use of cell lines expressing only one specific MHC class 1 allele. Homozygosity for the desired HLA allele improves the sensitivity of the assay, but heterozygous cells can also be employed. Finally, we applied this technique to a search for HLA-A*0301 binding peptides derived from carcinoembryonic antigen (CEA). Of a set of 34 CEA-specific peptides that fit with a specified HLA-A*0301-binding motif, we identified a set of six peptides with high binding affinity to this allele. These peptides can be regarded as potential CTL epitopes. Key Words: HLA-A*0301—CEA—Peptide binding—Elution—Binding affinity.

It is now generally agreed that only peptides with a high binding affinity to a certain MHC class I molecule can, potentially, function as a cytotoxic T lymphocyte (CTL) epitope (1-3). Both cellular and molecular binding assays have been developed to determine binding affinity of peptides to MHC class I molecules (4–11), each with its specific advantages and disadvantages, as reviewed subsequently. The peptide-reconstruction assay, as presented in this article, is the result of an effort to develop a simple assay combining the major advantages of the different assays.

Received April 27. 1995; accepted August 4, 1995.
Address correspondence and reprint requests to Prof. Dr. C. J. M. Melief, Department of Immunohematology and Blood Bank, Building 1 E3-Q, University Hospital Leiden, P.O. Box 9600, 2300 RC Leiden, The Netherlands.

The T2-binding assay, a simple cellular assay that measures upregulation of MHC class I on a peptide processing–defective cell line after overnight incubation with exogenous peptide, has been used successfully to detect peptides binding to human leukocyte antigen (HLA)-A*0201 (8,12,13). However, its use is limited to this allele, as it depends on the use of the processing-defective 174 CEM.T2 cell line. Although the HLA-A*0201 allele is one of the most frequently expressed class I alleles among the different ethnic populations (14), only inclusion of various other alleles will allow the development of CTL-mediated immunotherapy for the significant fraction of the population that does not express HLA-A*0201. Other assays are required that may be adopted for various HLA alleles to test peptide binding.

Recently a cellular binding assay was described (9) in which peptides presented in the MHC class I of C1R cell lines (immortalized B-cell lines modified to express only one HLA allele) were eluted according to a method first described by Sugawara et al. (15). Reconstitution of HLA-A*0201 was studied by staining with a monoclonal antibody against MHC class I, W6/32. This assay is simple to perform, and the principle can be applied to any HLA allele. However, its application is limited to those alleles of which a C1R transfectant is available.

A molecular binding assay has been described (11) in which peptides that are tested for binding to the purified class I molecules compete for this binding with specific radiolabeled standard peptides. As with other molecular binding assays (7), the principle can be employed for any class I molecule, but the assays involve the use of radioactive iodine and purified MHC class I molecules.

We developed a technique to use B-LCL lines for a cellular binding assay based on the same principle as previously described in the so-called peptide-reconstitution assay (9), because B-LCL lines expressing any desired HLA phenotype can be obtained rather easily by Epstein–Barr virus (EBV) transformation of peripheral blood lymphocytes of a blood donor expressing the desired phenotype. Under acid conditions, peptide is eluted from the MHC class I molecules, resulting in denaturation of the trimolecular complex consisting of HLA, peptide, and p-2 microglobulin (p2M). In the presence of p2M, exogenous peptide with adequate binding affinity will bind to empty MHC class 1 at the cell surface, thus reconstituting the trimolecular complex, and only such a configuration is recognized by monoclonal antibodies like BB 7.2 (for HLA-A2) and GAP A3 (for HLA-A3).

We compared the use of B-LCL lines, both homozygous and heterozygous for HLA-A*0201 and HLA-A*0301. For HLA-A*0301 we also compared these B-LCLs with the C1R A3 cell line.

Once optimal conditions had been established for the peptide-reconstitution assay in HLA-A*0201 and HLA-A*0301, results from our assay were compared with those obtained with identical peptides in other assays.

Carcinoembryonic antigen (CEA) is a physiologic glycoprotein that is grossly overexpressed in most colorectal cancers and many other carcinomas (16–18) because of a failure of excretion in malignant cells (19,20). This overexpression appears to be limited to carcinoma in adult humans (21). Because CEA might therefore be a very attractive target for CTL-mediated immunotherapy, we applied the technique for identification of CEA-derived peptides with high-affinity binding to HLA-A*0301.

MATERIALS AND METHODS

Cell Lines

The B-LCL lines used in the experiments were JY, an HLA-A*0201 homozygous cell line; EKR, an HLA-A*0301 homozygous cell line; and D-O, a heterozygous cell line expressing both HLA-A*0201 and HLA-A*0301. We used the C1R A3 cell line, an HLA-A and HLA-B knockout B-cell line transfected with HLA-A*0301. Furthermore, we used the human processing-defective cell line 174 CEM.T2 (T2) (22). (Both the C1R A3 and T2 cell lines were kindly provided by P. Creswell, Department of Immunology, Yale University, New Haven, CT, U.S.A.)

