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HLA-B27-restricted CTL responses to EBV are principally directed against two of the EBV nuclear Ags, EBNA3B and 3C. We have previously described a target epitope derived from EBNA3C (residues 258–266, sequence RRIYDLIEL) that is immunodominant in the context of at least three different B27 subtypes, including B*2705 and B*2702. In this study, we show that this peptide binds well to B*2705 and B*2702 in a cell surface binding assay, and that the two B27:peptide complexes are relatively stable, with $t_{1/2}$ of 20 and 37 h, respectively. We now identify another B27-restricted epitope derived from EBNA3B (residues 243–253, sequence RRARLSAERY), which again accords well with the B*2705/B*2702 consensus motifs, having an arginine residue at position 2 and a tyrosine residue at the carboxyl terminus. In this case, five of five B*2702-positive donors respond to the epitope, whereas there was no response in any B*2705-positive donor studied. This peptide binds at least as well to B*2705 as to its restriction element B*2702; however, the two class I:peptide complexes show marked differences in stability, with $t_{1/2}$ of 9 and 42 h, respectively. Thus, the stability of B27:peptide complexes can vary markedly between different B27 subtypes in ways that may not be apparent from cell surface binding assays and cannot be predicted from currently known peptide consensus motifs, yet which may critically influence CTL epitope choice. The Journal of Immunology, 1998, 161: 5252–5259.

Cytotoxic T lymphocytes recognize short peptide fragments, usually 8–11 amino acids in length, derived from the proteolysis of endogenous proteins and presented at the cell surface in association with MHC class I molecules (1–4). The range of peptides presented is dependent on the identity of the restricting class I molecule, and from the evidence of peptide elution and sequencing, allele-specific consensus motifs have been described (5). In this respect, one of the class I alleles that has been most extensively studied, due to its strong disease associations, is HLA-B27. To date, 12 subtypes of B27 have been identified, differing from the common B*2705 subtype by 1–7 amino acid residues concentrated within the peptide-binding groove (6). Sequence analysis of natural peptide ligands and defined CTL epitopes has allowed the description of a B27 peptide consensus motif (7, 8) that correlates with structural data from x-ray crystallography (9, 10). The dominant anchor residue in this motif is an arginine residue at position 2 of the peptide (7); this residue binds in a pocket within the peptide-binding groove that is conserved between all of the different B27 subtypes (9, 10). Subtype polymorphism influences the amino acid residues found at other positions within the peptide. For example, B*2702 differs from B*2705 by three amino acid residues, all of which are located within the C/F pocket that binds the peptide carboxyl-terminal residue (positions 77, 80, and 81 are N, I, A, respectively in B*2702, and D, T, L in B*2705). These changes decrease net negative charge within the B*2702 C/F pocket; consequently, while B*2705 accepts basic, aromatic, or aliphatic amino acids at the carboxyl terminus (L, F, Y, M, I, R, H, K), B*2702 only accepts aromatic or aliphatic amino acids (F, Y, I, L, W) (7, 8).

Despite the complexity of most intracellular pathogens, and hence the large number of protein Ags available for recognition, CTL responses are very often highly focused against just a small number of immunodominant peptides (11–13). The basis of this selectivity is not fully understood, but several contributing factors have been suggested: 1) the liberation of peptides by proteolytic degradation of proteins via the proteasome complex, a process probably requiring both appropriate flanking sequences and the absence of internal cleavage sites (14–16); 2) the transport of peptide fragments from the cytosol into the ER, via the TAP complex, which may have sequence/length specificity (17, 18); 3) the affinity of peptides for the available class I molecules (19, 20), which will determine their ability to compete effectively with other peptides in the ER; and 4) the stability of the class I:peptide complex and its $t_{1/2}$ at the surface of the APC (21, 22). In addition, for a presented peptide to be immunogenic, the host immune repertoire must contain T cells with TCRs capable of recognizing the class I:peptide complex (23, 24).

