# 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

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**Summary**

We have investigated the reactivity to two human histocompatibility leukocyte antigen (HLA) A11–restricted cytotoxic T lymphocyte (CTL) epitopes derived from amino acids 416-424 (IVTDFSVIK, designated IVT) and 399-408 (AVFDRKSVAK, designated AVF) of the Epstein-Barr virus (EBV) nuclear antigen (EBNA) 4. A strong predominance of CTL clones specific for the IVT epitope was demonstrated in polyclonal cultures generated by stimulation of lymphocytes from the EBV-seropositive donor BK with the autologous B95.8 virus-transformed lymphoblastoid cell line (LCL). This was not due to intrinsic differences of CTL efficiency since clones specific for the two epitopes lysed equally well A11-positive phytohemagglutinin blasts and LCLs pulsed with the relevant synthetic peptide. Irrespective of the endogenous levels of EBNA4 expression, untreated LCLs were lysed more efficiently by the IVT-specific effectors, suggesting that a higher density of A11–IVT complexes is presented at the cell surface. In accordance, 10–50-fold higher amounts of IVT peptides were found in high-performance liquid chromatography fractions of acid extracts corresponding to an abundance of about 350–12,800 IVT and 8–760 AVF molecules per cell. Peptide-mediated competition of CTL sensitization, transport assays in streptolysin-O permeabilized cells, and induction of A11 expression in the transporter associated with antigen presentation–deficient T2/A11 transfectant demonstrated that the IVT and AVF peptides bind with similar affinities to A11, are translocated with equal efficiency to the endoplasmic reticulum, and form complexes of comparable stability over a wide range of temperature and pH conditions. A rapid surface turnover of A11 molecules containing the AVF peptide was demonstrated in metabolically active T2/A11 cells corresponding to a half-life of ~3.5 as compared to ~2 h for molecules induced at 26°C in the absence of exogenous petpides and >12 h for IVT-containing complexes. This difference in persistence is likely to determine the representation of individual class I–restricted CTL epitopes within the cell surface pool of molecules, and may be an important factor contributing to their immunogenicity.

CTLs recognize short peptides that are derived from proteolytic degradation of intracellular antigens and are presented at the surface of virus-infected and tumor cells in association with self-MHC class I molecules (1). Antigenic peptides are usually 8–10 amino acids long and contain specific consensus motifs that allow binding to the presenting class I allele (2, 3). Despite the great complexity of most protein antigens, and the presence in each protein of numerous peptides that may potentially bind to a given class I allele, T cell responses are usually focused on a limited number of immunodominant epitopes (4, 5). Several mechanisms may account for the generation of this selective immunodominance. In different experimental models, T cell responses were shown to correlate with the efficiency of generation of the antigenic peptide by proteolytic degradation of native antigens (6–8), the affinity of binding to the relevant MHC molecules (9–11), or the stability of the corresponding MHC–peptide complex (12). In addition, a selectivity based on size and some sequence characteristics has been demonstrated in the activity of the transporters associated with antigen presentation (TAP)[1] which is likely to influence the availability

[1] *Abbreviations used in this paper:* AVF, AVFDRKSDAK; $\beta_2$m, $\beta_2$-microglobulin; EBNA, Epstein-Barr virus nuclear antigen; ER, endoplasmic reticulum; IVT, IVTDFSVIK; LCL, lymphoblastoid cell line; TAP, transporter associated with antigen presentation.

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of peptides for class I assembly in the endoplasmic reticulum (ER) (13, 14). It is not clear, however, to what extent these in vitro parameters influence determinant selection and immunodominance in class I–restricted CTL responses to pathogens in vivo.

In the present investigation we have examined the issue of immunodominance in the context of human CTL responses to EBV, a common herpesvirus that causes infectious mononucleosis and is associated with a variety of malignancies of lymphoid and epithelial cell origin (for a review see reference 15). Primary EBV infection usually occurs asymptomatically in childhood or adolescence and is followed by the establishment of a life-long carrier state whereby the virus persists in latently infected B lymphocytes. These virus-infected cells may give rise to EBV-carrying lymphoblastoid cell lines (LCL) upon in vitro culture of blood lymphocytes in the presence of T cell inhibitory drugs (16), and may cause lymphoproliferative disorders in severely immunosuppressed individuals such as organ transplant recipients and HIV patients (17, 18). CTLs play a major role in limiting the proliferative potential of EBV-transformed lymphoblasts. Studies in healthy virus carriers have shown that EBV-specific memory CTL responses are usually class I restricted and are directed to the five Epstein-Barr virus nuclear antigens (EBNA) 2–6, and the two latent membrane proteins 1 and 2 that are expressed in latently infected cells (for a review see reference 15). Several peptide epitopes have been identified in the primary sequences of these viral products, each recognized in the context of a specific class I allele. Thus, an individual's class I genotype strongly influences EBV-specific responses both in terms of antigen choice and quantitatively, in that strong CTL reactions are frequently restricted through certain class I alleles. HLA A11 provides a clear example of the phenomenon since effectors restricted through this determinant are usually the major component of polyclonal CTL cultures from A11-positive Caucasians (19, 20). We previously identified five A11-restricted CTL epitopes within residues 101-115, 396-410, 416-429, 481-495, and 551-564 of the viral EBNA4 protein (21), and mapped the minimal cognate peptides of two of these epitopes within EBNA4 residues 416-424 (IVTDFSVIK, designated IVT) and 399-408 (AVFDRKSDAK, designated AVF) (20, 21). Screening of polyclonal CTL cultures for peptide-specific reactivities demonstrated in all cases strong recognition of IVT and significantly weaker responses to AVF, whereas reactivities against other epitopes either tended to be lost on serial passages or, with some of the donors, were never detected. The possibility that such a hierarchy of responses may reflect differences in the immunogenicity of the epitopes was substantiated by the finding that mutations within the anchor residues of the IVT peptide abrogated CTL recognition in a series of 33 EBV isolates from Southeast Asia, where HLA A11 is expressed in >50% of the population, whereas only half of the isolates carried concomitant mutations within the AVF peptide (20). Thus, CTL responses to IVT appear to exert a stronger selective pressure in vivo.

We have now investigated the molecular basis for the different immunogenicity of the IVT and AVF epitopes. We report that a strong predominance of IVT-specific CTL clones in polyclonal cultures derived from autologous LCL stimulation correlates with a more efficient presentation at the surface of A11-positive LCLs and with a higher abundance of the peptide in acid extracts of virus-infected cells. Analyses of TAP-dependent translocation, A11–peptide complex stability in cell lysates, and turnover in metabolically active cells indicate that efficient antigen presentation and immunodominance may be accounted for by a longer life span of IVT-containing molecules at the surface of APCs.

## Materials and Methods

*Cell Lines.* B95.8 EBV-transformed LCLs were obtained by infection of lymphocytes from HLA class I–typed donors with culture supernatants of the virus producer B95.8 line (22) and are indicated in the text by the initials of the donor followed by a B, for B95.8, and a number corresponding to the transformation experiment from which the cell line was derived. All cell lines were maintained in RPMI 1640 supplemented with 100 μg/ml streptomycin, 100 IU/ml penicillin, and 10% FCS (complete medium).

*Synthetic Peptides.* Peptides, synthesized by the Merrifield solid phase method (23), were purchased from Alta Bioscience (The University of Birmingham, School of Biochemistry, Birmingham, UK). The peptides were dissolved in DMSO at a concentration of $10^{-2}$ M and were further diluted in PBS to obtain the indicated concentrations before the assays. The protein concentration of the DMSO stock solutions was determined by a Biuret assay (24).

