Cell lines transfected with the TAP inhibitor ICP47 allow testing peptide binding to a variety of HLA class I molecules


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Keywords: HLA class I ligands, HLA class I motifs, HLA class I surface expression

Abstract

The immediate early protein ICP47 of the Herpes simplex virus is known to block the human transporter associated with antigen processing (TAP), thereby creating a TAP-deficient phenotype in any human cell transfected with the corresponding cDNA. Exploiting this inhibitory activity, we constructed a selection of human cell lines each co-expressing one of the cDNAs of human leukocyte antigen (HLA) class I alleles HLA-A*1101, A24, A*3101, A*6601, B8 and B*1516, and the cDNA encoding the ICP47 molecule. The cell lines generated showed diminished HLA class I surface expression and the inhibition of the TAP function was confirmed in peptide translocation assays. The addition of specific exogenous peptide ligands restored the expression of the corresponding HLA class I molecules. Thus, the ICP47 transfectants provide us with a tool to closely examine peptide–HLA class I interactions, to confirm HLA class I ligand motifs and to test peptides predicted to bind.

Introduction

For therapeutic or prophylactic intervention in the cytotoxic T lymphocyte (CTL)-mediated immune response against tumors and infectious agents, knowledge about epitope selection from proteins associated with cell transformation or infection is a crucial prerequisite. In recent years much work has been directed towards elucidating the motifs of the peptides binding to the extremely polymorphic MHC products (1). MHC class I-presented peptides are between 8 and 10 amino acids long and bear two or three highly conserved residues at defined positions varying between the hundreds of alleles described so far (2). Prediction algorithms based on natural human leukocyte antigen (HLA) ligands and binding studies (2) use knowledge about the individual MHC peptide-binding motifs to screen proteins for fragments which potentially bind to MHC molecules. Since only a fraction of the predicted peptides bind with high affinity, actual binding has to be assessed experimentally.

Several methods to test such predicted peptides for their affinity towards MHC molecules have been described. In competition assays, the inhibition of the binding of labeled indicator peptides is tested on recombinant or stripped soluble MHC molecules (3). Alternatively, inhibition of peptide-specific T cell responses by competitor peptides can be measured (4). A third method relies upon the peptide-induced stabilization of empty class I molecules in cells with a transporter associated with antigen processing (TAP) deficiency (5) or cells where peptides have been removed from the MHC class I molecules by low pH treatment (6,7).

In this study, we established a system to test synthetic peptides for their binding properties towards, in principle, any human MHC class I molecule. The method described allows an easy induction of a TAP-deficient phenotype in any human cell line. Our approach will enlarge the spectrum of HLA class I molecules examinable with the peptide stabilization method now mainly restricted to the few cell lines deficient in TAP such as 721.174 (8), T2 (9) and STEMO (10), or mouse TAP-deficient cell lines (11) expressing HLA class I molecules (12). The immediate early protein ICP47 (13,14) of the Herpes simplex virus was chosen as an example for the TAP inhibitor as it is known to block TAP in any cell transfected with its cDNA (13,14). Other TAP-deficient cell lines have been described (3,5,7,11).
simplex virus, known to inhibit TAP function, was super-transfected into the human mutant B cell line C1R (15) that had been previously transfected with selected HLA class I cDNAs. C1R cells are deficient in endogenous HLA class I expression, with the exceptions of some residual B35 expres-sion (15) and naturally low levels of Cw4 (16). The double transfection of C1R cells with cDNAs for ICP47 and HLA class I molecules has the advantage that peptide binding to HLA alleles for which no specific mAb are available can be determined using the general anti-HLA class I mAb W6/32 (17) without the high background staining caused by HLA-A2 molecules in 721.174 or T2 cells.

In human cells, the 87 amino acid residue polypeptide ICP47 inhibits transport of proteosomally generated antigenic peptides from mostly cytosolic proteins into the endoplasmic reticulum (ER), where they would be loaded onto freshly synthesized HLA class I molecules. This inhibition is due to the 35 N-terminal amino acid residues (18) as the active part of the theoretical prediction of the HLA-A*6601 molecule. Screening of the transfectants for HLA class I reduction by flow cytometry and testing in stabilization assays with known peptide ligands resulted in cell lines deficient in the ability to transport a radio labeled model peptide from the cytosol into the ER in a translocation assay (20, 21).