Cells were cultured at 37°C, 5% CO_2, and RPMI 1640 Dutch modification (Gibco, Life Technologies, Paisley, Scotland), supplemented with 2 mM glutamine (referred to as RPMI medium), 10% fetal calf serum (FCS) and 5 x 10^{-5} *M* 2-mercaptoethanol (for the B-LCL lines) or Iscove's modification of Dulbecco's medium (IMDM; Gibco) supplemented with 10% FCS and 2 mM glutamine (for the C1R A3 and T2 cell lines). Cells were always supplied with fresh culture medium the day before an experiment, and adjusted to between 0.5 x 10^6 and 1 x 10^6 cells/ml.

Antibodies

The sources of the anti-HLA-A2 and -HLA-A3 antibodies were hybridoma culture supernatant of BB 7.2 (HB82-ATCC) and GAP A3 (HB 122-ATCC) [American Tissue Type Collection (ATCC), Rockville, MD, U.S.A.], respectively (23,24). To confirm allele specificity, al! cell lines used in the experiments were stained with both antibodies and a second-antibody-only negative control (GaM FITC).

Peptides

For control purposes, we used peptides with known HLA-A*0201 and HLA-A*0301 binding parameters, as shown in Table 1.

Our group previously described binding motif-guided computerized selection of peptides potentially binding to HLA-A*0201 (8,25). We used this computer scoring system and thus selected all CEA-specific peptides, eight to 11 amino acids long, that fit with the following binding motif for HLA-A*0301, based on the observations of Kubo et al. (26), and a comprehensive list of peptides naturally occurring in HLA-A*0301 (27,28): all peptides had L/I/V/M (one-letter amino acid code) at position 2 from the N-terminus, and K/R/Y at the C-terminus. These 34 peptides were synthesized and dissolved as described (8), adjusted to a pH between 6.5 and 7.5, aliquoted, and stored at −20°C.

Peptide-Reconstitution Assay

The peptide-reconstitution assay is a three-step procedure, consisting of elution (step 1), reconstitution of the trimolecular complex associated with a specific monoclonal antibody (step 2), and counterstaining with a fluorescent second antibody and fixation for flowcytometric analysis (step 3).

Step 1. 7 million cells were washed twice in 50 ml of phosphate-buffered saline (PBS). Supernatant was aspirated, the pellet resuspended in the residual PBS, and rested on ice for 10 min. Cells an ice were eluted by carefully rinsing with 3 ml of citrate phosphate buffer [0.131 *M* citric acid and 0.066 *M* Na_2HPO_4 (adjusted to the desired pH) for 90 s (unless stated otherwise)]. Then 47 ml of IMDM (0°C) without supplements was added, and cells were spun down immediately at 2,000 r/min (690 g) for 3 min, washed again in IMDM, and resuspended in 1.5 ml of RPMI medium.

Step 2. Wells of a 96-well U-bottom cell-culture plate (Costar, Cambridge, MA, U.S.A.) were filled with a saturating amount of 110 μl of first antibody, supplemented with β2M (final concentration of 1 μg/ml). Subsequently 25 μl of peptide solution diluted in RPMI medium (maximum final concentration, 400 μg/ml) and, finally, 15 μl of the cell suspension was added to each well (final volume, 150 μl).

Step 3. After 4-h incubation at room temperature, the cells were spun down at 1,300 r/min for 5 min,

TABLE 1. *Reference peptides used in the peptide-reconstitution assay*

Peptide (position)	Amino acid sequence	Species of origin	Reference
Binding to HLA-A*0201			
CEA 353–361	DVOPYECGI	Human	Our observation
CEA 308–318	ALTCEPEIQNT	Human "	Ourobservation
HIV pol 468–476	ILKEPVHGV	HIV	Walker et al., 1989
p53 25–35	LLPENNVLSPL	Human	Houbiers et al., 1993
p53 65–73	RMPEAAPPV	Human	Houbiers et al., 1993
p53 264–272	*LLGRNSFEV*	Human	Houbiers et al., 1993
p53 187–197	GLAPPQHLIRV	Human	Houbiers et al., 1993
Binding to HLA-A*030!			
HIV nef 73–82	QVPLRPMTYK	HIV	Culmann et al., 1989
?	KLYFKVYTYK	Unknown	Culmann et al., 1989
E6 125–133	HLDKKQRFH	HPV 16	Kas et al. 1994
E6 7-15	AMFQDPQER	HPV 16	Kas et al. 1994
E6 93–101	TTLEQQYNK	HPV 16	Kas et al. 1994
E6 89–98	*IVCPICDSQK*	HPV 16	Kas et al. 1994
E6 75–83	KFYSKISEY	HPV 16	Kas et al. 1994
E6 143-15!	AMSAARSSR	HPV 16	Kast et al. 1994
E6 42-50	QQLLRREVY	HPV 16	Kast et al. 1994