*Expression of EBNA4.* Aliquots of $10^7$ cells were dissolved in 1 ml electrophoresis sample buffer and sonicated. 100 μl of the total cell extracts was separated by discontinuous 7.5% polyacrylamide-SDS gel electrophoresis according to the method of Laemmli (25), and blotted onto nitrocellulose filters (26). The efficiency of the protein transfer and the position of molecular weight markers were visualized by staining with Ponceau-S (Sigma Chemical Co., St. Louis, MO). Excess protein binding sites were blocked by incubation for 1 h in PBS containing 5% dried skim milk and 0.1% Tween 20. The filters were subsequently incubated with a 1:50 dilution of a previously characterized human serum HR (vical capsid antigen, 1:5,120; EBNA1, 1:320; EBNA2A, 1:640; EBNA2B, 1:40; and EBNA6, 1:640) in PBS-milk for 2 h at room temperature or for 16 h at 4°C. After three washes in PBS-milk, the filters were incubated with horseradish peroxidase–conjugated goat anti–human antibodies, and the reaction was visualized by enhanced chemiluminescence (Amersham International, Amersham, Bucks, UK) according to the manufacturer's instructions. The intensity of the EBNA4-specific band was measured by densitometry using a laser spectrophotometer (LKB, Bromma, Sweden) equipped with a scanning attachment.

*Natural Peptide Extraction and Fractionation.* Cultured cells were harvested by centrifugation, washed in cold PBS, and stored in batches of $10^9$ at −70°C. Cell pellets were lysed by adding 10 ml 0.7% aqueous TFA on ice, homogenized in a Dounce homogenizer (Kontes, Vineland, NJ) with 50 strokes, and sonicated with 3 × 15-s bursts; additional TFA was added to bring the pH of the lysate below 2. The material was transferred to a Centriprep 10 ultracentrifugation device (Amicon, Beverly, MA) and subjected to centrifugation at 2,600 *g* for 3–5 h at 4°C until the volume of retained fraction was ~1 ml. The ultrafiltrate (molecular mass <10 kD) was fractionated by HPLC using the conditions de-

scribed by Falk et al. (3) with minor modifications. Briefly, 10 ml of ultrafiltrate was loaded on SuperPac™ Pep-S 5μ reverse phase column (Pharmacia, Uppsala, Sweden). Unbound material was eluted with 100% solvent A (0.1% TFA in $H_2O$) until the optical density of the flow through had reached baseline levels. Bound material was eluted on a linear gradient of 1.5% B/min at a flow rate of 1 ml/min where B = 0.1% TFA in acetonitrile. 40 fractions were collected at 1-min intervals in siliconized microfuge tubes, dried by SpeedVac (Pharmacia) and redissolved in 300 μl of PBS with vortexing.

*Peptide Translocation Assays.* Streptolysin-O–mediated transport assays were performed as described by Neefjes et al. (27) using a $^{125}$I-labeled synthetic peptide library constructed from the reference TYNTRALI peptide with fixed amino acids to allow iodination (Y) and glycosylation (N and T) (13). The peptide library was labeled by chloramine T–catalyzed iodination to a specific activity of 10 Ci/mmol. Briefly, $10^6$ LCL cells were permeabilized with 2 IU/ml streptolysin-O (Wellcome Diagnostics, Dartford, UK) for 10 min at 37°C. Iodinated peptide library (~66 ng) was added and incubation was continued for 10 min in the presence or absence of 10 mM ATP (Sigma Chemical Co.). The cells were then transferred to ice, lysed in buffer containing 1% NP-40, 150 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.5, and nuclei were removed by microcentrifugation at 14,000 rpm for 10 min. Translocated peptides which had been glycosylated in the ER were recovered by absorption to Con A–Sepharose beads (Pharmacia). The beads were washed five times in lysis buffer and the associated radioactivity was measured in a gamma counter (LKB, Uppsala, Sweden). Competition experiments were performed by mixing the indicated amounts of IVT or AVF peptides with the iodinated peptide library before addition to the streptolysin-O permeabilized cells.

*Stability and Turnover of HLA A11–Peptide Complexes.* The T2/A11 cell line, obtained by transfection of the transporter-deficient mutant T2 cell line (28) with a pHEBO vector-based HLA A11 expression vector (29), was maintained in medium containing 200 μg/ml hygromicin-B. "Empty" and peptide-loaded class I molecules were induced by culturing aliquots of $10^7$ cells overnight at 26°C in serum-free AIM-V® medium (GIBCO BRL, Gaithersburg, MD) alone or medium containing $10^{-6}$ M of IVT or AVF peptide. After extensive washing to remove the unbound peptides, surface $^{125}$I labeling was performed by the lactoperoxidase method. The temperature stability of iodinated class I molecules was assessed by incubating lysates of $10^6$-labeled cells at the indicated temperature for 2 h or at 37°C for the indicated time before immunoprecipitation with the W6/32 mouse mAb (30). The pH sensitivity was assessed by preincubating $10^6$ iodinated cells for 2 h at 4°C in 500 μl of buffer at the indicated pH before lysis. The immunoprecipitated complexes were resolved by SDS-PAGE and the specific class I heavy chain and $\beta_2$-microglobulin ($\beta_2$m) bands were visualized and quantitated by phosphoimager scanning. To determine the turnover of A11–peptide complexes at the surface of live cells, aliquots of $10^7$ T2/A11 cells were metabolically labeled by overnight culture at 26°C in methionine-free RPMI 1640 medium containing 100 μCi[$^{35}$S]methionine and 1% FCS with or without addition of $10^6$ M of the AVF or IVT peptides. The cells were then washed five times in PBS, resuspended in complete medium at a concentration of $0.5 \times 10^6$ ml, and cultured at 37°C. Aliquots were harvested at the indicated times, washed in PBS, and incubated for 1 h at 4°C with 2 μl of W6/32 ascites. After extensive washes to remove the unbound antibodies, the cells were lysed in 1 ml IEF lysis buffer as previously described (31). HLA class I–W6/32 complexes were precipitated from the cell lysates by 2× incubation with 50 μl of a 10% suspension of formalin-fixed *Staphylococcus aureus* (Sigma Chemical Co.), and the immunoprecipitates were digested for 2 h at 37°C with neuroaminidase (Sigma type VIII) at a concentration of 10 U/ml in a volume of 20 μl. The class I polypeptides were resolved by 1D-IEF and detected by autoradiography (31). The intensities of the $\beta_2$m and class I heavy chain bands were quantitated by densitometry from suitably exposed autoradiograms.

*Generation of CTL Cultures and Clones.* EBV-specific CTLs were obtained as previously described (32) by stimulation of lymphocytes from the EBV-seropositive donors BK (HLA A2, 11, B7, 35) with the autologous B95.8 virus-transformed LCL. After two or three consecutive restimulations, the cultures were expanded in complete medium supplemented with 10 U/ml recombinant IL-2 and 30% (vol/vol) culture supernatant from the gibbon lymphoma line MLA144 (33). Single cell cloning was done by limiting dilution in 96-well plates in 200 μl medium containing 30% MLA144 culture supernatant, 10 U/ml human recombinant IL-2, and $10^5$ irradiated (3,000 rad) allogeneic PHA-pulsed PBLs as feeder. Growing cultures were transferred into 24-well plates and were fed twice a week by replacing half of the medium without any further stimulation. The EBV specificity and class I restriction were investigated by testing the cytotoxic activity against a panel of EBV-positive and -negative targets, including the autologous LCLs, allogeneic LCLs matched through single class I alleles, at least two cell lines for each allele, PHA-activated blasts, HLA-mismatched LCLs, and the prototype NK-sensitive target K562.