We have addressed the issue of CTL epitope choice, and in particular the influence of B27 subtype polymorphism upon that choice, in the context of T cell responses to EBV. This B-lymphotropic herpesvirus, which is widespread in human populations, has cell growth-transforming ability, yet is carried by most individuals as a lifelong asymptomatic infection. The virus elicits strong CTL responses that persist over time and appear to play an important role in controlling the infection (25). Thus, memory...
CTLS can be reactivated from the blood of virus carriers by in vitro stimulation with the autologous EBV-transformed B-lymphoblastoid cell line (LCL) (26). Such LCLs express a limited range of viral latent gene products, namely six nuclear Ags, EBNA1s 1, 2, 3A, 3B, 3C, and -LP, and two membrane proteins, LMPs 1 and 2 (25). An individual’s CTL response will be determined by their class I genotype, both quantitatively in that certain alleles (including B27) mediate strong CTL responses, and qualitatively in terms of Ag choice. Interestingly, in the EBV system, memory CTL responses appear to be preferentially directed against the EBNA 3A, 3B, 3C family of proteins, irrespective of genotype (26, 27). Thus, one or more of these proteins constitute the immunodominant target(s) for CTL responses from almost all virus carriers analyzed to date. We have previously described an epitope derived from EBNA 3C residues 258-266, sequence RRIYDLIEL, that is presented in the context of at least three different B27 subtypes, including B*2705 and B*2702 (28). In this study, we define a B27-restricted epitope derived from EBNA 3B (29), which appears to be immunogenic only in the context of B*2702 and not B*2705. To study the factors that might influence these differences in epitope choice, we then compare these two B27 subtype molecules for their ability to bind the different epitope peptides, to present these peptides from endogenously expressed Ag, and to form stable B27-peptide complexes at the cell surface.

Materials and Methods

B27-positive donors

Nine HLA-B27-positive individuals, all previously infected with EBV, were used in this study: SC (A2, B*2705), RT (A2, A24, B*2705, B35), EN (A1, A2, B18, B*2705), AL (A3, B15, B*2705), LY (A1, A24, B*2702, B35), NW (A3, A31, B8, B*2702), Rov (A2, B14, B*2702), Kor (A24, A28, B*2702, B44), and Kla (A2, A3, B7, B*2702).

Target cells

LCLs were generated by in vitro transformation of B cells using either the standard type 1 EBV isolate B95.8 (29), or virus isolated from laboratory donors containing an R→K substitution at position 2 of the RRIYDLIEL epitope sequence. LCLs were cultured in RPMI 1640 containing 10% FCS and 2 mM glutamine (growth medium). Fibroblasts were established from skin biopsies and maintained in monolayer culture in DMEM supplemented as described above. PHA blasts were generated by culturing PBMCs in T cell medium (growth medium supplemented with 1% human AB serum) containing 100 µg/ml PHA, and maintained by twice weekly refueling with T cell medium containing 30% supernatant from the IL-2-secreting MLA 144 cell line (European Collection of Animal Cell Cultures, PHLS, Porton Down, U.K.).

Vaccinia virus recombinants

The recombinant vaccinia viruses (rVVs) encoding the EBV latent proteins (EBNAs 1, 2, 3A, 3B, 3C, -LP, and LMPs 1 and 2) have been previously described (27); the coding sequences were all of B95.8 strain origin.

Synthetic peptides

Peptides were synthesized using fluorescaminecarbonyl chemistry by Dr. J. Fox (Alta Bioscience, University of Birmingham, Birmingham, U.K.). They were dissolved in DMSO and assayed for protein concentration by a modification of the Brieuer assay (30).

Generation of EBV-specific CTL

EBV-specific CTLs were reactivated from the peripheral blood of donors by cocultivation of PBMCs with γ-irradiated cells of the autologous EBV (B95.8)-transformed LCL (responder/stimulator ratio of 40:1) in T cell medium. The resultant polyclonal CTL preparations were maintained by weekly refueling in T cell medium containing 30% MLA 144 supernatant, and by weekly restimulation with the γ-irradiated LCL. EBV-specific CTL clones were generated from cocultures either on day 4 poststimulation by seeding in semisolid agarose, as previously described (31), or on day 14 poststimulation by limiting dilution cloning at 0.3 and 3 cells/well in 96-well U-bottom plates, as previously described (32). T cell clones were subsequently maintained by weekly refueling with T cell medium containing 30% MLA 144 supernatant and 50 IU/ml of rIL-2, and by weekly restimulation with the γ-irradiated autologous LCL.

Chromium release assays

Effector CTLs were screened in standard 4–5 h 51Cr release assays, as previously described (32). For assays using rVV-infected target cells, monolayer cultures of fibroblasts or LCL cell pellets were exposed to rVV (multiplicity of infection 10) for 2 h, then additional culture medium was added for 16 h. Infected fibroblasts were then harvested by trypsinization. Cells were labeled with 51CrO4 for 1–2 h, washed twice, and used as targets in the standard CTL assay. In peptide sensitization assays, PHA blasts or LCLs were labeled with 51CrO4 for 2 h, washed twice, then incubated with peptide at twice the final required concentration in a volume of 100 µl. After 30 min, CTLs were added in a 100 µl vol to give final peptide concentrations, as stated in figure legends for the duration of the assay.