HLA, human leukocyte antigen; CEA, carcinoembryonic antigen; HIV, human immunodeficiency virus.

washed twice in 100 μl of PBS/BSA 1%, and incubated with 50 μl of FITC-conjugated goat anti-mouse immunoglobulin (GaM FITC, Boehringer Mannheim, Germany), dissolved 1:100 in PBS/BSA 1% per well at 4°C for 30 min. Cells were washed again, and finally cells were resuspended in 100 μl of 0.5% paraformaldehyde in PBS. Cells were analyzed using a Becton-Dickinson FACScan flowcytometer, and the mean fluorescence (mF) was determined.

The following values were calculated: fluorescence index (FI) was defined as the increase of mean fluorescence in the sample (eluted cells incubated with the peptide) over the background as a fraction of that background (background = eluted cells incubated with 25 μl of RPMI instead of peptide):

$$FI = (mF\ (sample) - mF\ (background)) / mF\ (background) \quad (1)$$

An FI < 0.5 was considered to indicate no significant binding affinity of the peptide for the allele concerned.

It is known from our experience with the T2 assay that serial dilution of the peptide results in an S-shaped curve when FI is plotted against peptide concentration on a logarithmic peptide concentration x axis. The level of the plateau (maximum FI) is correlated with the binding affinity of the peptide in the T2 assay (8,12).

The concentration at which half of the maximum FI is reached (the ½max value) is inversely correlated with binding affinity in the T2 assay (13). The ½max was determined by plotting the Fl against the concentration of peptide. A ½max in the same range as the values established for known CTL epitopes was considered indicative of high binding affinity.

RESULTS

Effect of pH and Length of Elution

Mild acid treatment of B-LCL for 60 s at pH 3.3 was reported sufficient for elution of HLA-A*0201 molecules (9). The same treatment applied even for 90 s resulted in a very moderate decrease of 35% (mF from 320 to 210) of cell surface HLA-A*0301 (Fig. 1), indicating a relatively low MHC class I dissociating effect of the treatment in both B-LCL and C1R A3.

At a fixed elution time of 90 s, decreasing the pH resulted in a progressive dissociation of the MHC class I complex. HLA-A*0201 complex dissociation occurred at a pH <5, reaching a plateau at pH 3.5. Dissociation of HLA-A*0301 required a pH <3.5, and at pH 2.9, only a hint of a plateau was seen (Fig, 1). However, down to a pH of 2.9, ~85% of cells survived acid treatment, whereas further lowering of the pH resulted in a sudden and sharp decrease in cell survival to 68% at pH 2.75, as determined by trypan blue exclusion (data not shown).

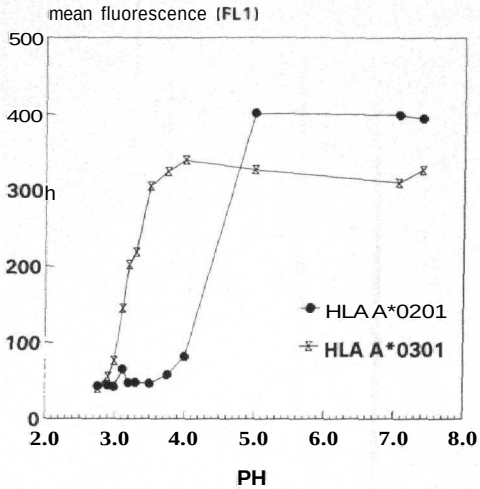

FIG. 1. Elution of HLA-A*0201 and HLA-A*0301 on the heterozygous B-LCL line D-O, measured as reduction of mean fluorescence al various pH values. Reequilibration in medium took place after 90 s.

Increasing the duration of acid treatment at a given pH also resulted in progressive dissociation. However, maximum effect was reached after 30 s in HLA-A*0201, whereas 90 s was required for HLA-A*0301 (Fig. 2).

Under conditions of optimal elution for each allele, an mF level was reached of ~10% of the ïeve! found with cells that had not been eluted (pH 7.4 in Fig. 1). Dissociation of the HLA-A*0301 complex required more vigorous acid treatment, both in pH and duration, possibly indicating stronger binding forces within the trimolecular complex of HLA-A*0301.

The allele specificity of the antibodies used in these experiments was confirmed by staining all cell lines used with both antibodies and a second-antibody-only negative control (GaM FITC), as shown in Fig. 3.

