

This information is current as
of April 13, 2017.

A Single Specific Amino Acid Residue in Peptide Antigens Is Sufficient to Activate Memory CTL: Potential Role of Cross-Reactive Peptides in Memory T Cell Maintenance

Eva Reali, Remo Guerrini, Mauro Marastoni, Roberto
Tomatis, Maria Grazia Masucci, Serena Traniello and
Riccardo Gavioli

J Immunol 1999; 162:106-113; ;

<http://www.jimmunol.org/content/162/1/106>

References This article **cites 50 articles**, 24 of which you can access for free at:
<http://www.jimmunol.org/content/162/1/106.full#ref-list-1>

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>

A Single Specific Amino Acid Residue in Peptide Antigens Is Sufficient to Activate Memory CTL: Potential Role of Cross-Reactive Peptides in Memory T Cell Maintenance¹

Eva Reali,^{2*} Remo Guerrini,[†] Mauro Marastoni,^{†‡} Roberto Tomatis,^{†‡} Maria Grazia Masucci,[§] Serena Traniello,* and Riccardo Gavioli*

In the present study, we examined the structural requirements of peptide Ags for productive interactions with the TCR of CTL. For this purpose, we used as a model a previously identified immunodominant epitope that represents the target of EBV-specific HLA-A11-restricted CTL responses. By the use of peptides having minimal sequence homology with the wild-type epitope, we demonstrated that it is possible to selectively expand and reactivate memory CTL precursors without triggering the lytic mechanisms of wild-type specific effectors. In fact, stimulation of PBL from EBV-seropositive donors by polyalanine analogues, sharing only the putative TCR contact residue with the natural epitope, exclusively induced clonal expansion and reactivation of EBV-specific memory CTL precursors. Interestingly, these polyalanine peptides failed to trigger the cytotoxic function of CTLs specific for the wild-type viral epitope. This clearly indicates that reactivation of memory CTL precursors and triggering of the cytotoxic function have different requirements. The same phenomenon was observed using as stimulators naturally occurring peptides carrying the appropriate TCR contact residue. These data strongly suggest that cross-reactive peptides may play an important role in the expansion and reactivation of CTL clones from the memory T cell pool, and may be involved in long-term maintenance of T cell memory. *The Journal of Immunology*, 1999, 162: 106–113.

The biologic effects of CD8⁺ T cell-mediated immune responses are controlled by the interaction between TCRs and their respective peptide ligands presented by MHC class I molecules (1, 2). The engagement of specific TCR by MHC/peptide complexes represents the central event in the initiation of the cytolytic T cell response against virus-infected or neoplastic cells.

Recent studies have demonstrated that this recognition may occur with a certain degree of flexibility (3–8). Indeed, it has been shown that a single TCR can productively interact with various MHC-associated peptides, defined as altered peptide ligands (APL).³ APL may interact with TCR with different affinities, which are in turn determined by the APL sequence homology with the wild-type epitope. Peptides with high structural similarity to the viral Ag can fully activate the specific TCR (agonistic peptides); analogues with intermediate affinity can induce partial triggering of a single transduction event (partial agonist); peptides with low sequence similarity, able to occupy the specific TCR without triggering T cell functions, may instead act as antagonists (9). Studies regarding CD4⁺ T cells have demonstrated that analogues of immunogenic peptides with partial agonistic activity do

not stimulate T cell clonal proliferation, but are capable of activating T cell-mediated cytokine production. Such an effect has been ascribed to differences in the activation pattern of signal-transduction events induced upon MHC/peptide recognition by the TCR (10).

Degeneracy in TCR recognition has been considered to be involved in the positive and negative selection of thymocytes, the pathogenesis of autoimmune diseases, and viral antagonism (10, 11). In addition, it has been speculated that it may play a role in memory T cell maintenance, the molecular bases of which are largely unknown.

At present it is not clear whether cross-reactivity phenomena are due to the TCR recognition of conformationally related antigenic surfaces, rather than interaction with specific amino acid side chains of MHC-bound peptides (3, 5, 6, 12). Crystallographic studies performed on purified MHC class I/peptide complexes have elucidated that the majority of peptide residues are buried in the MHC groove and that contacts with TCR are conferred by few side chains in the peptide center, called primary and secondary contact residues (11, 13–15).

In this investigation, we have studied the role of specific residues in the functional interaction with TCR expressed by CD8⁺ T cells, using as a model EBV-specific CTL responses.

EBV, a widespread herpes virus, usually infects humans asymptotically, and establishes a lifelong carrier state whereby the virus persists in latently infected B lymphocytes. The proliferative potential of virus-infected cells is specifically controlled by CTLs. Studies on healthy EBV-seropositive individuals have shown that EBV-specific memory CTL responses are usually MHC class I restricted, and are directed to the nuclear Ags EBNA2-6, and the latent membrane proteins 1 and 2 expressed in latently infected cells. Several peptide epitopes have been identified in the viral Ags, each recognized in the context of a specific class I molecule (16).

*Department of Biochemistry and Molecular Biology, [†]Department of Pharmaceutical Sciences, and [‡]Biotechnology Center, University of Ferrara, Ferrara, Italy; and [§]Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden. Received for publication June 8, 1998. Accepted for publication September 3, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This investigation was supported by grants awarded by Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), Associazione Italiana per la Ricerca sul Cancro (AIRC), and Istituto Superiore di Sanità (progetto AIDS).

² Address correspondence and reprint requests to Dr. Eva Reali, DIBIT, San Raffaele Scientific Institute, Via Olgettina, 58 Milan, Italy. E-mail address: reali.eva@hsr.it

³ Abbreviations used in this paper: APL, altered peptide ligand; HBV, hepatitis B virus; LCL, lymphoblastoid cell line.

We have identified previously the immunodominant epitope of EBV-specific HLA-A11-restricted CTL responses, which derives from EBNA4 and has been mapped within residues 416–424 (IVTDFSVIK, designed IVT) (17–20). Amino acid positions and characteristics important for HLA-A11 binding have been defined: these are represented by valine, threonine, or isoleucine residues at position 2, and by a lysine residue at position 9 or 10 (21, 22).

As concerns TCR contact, a preliminary study has suggested that the aspartic acid at position 4 of the IVT peptide may play a crucial role, since its substitution with alanine abrogates its capability to sensitize target cells to lysis by IVT-specific clones (23, 24). To specifically define the structural requirements of the antigenic peptide determining recognition by IVT-specific TCR and T cell activation, we analyzed a panel of HLA-A11-binding polyalanine analogues sharing with the IVT epitope only the aspartic acid residue, or carrying conservative, semiconservative, or nonconservative amino acid substitutions at position 4. These sequences were tested for their ability to induce IVT-specific memory CTL responses and to sensitize target cells to lysis. PBL from HLA-A11-positive EBV-seropositive donors were stimulated with the T2/A11 cell line, pretreated with IVT or with polyalanine IVT analogues (25). We found that IVT-specific CTLs could be reactivated not only by the wild-type IVT epitope, but also by polyalanine peptides sharing with the original epitope only the HLA-binding residues and a specific TCR contact residue at position 4. However, peptide analogues with high stimulatory capacity failed to sensitize target cells to IVT-specific lysis. Similar results were obtained with naturally occurring peptides carrying the relevant TCR contact amino acid at position 4. This indicates that TCR may have different affinity requirements for the reactivation of memory T cell responses and triggering of cytotoxic effectors, and suggests a role of cross-reactive environmental Ags in the maintenance of the memory T cell pool.