Here we show that the viral protein ICP47 represents an ideal tool to create cell lines which can be used as HLA class I-specific peptide sensors. The produced set of six double transfectants for HLA-A and HLA-B alleles was used to differentiate between binding and non-binding peptides. In addition, the C1R-A*6601/ICP47 cell line was applied to verify the theoretically predicted motif of the HLA-A*6601 molecule.

Methods

Cloning

The open reading frame of the Herpes simplex immediate early protein ICP47 was amplified by PCR, verified by DNA sequencing, and inserted into two eukaryotic expression vectors, pBJ1neo, a gift from M. M. Davis (22), and pKEX2XR, provided by G. Szczakiel, OKFZ, Heidelberg, Germany, containing the gene for the resistance to G418 or hygromycin B respectively. For mock transfections, the mentioned vectors were used without the ICP47 encoding insert, only containing the resistance gene. The HLA-A*6601 allele of Daudi cells (A*6601, A*0102) (23) was amplified from Daudi cell cDNA by PCR. The PCR product was cloned into a pBluescript KS+ vector and sequenced to exclude clones carrying PCR induced mutations. The insert was excised, purified and cloned into the vector pB36 (kindly provided by Dr W. Sugden, Madison, WI). This vector replicates in Epstein–Barr virus-transformed cell lines as an episome under continuous hygromycin B selection.

Cell lines and peptides

Stable HLA class I transfectants of the human HLA-A,B negative mutant B cell line C1R expressing different HLA class I cDNAs were used. HLA-A*1101 (24), HLA-A24 (kindly provided by M. Takiguchi), HLA-A3*101 (24), HLA-A*6601, HLA-B8 (kindly provided by M. Takiguchi) and HLA-B*1516 (25) expressing transfectants were maintained in RPMI 1640 medium containing 10% FCS and supplemented with l-glutamine (0.3 mg/ml), penicillin/streptomycin (100 U/ml) and the appropriate resistance drug, G418 (PAA Laboratories, Cölbe, Germany) (0.5 mg/ml) or hygromycin B (Boehringer Mannheim, Mannheim, Germany) (0.25 mg/ml). The human cell line T2 was grown in the same medium without any resistance drug. Peptides were synthesized on an ABI 432A Peptide Synthesizer (Applied Biosystems, Weiterstadt, Germany) applying Fmoc strategy, purified by reversed-phase HPLC (System Gold; Beckman Instruments, München, Germany) and analysed by matrix-assisted laser desorption ionization mass spectroscopy (G2025A; Hewlett-Packard, Waldbronn, Germany). The one-letter code for amino acids is used.

Stable transfections

C1R transfections were carried out with a Gene Pulser II (BioRad, München, Germany) at 220 V and 950 µF. Cells (5×10³) were suspended in 0.5 ml PBS in 0.4 cm electrode cuvettes and incubated on ice for 10 min. Then 10 µg of linearized recombinant plasmid DNA was added per cuvette. After electroporation, cells were resuspended in fresh culture medium and distributed into 24-well plates. Selection started 24 h after gene transfer, with G418 (0.5 mg/ml) for pBJ1neo or with hygromycin B (0.25 mg/ml) for pKEX2XR. Medium was changed every week, and after 3–4 weeks of selection drug-resistant cell populations which proliferated in many wells were analysed individually by indirect immunofluorescence and flow cytometry. Transfectant cell populations were stained using the general anti-HLA class I mAb W6/32 and FITC-conjugated goat anti-mouse Ig (Jackson Immuno-Research, West Grove, PA) as the second-step reagent. Stained cell populations were examined with a Becton Dickinson (Heidelberg, Germany) FACS Calibur flow cytometer using the CellQuest software. Cytometer calibration was performed using CaliBRITE beads with the aid of the FACSComp software.

Transfection with HLA-A*6601 cDNA was performed under similar conditions at 250 V and 960 µF. Hygromycin B (250 µg/ml)-resistant cells were pooled and analysed by FACSCalibur. The 10% brightest positive cells were sorted several times on a MOFlo high speed cell sorter.