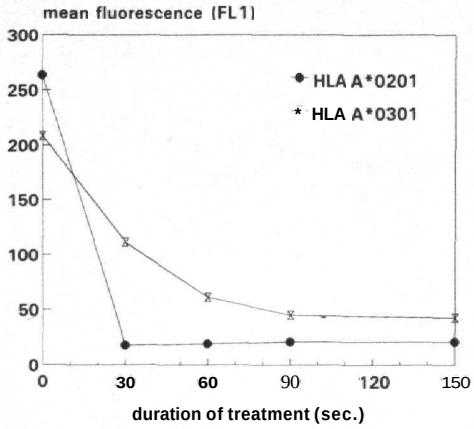

FIG. 2. Elution of HLA-A*0201 at pH 3.2 and HLA-A*0301 at pH 2.9 on the heterozygous fl-LCL line D-O, measured as reduction of mean fluorescence as a function of duration of acid treatment after which reequilibration in medium look place.

Influence of Homozygous Versus Heterozygous HLA Allele Expression on FI and ½max Values

The differences in HLA-A*020I and HLA-A*030I expression encountered when various cell lines were tested (Fig. 3) urged us to investigate the effect of these differences on the results of the peptide-reconstitution assay. This was done by testing B-LCL lines of both homozygous (JY, EKR) and heterozygous (D-O) HLA phenotypes when incubated with peptides of high or low binding affinity. To study HLA-A*0201 peptide elution, we used human immunodeficiency virus (HIV) pol 468-476 [ILKEPVHGV, an HLA-A*0201 restricted CTL epitope (29)], and CEA 308-318 [ALTCEPEIQNT, a peptide that never showed significant upregulation of HLA-A*0201 in the T2 assay (our observations)]. To study HLA-A*0301 peptide elution, we used HIV nef 73-82 [QVPLRPMTYK, an HLA-A*0301 restricted CTL epitope (30)], HIV pol 468-476 [one primary anchor and three amino acids on positions in which they are abundantly present in peptides eluted from HLA-A*0301 molecules (26)]. As a negative control peptide, we used CEA 353-361 (DVGPYECGI, a peptide that never showed any sign of binding to HLA-A*0201 or HLA-A*0301, either in T2 or in our peptide-reconstitution assays).

For all peptides, the FI curves were of identical shape, irrespective of the cell line used (homozygous, heterozygous, or C1R variant for the desired HLA alleie) (Figs. 4 and 5). However, ½max values for HIV pol 468-476 and CEA 308-318 were 2 and 20 μg/ml, respectively, when we used the homozygous ceil line, and 5 and 55 μg/ml when the heterozygous cell line was employed. For HIV nef 73-82 and HIV pol 468-476, the respective values were 3 and 25 μg/ml and 5 and 40 μg/ml, and by using the C1R A3 cell line, the respective ½max values were 2 and 20 μg/ml (Figs. 4 and 5).

This indicates that the assay may be carried out with cells with either homozygous or heterozygous expression of the HLA alleie tested, but sensitivity for binding at low peptide concentrations is higher in homozygous cell lines.

Validation of the Assay

The use of peptides with known binding affinity to HLA-A*0201 and HLA-A*0301 enabled us to validate our assay in its ability to quantify binding affinity of peptides. For HLA-A*0201, we tested p53 and CEA peptides, using the HLA-A*0201 homozygous JY cell line, and determined their maximum F! and ½max values, to compare these with the results from the T2 assay (12,13; our observa-

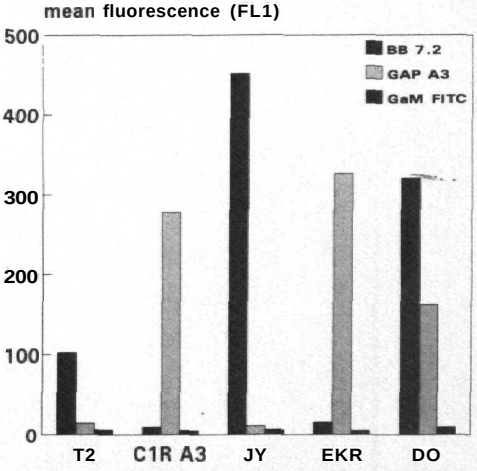

FIG. 3. Specificity with monoclonal antibodies against HLA-A2 (BB 7.2), HLA-A3 (GAP A3), and FITC-conjugated goat anti-mouse immunoglobulin (GaM FITC) only, and differences in expression of the respective HLA alleles, demonstrated on the processing-defective cell line T2, the C1R A3 cell line, expressing only HLA-A*0301, JY (B-LCL, homozygous for HLA-A*0201), EKR (B-LCL homozygous for HLA-A*0301), and D-O (B-LCL, HLA-A*0301, and HLA-A*0201).