Materials and Methods

Cell lines

The .174CEM.T2 cell line (T2), obtained by fusion of the peptide transporter mutant .174LCL with the T cell line CEM (26), was a kind gift from P. Cresswell (Yale University, New Haven, CT). An HLA-A11-positive subline (T2/A11) was obtained by transfection of a genomic *HindIII* fragment containing the HLA-A11-encoding sequence (21). T2/A11 cells were maintained in medium containing 200 $\mu\text{g}/\text{ml}$ hygromycin B (Sigma, St. Louis, MO). Lymphoblastoid cell lines (LCL) were obtained by transformation of purified B lymphocytes from HLA-typed donors with the B95-8-derived strain of EBV (27). Cell lines were maintained in RPMI 1640 supplemented with 2 mM glutamine, antibiotics, and 10% heat-inactivated FCS. PHA-activated blasts were obtained by stimulation of PBL with 1 $\mu\text{g}/\text{ml}$ of purified PHA (Wellcome Diagnostics, Dartford, U.K.) for 3 days, and expanded in medium supplemented with IL-2, as described (18). T cell clones were obtained from IVT-specific CTL cultures by limiting dilution. Single cells were seeded in 96-well plates in 200 μl of medium containing 10 U/ml human rIL-2 and 10^5 irradiated (3000 rad) allogeneic PHA-activated PBL as feeder. Growing cultures were transferred into 48-well plates and further expanded in IL-2-conditioned medium (25). Expanded clones were cryopreserved. After thawing, the clones were stimulated and kept in culture for 1 wk before use.

Synthetic peptides

Naturally presented peptides were synthesized by solid-phase method, as previously described (22). The following HLA-A11-binding peptides were used in this study: IVTDFSVIK (IVT), corresponding to amino acids 416–424 of the EBV nuclear Ag EBNA4 (18); AIFQSSMTK (AIF), corresponding to amino acids 325–333 of HIV pol Ag (21); ELNEALELK (ELN), corresponding to amino acids 343–351 of wild-type p53 (21); and YVNVNMGGLK (YVN), corresponding to amino acids 88–96 of HBV nucleocapsid Ag (28). Crude deprotected peptides were purified by HPLC; purity was >98%.

Polyalanine peptides were synthesized in two steps. The C-terminal pentapeptide was prepared by solid-phase synthesis performed in a Milli-

gen 9050 synthesizer, using F-moc-Lys(Boc)-Wang resin (Novabiochem, L aufelfingen, Switzerland). Pentapeptides were assembled using F-moc-protected amino acids and diisopropylcarbodiimide and 1-hydroxybenzotriazole (HOBt) as coupling agents. The preparation of N-terminal tetrapeptide sequences was performed by conventional solution methods and condensed to linked resin pentapeptide by HOBt and water-soluble carbodiimide as coupling agents. The nonamers were cleaved from the solid support by 1 h of trifluoroacetic acid (TFA)/H₂O/phenol/ethanedithiol/thioanisole treatment at room temperature. Crude peptides were purified by preparative reverse-phase HPLC using a Water Delta Prep 4000 system.

Peptide sequences were confirmed by amino acid analysis and mass-spectroscopy properties. All analogues showed less than 1% impurities. Peptide stocks were prepared in DMSO at a concentration of 10^{-2} M and were diluted in PBS before use.

Generation of CTL cultures

Monocyte-depleted PBL from the EBV-seropositive donors BEN (HLA-A11,24 and HLA-B35,57), PRI (HLA-A3,11 and HLA-B7,16), PF (HLA-A11,24 and HLA-B35,44), POL (HLA-A11,29 and HLA-B35), TF (HLA-A2,11 and HLA-B35), MU (HLA-A2,11 and HLA-B39,50), FG (HLA-A11,24 and HLA-B35), ZL (HLA A3, A4 and HLAB35), and GB (HLA-A11,24 and HLA-B35,44) were stimulated with mitomycin-treated T2/A11 cells incubated overnight at 26°C and pulsed with 10^{-4} M synthetic peptides for 1 h at 37°C, at stimulator: responder ratio of 1:20 (25). The first stimulation was performed in AIM-V medium (Life Technologies, Gaithersburg, MD), and serum was added to the cultures after 3 days. The second and third stimulations were performed in the same conditions on days 7 and 14. The medium was supplemented from day 8 with 10 U/ml rIL-2 (Proleukin, Chiron, Milan, Italy).

Detection of peptide binding to HLA-A11 molecules by immunofluorescence

Aliquots of 1×10^6 T2/A11 cells, incubated overnight at 26°C in 1 ml of AIM-V medium, were treated for 3 h at 37°C with 10^{-4} M concentration of synthetic peptides. After washing, the cells were stained by indirect immunofluorescence using the AUF5.13 mAb, specific for HLA-A3 and -A11 molecules, and the W6.32 mAb, specific for HLA-A, -B, and -C molecules (23). Mean fluorescence intensity was determined by FACS analysis (22).

Cytotoxicity tests

Cytotoxic activity was tested in standard 4-h ^{51}Cr release assays (17). LCL and PHA blasts were labeled with 0.1 mCi/ 10^6 cells of $\text{Na}_2^{51}\text{CrO}_4$ for 1 h at 37°C. For the peptide sensitization assays, 4×10^5 PHA blasts were placed in triplicate wells of 96 V-shaped well plates. Peptides were added to each well, and the plates were then incubated for 1 h at 37°C before adding the effectors (18). Peptide toxicities were checked in each assay and were always $\leq 3\%$. The cytotoxicity tests were routinely run at 10:1, 3:1, and 1:1 E:T ratios in triplicate. Percentage of specific lysis was calculated as $100 \times [(\text{cpm sample} - \text{cpm medium}) / (\text{cpm Triton X-100} - \text{cpm medium})]$.

T cell proliferation assay

T cell proliferation assay was performed using the T2/A11 cell line as APC. T2/A11 cells were incubated overnight at 26°C, treated with mitomycin, and then pulsed with 10^{-4} M peptides for 1 h at 37°C. After extensive washing to remove the unbound peptide, 25×10^3 cells were added to 50×10^3 CTLs in triplicate wells of round-bottom 96-well microtiter plates. After 3 days, T cell proliferation was determined by adding 1 μCi of [^3H]thymidine to each well for the last 18 h of culture. Cells were then harvested, and incorporated radioactivity was measured by scintillation counting.

TCR antagonism assay

^{51}Cr -labeled HLA-A11-positive PHA blasts were pulsed with the IVT peptide at concentrations ranging from 10^{-4} – 10^{-14} M. The cells were then washed three times to remove the unbound peptide, seeded in 96 V-bottom microtiter plates (4×10^3 cells/well), and treated for 1 h at 37°C with 2-9 or 2-4D-9 polyalanine peptides in a concentration range of 10^{-4} – 10^{-8} M. Effector IVT-specific CTL clones were then added to each well to give an E:T ratio of 5:1, and the percentage of specific lysis was detected as described above.