HLA stabilization assays

C1R double transfectants (2×10⁵) were incubated in FCS-free RPMI in the presence or absence of 100 µM peptide for 16 h at 37°C. HLA class I surface expression was analysed by indirect immunofluorescence and flow cytometry (see above). The fluorescence ratio (FR) was calculated from the mean fluorescence intensities (MFI) as follows: FR = (MFI after incubation with peptide)/(MFI after incubation without peptide).
Table 1. Effect of ICP47 transfection on MHC class I surface expression

<table>
<thead>
<tr>
<th>C1R-transfectant expressing the ICP47 transgene</th>
<th>Residual MFI (%)</th>
<th>Average residual MFI (%)</th>
</tr>
</thead>
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<tr>
<td>HLA-A*1101</td>
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<td>2.0</td>
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<td>4.4</td>
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<td>HLA-B8</td>
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<td>12.5</td>
</tr>
<tr>
<td>HLA-B*1516</td>
<td>7.0</td>
<td>7.8</td>
</tr>
</tbody>
</table>

*Residual MFI is indicated as the percentage of MHC class I expression of ICP47 transfectants compared to ICP47-mock transfectants.

125I-labeling of peptides

Aliquots of 10 µl of peptide TNKTRIDGQ(Tpa)Y (1 mg/ml in DMSO), 30 µl of water, 10 µl of chloramine T (1 mg/ml in water) and 10 µl of Na2S2O5 (1 mg/ml in water), the reaction mixture was incubated for 1 min at room temperature. The reduced 125I anions were removed by two cycles of Dowex (Serva, Heidelberg, Germany) extraction. The concentrations of the labeled peptide were usually ~20 ng/µl with specific activities ranging from 2×103 to 104 c.p.m./ng peptide.

Translocation assays

Cells (2.5×10^6) were washed once with transport buffer (130 mM KCl, 10 mM NaCl, 1 mM CaCl2, 2 mM EGTA, 2 mM MgCl2 and 5 mM HEPES, pH 7.3) at 4°C and permeabilized at 37°C for 10 min in 50 µl of transport buffer containing 2 U/ml DTT-activated streptolysin O (Bio Merieux, Lyon, France), as described (20,21). The permeabilized cells were transfected on ice for addition of peptide [10 µl of 125I-labeled TNKTRIDGQ(Tpa)Y], ATP (15 µl of 100 mM in water) and transport buffer to a final volume of 100 µl. After incubation at 37°C for 15 min, cells were lysed for 30 min at 4°C by adding 900 µl of lysis buffer (0.1% NP-40 in PBS containing 10 mM iodoacetamide and 0.23 mM PMSF). Following centrifugation, the glycosylated peptides were extracted for 1 h at 4°C by adding 50 µl concanavalin A (Con A)–Sepharose beads (Pharmacia, Biotech, Uppsala, Sweden) to the supernatants. After extensive wash of the beads with lysis buffer, the radioactivity of the Con A-superantam and the Con A-pellet was measured γ-spectroscopically. Translocation efficiencies were calculated according to the formula: translocation efficiency = c.p.m. (glycosylated radioactive peptide)/c.p.m. (total amount of radioactive peptide used).

Results

Six C1R transfectants, each expressing a different HLA class I molecule, were transfected with the Herpes simplex virus protein ICP47, known to block the human TAP transporter. The transfected clones show a down-regulated HLA class I surface expression as identified by flow cytometry. The degree of this reduction is listed in Table 1 for each of the ICP47 transfectants. The values were calculated from two individual experiments and represent the MFI in comparison to the corresponding cell lines transfected with the plasmids lacking the ICP47 encoding insert (mock transfectants). The average residual MFI varies between 2.5 and 54.5% for the different C1R transfectants (Table 1), and the results were consistent between different drug-resistant clones from the same transfection (data not shown). These first results indicate that the HLA class I surface expression is reduced by ~90% through the effect of ICP47 reaching approximately the MFI of untransfected C1R cells (data not shown), except for HLA-A24 which demonstrates a 50% decrease only and seems to be therefore less affected by the TAP inhibition.