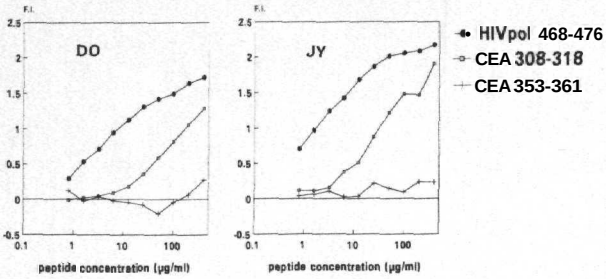

FIG. 4. Reconstitution with peptides of high (HtV pol 468-476, ILKEPVHGV), intermediate (CEA 308-318, ALTCEPEIQNT), and very low (CEA 353-361, DVGPYECGI) binding affinity to HLA-A*020I on JY (homozygous for the allele) and D-0 (heterozygous, HLA-A*0201 and HLA-A*0301) B-LCL.

tions). ½Max values in both tests did not differ significantly (Table 2).

For HLA-A*0301, we tested seven peptides derived from HPV 16 E6 and E7, of which the binding affinities to HLA-A*0301 were previously published (1; Table 2 ranked to the published IC_{50} values, the peptide concentration resulting in 50% binding inhibition of the reference peptide). The peptides with the lowest IC_{50} (HPV 16 E6 125-133 and HPV 16 E 6 7-15) had ½max values at the low end of the range we found (5 and 6 µg/ml, respectively). The peptides with the highest IC_{50} (HPV 16 E 6 143–151 and HPV 16 E6 42-50) were at the high end of the range encountered (35 and 55 µg/ml, respectively).

Thus the results in these different assays led to a similar hierarchy of binding affinity.

Binding Affinity for HLA-A*0301 of Selected CEA Peptides

The selected 34 CEA specific peptides complying with the HLA-A*0301 peptide-binding motif described were tested in peptide-reconstitution assay in serial dilution on the HLA-A*0301 homozygous EKR cell line (Table 3). Eight peptides did not show any significant binding affinity, illustrated by an ½max 3=400 µg/ml (Table 3) in combination with a very low FI (≤0.5, data not shown). Half max values of 19 peptides were in the same range as the HPV-derived binding peptides tested (½max ≤55 µg/ml), six of which were in the same range (½max ≤16 µg/ml) as the three best binding HPV-derived peptides tested (IC_{50}) (Tables 2 and 3).

This indicates that six CEA-specific peptides

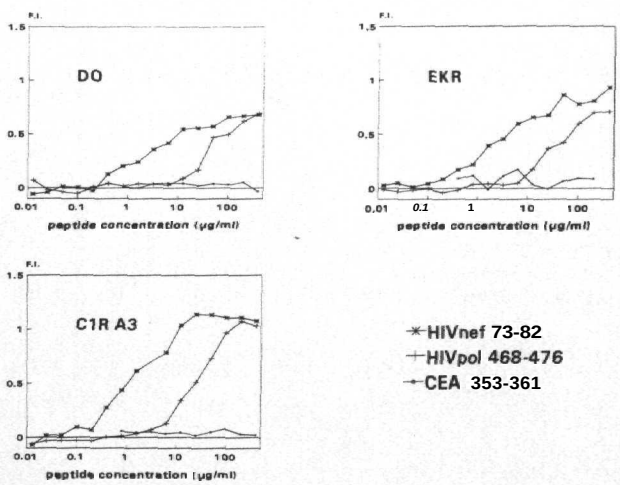

FIG. 5. Reconstitution with peptides of high (HIV nef 73-81, QVPLRPMTYK), intermediate (HIV pol 468-476, ILKEPVHGV), and very low (CEA 353-361, DVGPYECGl) binding affinity to HLA-A"0301 on EKR (B-LCL homozygous for the allele), D-O (B-LCL, heterozygous, HLA-A*0201 and HLA-A*0301), and C1R A3 (HLA-A*0301 only) cell lines.

TABLE 2. *Validation of the peptide-reconstitution assay*

Peptide	Reference assay	Peptide-reconstitution assay
HLA-A*0201	T2 assay ½max. (μg/ml)	½max (μg/ml)
p53 25–35	6	5
HIV pol 468–476	8	2
p53 187–197	8	25
p53 65–73	12	10
p53 264–272	26	8
CEA 353–361	>100	>400
HLA-A*0301	Molecular binding assay IC_{50} (n*M*)	½max (μg/ml)
E6 125–133	68	5
E6 7-15	290	6
E6 93–101	384	16
E6 89–97	1,111	5
E6 75–83	3,000	12
E6 143–151	4,285	35
E6 42-50	5,000	55

Human p53-, CEA-, and HIV-derived HLA-A*0201 binding peptides tested in the T2 assay and the peptide-reconstitution assay. Stated are the position of the peptide and ½max values for both assays. HPV 16-derived peptides were tested in the peptide-reconstitution assay and the molecular-binding assay. Stated are the 50% inhibition by competition (IC_{50}) values in the molecular-binding assay (Kast et al., 1994) and the ½max values in the peptide-reconstitution assay for binding to HLA-A*0301.