Table I. Peptide sequences

Peptides	Code
I V T D F S V I K	IVT
A V A A A A A A K	2-9
A V A D A A A A K	2-4D-9
A V A E A A A A K	2-4E-9
A V A K A A A A K	2-4K-9
A V A Q A A A A K	2-4Q-9
A V A V A A A A K	2-4V-9
A V A F A A A A K	2-4F-9
A I F Q S S M T K	AIF
E L N E A L E L K	ELN
Y V N V N M G L K	YVN

Results

IVT-specific CTL reactivation by synthetic peptides with minimal homology with the wild-type IVT epitope

We have demonstrated previously that efficient EBV-specific HLA-A11-restricted CTL responses can be reactivated in vitro by stimulating lymphocytes from HLA-A11-positive EBV-seropositive individuals with T2/A11 cells pulsed with the relevant synthetic epitopes (25). To investigate this phenomenon further, we have now characterized the peptide residues required for triggering the activation of memory CTL responses.

For this purpose, PBL from the HLA-A11-positive EBV-seropositive donors BEN, ZL, PF, POL, TF, and FG were stimulated with T2/A11 cells pulsed with IVTDFSVIK (IVT) or 2-4D-9 peptides (Table I). The 2-4D-9 represented an IVT analogue in which all amino acids were substituted with alanine, with the exceptions of valine and lysine at positions 2 and 9, required for binding to A11 molecules (21), and of the aspartic acid at position 4, a putative TCR contact residue (23, 24). As a control, we performed stimulations with T2/A11 cells, either untreated or pulsed with a polyalanine peptide containing only the HLA-A11-binding residues at positions 2 and 9 (2-9 peptide).

Immunofluorescence experiments indicated that the 2-9 and 2-4D-9 peptides efficiently bound to HLA-A11 molecules, as they both induced surface HLA-A11 expression in the transfected T2/A11 mutant cell line at the same level as the synthetic IVT peptide (Table II).

Cytotoxic activity of the cultures was tested after three consecutive stimulations on a panel of targets including HLA-A11-pos-

itive PHA blasts either untreated or pulsed with 10^{-7} M IVT peptide, and HLA-A11 single-matched or mismatched LCL. Representative results obtained from donor BEN are reported in Fig. 1. As previously observed, stimulation with the wild-type IVT peptide efficiently induced IVT-specific CTLs. Indeed, the culture lysed both IVT-pulsed PHA blasts and HLA-A11 single-matched LCL, expressing the endogenously processed viral epitope (25). Interestingly, a similar pattern of reactivity was demonstrated in cultures stimulated with T2/A11 cells pulsed with the minimally homologous 2-4D-9 peptide. Conversely, CTL cultures stimulated with T2/A11 cells pulsed with the 2-9 peptide did not show any IVT specificity. Control cultures stimulated with untreated T2/A11 cells were also inactive (not shown), thus excluding the possibility that CTL reactivation might be a nonspecific effect due to the release of soluble factors, such as cytokines, by APCs.

Similar results were obtained with PBL from five different HLA-A11-positive donors (ZL, PF, POL, TF, and FG, not shown).

These data indicate that IVT-specific CTL precursors can be reactivated not only by the original viral determinant, but also by the structurally related 2-4D-9 peptide, sharing with the wild-type epitope only the HLA-A11-binding residues and the TCR contact residue at position 4.

IVT-specific CTL proliferation by polyalanine peptides

We further investigated the capacity of 2-4D-9 peptide to stimulate the proliferation of IVT-specific T cell clones. T2/A11 cells were treated with different concentrations of IVT, 2-4D-9, and 2-9 peptides in a range of 10^{-4} – 10^{-8} M and used as stimulators of IVT-specific clones isolated from different donors. Mitomycin-treated stimulators were added to 50×10^3 CTLs in the ratio 1:2. After 3 days of coculture in serum-free medium, CTL proliferation was determined by adding [3 H]thymidine for the last 18 h. Fig. 2 shows a representative experiment performed with the IVT-specific clone ZAN-19. The 2-4D-9 peptide efficiently induced proliferation, with a stimulatory capacity comparable with the original IVT peptide. Conversely, the 2-9 analogue did not significantly enhance T cell proliferation as compared with CTL stimulated with untreated T2/A11. Similar results were obtained using other IVT-specific clones (not shown).

Triggering of IVT-specific cytotoxic activity by polyalanine peptides

To establish whether the cross-reactivity between the IVT peptide and its minimally homologous 2-4D-9 peptide could occur also in the recognition of target cells, we tested the cytotoxic activity of IVT-specific clones against PHA blasts pulsed with concentrations of IVT, 2-9, or 2-4D-9 peptides ranging from 10^{-4} to 10^{-10} M. As shown in Fig. 3, only PHA blasts pulsed with the wild-type peptide were lysed, whereas blasts pulsed with 2-4D-9 or 2-9 peptides were not.

The data indicate that CTLs strictly require the wild-type epitope to trigger the cytolytic mechanism, whereas a peptide with minimal homology is sufficient to induce CTL reactivation and clonal expansion.

IVT-specific TCR antagonism by polyalanine analogues

It is well established that variants of antigenic MHC/peptide complexes can act as TCR antagonists or partial agonists (10, 29). To evaluate the antagonistic activity of the polyalanine analogues, we evaluated their capacity to inhibit CTL cytotoxicity induced by the IVT peptide.

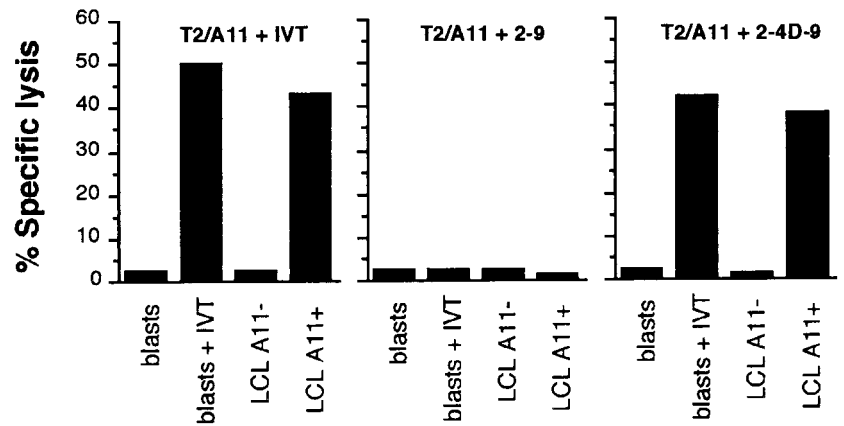
For this purpose, the cytotoxic activity of IVT-specific clones was tested against HLA-A11-positive PHA blasts, pretreated with different concentrations of IVT peptide, in the absence or presence

Table II. Induction of surface HLA class I expression by peptides^a

Peptide Treatment	MFI W6.32	MFI AUF5.13
None	8 ± 4	238 ± 22
IVT	103 ± 14	521 ± 29
2-9	223 ± 20	587 ± 27
2-4D-9	203 ± 14	575 ± 32
2-4E-9	140 ± 24	478 ± 25
2-4K-9	175 ± 16	502 ± 36
2-4Q-9	150 ± 12	504 ± 26
2-4V-9	189 ± 20	526 ± 22
2-4F-9	91 ± 22	500 ± 27
AIF	100 ± 12	488 ± 23
ELN	103 ± 22	478 ± 32
YVN	156 ± 9	538 ± 36

^a T2/A11 cells were incubated overnight in medium alone or medium containing 100 μ M concentrations of the indicated peptides. HLA class I expression was measured by FACS analysis after indirect immunofluorescence staining with the AUF5.13 mAb, which is specific for HLA-A3 and -A11 molecules, and W6.32 mAb, which is specific for HLA-A, -B, and -C molecules. Mean of \pm SD of three experiments. MFI, mean fluorescence intensity.