To exclude reduction of HLA class I expression due to loss of the HLA class I CDNA and to confirm the specific effect of the ICP47 molecule on the ATP-dependent human TAP heterodimer, translocation assays and peptide stabilization assays were performed. The 125I-labeled model peptide TNKTRIDGQ(Tpa)Y, a peptide whose transport is known to be absolutely dependent on TAP (26) and which possesses an N-glycosylation site (NKT), was used for the translocation assays. The translocation efficiencies for the different transfectants and for the TAP-negative cell line T2 are depicted in Fig. 1. The strict ATP dependency of translocation in the mock transfectants proves the measurements to be specific and not due to any leakage or permeabilization of the glycosylating ER compartment. All translocation experiments were performed twice with very similar results. No significant difference was measured between the mock transfected and the corresponding non-transfected mother cell line C1R-A*1101 (Fig. 1). The ICP47 transfectants show low levels of peptide translocation comparable to the levels obtained with T2 cells. The residual transport efficiencies are all <5% as compared to the mock-transfected controls, indicating an efficient TAP inhibition in all C1R-HLA class I/ICP47 transfectants.
Peptide binding to ICP47-transfected human cells

Fig. 2. Flow cytometric analysis of peptide stabilization assays using known specific HLA class I ligands. The profiles on the far left of each panel represent the staining of the ICP47 transfectants with an isotype-matched control mAb and the other profiles represent the fluorescence intensity after staining the cells with the W6/32 mAb; 10,000 cells were counted for each profile. The mock transfectants are depicted as a bold line and the ICP47 transfectants without peptide as a dotted line. The sequences of the specific ligands used to stabilize the different ICP47 transfectants are listed next to each panel.

To further characterize the ICP47 transfectants, all of them were tested in peptide stabilization assays for their ability to up-regulate their individual HLA class I molecules after incubation with different peptides previously described to be ligands (2,25). The five double transfectants expressing ICP47 and one of the HLA types A*1101, A24, A*3101, B8 or B*1516 with known peptide motifs increased their HLA surface expression following incubation with 100 µM of specific HLA ligands (Fig. 2). The HLA-A*1101 peptides, all being naturally processed ligands, were strong inducers of the HLA-A*1101 surface expression when added exogenously to the double transfectant C1R-HLA-A*1101/ICP47 which almost reached the HLA surface expression of the C1R-HLA-A*1101 mock transfectant (Fig. 2). Natural ligands of HLA-A*3101, B*1516 or B8 were similarly shown to bind to their appropriate HLA molecules. The HLA class I surface expression increased significantly for all the peptides tested. The peptide stabilization test involving HLA-A24 was less sensitive. In this case, the three tested natural ligands only induced a slight increase in HLA-A24 surface expression (Fig. 2).

To prove that up-regulation of HLA class I on the different cells is a ligand-specific effect, a set of ligands from other HLA class I molecules was tested on all double transfectants. With the exception of two peptides among those tested for binding to HLA-A*1101 molecules and one peptide for HLA-A*3101, the double transfectants possessed the ability to distinguish between ligands and non-ligands in the stabilization assays (Fig. 3). All peptide binding data are summarized in Table 2. Increases in MFI detected by flow cytometry were converted into FR according to the formula described in Methods. As shown in Table 2, the values indicated in bold are the results of HLA class I ligands tested on their appropriate C1R-HLA transfectants and generally represent much higher FR values than those observed when the ligands were tested on control HLA transfectants.