*Cytotoxicity Tests.* Cytotoxic activity was measured in standard 4-h $^{51}$Cr release assays (32). The targets were labeled with $Na^{51}CrO_4$ (0.1 μCi/$10^6$ cells) for 1 h at 37°C. The cytotoxicity tests were routinely run at 10:1, 3:1, and 1:1 E/T ratios in triplicate. Peptide titration, competition assays, and tests of HPLC fractions were performed by adding 20 μl of the peptide preparations to $4 \times 10^3$ labeled targets (in 20 μl complete medium) in triplicate wells of 96 V-shaped well plates. The plates were incubated for 1 h at 37°C before addition of the CTLs. Peptide toxicities were checked in each assay and were always ≤3%. In all other assays, peptide pulsing was performed by incubating the targets in the presence of $10^{-9}$ M of peptide during labeling followed by extensive washing before the cytotoxicity tests.

## Results

*IVT-specific CTLs Dominate HLA A11–restricted Responses to EBNA4.* We have previously observed that polyclonal HLA A11–restricted CTL responses to EBNA4 are dominated by reactivities to the nonamer peptide IVT, corresponding to residues 416-424 of the prototype B95.8 sequence, whereas a weaker response is usually detected against the 10-mer AVF derived from amino acid residues 399-408 (21). We have now examined this issue at the clonal level and have compared the frequencies of CTL clones specific for the AVF and IVT peptides in polyclonal cultures obtained by stimulation of lymphocytes from the A11-positive donor BK with the autologous B95.8 virus-transformed LCL. A compilation of the results obtained from the analysis of 173 clones derived from four CTL reactivation experiments performed over a period of 30 mo is shown in Table 1. 85% of the clones (146/173) lysed the autologous stimulator LCL, and the majority (135/146,

**Table 1.** *Specificity of CTL Clones Generated by Autologous LCL Stimulation of Lymphocytes from Donor BK*

| Expt. | No. of clones | Cytotoxic clones | HLA restriction | | Peptide specificity | |
|---|---|---|---|---|---|---|
| | | | All | Other | IVT | AVF |
| 1 | 32 | 25 | 23 | 2* | 22 | 1 |
| 2 | 19 | 16 | 14 | 2 | 13 | 1 |
| 3 | 59 | 59 | 55 | 4 | 46 | 9 |
| 4 | 63 | 46 | 43 | 3 | 28 | 15 |
| Total | 173 | 146/173 | 135/146 | 11/146 | 109/135 | 26/135 |
| (%) | | (85) | (92) | (8) | (81) | (19) |

Polyclonal CTL cultures from donor BK (HLA A2, A11 B7, B62) were cloned by limiting dilution. The specificity of the derived clones was assessed on a panel of targets including the autologous LCL and allogeneic A11-matched or-mismatched LCLs. A11-restricted clones were further tested for lysis of A11-matched PHA blasts preincubated with $10^{-9}$ M of the IVT or AVF peptides.

*EBV-specific CTL clones restricted through other class I alleles expressed in the donor and nonspecific CTL clones that lysed class I–mismatched LCLs and the prototype NK-sensitive target K562.

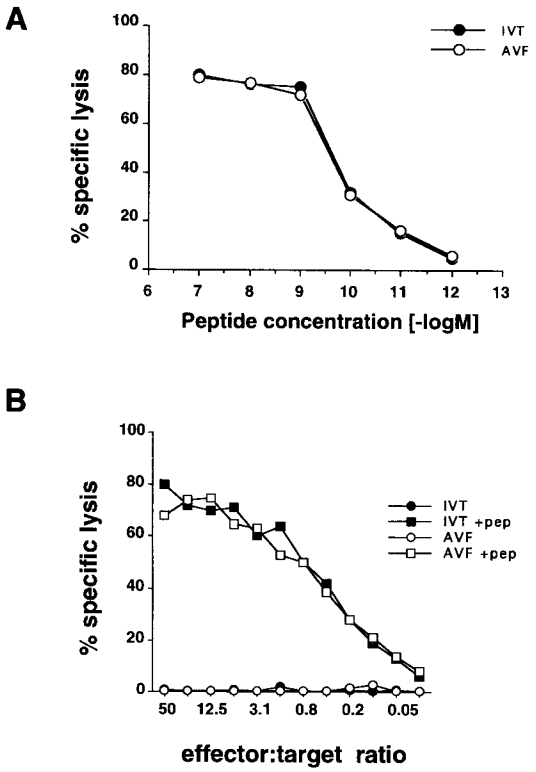


**Figure 1.** CTL clones specific for the IVT and AVF epitopes lyse equally well PHA blasts pulsed with synthetic peptides. (*A*) HLA A11–positive PHA blasts were preincubated for 1 h with 10-fold serial dilutions of the IVT or AVF peptides and were then used as targets for the AVF-specific CTL clones BK280 (*open symbols*) or IVT-specific CTL clones BK102 (*closed symbols*). The percent specific lysis recorded at a 10:1 E/T ratio in one representative experiment out of three is shown. (*B*) Untreated (*circles*) and peptide-pulsed (*squares*) blasts were tested for sensitivity to lysis by the IVT-specific clone BK289 (*filled symbols*) and AVF-specific clone BK280 (*open symbols*) in standard 4-h $^{51}Cr$ release assays. The targets were preincubated with $10^{-9}$ M of the indicated peptide for 1 h during labeling and extensively washed to remove unbound peptides before the assays. One representative experiment out of three is shown.

93%) were EBV specific and A11 restricted in that they lysed allogeneic A11-matched LCLs, but not A11-positive PHA blasts, mismatched LCLs, or LCls matched through other class I specificities. Only 8% of the cytotoxic clones (11/146) were either EBV specific and restricted through other class I alleles expressed in the donor, or exhibited LAK-type cytotoxicity. In accordance with the results obtained with polyclonal cultures, the A11-restricted response was strongly dominated by CTL clones specific for the IVT epitope (109/135, 81%) whereas only 19% of the clones (26/135) recognized the AVF epitope.

Wide variations have been reported in the amounts of synthetic peptides required to trigger lysis by different CTL clones, probably reflecting differences in the avidity of their TCRs. We have therefore compared IVT- and AVF-specific CTLs for their capacity to lyse PHA blasts in peptide titration assays. As illustrated in Fig. 1 *A*, where target sensitization was tested across a $10^{-7}$ to $10^{-12}$ M range of peptide concentrations, both peptides were active down to picomolar concentrations, reaching half-maximal lysis points at $5 \times 10^{-10}$ M. Similar dose–response curves were obtained in experiments performed with more than 10 individual IVT- and AVF-specific clones (not shown). We then compared lysis of blasts pulsed with $10^{-9}$ M of the relevant peptide for lysis by IVT- and AVF-specific CTLs over a wide range of E/T ratios (Fig. 1 *B*). Equal levels of killing were observed at all ratios, confirming the similar efficiency of the effectors.

*The IVT Epitope Is Presented More Efficiently than AVF.* During the course of the above screening a consistent difference was observed in the capacity of AVF- and IVT-specific CTLs to lyse different types of targets. Whereas peptide-pulsed blasts were lysed equally well, A11-positive LCLs were regularly more sensitive to IVT-specific cytotoxicity. A compilation of the mean percent specific lysis recorded at a 10:1 E/T ratio in at least 3 and up to 10 independent experiments performed with a panel of 12 B95.8 virus-transformed LCLs from A11-positive individuals is shown in Fig. 2 *A*. Reproducible levels of killing were obtained in experiments performed with each cell line using different IVT- and AVF-specific clones. All LCLs tested were lysed more efficiently by the IVT-specific effectors, although significant differences in the levels of killing were detected with individual cell lines.