FIGURE 1. IVT-specific CTL reactivation by synthetic peptides. Freshly isolated PBL derived from the HLA-A11-positive, EBV-seropositive donor BEN (HLA-A11,24 and HLA-B35,57) were stimulated with T2/A11 cells pulsed with 10^{-4} M IVT, 2-9, or 2-4D-9 peptides. CTL cultures obtained after three consecutive stimulations were tested in cytotoxicity assays on a panel of targets including HLA-A11-positive PHA blasts, pulsed or not with 10^{-7} M IVT peptide for 1 h before the assay, and HLA-A11 single-matched or mismatched LCL. The percentage of specific lysis recorded at E:T 10:1 in one representative experiment is shown.



of increasing amounts of 2-9 or 2-4D-9 peptides. The results clearly show that both peptides inhibited the CTL recognition of IVT-pulsed PHA blasts in a concentration-dependent way (Fig. 4). Maximal inhibition was observed with suboptimal concentrations of IVT peptide. Similar data were obtained, using as effectors IVT-specific polyclonal T cell cultures (not shown). From these experiments we conclude that 2-9 and 2-4D-9 peptides possess antagonistic activity on IVT-specific cytotoxicity.

CTL stimulation with 2-4D-9 peptide analogues, modified at position 4

We next investigated whether IVT-specific CTL reactivation requires peptides exclusively with the aspartic acid residue at position 4, or also occurs with peptides carrying a different amino acid residue.

For this purpose, we synthesized a panel of polyalanine analogues carrying conservative, semiconservative, or nonconservative amino acid substitutions at position 4 (peptide sequences shown in Table I).

In accordance with the demonstration that residue 4 of the IVT peptide points away from the peptide-binding groove (23), immunofluorescence analysis revealed that 2-4E-9, 2-4Q-9, 2-4K-9, 2-4F-9, and 2-4V-9 peptides all bound to HLA-A11 molecules, since they induced surface HLA-A11 molecules on T2/A11 cells at levels comparable with those obtained with the IVT peptide (Table II).

The modified polyalanine analogues were compared with the 2-4D-9 peptide for their capacity to reactivate IVT-specific CTL responses. Fig. 5 shows the results of one representative experiment of four performed with CTL cultures obtained from different donors (PF, TF, MU, and GB). CTL cultures obtained after three consecutive stimulations with T2/A11 cells pulsed with the different peptides were tested for specificity in cytotoxic assays against HLA-A11-positive

PHA blasts, untreated or treated with the IVT peptide. Interestingly, the two analogues carrying the glutamic acid or glutamine at position 4 were found to be potent activator of IVT-specific CTLs, whereas analogues carrying lysine, phenylalanine, or valine were not.

This indicates that conservative and semiconservative amino acid substitutions are tolerated at position 4, suggesting that TCR-mediated reactivation of IVT-specific CTL precursors may occur by interaction with different peptide ligands.

Peptide specificity of CTL cultures reactivated by polyalanine peptides

CTL cultures obtained by stimulation with 2-4D-9, 2-4E-9, or 2-4Q-9 peptides were tested for specificity against HLA-A11-positive PHA blasts pulsed with the IVT peptide or with the correspondent stimulating peptide in a concentration range of 10^{-4} – 10^{-10} M. The data clearly show that CTL cultures exclusively lysed PHA blasts pulsed with the wild-type IVT peptide, but failed to recognize the polyalanine analogues used as stimulators in CTL reactivation (Fig. 6).

These results further confirm that TCR has different activation requirements for the triggering of memory CTL responses and target cell lysis. In particular, target cell lysis strictly requires the viral peptide sequence bound to HLA-A11 molecules, whereas epitope-specific CTL reactivation and growth can be triggered by cross-reactive peptide ligands.

Identification of IVT cross-reactive peptides of cellular and viral origin

Having established that the residue in position 4 is sufficient to trigger the activation of IVT-specific CTLs, we searched for natural peptides of viral and cellular origin containing the amino acid residues required for both MHC-binding and TCR activation.

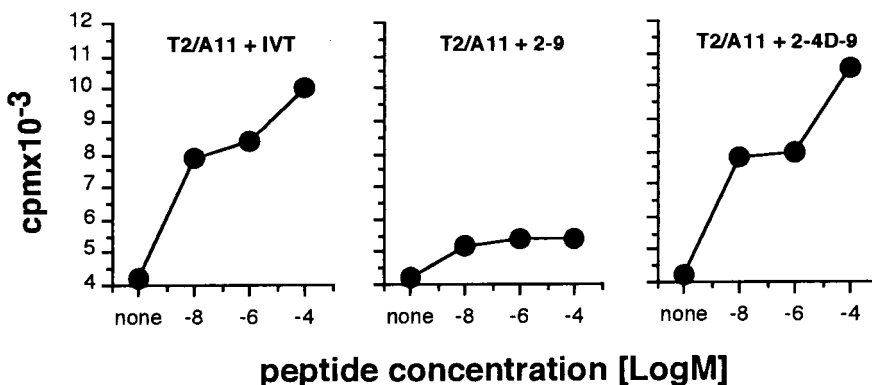


FIGURE 2. IVT-specific CTL clone proliferation by synthetic peptides. T2/A11 pulsed or not with 10^{-4} , 10^{-6} , 10^{-8} M IVT, 2-9, 2-4D-9 peptides were mitomycin C treated and used as stimulators of the IVT-specific clone ZAN-19. Stimulators were added to 50×10^3 CTLs in a ratio of 1:2. After 3 days of coculture in serum-free medium, CTL proliferation was determined by adding [3 H]thymidine for the last 18 h. Results are expressed as $\text{cpm} \times 10^{-3}$ of incorporated [3 H]thymidine. One representative experiment is shown.