Epitope identification by direct visualization of CTL-CTL killing

The peptide specificity of novel CTL clones was determined using a visual assay of CTL-CTL killing (33) with modifications. CTL clones were incubated overnight in 96-well U-bottom plates (300 cells/well) in 200 µl of T cell medium containing individual 15-mer peptides spanning the EBNA 3B sequence (peptide concentration, 2 µM). Cell viability was assessed the following day using an inverted phase microscope.

Cell surface class I peptide-binding assay

Peptide binding to cell surface B*2705 and B*2702 was measured using a T2 FACS peptide-binding assay (34). TAP-deficient T2 cells transfected with B*2705 (35) or B*2702 were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM glutaminie, and 500 µg/ml G418. For peptide loading, cells were washed twice with RPMI 1640 and resuspended in EX-CELL 301 defined serum-free medium (JRH Biosciences, Lenexa, KS) supplemented with 2 mM glutamime, nonessential amino acids, and sodium pyruvate. A total of 5 x 10^6 cells was incubated with peptide, or diluent alone, at varying concentrations (final vol 200 µl), in a 96-well U-bottom plate for 16–18 h, in a humidified 5% CO2 incubator. Peptides were dissolved in DMSO at a concentration of 10 mg/ml, then diluted in RPMI 1640; for each peptide sample, a control with the same amount of DMSO was run. Assessed cell surface B27 molecules were detected by staining with ME.1 (specific for HLA-B27, HLA-B7, and HLA-Bw22 complexed with peptides), then affinity-purified FITC-F(ab)2 goat anti-mouse IgG (Fc specific; Organon Technika, Westchester, PA), followed by FACS analysis using a FACSscan (Becton Dickenson, Palo Alto, CA) flow cytometer. Mean fluorescence intensity (MFI) of a minimum of 5000 events was calculated for each sample using Lysis II software. The relative binding of each peptide is inferred from the change in fluorescence, and was determined as follows: Percent of maximum fluorescence = [MFIsample peptide - MFIβ2m peptide] / [MFIβ2m peptide] x 100. The concentration of peptide required for 50% maximum fluorescence (EC50) is used for comparison.

Cell surface class I peptide complex stability assay

To assess the stability of B*2705 and B*2702:peptide complexes, transfected T2 cells were incubated with peptide (100 µM) or diluent control for 16–18 h, as described for the peptide-binding assay, washed, and incubated in Brefeldin A (Sigma, St. Louis, MO) (10 µg/ml) to block egress of newly synthesized class I molecules (36). Following a 1-h incubation at 37°C, cells were washed, and incubations continued in the presence of Brefeldin A (0.5 µg/ml) at 37°C in a humidified 5% CO2 incubator. Aliquots were removed at the indicated times, and cells were stained with ME.1, as described above. The decay of B27 complexes was determined as follows: Percentage of immunoreactivity remaining (at T = X) = [MFIβ2m T=∞(peptide) - MFIβ2m T=X(peatide)] / [MFIβ2m T=∞(peptide) - MFIβ2m T=∞(peptide)] x 100.

Results

Target epitopes for B27-restricted, EBV-specific CTL responses

In an initial set of experiments, we mapped the target Ags recognized by EBV-specific polyclonal CTL populations from B*2705- and B*2702-positive donors. CTLs were screened in chromium release assays against B27-matched fibroblasts expressing individual EBV latent proteins from rVVs. The pattern of results obtained is illustrated in Fig. 1. Polyclonal CTLs from three B*2705-positive donors, SC, RT, and EN, all showed dominant reactivity against B*2705-positive fibroblasts expressing EBNA 3C (Fig. 1A). This EBNA 3C-specific response included a major component
recognizing the previously defined RRIYDLIEL epitope (designated RRIY) (data not shown). Note that all three B*2705-positive donors also shared HLA-A2, and the small but reproducible LMP 2-specific response was directed against a previously defined A2-specific component (26; data not shown). Polyclonal CTLs from B*2702-positive donors displayed two distinct patterns of recognition (Fig. 1B). CTL preparations from donor LY showed strong killing of fibroblasts expressing EBNA 3C (reflecting the presence of T cells reactive to the same RRIY epitope as seen by B*2705-positive donors; data not shown), but also contained an EBNA 3B-specific component. In contrast, polyclonal CTLs from B*2705-positive donors also shared HLA-A2, and the small but reproducible LMP 2-specific response was directed against a previously defined A2-specific component seen within the polyclonal CTLs from all B*2702-positive donors (data not shown).