To assess whether these differences could be ascribed to variations in the levels of endogenous EBNA4 expression, this parameter was measured in parallel with IVT- and AVF-specific killing in a representative panel of seven A11-positive LCLs. EBNA4 expression was quantitated by densitometry in immunoblots of total extracts from $10^6$ cells probed with a previously characterized human serum containing high antibody titers to all EBNAs. Similar levels of

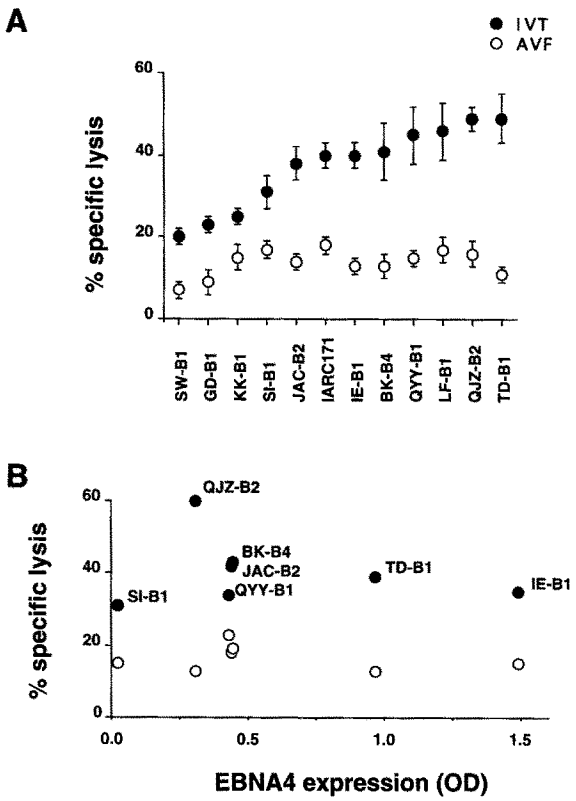


**Figure 2.** HLA A11–positive LCLs are lysed more efficiently by IVT-specific effectors independently of the endogenous levels of EBNA4 expression. B95.8 virus-transformed LCLs from the HLA A11–positive individuals SW (A11, A24, B44, B55); GD (A2, A11, B35, B41); KK (A11, A28, B14, B40); JAC (A1, A11, B49, B55); IARC 171 (A11, A32, B35, B49); IE (A3, A11, B7, B35); BK (A2, A11, B7, B62); QYY (A2, A22, B13, B38); LF (A2, A11, B7, B37); QJZ (A11, B13, B51); and TD (A2, A11, B44, B55) were tested for sensitivity to lysis by the IVT-specific clones BK102, BK263, and BK250 (*closed symbols*) and AVF-specific clones BK248, BK280, and BK203 (*open symbols*). Each symbol corresponds to the mean ± SE of the percent specific lysis recorded at a 10:1 E/T ratio in at least three experiments performed with each E/T combination. (*B*) Protein extracts from total cells were separated by SDS-PAGE and immunoblots were probed with a previously characterized human serum with high antibody titers to all EBNAs (HR: VCA, 1:5,120; EBNA1, 1:320; EBNA2A, 1:640; EBNA2B, 1:40; and EBNA6, 1:640). The EBNA4-specific band was identified by comparison with the B95.8 reference cell line and the intensity was quantitated by densitometry. The results of one representative experiment out of three where EBNA4 expression and sensitivity to AVF (*open circles*) and IVT-specific lysis (*closed circles*) were tested in parallel are shown.

expression were detected on repeated testing of the same LCL, but wide variations were observed when the intensity of the EBNA4-specific band was compared in different LCLs. As illustrated by the representative experiment shown in Fig. 2 *B*, there was no correlation between the level of EBNA4 expression and the sensitivity to AVF- or IVT-specific cytotoxicity. All cell lines were lysed poorly by the AVF-specific effector in spite of greater than 10-fold differences in the expression of EBNA4.

To explore this phenomenon further, untreated and peptide-pulsed LCLs were compared for sensitivity to lysis over a broad range of E/T ratios. Representative experiments, where the IVT-specific clone BK289 and AVF-specific clone BK280 were titrated by twofold dilutions against a fixed number of targets, are shown in Fig. 3. Equal levels of IVT- and AVF-specific killing were observed with peptide-pulsed targets over E/T ratios ranging from 50:1 down to 0.025:1. In contrast, untreated LCLs remained less sensitive to AVF-specific lysis even at E/T ratios as high as 50:1. It is noteworthy that plateau levels of killing were achieved against all targets with E/T ratios significantly lower than 50:1, confirming that the effectors were not limiting in the assays. Because the dose–response curves of the IVT- and AVF-specific CTLs were identical against peptide-pulsed targets, discrepancies in their activity against untreated cells are probably due to differences in the efficiency of presentation of the epitopes. Thus, presentation of the endogenous AVF epitope varied in different cell lines between 20 and 30% of that required to trigger maximal lysis achieved with peptide-pulsed cells, whereas presentation of the endogenous IVT epitope was between 60 and 80%. The unequal presentation of these determinants is likely to reflect the relative abundance of the corresponding class I–peptide complexes in virus-infected cells and may be influenced by selective peptide transport, differences in the affinity of binding, or unequal persistence of the complexes in APCs.

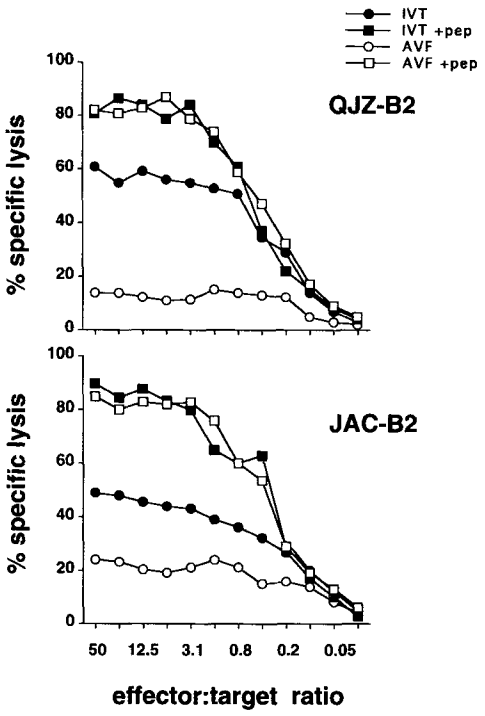


**Figure 3.** The endogenous IVT and AVF epitopes are presented with different efficiency at the surface of HLA A11–positive LCLs. Untreated (*circles*) and peptide-pulsed (*squares*) LCL cells were tested for sensitivity to lysis by the IVT-specific clone BK289 (*filled symbols*) and AVF-specific clone BK280 (*open symbols*) in standard 4-h $^{51}Cr$ release assays. The targets were preincubated with $10^{-9}$ M of the indicated peptide for 1 h during labeling and extensively washed to remove unbound peptides before the assays. One representative experiment out of three is shown.

These parameters were therefore compared in A11-positive LCLs and in the TAP-deficient mutant T2/A11 transfectant.