HLA, human leukocyte antigen; CEA, carcinoembryonic antigen; HIV. human immunodeficiency virus; HPV, human papilloma virus.

have such binding affinity that they can be designated as being potential HLA-A*0301-restricted CTL epitopes.

DISCUSSION

In the search for MHC class I binding peptides as targets for CTL-mediated immunotherapy, the potential effectiveness of which has been demonstrated in both murine models (31-33) and made likely in humans (34,35), there is a need to identify potential CTL epitopes binding to a great variety of HLA alleles to cover a majority of the population.

B-LCL with any HLA expression can be obtained rather easily. Therefore, we adapted the peptide-reconstitution assay as originally described (9) for use with such B-LCL lines and established the practical application of this assay for the HLA-A*0301 allele. Of course, having established the optimal circumstances for elution of this molecule, the technique might also be used to prepare antigen-presenting cells for primary CTL response induction.

The peptide reconstitution assay combines a number of advantages of the T2-binding assay with the advantages of molecular binding assays. The assay does not involve radioactive components, and apart from a flow cytometer, no specialized equipment in the laboratory is required. Because B-LCL can be applied, it is possible to study peptide-binding affinities for any HLA allele, provided an allele-specific antibody is available.

Whether peptide binding to isolated MHC class 1 molecules differs from binding to the MHC class I in its native biological context, the intact cell (-surface), has not been established yet. If such differences do occur, it seems likely that a cellular bind-

TABLE 3. *Reconstitution of HLA-A*0301 with exogenous CEA-derived peptides after elution at pH 2.9*

Amino acid sequence	Position	½Max (μg/ml)
TLTLFNVTR	521	<2
HLFGYSWYK	27	9
RVYPELPK	105	9
RLQLSNDNR	334	11
TLFNVTRNDAR	523	12
RVDGNRQI1GY	38	15
TISPSYTYY	385	19
TITVYAEPPK	281	20
TVYAEPPK	283	20
ELFISNITEK	427	22
LFISNITEK	428	23
TISPLNTSY	207	25
HLFGYSWY	27	25
TITVSAELPK	459	29
KITPNNNGTY	610	38
NLPQHLFGY	23	40
FVSNLATGR	622	40
TVSAELPK	461	42
FISNITEK	429	55
IIQNDTGFY	78	65
NVTRNDAR	526	70
SVTRNDVGPY	348	75
PVILNVLY	372	80
1ISPPDSSSY	563	85
GIQNSVSANRS	538	100
TISPSYTY	385	110
NVTRNDTASY	170	200
NVTRNDARAY	526	>400
N1TEKNSGLY	432	>400
TVTTITVY	278	>400
SISSNNSK	470	>400
SISSNNSK	114	>400
PVTLDVLY	550	>400
P1ISPPDSSY	562	>400
SVILNVLY	194	>400

½Max values of carcinoembryonic antigen (CEA)-derived peptides for binding to HLA-A*0301 in the peptide-reconstitution assay in micrograms per milliliter. Stated are the amino acid sequence in one letter code, and the position of the first amino acid from the N-terminus of the protein.

HLA, human leukocyte antigen; CEA, carcinoembryonic antigen.

ing assay gives more realistic information about potential immunogenicity of the peptide when presented by the MHC class 1 molecule to the CTL.

To take advantage of the possibilities of the peptide-reconstitution assay, we adapted the assay for use on another HLA allele than the HLA-A*0201 described originally. Applying this strategy, in principle, all types of HLA alleles can be tested for peptide binding. Considerable differences in acid treatment conditions required for elution of HLA-A*0201 and HLA-A*0301 were encountered. These differences were allele specific and not cell-line dependent. The required acid treatment conditions for either of the alleles tested were identical in any of the cell lines tested, whether homozygous B-LCL, heterozygous B-LCL, or C1R variant.

Further investigation is needed to establish whether the differences in acid treatment conditions required for optimal elution of peptide from the respective MHC class I molecules is reflected in differences in immunogenicity of peptides presented by these alleles. Such a relation is not unlikely, because so far, peptide-binding affinity is closely related to immunogenicity (2).

When identical peptides were tested on different cell lines in the peptide reconstitution assay, FI curves for each peptide were identical in shape (Figs. 4 and 5), resulting in a comparable discrimination between strong, weak, and nonbinding peptides, irrespective of the use of homozygous, heterozygous, or the C1R A3 cell line.

When the homozygous and C1R A3 cell lines were used, the range of ½max values was wider and, therefore, supposedly more accurate differentiation between binding affinities of the peptides tested is possible. ½Max values were ~2 to 3 times lower, indicating a higher sensitivity for binding at low peptide concentrations when homozygous cells are used.