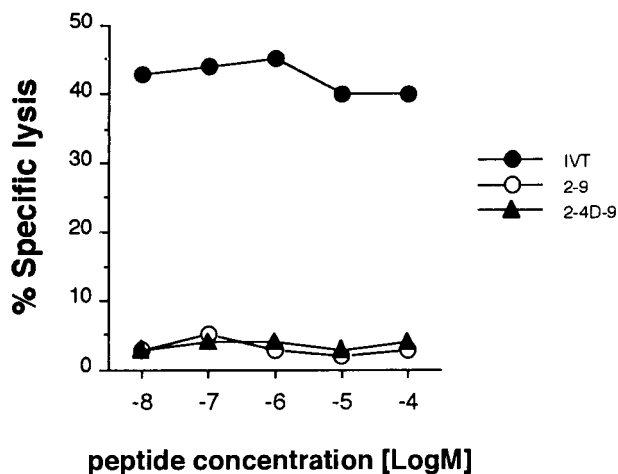


FIGURE 3. 2-9 and 2-4D-9 peptide recognition by an IVT-specific CTL clone. The CTL clone ZAN-43, obtained from a polyclonal culture activated by the autologous LCL, was tested for the ability to lyse HLA-A11-matched PHA blasts pulsed with different concentrations of IVT, 2-9, and 2-4D-9 peptides for 1 h before CTLs were added to the assay. The percentage of specific lysis recorded at E:T 10:1 is shown.

We chose two previously identified HLA-A11-binding non-peptides: ELNEALELK (ELN), derived from p53 cellular protein, and AIFQSSMTK (AIF), derived from HIV pol Ag, in which position 4 is occupied respectively by a glutamic acid or a glutamine residue. As a control, we chose the peptide sequence YVN VNMGLK (YVN), derived from the HBV nucleocapsid Ag, carrying at position 4 a valine residue, which has been shown to be ineffective in IVT-specific CTL reactivation. The experiments were performed with PBL from three different donors (ZL, PRI, and FG), stimulated with T2/A11 cells treated with 10^{-4} M ELN, AIF, or YVN peptides. The data in Fig. 7 show that ELN and AIF peptides were both capable of eliciting an IVT-specific CTL response directed to both IVT-pulsed PHA blasts and HLA-A11-positive LCL, whereas the YVN peptide was not. CTL cultures activated by cross-reactive Ags selectively lysed PHA blasts pulsed with the original viral peptide IVT, but not blasts treated with the ELN or AIF peptides.

Discussion

The study reported in this work demonstrates that minimal homologues of the EBV-derived IVT epitope, presented by HLA-A11

FIGURE 5. IVT-specific CTL reactivation by synthetic analogues of 2-4D-9 peptide. Freshly isolated PBL derived from the HLA-A11-positive, EBV-seropositive donor PF (HLA A11, 24 and B35, 44) were stimulated with T2/A11 cells pulsed with 10^{-4} M concentrations of the indicated peptides. CTL cultures obtained after three consecutive stimulations were tested in cytotoxicity assays on HLA-A11 single-matched PHA blasts, pulsed or not with 10^{-7} M IVT peptide for 1 h before the assay. The percentage of specific lysis recorded at E:T 10:1 in one representative experiment is shown.

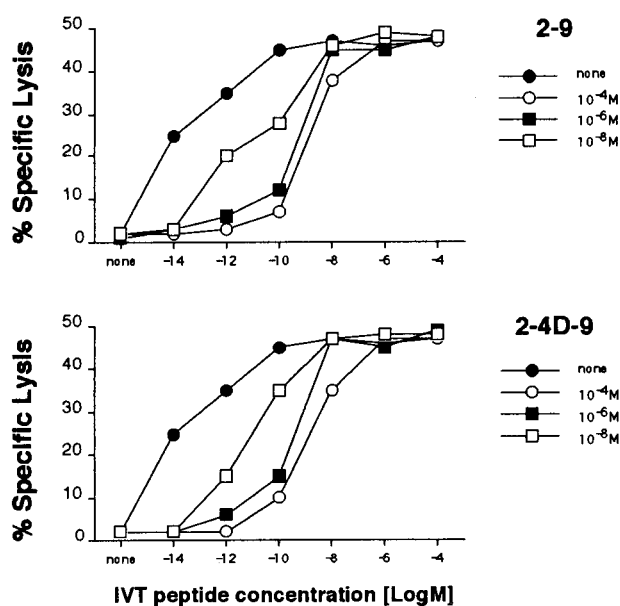
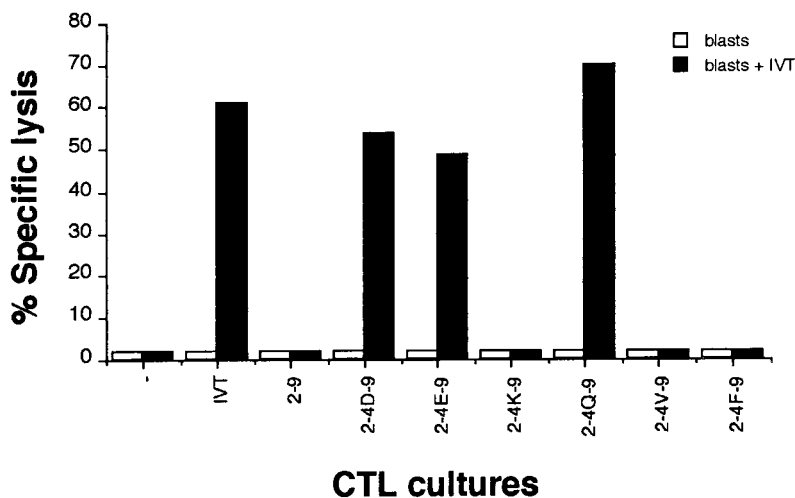


FIGURE 4. Antagonistic effect of 2-9 and 2-4D-9 peptides on IVT-specific CTL clones. HLA-A11-positive PHA blasts were pulsed with different concentrations of IVT peptide for 1 h at 37°C, washed to remove unbound peptide, and used as targets of IVT-specific CTL clones. IVT-pulsed target cells were treated or not with different concentrations of 2-9 and 2-4D-9 peptides for 1 h before adding CTLs to the assay. The percentage of specific lysis recorded at E:T 5:1 is shown. One representative experiment performed with the IVT-specific clone ZAN-62 is shown in the figure.

molecules, are capable of effectively reactivating IVT-specific CTL responses in EBV-immune donors. Indeed, the polyalanine analogue carrying the putative TCR contact residue at position 4 and the anchor residues at positions 2 and 9 (2-4D-9 peptide) induced reactivation and clonal expansion of CTLs specific for the IVT viral epitope. In contrast, the polyalanine analogue carrying only the anchor residues 2V and 9K did not induce any CTL response (2-9 peptide). This result indicates that a single, specific amino acid residue is sufficient to productively interact with the TCR of IVT-specific memory CTL precursors. However, the aspartic acid at position 4 is not the only residue capable of IVT-specific CTL reactivation, since polyalanine analogues carrying the glutamic acid or glutamine in 4 (2-4E-9, and 2-4Q-9 peptides) were shown to be potent stimulators of IVT-specific CTLs. It is likely that the carboxylic group on the side chain of these amino