In order to demonstrate that the ICP47 approach can also be used to determine unknown HLA class I peptide motifs, we produced a C1R transfectant expressing ICP47 and HLA-A*6601 for which the peptide motif is still unknown. A motif prediction was performed by analyzing the polymorphic amino acid residues facing the A, B and F pockets of different related HLA molecules (Table 3). The three mentioned pockets are assumed to interact with the N-terminal, the second and the C-terminal residue within the HLA class I ligand. We compared these pockets of the HLA-A*6601 molecule with those of a selection of related HLA class I alleles with known peptide motifs (2). Sequence identity or similarity between the pocket-forming residues suggest similar preferences for the anchor residues within the ligand. For the A pocket, the sequence RN for residues 62 and 63 of the α chain has recently been connected with a strong preference for acidic residues at the N-terminus of HLA-A ligands (T. Dumrese et al., manuscript submitted). The A pocket composition of HLA-A*6601 is identical to HLA-A*2601 and differs from HLA-A*6901 by a T163R exchange (Table 3A). For HLA-A*6601, the preference for acidic residues at the N-terminus of the ligands can be explained by electrostatic interactions with the two arginines within the A pocket (amino acids 62 and 163). A similar comparison of the B pocket favoured the amino acid residues V, T, I and A at position 2 of the ligands due to close similarities with the alleles HLA-A*2601 and A*6901 (Table 3B). Concerning the F pocket, the presence of the three acidic residues D74, D77 and D116 within the polymorphic positions is well correlated with high affinity towards basic amino acids at the C-terminus of the ligand (27). This is the case for the F pocket of, for example, HLA-A*6601, A*3303, B*27052 and A*31012 (not shown).

Having defined the probable anchors within ligands of HLA-A*6601 being D or E at the N-terminus, I, V, T or A at position 2 and R or K at the C-terminus, six ligands of known HLA restriction possessing two of the three predicted anchor residues were chosen for stabilization assays as well as two
Peptide binding to ICP47-transfected human cells

Discussion

Using C1R HLA class I transfectants as parental cell lines, HLA ligand-specific sensors were produced by co-transfection of ICP47. The advantage of those transfectants as parental cells for introducing the ICP47 transgene lies in their very low endogenous HLA class I expression (15) at the cell surface. Consequently, the analysis of peptide binding to the HLA molecules expressed by C1R-HLA transfectants can be done using a general anti-HLA class I antibody and no allele-specific mAb is required for immunofluorescence analysis.

The introduction of ICP47 into C1R-HLA transfectants induces down-regulation of HLA class I surface expression in an allele-specific manner. Whereas the expression of most of the HLA class I molecules is reduced to residual values ranging between 3 and 14%, HLA-A24 molecules are affected to a far lesser extent and still show 55% residual expression, although the degrees of TAP inhibition shown by translocation assays are equally high in all cases. This weak effect of ICP47 on HLA-A24 expression could be explained by the presence of two different populations of potential HLA-A24 ligands in the ER. The first population would be made up of peptides transported by TAP and is deplete by ICP47, whereas the second population contains peptides which should cross the ER membrane independently of TAP, either as signal sequences or by means of a different transporter. Presumably different HLA class I molecules acquire their ligands to different extents from the two peptide sets, leading to differential effects of ICP47 on their loading and surface expression. The HLA-A2 molecule is known to show similar effects as HLA-A24 in TAP-deficient cells. This is due to the high affinity of HLA-A2 molecules to hydrophobic signal sequence-derived peptides (28) which enter the ER without the action of TAP, leading to a 30–40% residual expression in T2 (9).

The strong effect of ICP47 on the expression of HLA class I alleles allowed us to use the C1R double transfectants as sensitive peptide sensors for HLA ligands. The stabilization assays summarized in Table 2 gave FR values varying between 1.6 and 6.6 when HLA class I ligands were incubated with their corresponding C1R-HLA class I/ICP47 transfectant. In contradiction, the HLA-A24 molecules were only weakly up-regulated, showing FR values of 1.2 in the stabilization assays. This poor up-regulation is invariably connected to the weak effect of ICP47 on HLA-A24 expression, the reason for which is presently investigated.