To validate the assay, p53-derived HLA-A*0201-binding peptides were tested in the peptide-reconstitution assay. Compared with the results from the T2 assay, the outcome of testing HLA-A*0201-binding p53 peptides in the peptide-reconstitution assay was mainly similar. Comparison of the HPV-derived peptides with different binding affinity known from the molecular binding assay (1) shows good correlation with the ½max values established using the peptide-reconstitution assay. One peptide (p53 264–272) appeared to have relatively lower binding affinity in the T2 assay than in the peptide-reconstitution assay. A similar phenomenon was noted for HLA-A*0301 when the IC_{50} values of the HPV-derived peptides in the molecular-binding assay were compared with the findings in the peptide-reconstitution assay. Similar discrepancies in ranking of binding affinity were found before when p53 peptide binding was studied in the T2 binding and T2 competition assay (13).

Such discrepancies might be caused by the considerable differences in incubation time between the peptide-reconstitution assay and the other test: whereas association rate and dissociation rate together cause an equilibrium (molecular binding) or stable effect (T2) reached during overnight incubation, a high association rate could cause features of high binding affinity in the peptide-reconstitution assay. The presence of the native biological context might also contribute to such discrepancies.

The peptide-reconstitution assay is a valid and useful instrument in determining peptide-binding affinity to MHC class I; by the application of B-LCL, any phenotype can be studied, provided an allele-specific antibody is available. We demonstrated here that the used cells are preferably homozygous for the HLA allele studied, because this results in higher sensitivity, especially at low peptide concentrations; however, in the unlikely event that a homozygous B-LCL cannot be made available, a heterozygous cell line may be used. Therefore, it may be a very useful tool in the development of CTL-mediated immunotherapy.

We applied the peptide-reconstitution assay using a B-LCL line homozygous for HLA-A*0301 to test binding affinity of 34 binding-motif-selected CEA-specific peptides. Six of these had binding affinities in the same range as high-affinity binding peptides of viral origin and can therefore be designated to be potential CTL epitopes.

Acknowledgment: The study was supported by grants from the Dutch Cancer Society KWF and the National Institutes of Health (grant RO1 CA579333, to W. M. Kast). We thank Dr. J. W. Drijfhout for synthesizing peptides. Dr. W. M. Kast is a senior fellow of the Royal Netherlands Academy of Arts and Sciences (KNAW).

REFERENCES

1. Kast WM. Brandt RM, Sidney J, et al. Role of HLA-A motifs in identification of potential CTL epitopes in human papilloma virus type 16 E6 and E7 proteins. *J Immunol* 1994; 152(8):3904–12.
2. Feltkamp MCW, Vierboom MPM, Kast WM, Melief CJM. Efficient MHC class I-peptide binding is required but does not ensure MHC class I-restricted immunogenicity. *Mol Immunol* 1994;31(18):1391–401.