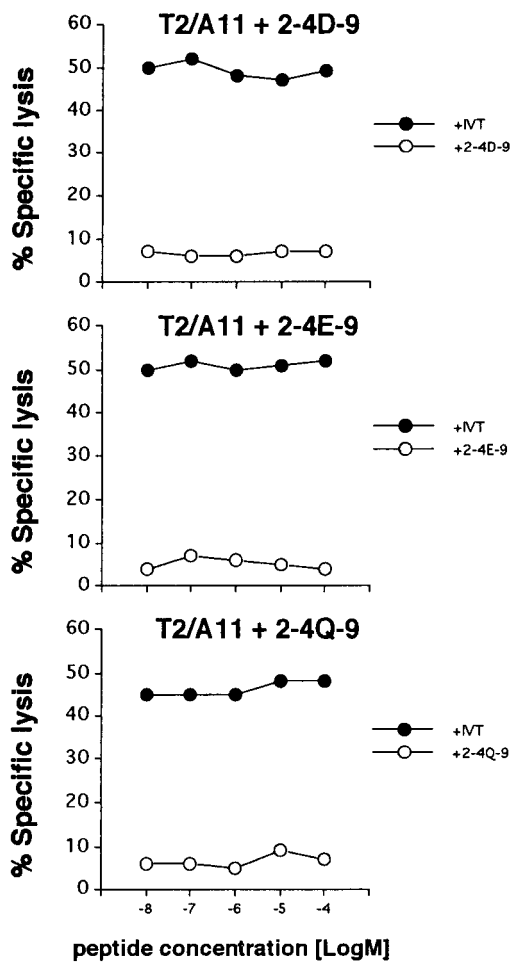


FIGURE 6. Peptide recognition by polyclonal cultures obtained by different peptide stimulations. Polyclonal cultures obtained from freshly isolated PBL of donor PF (HLA-A11,24 and HLA-B35,44) stimulated with T2/A11 pulsed with 10^{-4} M 2-4D-9, 2-4E-9, 2-4Q-9 peptides were tested against HLA-A11 single-matched PHA blasts treated with different concentrations of IVT or of the stimulating peptide. The percentage of specific lysis recorded at E:T 10:1 is shown.

acids is responsible per se for TCR engagement and activation. It has in fact been shown in different experimental systems that functional interactions with TCRs require the presence of specific key residues (3, 5, 6, 21, 23). The specificity of TCR/MHC-peptide

interactions was further confirmed by the failure of polyalanine analogues carrying nonconservative amino acid substitutions in 4 to induce IVT-specific CTL reactivation.

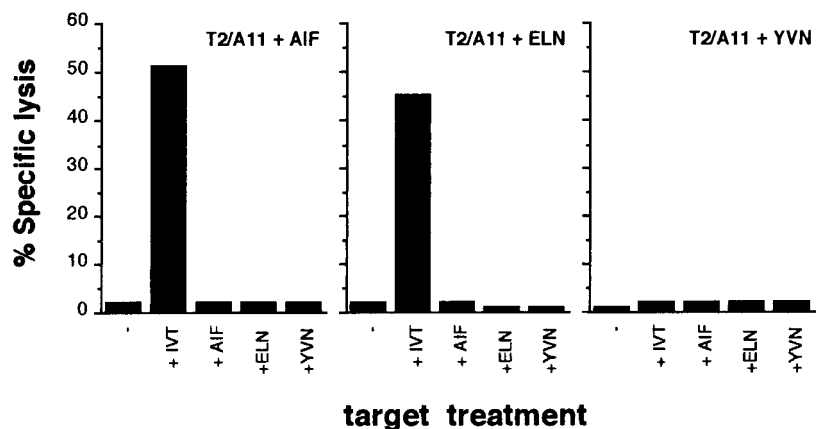
Interestingly, the 2-4D-9, 2-4E-9, and 2-4Q-9 peptides failed to sensitize HLA-A11 PHA blasts to lysis by IVT-specific CTL clones, indicating that the polyalanine analogues cannot sufficiently mimic the viral epitope on target cells to the point of inducing IVT-specific killing. It should be noted that none of these modified analogues sensitized target cells to lysis, even when CTL cultures obtained by polyalanine peptide stimulations were used as effectors. This finding clearly demonstrates the existence of a different requirement in TCR stimulation to obtain the induction of memory CTL reactivation and/or the triggering of the cytolytic mechanisms. As concerns the triggering of cytotoxicity, we and others have shown that a single conservative amino acid substitution or conformational changes are sufficient to abrogate the capacity of immunogenic epitopes to sensitize target cells to lysis by CTLs specific for the wild-type epitope (Refs. 21 and 23; and R. Gavioli et al., unpublished results). This suggests that the reaching of the threshold required for activating the lytic mechanism may occur on the basis of stringent interactions between TCR and the side chains of the primary and secondary TCR contact residues. In contrast, the reactivation of memory T cell precursors can be effectively induced by a peptide with a minimal sequence homology with the immunogenic epitope.

Different TCR activation thresholds accounting for the stimulation of a subset of T cell functions have recently been described (9, 30, 31). Altered peptide ligands with intermediate affinity for TCR molecules, defined as partial agonists, have been shown to induce only partial T cell activation, following the triggering of a single signal transduction pathway (32).

From our antagonism experiments, it was demonstrated that 2-4D-9 and 2-9 peptides equally inhibited the cytotoxic activity of IVT-specific clones; however, while the 2-4D-9 peptide was able to stimulate memory CTL responses, thus acting as a partial agonist, the 2-9 peptide inhibited the IVT-specific cytotoxicity without activation of T cell functions, thus behaving as a pure antagonist (9, 10).

The characterization of peptides with partial agonistic activity may be relevant for the design of peptides suitable for vaccination or treatment of viral diseases and immunogenic malignancies. The possibility of employing peptides, which selectively induce memory CTL reactivation, without sensitizing the peptide-pulsed cells to lysis, may increase the safety and the efficacy of peptide-based immunotherapies.

FIGURE 7. IVT-specific CTL reactivation by synthetic peptides of different origin. Freshly isolated PBL derived from the HLA-A11-positive, EBV-seropositive donor ZL (HLA-A3,11 and HLA-B35) were stimulated with T2/A11 cells pulsed with 10^{-4} M AIF, ELN, or YVN peptides. CTL cultures obtained after three consecutive stimulations were tested in cytotoxicity assays on a panel of targets including HLA-A11-positive PHA blasts pulsed or not with 10^{-7} M IVT, AIF, ELN, YVN peptides for 1 h before the assay. The percentage of specific lysis recorded at E:T 10:1 in one representative experiment of three is shown.



Finally, we extended our investigation to naturally occurring peptides, which possess the structural requirements for reactivation and expansion of IVT-specific cytotoxic precursors. For this purpose, we used previously identified peptide sequences that bind to HLA-A11 molecules and carry the relevant residue at position 4: the AIFQSSMTK peptide, derived from pol protein of HIV, and the ELNEALELK peptide, derived from the cellular p53 protein. As a control, we chose the YVNVNMGK peptide derived from the HBV, carrying the ineffective amino acid valine at position 4. The AIF and ELN peptides reactivated IVT-specific CTL responses, whereas YVN did not, thus confirming the key role of the carbonylic group of the amino acid side chain in position 4. Importantly, AIF- and ELN-pulsed PHA blasts failed to be recognized by IVT-specific CTL cultures. We have in fact previously shown that the activation protocol used in these experiments is not adequate for the induction of primary CTL responses (25).