*Abundance of Naturally Processed IVT and AVF Peptides in LCL Cells.* Cytosolic peptides are rapidly degraded unless they are protected by binding to class I molecules (34). Thus, the recovery of peptides from acid extracts of total cell lysates can be taken as a good correlate of the amount of complexes present in a given cell. To determine the abundance of class I molecules containing the naturally processed IVT and AVF peptides, A11-positive LCLs were expanded and the MHC-bound peptides from $10^9$ cells were isolated by TFA extraction, ultrafiltration, and reverse phase HPLC fractionation. As control, peptides were extracted under the same conditions from A11-negative LCLs and from EBV-negative A11-positive or -negative BL lines and PHA blasts. As illustrated in the representative experiments shown in Fig. 4, HPLC fractions from EBV-negative cells did not sensitize A11-positive blasts to lysis by AVF- or IVT-specific effectors (Fig. 4 *A*). When HPLC fractions from A11-positive LCLs were tested in cytotoxicity assays, a peak of AVF-specific reactivity was recovered in fractions 15 and 16, whereas IVT-specific reactivity was detected in fractions 23 and 24 and occasionally fraction 22 (Fig. 4 *B*). The retention times of the naturally processed peptides were identical to those observed in spiking experiments where 100 pg of synthetic IVT and AVF peptides were added to $10^9$ EBV-negative cells immediately before extraction and fractionation (Fig. 4 *C*). Similar retention times were also observed when synthetic IVT and AVF peptides were fractionated according to the same HPLC conditions (not shown). To estimate the abundance of naturally processed peptides, the active HPLC fractions were titrated in cytotoxicity assays and the percent specific lysis was compared with standard curves from cytotoxicity assays carried out under the same conditions with synthetic peptides (Fig. 5). A compilation of the estimated content of IVT and AVF in A11-positive and -negative LCLs and in control EBV-negative cells is shown in Table 2. IVT- and AVF-specific reactivities were regularly detected in peptide extracts from A11-positive LCLs, only in some extracts from A11-negative LCLs, and in none of the extracts from EBV-negative cells. Empty runs tested before each peptide fractionation were always negative, confirming that no cross-contamination had occurred between runs. The recovery of peptides varied considerably between different cell lines, but similar levels were detected in repeated experiments performed with the same cell line. The recovery of IVT peptides was in each case higher than the AVF recovery with calculated abundances ranging between about 350 and 12,800 and about 8 and 760 molecules per cell, respectively. The percent specific lysis induced by the active fractions from three spiking experiments with known amounts of synthetic peptides corresponded to an average efficiency of recovery of ~30% for both AVF and IVT (Fig. 4 *C*, and data not shown), confirming that higher yield of IVT in extracts from EBV-positive cells is not an artefact of the fractionation procedure.

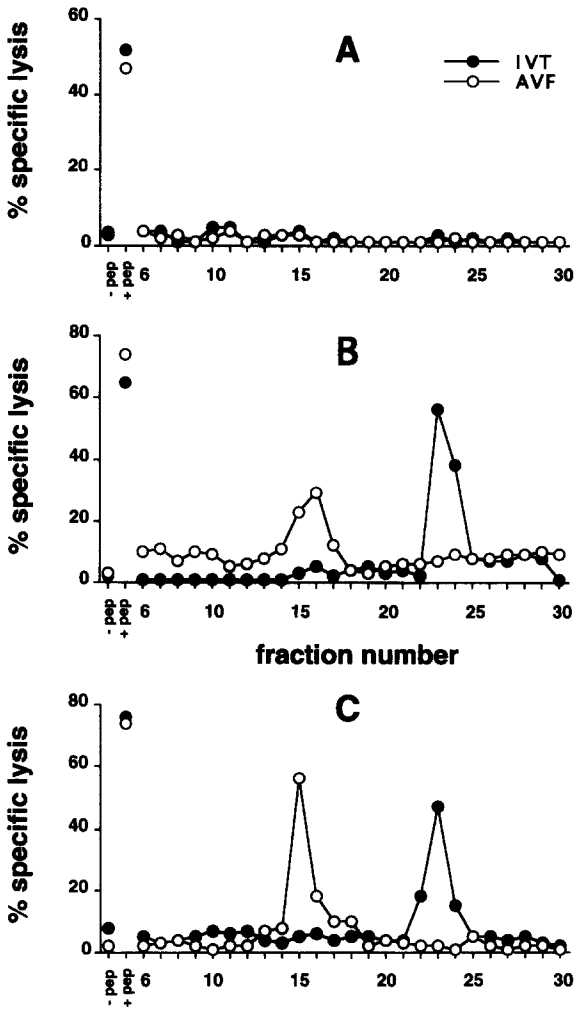


**Figure 4.** Isolation of naturally processed IVT and AVF peptides from EBV transformed LCLs. $10^9$ cells from the HLA A11–positive EBV-negative line BL28 (*A*), the HLA A11–positive LCL BK-B4 (*B*), or BL28 cells spiked with 10 pg IVT and AVF peptides (*C*) were lysed in 1% TFA, homogenized with a Dounce homogenizer, subjected to ultrafiltration in order to isolate peptide containing fractions (<10 kD), and fractionated by C18 HPLC using a gradient of 1.5% acetonitrile/min at a flow rate of 1 ml/min. Fractions were collected at 1-min intervals, dried, and tested for their ability to sensitive HLA A11–positive PHA blasts to lysis by the IVT-specific clone BK112 (*filled symbols*) and AVF-specific clone BK280 (*open symbols*) in standard 4-h $^{51}Cr$ release assays. Toxicity controls consisting of target cells incubated in the absence of CTLs were negative (not shown).

*TAP-dependent Transport.* Differences in the efficiency of TAP-dependent translocation to the site of class I assembly could result in preferential formation of IVT-containing complexes. We compared, therefore, the capacity of synthetic IVT and AVF peptides to compete the transport of a reference peptide library in streptolysin-O permeabilized LCL cells. A $^{125}I$-labeled peptide library, containing 2,304 peptides with variable COOH termini was used to assess TAP- and ATP-dependent translocation by virtue of N-linked glycosylation and consequent retention in the ER lumen (13). Translocation of the peptide library was competed by addition of cold IVT or AVF peptides in a dose-

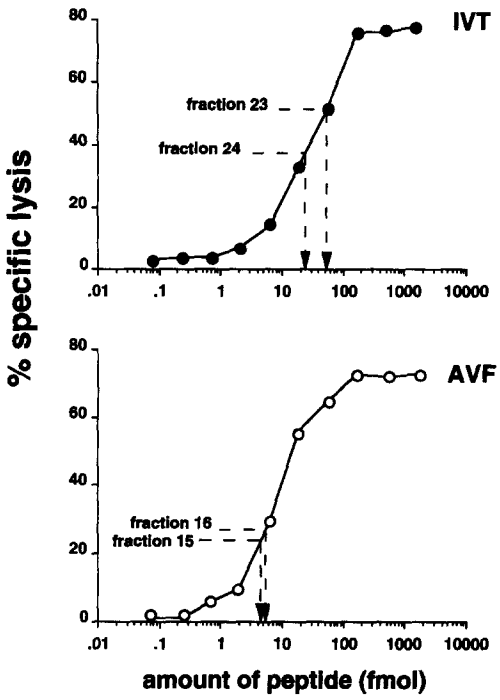


**Figure 5.** Calculation of peptide recovery. $10^9$ JL-B1 (HLA A11, A28, B35, B44) cells were fractionated as described in Fig. 4. The active HPLC fractions recognized by BK298 (fractions *23* and *24*) and BK280 (fractions *15* and *16*) CTL were titrated in standard cytotoxicity assays using HLA A11–positive blasts as targets. The percent specific lysis values obtained using $5 \times 10^7$ cells equivalent per well (i.e., 5% of the active fraction) are plotted on titration curves performed with known amounts of synthetic IVT or AVF peptides in the same assays. The estimated content of IVT peptide was 49 fmol in fraction 23 and 22 fmol in fraction 24, and the content of AVF peptide was 4.5 fmol in fraction 15 and 5.5 fmol in fraction 16. This corresponds to a calculated recovery of ≈1,050 IVT and ≈200 AVF molecules per JL-B1 cells.