We then conducted a clonal CTL analysis on all of the above donors to determine whether the patterns of Ag specificity seen in polyclonal T cell populations were reproduced, and to check whether any additional reactivities could be identified. CTL clones were isolated from four B*2705-positive donors, SC, RT, and EN, and B*2702-positive donors, LY, NW, and Rov. The minimum epitope recognized by all clones is the 11-mer sequence RRARSLSAERY (designated RRAR), representing EBNA 3B residues 243–253. This was subsequently confirmed as the target epitope for the EBNA 3B-specific component seen within the polyclonal CTLs from all B*2702-positive donors (data not shown).

Table I. Definition of the minimal epitope recognized by B*2702-positive, EBNA 3B-specific CTLs

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<tr>
<th>Peptide Sequence</th>
<th>LY c27</th>
<th>LY c64</th>
<th>NW c18</th>
<th>NW c20</th>
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<td>24</td>
<td>24</td>
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<td>24</td>
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<td>29</td>
<td>26</td>
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</table>

No peptide 2 1 3 1 2

* Results are expressed as percent specific lysis.
* Peptides were screened for their ability to sensitize B*2702-positive PHA blasts to CTL-mediated lysis in chromium release assays at a concentration of 10^{-3}M.

* Denotes the number of clones tested which were specific for EBV latent Ags other than RRIY or RRAR.

NT not tested; in these experiments clones were only screened against the RRIY and RRAR epitope peptides.
AL, and EN, where they constituted up to ∼50% of all CTL clones recognizing EBV latent Ags. No RRAR-specific CTL clones were identified in any of these B*2705-positive donors. In contrast, three of the five B*2702-positive donors tested, LY, Kor, and Kla, yielded both RRIY-specific and RRAR-specific clones, with the former being the more frequent reactivity. The two remaining B*2702-positive donors, NW and Rov, never gave RRIY-specific responses. Instead, all of their B27-restricted clones recognized the RRAR epitope; in fact, donor NW’s CTL response to the EBV latent Ags appears to be focused solely on this epitope. Thus, clonal analysis of B*2705- and B*2702-restricted EBV-specific CTL responses identified similar reactivities to those observed in polyclonal CTL populations.

**Binding of the RRIY/RRAR epitopes to B*2705 and B*2702**

Several studies have suggested that there is a positive correlation between peptide binding to class I molecules and immunogenicity, such that defined CTL epitopes usually have a high affinity for their restriction determinant (19, 20). The next experiments were therefore designed to measure the relative binding of the RRIY and RRAR epitopes to B*2705 and to B*2702. Peptide binding was assessed using B*2705 and B*2702 transfectants of the T2 cell line in a cell surface class I-binding assay (34). Since T2 cells do not express TAP, and therefore do not load endogenous peptides efficiently, class I molecules expressed in these cells are available for the binding of appropriate exogenously added peptides; B27:peptide complexes can then be detected using a conformation-dependent mAb.

T2:B*2705 and T2:B*2702 cells were incubated overnight at 37°C in serum-free medium containing the RRIY or RRAR peptides at concentrations between 10⁻⁴ and 10⁻⁹ M; cells were then stained with the B27/B7-specific mAb ME.1, and the staining was quantitated by flow cytometry. Results are expressed as percentage of maximum fluorescence, in which 100% represents the fluorescence of T2:B*2705 cells incubated with the RRIY peptide at the highest concentration (10⁻⁴ M). The data presented in Fig. 2 are the mean of four (B*2705) and three (B*2702) independent experiments. As shown in the upper panel of Fig. 2, the RRIY peptide appeared to bind equally well to both B*2705 and B*2702; peptide concentrations required to achieve 50% maximum fluorescence (EC₅₀) were ∼4 and 6 μM, respectively. The RRAR peptide also appeared to bind to both B*2705 and B*2702 (Fig. 2, lower panel), although this required higher peptide concentrations than for the RRIY peptide. Interestingly, the RRAR peptide actually appeared to bind better to B*2705 than to its restriction element B*2702. Thus, the absence of a B*2705-restricted CTL response to the RRAR epitope does not appear to be due to an inability to bind this class I molecule.