Cross-reactivity phenomena in TCR recognition have already been described, and several physiological implications have been postulated (5, 7, 8, 33). These phenomena may be involved in the pathogenesis of autoimmune encephalomyelitis and multiple sclerosis (3, 4, 34–36). This hypothesis was based on the epidemiologic evidence that infections often precede autoimmune reactions (34). The activation and clonal expansion of self-reactive cells may be due to cross-reactive viral or bacterial peptides that have sufficient sequence similarity with the myelin-derived epitope. Activated T lymphocytes specific for a self Ag may therefore cross the blood brain barrier and initiate the autoimmune destruction of myelin (4). Other authors have reported that degeneration in T cell recognition may be involved in thymic positive selection and lymphocyte differentiation (37, 38). In addition, variant epitopes of viral origin may act as antagonists of their own CTLs; in this case, APL may represent an additional mechanism of immune evasion by viruses, such as HBV and HIV (39, 40).

APL have moreover been postulated to be involved in the maintenance of the memory T cell pool, but this hypothesis is not supported by clear experimental evidence (41, 42). Different mechanisms have been proposed to account for the persistence of memory (43, 44). The first suggests that Ags induce the responding lymphocytes to differentiate into a memory cell that has a very long life span compared with its virgin precursor. The second attributes memory to the Ag rather than to the responding cell, suggesting that the original Ag is periodically reintroduced by recurring infection, or else persists in specialized reservoirs, causing continued stimulation of memory T cell clones (45). The third proposes that the difference between a memory cell and its precursor does not reside in life span, but in the threshold for stimulation. As a memory cell has altered levels of adhesion molecules, it can be more easily stimulated than the precursors, and will be maintained by periodic activation of cross-reactive environmental Ags or antiidiotypic interactions (46, 47). It has been observed that memory T cells from previous infection may be reactivated by unrelated viruses and that they contribute to the primary response directed to the unrelated infectious agent. This suggests that prior immunity to one virus might provide some level of protective natural immunity to an unrelated infectious agent (48, 49). It is noteworthy that a recent report on *in vivo* mouse models has defined the requirements of naive and memory T cells for survival and proliferation. In particular, naive T cell survival has been shown to require the presence of the restricting MHC allele independently of the Ag bound, whereas expansion also necessitates the encounter of the specific peptide Ag. Conversely, for memory T cell survival, the presence of a nonspecific MHC class I molecule suffices,

whereas expansion requires the correct restricting element, but is independent of the bound peptide Ag (50).

From our experiments, we consider that cross-reactivity may be an important physiologic mechanism involved in the regulation of immune responses. In particular, this phenomenon is likely to be involved in memory T cell maintenance. Periodic stimulation with cross-reactive environmental Ags, having minimal sequence homology with the wild-type epitope can induce the reactivation and expansion of resting memory circulating precursors that maintain the specificity for the wild-type pathogen-derived epitope. Cross-reactivity may be highly advantageous for the induction of those T cell activities responsible for the maintenance of protective immune responses against pathogens. At the same time, the high activation threshold of the cytotoxic function would hinder the autoaggressive immune reactions potentially induced by CTL recognition and killing of target cells expressing peptide Ags other than the specific pathogen-derived epitope.

The capacity of structurally modified epitopes to stimulate wild-type specific memory CTL responses has been extended to epitopes other than IVT (R. Gavioli et al., unpublished results). We have observed that CTL responses directed to immunodominant epitopes are reactivated *in vitro* by peptide analogues having minimal homology with the relevant epitope, while responses to subdominant epitopes require stimulating peptides with stringent sequence homology. This suggests that cross-reactive peptides may play a role in determining the immunodominance of an epitope.

Acknowledgments

We thank the donors whose cytotoxic T lymphocytes are described in this paper, and Banca del Sangue of Ferrara for supplying fresh blood.

References

- Zinkernagel, R. M., and P. C. Doherty. 1974. Immunological surveillance against altered self components by sensitized T lymphocytes in lymphocytic choriomeningitis. *Nature* 251:547.
- Townsend, A. R. M., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 44:959.
- Bhardwaj, V., V. Kumar, H. M. Geysen, and E. E. Sercarz. 1993. Degenerate recognition of a dissimilar antigenic peptide by myelin basic protein-reactive T cells. *J. Immunol.* 151:5000.
- Wucherpfennig, K. W., and J. L. Strominger. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 80:695.
- Evavold, B. D., J. Sloan-Lancaster, K. J. Wilson, J. B. Rothbard, and P. M. Allen. 1995. Specific T cell recognition of minimally homologous peptides: evidence for multiple endogenous ligands. *Immunity* 2:655.
- Nanda, N. K., K. K. Arzoo, H. M. Geisen, A. Sette, and E. E. Sercarz. 1995. Recognition of multiple peptide cores by a single T cell receptor. *J. Exp. Med.* 182:531.
- Zügel, U., B. Schoel, S. Yamamoto, H. Hengel, B. Morein, and S. H. E. Kaufman. 1995. Crossrecognition by CD8 T cell receptor $\alpha\beta$ cytotoxic T lymphocytes of peptides in the self and mycobacterial hsp60 which share intermediate sequence homology. *Eur. J. Immunol.* 25:451.
- Chen, Y.-Z., S. Matsushita, and Y. Nishimura. 1996. Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide. *J. Immunol.* 157:3783.
- Sloan-Lancaster, J., and P. M. Allen. 1996. Altered peptide ligand-induced partial activation: molecular mechanisms and role in T cell biology. *Annu. Rev. Immunol.* 14:1.
- Jameson, S. C., and M. J. Bevan. 1995. T cell receptor antagonists and partial agonists. *Immunity* 2:1.
- Evavold, B. D., J. Sloan-Lancaster, and P. M. Allen. 1993. Tickling the TCR: selective T-cell functions stimulated by altered peptide ligands. *Immunol. Today* 14:602.
- Quarantino, S., C. J. Thorpe, P. J. Travers, and M. Londei. 1995. Similar antigenic surfaces, rather than sequence homology, dictate T-cell epitope molecular mimicry. *Proc. Natl. Acad. Sci. USA* 92:10398.
- Björkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* 54:505.
- Fremont, D. H., M. Matsumura, E. A. Stura, P. A. Peterson, and I. A. Wilson. 1992. Crystal structures of two viral peptides in complex with murine MHC class I H-2k^b. *Science* 257:919.