**Table 2.** *Estimated Content of IVT and AVF Peptides*

| Cell type | No. of experiments | Peptide molecules/cell IVT | Peptide molecules/cell AVF |
|---|---|---|---|
| EBV$^+$ HLA A11$^{+*}$ | | | |
| EA-B1 | 4 | 12,800* | 760 |
| SI-B1 | 1 | 350 | 8 |
| JL-B1 | 1 | 1,050 | 200 |
| LF-B1 | 1 | 1,300 | nt |
| BK-B4 | 3 | 10,000 | 10‡ |
| EBV$^+$ HLA A11$^-$ | | | |
| GK-B2 | 1 | 263 | 119 |
| JM-B2 | 1 | 550 | nt |
| LS-B1 | 1 | <1 | nt |
| SN-B1 | 1 | <1 | nt |
| EBV$^-$ | | | |
| BK PHA blasts | 1 | <1 | <1 |
| BJAB | 3 | <1 | <1 |
| BL28 | 1 | <1 | <1 |

*Peptide recovery was calculated for each separation as described in Fig. 5. The values represent the estimated recovery of individual separation experiments or the average of repeated separations performed with each cell line.
‡AVF recovery was calculataed only in one of the three experiments.
*nt*, not tested.

dependent manner (Fig. 6). Similar dose–response curves were obtained with the two peptides, indicating that there is no selective transport into the ER/pre-Golgi compartment.

*The IVT and AVF Peptides Bind Equally Well to HLA A11.* We next examined whether the higher abundance of IVT could be ascribed to differences in the ability of the peptides to associate with A11 or in the stability of the corresponding complexes. First, the capacity of synthetic IVT and AVF to reciprocally inhibit T cell recognition was tested in functional assays using specific CTLs as reporters of A11–peptide association. HLA A11–positive blasts were pulsed for 1 h at room temperature with a mixture of $10^{-9}$ M of the reporter peptide and graded concentrations of the competitor peptide to estimate the concentration required for 50% inhibition of CTL responses (Fig. 7). In several independent experiments, equal concentrations of competitor peptide were required to inhibit IVT- and AVF-specific lysis. Thus, $\sim 10^{-6}$ M of IVT peptide was required for 50% inhibition of cytotoxicity induced by $10^{-9}$ M of AVF and vice versa, suggesting that peptides have a similar affinity for A11 molecules.

The relative stability of A11–peptide complexes was estimated by measuring their rate of dissociation in lysates of surface-iodinated T2/A11 cells as a function of temperature, time at 37°C, and pH. A11 molecules loaded with the IVT or AVF peptide were induced at the surface of T2/A11 cells by overnight incubation at 26°C in serum-free medium containing $10^{-6}$ M of synthetic peptides. After extensive washes to remove the unbound peptides and surface iodination, lysates of $10^6$ cells were incubated at increasing temperature for 2 h, or at 37°C for different periods of time. Class I molecules were then precipitated with the W6/32 mAb and resolved by SDS-PAGE. As expected, class I molecules induced by incubation of T2/A11 cells in the absence of exogenous peptides were unstable at temperatures higher than 25°C (Fig. 8 *A*). In contrast, the majority of molecules induced by incubation in the presence of both IVT and AVF peptides was stable at 37°C and 42°C, with recoveries varying between 70 and 90 and 50 and 70% of that observed at 25°C, respectively. Virtually all complexes were disrupted after incubation for 2 h at 50°C. Comparable dissociation kinetics of IVT- and AVF-containing complexes were also demonstrated after incubation at 37°C for prolonged periods of time (Fig. 8 *B*). To test the stability of the complexes as a function of pH, iodinated T2/A11 cells were preincubated for 2 h at 4°C in citrate

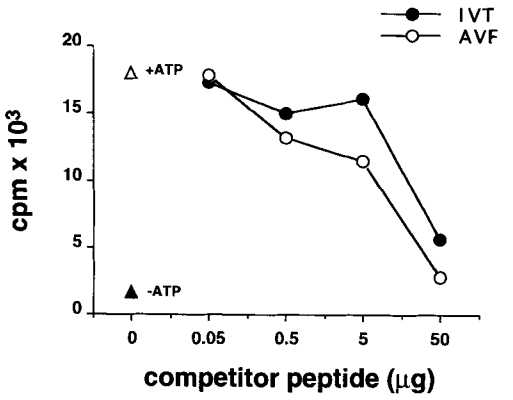


**Figure 6.** The IVT and AVF peptides compete equally well for TAP-dependent translocation. $10^6$ BK-B4 cells were permeabilized with 2 IU/ml streptolysin-O for 10 min at 37°C and further incubated for 10 min with the iodinated peptide library with (*open triangle*) or without (*filled triangle*) addition of 10 mM ATP. Translocated peptides that had been glycosylated in the ER were recovered by absorption to Con A–Sepharose beads and the radioactivity was measured in a gamma counter. The indicated amounts of synthetic IVT (*filled symbols*) or AVF peptides (*open symbols*) were mixed with the iodinated peptide library before addition to the streptolysin-O permeabilized cells. One representative experiment out of three is shown.

phosphate buffers of pH ranging from 7 to 4 before lysis and immunoprecipitation with the W6/32 mAb (Fig. 8 *C*). Class I molecules induced in T2/A11 cells in the absence of exogenous peptides were unstable at pH higher than 5.5, whereas both IVT- and AVF-containing complexes were unaffected at pH 4.5 and ~20% of the complexes was still detectable after incubation at pH 4.

*Rapid Turnover of AVF-containing Complexes in Metabolically Active Cells.* In the final set of experiments, the life span of class I molecules loaded with IVT or AVF was compared in intact cells incubated at 37°C. T2/A11 cells were metabolically labeled overnight at 26°C either alone

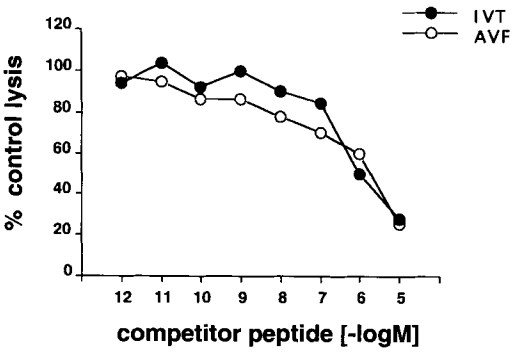


**Figure 7.** The IVT and AVF peptides compete equally well in target sensitization assays. HLA A11–positive blasts were preincubated for 1 h at room temperature in the assay well with a mixture of $10^{-9}$ M of the IVT or AVF peptide and the indicated molar concentration of competitor peptide before addition of the IVT-specific clone BK289 or AVF-specific clone BK280 at a 10:1 E/T ratio. (*Closed symbols*) AVF-specific lysis competed by IVT peptide; (*open symbols*) IVT-specific lysis competed by AVF peptide. The percent IVT- and AVF-specific lysis in the absence of competitor peptide was 61 and 47%, respectively. One representative experiment out of three is shown.