**Processing and presentation of the RRIY epitope by B*2705 and B*2702**

There are several other steps in the Ag processing and presentation pathways that may influence whether a particular peptide sequence is presented at the cell surface in association with a particular class I allele (13–18, 21, 22). We first examined the efficiency of processing/presentation of the EBNA 3C-derived RRIY epitope in B*2705- versus B*2702-positive cell backgrounds. As APCs for these experiments, we chose LCLs transformed with EBV isolates carrying an epitope-loss mutation of the RRIY sequence (R→K at position 2) so that there was no baseline recognition of the resident EBV-encoded EBNA 3C protein. As a source of CTLs, we chose T cell clones from B*2702-positive donors that recognized the RRIY peptide equally well in the context of B*2705 and in the context of their natural restriction element B*2702. Fig. 3A shows such a validation experiment in which two representative RRIY-specific clones (from donors LY and Kor) were tested against B*2705- and B*2702-positive target cells that had been exogenously preloaded with RRIY epitope peptide over a wide range of peptide concentrations. Reproducibly, the concentration of exogenous peptide required to achieve half-maximal lysis was ∼10⁻⁹ M on both B27 subtype backgrounds.

These same clones were then tested on B*2705- and on B*2702-positive targets expressing EBNA 3C (B95.8 sequence) endogenously from a rVV vector. As shown in Fig. 3B, the two types of target were equally well recognized in assays in which, as a control, there was no lysis of the targets infected with an irrelevant rVV (rVV-EBNA 3B). Such results are in line with those observed in earlier work using LCL targets transformed with the standard B95.8 EBV strain; there we found that the majority of RRIY-specific CTL clones from B*2702-positive donors could recognize B*2705- and B*2702-positive LCLs equally well (28).

We also conducted reciprocal experiments using the same target cells as those shown in Fig. 3, but this time with RRIY-specific CTL clones established from B*2705-positive donors and selected for their ability to detect the exogenously loaded RRIY peptide with equal efficiency on B*2705- and on B*2702-positive APCs. These effectors again showed equally strong lysis of B*2705 and B*2702 LCL targets following infection with the EBNA 3C-expressing rVV (data not shown). Together these results suggest that the RRIY epitope is processed and presented in a very similar manner by both B*2705- and B*2702-positive APCs; this is consistent with this epitope being immunogenic in the context of both subtype molecules.
Processing and presentation of the RRAR epitope by B*2705 and B*2702

In a parallel set of experiments, we used epitope-specific CTLs as probes to compare processing/presentation of the RRAR epitope (i.e., an epitope only immunogenic in the context of B*2702) in B*2705- versus B*2702-positive cell backgrounds. A proportion of the RRAR-specific CTL clones from B*2702-positive donors (here represented by LY c64 and LY c22) recognized exogenously loaded epitope peptide equally well on B*2705- and B*2702-positive targets; as shown in Fig. 4A, a peptide concentration of \(10^{-9}\) M mediated half-maximal lysis on both B27 subtype backgrounds. We then tested these same CTLs on targets expressing the EBNA 3B protein endogenously from a rVV vector, the results being shown in Fig. 4B. There was strong recognition of rVV-EBNA 3B-infected B*2702-positive targets, but no significant lysis of the corresponding B*2705-positive targets. Clearly, processing/presentation of the RRAR epitope was either completely abrogated or at least severely impaired in the B*2705 cell background.

Subsequent experiments with other B*2702-positive clones suggested that the latter explanation is correct. Thus, the majority of RRAR-specific CTL clones obtained from B*2702-positive donors (here represented by LY c29 and NW c20) actually recognized the epitope, when provided as exogenous peptide, at approximately 10-fold lower peptide concentrations on B*2705, than on the natural restriction element B*2702; peptide concentrations required to achieve half-maximal lysis were \(5 \times 10^{-10}\) M and \(5 \times 10^{-9}\) M, respectively (Fig. 4C). In contrast to the results with the previous clones, these more sensitive effectors did show recognition of B*2705-positive as well as B*2702-positive LCLs expressing EBNA 3B from a rVV (Fig. 4D). Note, however, that the levels of CTL-mediated lysis of B*2705-positive targets were reproducibly lower than those of B*2702-positive targets.