The stabilization assays with double transfectants were used to analyse the class I peptide binding specificity. Three peptides show cross-reactivity when tested on HLA class I molecules other than the allele they were eluted from as natural ligands. These cross-reactivities can be explained by examining their anchor positions with reference to the required motif. In every case the binding activity could be assigned to similarities in at least one or two anchor residues. The HLA-A*3101 and A*3303 ligands, KVFPGPHER and ESGPSVHR respectively, contain typical anchors at position 9 (shown in bold) and auxiliary anchors (underlined) of the HLA-A*1101 motif, explaining the FR values of 1.8 and 4.7 listed in Table 2. The analysis of the HLA-A24 ligand KYPENFLLL shows two auxiliary anchors for HLA-A*3101, explaining the FR value of 2.5.
Table 2. Peptide-induced stabilization of MHC class I molecules in ICP47 transfectants

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<th>Peptide sequence</th>
<th>HLA</th>
<th>FR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>A*1101</th>
<th>A24</th>
<th>A*3101</th>
<th>A*6601</th>
<th>B8</th>
<th>B*1516</th>
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<sup>a</sup>The tested peptides are natural ligands of the HLA type indicated in the table.
<sup>b</sup>FR indicates the fluorescence ratio determined for the different peptides after incubation with the ICP47 transfectants carrying the various HLA class I molecules. Values were calculated as described in Methods.
<sup>c</sup>Values for the combination of natural ligand with its corresponding HLA class I allele are printed in bold.
<sup>d</sup>Mixture 1 contains three peptides (EGGPSIVHR, EGGPSIVHR, EGGPSIVHR) as modifications of the HLA-A*3303 natural ligand ESGPSIVHR at position 2 (modified amino acids are underlined).
<sup>e</sup>Mixture 2 contains two peptides (EVFGPIHER, EVFGPIHER) as modifications of the HLA-A*3101 natural ligand KVFGPIHER at position 1 (modified amino acids are underlined).

Table 3. Amino acid alignments of polymorphic amino acids forming the A and B pockets of HLA-A*6601 molecules and related HLA class I alleles

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<th>Allele</th>
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<td>W</td>
<td>Y</td>
<td>+++</td>
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<td>E</td>
<td>K</td>
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<td>T</td>
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<td>Y</td>
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<tr>
<td>A*31012</td>
<td>Q</td>
<td>E</td>
<td>N</td>
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<td>T</td>
<td>W</td>
<td>Y</td>
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<sup>a</sup>Amino acid position in mature HLA class I α-chain.
<sup>b</sup>A-pocket residues depicted in bold contribute to the electrostatic interactions causing the specificity towards acidic residues.
<sup>c</sup>T. Dumrese et al.; manuscript submitted.
<sup>d</sup>Crosses indicate very high (+++) and high (+) preference, dashes indicate disfavour (--) and strong disfavour (---) for acidic residues.

<table>
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<td>M</td>
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<td>Y</td>
<td>T</td>
<td>W</td>
<td>V T A</td>
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<sup>a</sup>A*6601 was predicted according to HLA pocket structure analysis, and we suggested that this class I molecule would prefer I, V, T or A as anchor at position 2, R or K as second
field of tumor therapy, the transfection of Epstein–Barr virus-transformed patient cells with ICP47 will provide a unique tool to screen for and define potential CTL epitopes from the individual tumor-associated proteins.

Acknowledgements

We thank J. G. Wesseling for providing the cDNA of ICP47. The cell lines C1R-HLA-A*1101, C1R-HLA-A24, C1R-A*3101 and C1R-HLA-B8 were gifts from M. Takiguchi. The C1R-HLA-B*1516 cells were provided by H. de la Salle. This work was supported by grants from the European Union (Biomed CT95-1627), the Deutsche Forschungsgemeinschaft (Leibnizprogramm, Ra 369/4-1) and Merck KGaA.

Abbreviations

Con A concanavalin A  
CTL cytotoxic T lymphocytes  
ER endoplasmic reticulum  
FR fluorescence ratio  
HLA human leukocyte antigen  
ICP infected cell protein  
MFI mean fluorescence intensity  
TAP transporter associated with antigen processing  
Tpa 4′-(trifluoromethyl-diazirinyl)-phenylalanine

References

8. DeMars, R., Chang, C. C., Shaw, S., Reitnauer, P. J. and Sondel, P. M. 1984. Homozygous deletions that simultaneously eliminate expressions of class I and class II antigens of EBV-transformed B-lymphoblastoid cells. I. Reduced proliferative responses of autologous and allogeneic T cells to mutant cells that have decreased expression of class II antigens. Hum. Immunol. 11:77.
Peptide binding to ICP47-transfected human cells