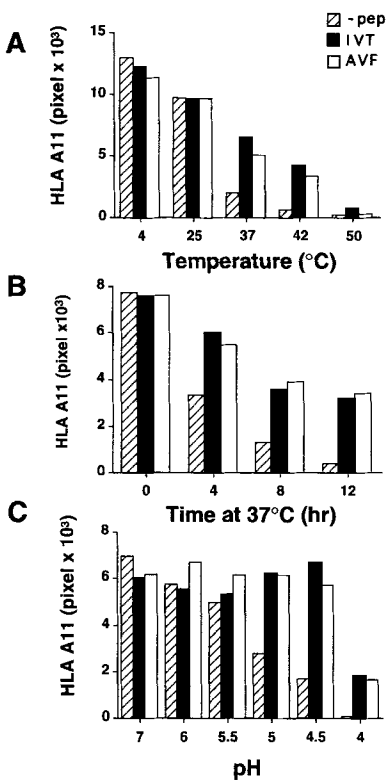


**Figure 8.** HLA A11 molecules containing the IVT and AVF peptides are equally stable over a wide range of temperature and pH conditions. Surface HLA A11 molecules were induced in T2/A11 cells by overnight incubation at 26°C in serum-free medium alone (*open bars*), medium containing $10^{-6}$ M IVT (*filled bars*), or AVF peptides (*hatched bars*). After extensive washes to remove the unbound peptides, the cells were surface iodinated. The temperature stability of class I molecules was assessed by incubating lysates of $10^6$ cells at the indicated temperature for 2 h (*A*) or at 37°C for the indicated time (*B*) before immunoprecipitation. (*C*) The pH sensitivity of the complexes was assessesd by preincubation of $10^6$ labeled cells for 2 h at 4°C in 500 μl buffer at the indicated pH before lysis. Class I molecules were immunoprecipitated using the W6/32 mAb and the complexes were resolved by SDS-PAGE. The specific class I heavy chain and $\beta_2$m bands were visualized and quantitated by phosphoimager scanning. One representative experiment out of three is shown.

or in the presence of $10^{-6}$ M of the peptides. After extensive washes, the cells were placed back in medium alone and surface class I molecules were chased at 37°C for different times. As expected, A11 molecules induced in the absence of exogenous peptides were rapidly cleared from the cell surface with an approximate half-life of 2.5 h (Fig. 9, *A* and *B*). The average half-life was prolonged for A11 molecules induced in the presence of peptides but a significant difference was observed in the life span of IVT- and AVF-containing complexes (Fig. 9). Over 50% of the A11 molecules containing AVF had been disrupted after incubation at 37°C for 4 h and only 20% of the complexes were still detected after 12 h, corresponding to an approximate half-life of 3.5 h. In contrast, after an initial decrease observed during the first 4 h, the majority of IVT-induced complexes remained stable with a half-life exceeding the 12-h observation time.

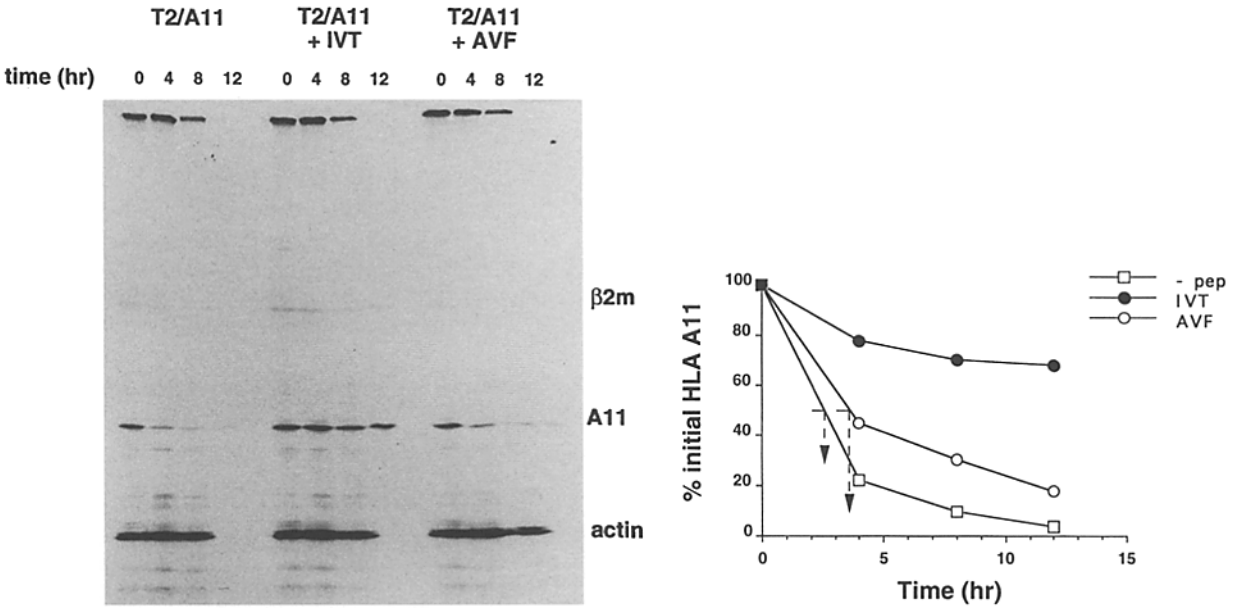


**Figure 9.** HLA A11 molecules containing the AVF peptide are rapidly cleared from the cell surface whereas IVT-containing complexes are stable. (*A*) T2/A11 cells were metabolically labeled by culturing overnight at 26°C in methionine-free RPMI containing 100 μCi [$^{35}$S]methionine and 1% FCS either alone or in the presence of $10^{-6}$ M IVT or AVF peptides. The cells were then washed five times in PBS to remove the excess of unbound peptides, and incubated at 37°C in peptide-free medium for the indicated times. Precipitation of surface class I molecules and separation by 1D-IEF were performed as described in Materials and Methods. The specific class I heavy chain and $\beta_2$m bands were identified by comparison with a panel of previously characterized LCL of known HLA class I type. The HLA A2, B5, and $\beta_2$m polypeptides, which are hardly visible in the figure, were clearly detected on longer exposures of the autoradiograms. One representative experiment out of four is shown. (*B*) The intensity of the A11-specific bands was quantitated by densitometry and the percent residual A11 was calculated as the ratio between the value observed at the indicated time after incubation at 37°C and the initial value. Shown is the mean result of four experiments.

## Discussion

The question of immunodominance in the context of class I–restricted CTL responses to intracellular pathogens and tumor cells has attracted considerable attention since immunodominant epitopes are likely to elicit effective responses in vivo and may therefore be preferential targets for immunization or passive immunotherapy. Very little is known about the factors influencing immunodominance although, in different model systems, correlations have emerged between immunogenicity and the chemistry of peptide binding or the efficiency of antigen processing. We have now examined this issue with regard to human CTL responses to a common herpesvirus, EBV, that establishes life-long persistent infections and is associated with a variety of lymphoid and epithelial cell malignancies. We have shown that the relative immunogenicity of two HLA A11–restricted epitopes derived from EBNA4 correlates with the efficiency of presentation in virus-infected cells. This parameter appears to be strongly influenced by a peptide-driven persistence of the antigenic complexes at the cell surface.