**FIGURE 3.** CTL recognition of the RRIYDLIEL epitope presented by B*2705- and B*2702-positive APCs. A, Representative B27-restricted, RRIY-specific CTL clones from B*2702-positive donors LY and Kor were tested in peptide sensitization assays against B*2702- and B*2705-matched LCL targets preincubated with the RRIY epitope at concentrations between \(10^{-11}\) and \(10^{-9}\) M. E:T ratio was 5:1 for both clones. Lysis of target cells in the absence of peptide (DMSO solvent alone) was always below 6%. B, The same clones as in A were tested against B*2702- and B*2705-matched LCL targets expressing either EBNA 3C or EBNA 3B (control) from a rVV (vacc-). E:T ratio was 2:1 for both clones. All results are expressed as percentage of specific lysis and are illustrative of those seen in several repeated assays.

**FIGURE 4.** CTL recognition of the RRARSLSAERY epitope presented by B*2702- and B*2705-positive APCs. A and C, Representative B27-restricted, RRAR-specific CTL clones from B*2702-positive donors LY and NW were tested in peptide sensitization assays against B*2702- and B*2705-matched LCL targets preincubated with the RRAR epitope at concentrations between \(10^{-11}\) and \(10^{-9}\) M. E:T ratios were between 3:1 and 5:1. Lysis of target cells in the absence of peptide (DMSO solvent alone) was always below 16%. B and D, The same clones as in A and C were tested against B*2702- and B*2705-matched LCL targets expressing either EBNA 3B or EBNA 3C (control) from a rVV (vacc-). E:T ratios were between 3:1 and 5:1. All results are expressed as percentage of specific lysis and are illustrative of those seen in several repeated assays.
lower than those of B*2702-positive targets, although absolute values did vary between individual cell lines in each group. Together these results suggest that the RRAR epitope can be processed and presented by B*2705-positive APCs, but that the number of B*2705:peptide complexes presented at the APC surface is at least 10-fold lower than the number of B*2702:peptide complexes. Note that throughout the work with RRAR-specific T cells (Fig. 4), there was little, if any, baseline killing of unmanipulated targets, even though the resident virus isolate in these cells does express an EBNA 3B protein containing the RRAR epitope. This poor recognition of LCL targets is not unique to RRAR effectors, however, but has been observed with several types of epitope-specific CTLs in the EBV system (32, 37) and may well be a consequence of the particular in vitro cloning conditions used.

Stability and \( t_{1/2} \) of B*2705 and B*2702:peptide complexes

From the above results, there seemed to be a discrepancy between the apparent affinity of B27:peptide binding as measured in the cell surface peptide-binding assay (Fig. 2) and the level at which the epitopes are presented on the surface of infected cells (Figs. 3 and 4). Because differences in the stability of the B27:peptide complexes might also be a factor influencing peptide representation on the cell surface (21, 22), we measured the \( t_{1/2} \) of B27:RRIY and B27:RRAR complexes using a cell surface class I peptide complex stability assay (36). In this case, T2:B*2705 and T2:B*2702 cells were loaded exogenously with peptide overnight, then the cells were washed into fresh medium containing brefeldin A to block the egress of newly synthesized class I molecules from the ER. Loss of the existing B27:peptide complexes on the cell membrane was then followed over a period of 28 h by staining aliquots of cells with the B27/B7 conformation-dependent mAb ME.1 and quantitating staining by flow cytometry. Results shown in Fig. 5 represent the mean of three experiments for each B27:peptide combination tested and are expressed as the percentage of immunoreactivity remaining at different times; the \( t_{1/2} \) values calculated from these data are summarized in Table III. The RRIY epitope formed relatively stable complexes with both B*2705 and B*2702, giving \( t_{1/2} \) values of 20 and 37 h, respectively. In contrast, the RRAR epitope formed very stable complexes with its natural restriction element B*2702, \( t_{1/2} \) 42 h, but comparatively unstable complexes with B*2705, \( t_{1/2} \) 9 h.

Discussion

To learn more about the determinants of CTL epitope choice, we have studied T cell responses restricted through two closely related class I alleles, B*2705 and B*2702, for which peptide consensus motifs are well established (7, 8). Our earlier work had suggested that the EBNA 3C-derived RRIY peptide was selected as an immunodominant CTL epitope presented in the context of both B*2705 and B*2702 (28); this is in accordance with the consensus motifs for both subtype molecules (R at position 2, and L at the carboxyl terminus) (7, 8). In the present work, we have extended this analysis to more donors, in each case challenging their CTL repertoire with an autologous LCL carrying the standard B95.8 EBV strain that contains the RRIY epitope. These studies confirmed the immunodominance of the RRIY epitope in B*2705-positive donors, and also in three of five B*2702-positive donors analyzed. The absence of a CTL response to this epitope from one of the two nonresponsive B*2702-positive donors, NW, may be explained by the fact that this donor’s endogenous virus isolate has a point mutation in the EBNA 3C gene within the region encoding the RRIY sequence (J. M. Brooks, manuscript in preparation). This results in an R→K substitution at position 2 of the epitope, which is the primary anchor position for presentation on B27 molecules; accordingly, LCLs carrying this virus isolate are not recognized by RRAR-specific effectors. This explanation may also hold for the second nonresponsive B*2702-positive donor Rov, but no endogenous isolate was available for analysis in this case.