15. Madden, D. R., D. N. Garboczi, and D. C. Wiley. 1993. The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. *Cell* 75:693.
16. Rickinson, A. B., and D. J. Moss. 1997. Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection. *Annu. Rev. Immunol.* 15:405.
17. Gavioli, R., P. O. de Campos-Lima, M. G. Kurilla, E. Kieff, G. Klein, and M. G. Masucci. 1992. Recognition of the EBV encoded nuclear antigens EBNA-4 and EBNA-6 by HLA A11 restricted cytotoxic T-lymphocytes: implications for the down-regulation of HLA A11 in Burkitt's lymphoma. *Proc. Natl. Acad. Sci. USA* 89:5862.
18. Gavioli, R., M. Kurilla, P. O. de Campos-Lima, L. Wallace, R. Dolcetti, R. J. Murray, A. B. Rickinson, and M. G. Masucci. 1993. Multiple HLA A11-restricted cytotoxic T-lymphocyte epitopes of different immunogenicities in the Epstein-Barr virus-encoded nuclear antigen 4. *J. Virol.* 67:1572.
19. de Campos-Lima, P. O., V. Levitsky, J. Brooks, S. P. Lee, L. F. Hu, A. B. Rickinson, and M. G. Masucci. 1994. T cell responses and virus evolution: loss of HLA A11-restricted CTL epitopes in Epstein-Barr virus isolates from highly A11-positive populations by selective mutation of anchor residues. *J. Exp. Med.* 179:1297.
20. Levitsky, V., Q.-J. Zhang, J. Levitskaya, and M. G. Masucci. 1996. The life span of major histocompatibility complex-peptide complexes influences the efficiency of presentation and immunogenicity of two class I-restricted cytotoxic T lymphocyte epitopes in the Epstein-Barr virus nuclear antigen 4. *J. Exp. Med.* 183:915.
21. Zhang, Q.-J., R. Gavioli, G. Klein, and M. G. Masucci. 1993. An HLA-A11-specific motif in nonamer peptides derived from viral and cellular proteins. *Proc. Natl. Acad. Sci. USA* 90:2217.
22. Gavioli, R., Q.-J. Zhang, M. Marastoni, R. Guerrini, E. Reali, R. Tomatis, M. G. Masucci, and S. Traniello. 1995. Effect of anchor residue modifications on the stability of HLA-A11/peptide complexes. *Biochem. Biophys. Res. Commun.* 206:8.
23. Zhang, Q.-J., Y. Lindquist, V. Levitsky, and M. G. Masucci. 1996. Solvent exposed side chains of peptides bound to HLA*A1101 have similar effects on the reactivity of alloantibodies and specific TCR. *Int. Immunol.* 8:927.
24. de Campos-Lima, P.-O., V. Levitsky, M. P. Imreh, R. Gavioli, and M. G. Masucci. 1997. Epitope-dependent selection of highly restricted or diverse T cell receptor repertoires in response to persistent infection by Epstein-Barr virus. *J. Exp. Med.* 186:83.
25. Reali, E., R. Guerrini, B. Giori, M. Borghi, M. Marastoni, R. Tomatis, S. Traniello, M. G. Masucci, and R. Gavioli. 1996. Activation of epitope-specific memory cytotoxic T lymphocyte responses by synthetic peptides. *Clin. Exp. Immunol.* 105:369.
26. Salter, R. D., and P. Cresswell. 1986. Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. *EMBO J.* 5:943.
27. Miller, G., and M. Lipman. 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. *Proc. Natl. Acad. Sci. USA* 70:190.
28. Tsai, S.-L., M.-H. Chen, C.-T. Yeh, C.-M. Chu, A.-N. Lin, F.-H. Chiou, T.-H. Chang, and Y.-F. Liaw. 1996. Purification and characterization of a naturally processed hepatitis B virus peptide recognized by CD8⁺ cytotoxic T lymphocytes. *J. Clin. Invest.* 97:577.
29. De Magistris, M. T., J. Alexander, M. Coggeshall, A. Altman, F. C. A. Gaeta, H. M. Gray, and A. Sette. 1992. Antigen analog-major histocompatibility complexes act as antagonist of the T cell receptor. *Cell* 68:625.
30. Cao, W., S. S. Tykodi, M. T. Esser, V. L. Braciale, and T. J. Braciale. 1995. Partial activation of CD8⁺ T cells by a self-derived peptide. *Nature* 378:295.
31. Vergelli, M., B. Hemmer, U. Utz, A. Vogt, M. Kalbus, L. Tranquill, P. Conlon, N. Ling, L. Steinman, H. F. McFarland, and R. Martin. 1996. Differential activation of human autoreactive T cell clones by altered peptide ligands derived from myelin basic protein peptide (87-99). *Eur. J. Immunol.* 26:2624.
32. Sloan-Lancaster, J., T. H. Steimberg, and P. M. Allen. 1996. Selective activation of the calcium signaling pathway by altered peptide ligands. *J. Exp. Med.* 184:1525.
33. Harris, D. P., H.-M. Vordermeier, M. Singh, C. Moreno, S. Jurcevic, and J. Ivanyi. 1995. Cross-recognition by T cells of an epitope shared by two unrelated mycobacterial antigens. *Eur. J. Immunol.* 25:3173.
34. Barnett, L. A., and R. S. Fujinami. 1992. Molecular mimicry: a mechanism for autoimmune injury. *FASEB J.* 6:840.
35. Baum, H., H. Davies, and M. Peakman. 1996. Molecular mimicry in the MHC: hidden clues to autoimmunity? *Immunol. Today* 17:64.
36. Barnaba, V., and F. Sinigaglia. 1997. Molecular mimicry and T cell-mediated autoimmune disease. *J. Exp. Med.* 185:1529.
37. Pfeiffer, C., J. Stein, S. Southwood, H. Ketelaar, A. Sette, and K. Bottomly. 1995. Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. *J. Exp. Med.* 181:1569.
38. Chidgey, A., and R. Boyd. 1997. Agonist peptide modulates T cell selection thresholds through qualitative and quantitative shifts in CD8 co-receptor expression. *Int. Immunol.* 9:1527.
39. Bertoletti, A., A. Sette, F. V. Chisari, A. Penna, M. Levrero, M. De Carli, F. Fiaccadori, and C. Ferrari. 1994. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature* 369:407.
40. Klenerman, P., U.-C. Meier, R. E. Phillips, and A. J. McMichael. 1995. The effects of natural altered peptide ligands on the whole blood cytotoxic T lymphocyte response to human immunodeficiency virus. *Eur. J. Immunol.* 25:1927.
41. Bradley, L. M., M. Croft, and S. L. Swain. 1993. T-cell memory: new perspectives. *Immunol. Today* 14:197.
42. Beverley, P. C. L. 1990. Is T-cell memory maintained by cross-reactive stimulation? *Immunol. Today* 11:203.
43. Sprent, J., and D. F. Tough. 1994. Lymphocyte life-span and memory. *Science* 265:1395.
44. Sprent, J. 1994. T and B cell memory. *Cell* 76:315.
45. Gray, D., and P. Matzinger. 1991. T cell memory is short-lived in the absence of antigen. *J. Exp. Med.* 174:969.
46. Lau, L. L., B. D. Jamieson, T. Somasundaram, and R. Ahmed. 1994. Cytotoxic T-cell memory without antigen. *Nature* 369:648.
47. Tough, D. F., and J. Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med.* 179:1127.
48. Nahill, S. R., and R. M. Welsh. 1993. High frequency of cross-reactive cytotoxic T lymphocytes elicited during the virus-induced polyclonal cytotoxic T lymphocyte response. *J. Exp. Med.* 177:317.
49. Selin, L. K., S. R. Nahill, and R. M. Welsh. 1994. Cross-reactivities in memory cytotoxic T lymphocyte recognition of heterologous viruses. *J. Exp. Med.* 179:1933.
50. Tanchot, C., F. A. Lemonnier, B. Pérarnau, A. A. Freitas, and B. Rocha. 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science* 276:2057.