The possibility that the IVT epitope may be the immunodominant target of HLA A11–restricted responses to EBV-infected cells in vivo had been previously surmised based on the reactivity of polyclonal CTL cultures from EBV-immune donors and on the regular detection of mutations affecting this peptide in EBV isolates from Southeast Asia where HLA A11 is expressed in more than 50% of the population (20). We have now substantiated this assumption by demonstrating that ~80% of the A11-restricted CTL clones reactivated by autologous LCL stimulation of lymphocytes from the EBV-immune donor BK are specific for this epitope, whereas only 20% of the clones recognize the subdominant epitope AVF. A similar pattern of IVT predominance was observed in four CTL reactivation experiments performed over a period of 30 mo and is therefore a stable feature of EBV-specific responses in this donor (Table 1). The significance of this finding was corroborated in less extensive screenings performed with two additional A11-positive individuals which also yielded a similar prevalence of IVT-specific clones (Levitsky, V., unpublished results). Dose–response experiments performed over a wide range of synthetic peptide concentrations and E/T ratios confirmed that the differences cannot be ascribed to an intrinsically lower efficiency of the AVF-specific effectors which could have hampered the expansion of these cells during in vitro restimulation (Fig. 1). It is noteworthy that comparable, albeit very low, frequencies of IVT- and AVF-specific precursors were demonstrated after autologous LCL stimulation of lymphocytes from EBV-seronegative donors under limiting dilution conditions (Frisan, T., and M.G. Masucci, unpublished results). Thus, differences in the relative immunogenicity of the IVT and AVF epitopes

are unlikely to be due to obvious differences in the precursor T cell repertoire.

The derivation of IVT and AVF from the same viral protein and restriction through the same class I allele, together with the similar efficiency of the specific CTL, allowed us to directly compare the presentation of the two determinants in EBV-infected cells. Preferential killing by IVT-specific effectors was demonstrated in a series of 12 LCLs derived from HLA A11–positive individuals. Equal sensitivity after pulsing with the relevant synthetic peptide indicates that lower amounts of AVF-containing class I molecules may be available for CTL recognition at the surface of untreated cells (Fig. 3). To address this issue directly, we applied the technique described by Falk et al. (3) to determine the abundance of naturally processed IVT and AVF peptides in EBV-infected cells. From the titration curves of synthetic peptides we could estimate that a minimal recovery of one peptide per infected cell would be detected as a positive signal in target sensitization assays performed with HPLC fractionations from $10^9$ cells, taking into account an efficiency of isolation of ~30%. The abundance of naturally processed IVT peptides was found to vary in different cell lines between about 200 and more than 12,000 molecules per cell, whereas the recovery of AVF peptides was 10–20-fold lower on average (Figs. 4 and 5, and Table 2). These estimated values are well within the range of those reported for other class I–restricted CTL target epitopes derived from cellular proteins, e.g., between 12,000 and 400,000 molecules per cell for two epitopes from the enzyme α-ketoglutarate recognized by alloreactive CTLs (35, 36), viral antigens, e.g., between 10 and 1,500 molecules per cell of antigenic peptides from HIV and influenza virus antigens (37, 38), or bacterial proteins, e.g., more than 3,000 molecules per cell of a peptide epitope from *Lysteria monocytogenes* (39). In contrast to the findings of Rammensee et al. (34) that naturally processed peptides accumulate in detectable levels only in cell lines expressing the relevant class I molecules, we could demonstrate that IVT and AVF peptides are also in A11-negative LCLs, albeit with relatively low abundance. This discrepancy is probably explained by the fact that both GK and JM express the A24 and Aw68 alleles that belong to the A11 family. We have indeed observed that synthetic IVT and AVF stabilize the expression of Aw68 in transfected T2 cells and AVF-pulsed, Aw68-positive targets are recognized by AVF-specific CTLs (Levitsky, V., unpublished results). The large differences in the abundance of IVT and AVF peptides in LCLs may be partially explained by experimental variations in the efficiency of recovery, although comparable values were obtained in repeated experiments performed with the same cell line. Variations in the levels of expression of native antigens are likely to play a major role in determining the abundance of the processed product, but this cannot explain the different recovery of IVT and AVF since they are derived from the same viral protein. We cannot rule out the possibility that differences in generation of individual peptides by proteolytic degradation of EBNA4 may contribute to our findings. Indeed, preferential processing was suggested to underlie the different immunogenicity of two H-2K$^b$–restricted epitopes in chicken albumin (8). Whereas experiments addressing this question in the context of EBNA4-specific responses are presently in progress, it is important to stress that the relationship between IVT and AVF presentation was maintained over a more than 10-fold difference in the level of endogenous EBNA4 expression (Fig. 2 *B*). Because the density of peptide epitopes at the cell surface is likely to influence the affinities of antigen-specific T cell responses, our results predict that TCR affinity will be higher in AVF-compared to IVT-specific CTLs. This correlation was in fact demonstrated in CD8 blocking experiments performed with a large panel of CTL clones derived from four A11-positive donors (de Campos-Lima, P.O., V. Levitsky, M. Imzeh, and M.G. Masucci, manuscript in preparation).

Numerous investigations have evaluated the contribution of class I binding affinity to epitope selection and immunodominance. Positive correlations have been demonstrated using different biochemical methods including biosensor-based technologies that employ surface plasmon resonance to monitor binding of immobilized peptide to soluble class I molecules (10), peptide competitions with radiolabeled standard peptide bound to detergent-solubilized class I (11, 40), or peptide-driven association and dissociation rates of soluble class I–$\beta_2$-m complexes (41–42). Since recombinant A11 molecules are not available, and since we also lack conformation-dependent mAbs that would selectively recognize A11–peptide complexes, our analysis of IVT and AVF binding affinity had to rely on biologic assays performed with live LCLs and A11-transfected, TAP-deficient mutants. We have shown that IVT and AVF peptides compete equally well for TAP- and ATP-dependent translocation in streptolysin-O permeabilized cells (Fig. 6), suggesting that comparable amounts could be available for loading into nascent class I molecules in vivo. The two peptides were also equally potent competitors in CTL target sensitization assays (Fig. 7), indicating that they have similar affinities for A11. In line with this observation is the finding that peptide-loaded molecules induced at the surface of T2/A11 cells by incubation at low temperature were equally stable in cell lysates exposed to a wide range of temperature and pH conditions (Fig. 8). It is therefore very significant that AVF-induced complexes were rapidly cleared from the surface of T2/A11 cells incubated at 37°C with a half-life only slightly better than A11 molecules induced at low temperature in the absence of exogenous peptides. This cannot be explained by a lower binding of the complexes to the mAb used for immunoprecipitation since W6/32 was used in all assays.

Taken together, our results imply that, as previously demonstrated for class II (43), peptides influence the representation of class I molecules at the surface of APCs. Differences in persistence will select for long-lived complexes and are likely to affect both the potency and immunodominance of T cell epitopes. The factors regulating such persistence are unknown. Conceivably, minor conformational changes could be sensed in live cells resulting in differential

handling of the complexes. There is now consistent evidence suggesting that class I molecules recycle through the endocytic compartment. In the study of Reid and Watts (44), ~2% of the surface class I molecules in the EBV-transformed A46 cell line were endocytosed over a period of 1 min and recycled to the cell surface with a half-life of about 2–3 min. Some complexes could be selectively lost during this extensive recycling because of a higher dissociation rate in the endosomal environment. We could not demonstrate any significant difference in the stability of IVT and AVF complexes at pH as low as 4.5 (Fig. 8 *C*), indicating that they could survive equally well the mildly acidic pH of the endosomes. Other factors may contribute, however, to a quality control in this cellular compartment, including the presence of some proteolytic enzymes (45). It is noteworthy that substitutions of solvent-exposed residues within class II–restricted peptide epitopes were shown to influence the half-life of the complexes without significant effect on binding affinity or peptide dissociation rates (43). The findings presented in this paper have important implications for the design of peptide-based vaccines and for the choice of peptide-specific CTLs for adoptive immunotherapy trials. The most efficient vaccines are likely be those able to elicit CTL responses against antigenic epitopes that are efficiently expressed at the surface of virus-infected or tumor cells. The demonstration that this property is strongly dependent on the life span of the presenting class I molecules in live cells indicate that prediction of such epitopes cannot be reliably based on biochemical analysis of binding affinity or on the stability of isolated MHC–peptide complexes.

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