In the course of the above work, we consistently detected a B*2702-restricted reactivity to EBNA 3B that could be mapped to a novel 11-mer epitope, RRAR; interestingly, we never found this reactivity in any of the B*2705-positive donors studied. In this case, the lack of response cannot be ascribed to epitope sequence variation; endogenous virus isolates from two of the B*2705-positive donors studied (SC and RT) and two B*2702-positive donors (LY and NW) have been sequenced across a 170-bp fragment of

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<th>Subtype</th>
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<td>B*2705</td>
<td>RRIYDLIEL</td>
<td>20</td>
</tr>
<tr>
<td>B*2702</td>
<td>RRIYDLIEL</td>
<td>37</td>
</tr>
<tr>
<td>B*2705</td>
<td>RRARLSAERY</td>
<td>9</td>
</tr>
<tr>
<td>B*2702</td>
<td>RRARLSAERY</td>
<td>42</td>
</tr>
</tbody>
</table>

\(^a\) Half-lives were estimated from decay curves by solving % immunoreactivity remaining = \( ae^{-bt} \), in which \( t \) = time (h), \( a \) = the y intercept at \( t = 0 \), and \( b \) = the slope of the line. Results represent the mean of three experiments, with error bars indicating the SEM; where bars are not visible, the error is smaller than the symbol.
the EBNA 3B gene spanning the coding region for the RRAR epitope, and all were identical to the B95.8 prototype in this region (R. I. Tierney, unpublished data). Rather, the results appear to reflect a real difference in the immunogenicity of the RRAR peptide between B*2705- and B*2702-positive donors. Such a difference would not be expected from the peptide consensus motifs of these B27 subtype molecules, since these predict that the RRAR epitope (having an R at position 2 and a Y at the carboxyl terminus) should be presented by both subtypes (7, 8). Furthermore, although the RRAR 11-mer exceeds the standard 9-mer length of most B27-associated peptides, there is no a priori reason to expect longer peptides to be presented more efficiently by B*2702 than by B*2705. Indeed, natural peptide ligands of 11 or more amino acids have been eluted from B*2705 molecules in several studies (8, 38, 39). In this context, we noted that the 11-mer RRAR epitope contained a 9-mer sequence (ARSLSAERY), which also conforms to both the B*2705 and the B*2702 consensus motifs; however, this was not recognized as an epitope by donors of either subtype (Tables I and II), and in fact appeared to bind poorly to B*2705 (J. M. Brooks, unpublished data). The differential immunogenicity of the RRIY and RRAR epitopes in the context of these two closely related B27 subtype molecules then enabled us to analyze the relative importance of several parameters in determining CTL epitope choice.

A number of studies have evaluated the contribution that peptide affinity for class I molecules makes to CTL epitope selection (13, 19, 20). A positive correlation has been observed in that all epitope peptides bind with moderate to high affinity; however, binding is not the only factor, since other peptides may bind equally well or even better, yet not be immunogenic. We assessed the relative binding of the RRIY and RRAR epitopes to B*2705 and B*2702 molecules expressed on the surface of T2 cell transfectants (34). Note that this assay probably reflects the efficiency of peptide loading onto cell surface class I molecules (i.e., the on-rate), rather than actual binding affinity (which also encompasses the off-rate), since the assay is performed for a relatively short time period and in the continual presence of peptide. The RRIY peptide appeared to bind well to both B27 subtype molecules in this assay (Fig. 2, upper panel), consistent with it being an immunodominant CTL epitope in the context of either subtype. Surprisingly, however, the RRAR peptide also appeared to bind to both subtypes, and in fact bound better to B*2705 than to its natural restriction element B*2702 (Fig. 2, bottom panel). Similar results have been obtained as part of an independent study analyzing binding of the RRIY and RRAR epitopes and various analogues to a wider spectrum of B27 subtype molecules (40). The absence of a B*2705-restricted, RRAR-specific CTL response therefore does not appear to be due to an inability of this B27 subtype molecule to bind the peptide.