3. Sette A, Sidney J, del Guercio MF, et al. Peptide binding to the most frequent HLA-A class I alleles measured by quantitative molecular binding assays. *Mol Immunol* 1994;31(11): 813–22.
4. Cerundolo V, Elliott T, Elvin J, Bastin J, Rammensee HG. Townsend A. The binding affinity and dissociation rates of peptides for class I major histocompatibility complex molecules. *Eur J Immunol* 1991;21:2069–75.
5. Reyes VE, Lu S, Humphreys RE. Binding of radioiodinated influenza virus peptides to class I MHC molecules and to other cellular proteins as analyzed by gel filtration and photoaffinity labeling. *Mol Immunol* 1991;28(4–5):341–8.
6. Stuber G, Modrow S, Hoglund P, et al. Assessment of major histocompatibility complex elevation of membrane H-2 and HLA in peptide loading-deficient cells. *Eur J Immunol* 1992; 22(10):2697–703.
7. Elvin J, Potter C, Elliott T, Cerundolo V, Townsend A. A method to quantify binding of unlabeled peptides to class I MHC molecules and delect their allele specificity. *J Immunol Methods* 1993:158(2): 161–71.
8. Nijman HW, Houbiers JGA, Vierboom MPM, et al. Identification of peptide sequences that potentially trigger HLA A2.1 restricted cytotoxic T lymphocytes. *Eur J Immunol* 1993;23:1215–9.
9. Zeh HJ, Leder GH, Lotze MT, et al. Flow cytometric determination of peptide class I complex formation. Identification of p53 peptides that bind to HLA-A2, *Hum Immunol* 1994;39(2):79–86.
10. Ruppert J, Sidney J, Celis E, Kubo RT, Grey M, Sette A. Prominent role of secondary anchor residues in peptide binding to HLA A2.1 molecules. *Cell* 1993;74(5):929–37.
11. Chen Y, Sidney J. Southwood S. et al. Naturally processed peptides longer than nine aminoacid residues bind to the class I MHC molecule HLA A2.1 with high affinity and in different conformation. *J Immunol* 1994;152(6):2874–81.
12. Houbiers JGA, Nijman HW, van der Burg SH, et a). In vitro induction of human cytotoxic T lymphocyte responses against peplides of mutant and wild-type p53. *Eur J Immunol* 1993;23:2072–7.
13. Nijman HW, van der Burg SH. Vierboom MPM, Houbiers JGA, Kast WM, Melief CJM. P-53, a potential target for tumor-directed T cells. *Immunol Lelt* 1994;40(2): 171–8.
14. Imanishi T, Akaza T, Kimura K, Tokunaga K, Gojobori T. *HLA 1991 Proceedings of the Eleventh International Histocompatibility Workshop and Conference.* Tokyo: Oxford University Press. 1991, 1066.
15. Sugawara S, Abo T, Kumagai K. A simple method to eliminate the antigenicity of surface class I MHC molecules from the membrane of viable cells by acid treatment at pH 3. *J Immunol Methods* 1987; 100:83–90.
16. Boucher D, Cournoyer D. Stanners CP. Fuks A. Studies on the control gene expression of the carcinoembryonic antigen family. *Cancer Res* 1989;49:847–52.
17. Simon B, Podolsky DK, Moldenhauer G, Isselbacher KJ, Gattoni-Celli S, Brand SJ. Epithelial glycoprotein is a member of a family of epithelial cell surface antigens homologous to nidogen, a matrix adhesion protein. *Proc Natl Acad Sci USA* 1990;87(7):2755–9.
18. Thompson JA, Grunert F, Zimmermann W. Carcinoembryonic antigen gene family: molecular biology and clinical perspectives. *J Clin Lab Anal* 1991;5(5):344–66.
19. Kuroki M, Skinoda F, Takayasu T, Koga Y, Matsuoka Y. Jmmunological characterization and structural studies of normal fecal antigen-1 related to carcinoembryonic antigen. *Mol Immunol* 1982;19:399–406.
20. Kuroki M, Arakawa F, Yamahoto H, Shiramura H, Ikehara Y, Matsuoka Y. Active production and membrane anchoring of carcinoembryonic antigen observed in normal colon mucosa. *Cancer Lett* 1988;43(1–29): 151-7.
21. Benchimol S, Fuks A, Jothy S, Beauchemin N, Shirota K, Stanners CP. Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. *Cell* 1989;57:327–34.
22. Salter RD, Cresswell P. Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. *EMBO J* 1986;5(5):943-9.
23. Parham P, Brodsky FM. Partial purification and some properties of BB 7.2, a cytotoxic monoclonal antibody with specificity for HLA A2 and a variant of HLA A28. *Hum Immunol* 1981;3(4):277–99.
24. Berger AE, Davis JE, Creswell P. Monoclonal antibody to HLA A3. *Hybridoma* 1982;1(2):87–90.
25. D'Amaro J, Houbiers JGA, Drijfhout JW, et al. *Hum Immunol* 1995;43:13–8.
26. Kubo RT. Sette A. Grey HM, et al. Definition of specific peptide motifs for four major HLA A alleles. *J Immunol* 1994;152(8):3913-24.
27. Engelhard VH. Structure of peptides associated with MHC class I molecules. *Curr Opin Immunol* 1994:6(1): 13–23.
28. DiBrino M, Parker KC, Shiloach J, et al. Endogenous peptides bound to HLA A3 possess a specific combination of anchor residues that permit identification of potential antigenie peptides. *Proc Natl Acad Sci USA* 1993;90:15–27.
29. Walker BD. Flexner C. Birch-Limberg K, et al. Long term culture and fine specificity of human cytotoxic T lymphocyte clones reactive with human immunodeficiency virus. *Proc Natl Acad Sci USA* 1989;86(23):9514–8.
30. Culmann B, Gomard E, Kieny MP, et al. An antigenic peptide of the HIV-1 NEF protein recognised by cytotoxic T lymphocytes of seropositive individuals in association with different HLA-B molecules. *Eur J Immunol* 1989;19(12): 2383–6.
31. Kast WM, Offringa R, Peters PJ, et al. Eradication of adenovirus El-induced tumors by E1A specific cytotoxic T lymphocytes. *Cell* 1989;59:603–14.
32. Melief CJM. Tumor eradication by adoptive transfer of cytotoxic T lymphocytes. *Adv Cancer Res* 1992;58:143–75.
33. Greenberg PD. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. *Adv Immunol* 1991;49:281–354.
34. Koo AS, Tso CL, Shimabukouro T. Peyret C, deKernion JB, Belldegrun A. Autologous tumor-specific cytotoxicity of tumor infiltrating lymphocytes derived from human renal cell carcinoma. *J Immunother* 1991;10(5):347–54.
35. Cerottini JC, von Fleidner V, Boon T. Recognition of tumor associated antigens by T lymphocytes: from basic concepts to new approaches. *Ann Oncol* 1992;3:11–6.