The efficiency of Ag processing and presentation has been shown to correlate with immunogenicity in several systems (13–18, 21, 22). We have analyzed the processing and presentation of the two EBV-derived epitopes using CTL detection as the readout; this was possible because B*2702-positive CTL clones specific for either the RRIY or the RRAR epitope recognized their cognate epitope in the context of B*2702 and B*2705. Thus, RRIY-specific, B*2702-positive CTL clones that recognized exogenously added peptide equally well on B*2702 or B*2705 (Fig. 3A) also showed equal recognition of B*2702- or B*2705-positive targets expressing EBNA 3C from a rVV (Fig. 3B), or from the resident EBV genome (28). The efficient processing/presentation of the RRIY epitope by both B27 subtypes is consistent with this epitope being immunodominant in both B*2705- and B*2702-positive donors.

In contrast, although all RRAR-specific, B*2702-positive CTL clones recognized their epitope in the context of either subtype molecule when it was provided as exogenous peptide (Fig. 4, A and C), there was a clear difference in recognition of the same target cells when the epitope had to be processed and presented from endogenously expressed Ag (Fig. 4, B and D). Comparison of CTL recognition of the exogenous RRAR peptide versus the endogenous rVV-encoded Ag (cf Fig. 4, A, B with C, D) suggested that there were at least 10-fold lower numbers of B*2705:RRAR complexes at the cell surface compared with B*2702:RRAR complexes. Thus, the differential processing/presentation of the RRAR epitope on B*2705 versus B*2702 correlates with differential immunogenicity on these subtypes. We reasoned that this differential processing/presentation is unlikely to reflect differences in proteolysis or peptide transport between individual donors who are either B*2705 or B*2702 positive. Although the proteasome components, LMP2 and LMP7, and the human TAP1/TAP2 transporter subunits display limited polymorphism, studies to date have not shown such polymorphism to have functional significance (41, 42). Instead we would argue that differences in epitope processing/presentation might reflect the different stabilities of the B27:peptide complexes. Thus, the results of the complex stability assays (Fig. 5 and Table III) suggest that the lower representation of the RRAR epitope at the surface of B*2705-positive APCs compared with B*2702-positive APCs is determined by the relatively short t1/2 of B*2705:RRAR complexes. Although the RRAR epitope loads more efficiently onto B*2705 than onto B*2702 (Fig. 2, lower panel), the t1/2 of B*2705:RRAR peptide complexes at the cell surface is only approximately one-fifth of the t1/2 of B*2702:RRAR complexes (9 and 42 h, respectively). Two other recent studies have suggested that the life span of MHCC:peptide complexes at the cell surface of APCs may be the critical factor determining immunogenicity (21, 22). It is not known what t1/2 is required for any particular MHC:peptide complex to be immunogenic, and this may well vary significantly for different epitopes; however, the data presented in this work suggest that for B27:RRAR complexes, it is longer than 9 h. In the same assay, the RRIY epitope, which is immunogenic in the context of both B*2702 and B*2702, forms complexes with t1/2 of 20 and 37 h, respectively. These t1/2 are comparable with those measured for a natural peptide ligand derived from cellular protein; thus, the KRYKSIVKY peptide (derived from human farnesyl pyrophosphate synthetase) that has been eluted from B*2702 (8) and B*2703 (43) forms complexes with B*2705 and B*2702 that have t1/2 of 37 and 47 h, respectively (R. A. C., unpublished observations).

It is not clear at present exactly what factors determine the t1/2 of MHC:peptide complexes at the cell surface, but this does not appear to correlate directly with how efficiently a peptide can be loaded, or with the temperature or pH stability of isolated MHC:peptide complexes (22). Recent studies have suggested that an undefined mechanism may operate in the ER to enhance the dissociation of labile MHC class I:peptide complexes, thus reducing their presentation at the cell surface and making available a greater number of peptide-receptive class I molecules for presentation of long-lived epitopes (44, 45).

In summary, the present work has focused on two viral epitope peptides, one of which (RRIY) is immunogenic in the context of both B*2702 and B*2702, the other of which (RRAR) is immunogenic only in the context of B*2702. These differences in immunogenicity correlated with unexpected differences in the t1/2 of the relevant B27:peptide complexes. It seems that the stability of B27:peptide complexes can vary markedly between different B27
subtypes in ways that may not be apparent from surface binding
assays and cannot be predicted from current peptide consensus
motifs, yet which may critically influence CTL epitope choice.

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References


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