Hierarchy of Epstein-Barr Virus-Specific Cytotoxic T-Cell Responses in Individuals Carrying Different Subtypes of an HLA Allele: Implications for Epitope-Based Antiviral Vaccines

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Major histocompatibility complex class I-restricted Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes (CTLs) in healthy virus carriers constitute a primary effector arm of the immune system in controlling the proliferation of virus-infected B cells in vivo. These CTLs generally recognize target epitopes included within the latent antigens of the virus. For example, CTLs from HLA B44+ healthy virus carriers often recognize peptide EENLLDVFRM from EBV nuclear antigen 6. However, the strength of this response directly correlates with the HLA B44 subtype expressed by the individual donor. Indeed, HLA B*4405+ virus carriers consistently show a very high frequency of CTL precursors for the EENLLDVFRM epitope, while a much weaker response is seen in HLA B*4403+ and HLA B*4402+ individuals. This disparity is not due to an intrinsic difference in the CTLs generated by individuals carrying different subtypes of HLA B44. In fact, virus-specific CTLs recognize EENLLDVFRM peptide-sensitized HLA B*4405+ target cells more efficiently than B*4402+ or B*4403+ target cells irrespective of the HLA B44 subtype expressed by the donors from whom these effectors were isolated. This effect is evident whether the CTL epitope is endogenously processed or exogenously presented. In addition, a comparison of the intracellular transport kinetics of different B44 subtypes revealed that the B*4405 allele is rapidly assembled and arrives in the trans-Golgi compartment at a faster rate than B*4402 or B*4403. Based on these results, we propose that HLA class I alleles that are capable of binding peptides more efficiently from the intracellular pool, and are rapidly assembled and transported, may confer a protective advantage against viral infection.

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seen in individuals carrying the HLA B*4403 and HLA B*4402 alleles. We have now investigated the molecular basis of this hierarchy. The data presented in this study show that a strong CTL response to EENLLDFRVM in HLA B*4405+ virus carriers correlates with more efficient presentation of the peptide and intracellular transport kinetics of this allele than of HLA B*4402 and HLA B*4403.

MATERIALS AND METHODS

Establishment and maintenance of cell lines. EBV-transformed lymphoblastoid cell lines (LCLs) from seropositive donors DM (HLA A24, A29, B7, B*4405), IT (HLA A1, A3 B7 B*4404), CM (HLA A11, A24, B7, B*4405), PM (HLA A12, A26, B*4402), CS (HLA A3, A25, B*4403), SE (HLA A2, A29, B60, B*4403), BK (HLA A1, A2, B8, B*4403), DY (HLA A11, A32, B35, B*4402), CF (HLA A1, A2 B8, B*4402), and KE (HLA A32, A26, B49, B*4402) were established by exogenous virus transformation of peripheral B cells, using EBV derived from the B95.8 cell line (19). All LCLs were routinely maintained in RPMI 1640 containing 2 mM glutamine, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 10 to 20% fetal calf serum (FCS) (growth medium).

To generate phophometagglutinin (PHA) blasts, unfractionated mononuclear (UM) cells were stimulated with PHA (Commonwealth Serum Laboratories, Melbourne, Australia), and after 3 days, growth medium containing MLA-144 supplemented with recombinant interleukin 2 (IL-2) (2) was added. T-cell growth medium (4). PHA blasts were propagated with biweekly replacement of IL-2 and MLA-144 supernatant (no further PHA added) for up to 6 weeks.

Source and generation of EBV-specific CTL clones. HLA B44-restricted EBV-specific CTL clones recognizing the CTL epitope, EENLLDFRVM, were established from healthy seropositive donors DM (B*4405*), CS (B*4403*), SE (B*4403*), IT (B*4402*), KE (B*4402*), and CF (B*4402*) as described earlier (2, 19). Briefly, 2 × 106 peripheral blood lymphocytes from these donors were stimulated in 2 ml of growth medium with autologous γ-irradiated (8,000 rads) B95.8-transformed LCLs (responder/stimulator ratio of 50:1). After 3 days, cells were dispersed and seeded in 0.35% agarose (SeaPlaque; FMC Corp., rads) B95.8-transformed LCLs (responder/stimulator ratio of 50:1). After 3 days, growth medium containing MLA-144 supplemented with recombinant interleukin 2 (IL-2) (2) was added. CTL clones specific for peptide EENLLDVFRM were used as target cells in a chromium release assay.

RESULTS

Individuals carrying different subtypes of HLA B44 display hierarchy in their responses to the EBV EENLLDFRVM epitope. Preliminary studies with polyclonal EBV-specific CTLs from HLA B*4405* individuals consistently showed a very strong response to the EENLLDFRVM epitope, while a weak response was seen in HLA B*4402* and HLA B*4403* virus carriers (11a). To examine this issue at the clonal level, we compared the frequencies of CTL precursors (CTLp) specific for the EENLLDFRVM epitope in HLA B*4402, B*4403, and B*4405 individuals by stimulating UM, LCLs, and recombinant vaccinia virus constructs encoding individual EBV antigens (12, 14, 15). The peptide specificity of each CTL clone was determined by using synthetic peptides (4, 18).

Generation of B*4402/B*4405 and B*4403/B*4405 chimeras. To generate B*4402/B*4404 and B*4403/B*4405 chimeric LCLs from donors CF (B*4402*), and BK (B*4403*) were fused with LCLs from donor CM (B*4405*), using polyethylene glycol (molecular weight, 2,000). These fused cells were cultured for 10 weeks. Each CTL microculture was split into two replicates, and the cells were expanded with 20 U of rIL-2 and 30% (vol/vol) supernatant from MLA-144 cultures. On day 10, each CTL microculture was split into two replicates, and the cells were used as effectors in a standard 5-h 3Cr release assay against autologous PHA blasts precoated with peptide EENLLDFRVM or left uncoated. Wells were scored as positive when the percent specific chromium release exceeded the mean release from control wells by 3 standard deviations. Limiting dilution analysis (LDA) was performed by the method of maximum likelihood estimation (7). From all experiments were compatible with the hypothesis of single-hit kinetics (P > 0.4), and precursor estimates are given with 95% confidence limits.

ID-IEF. To analyze intracellular transport of EBV B44 subtypes, 3 × 105 LCLs from donors CF (B*4402*), CS (B*4403*), and CM (B*4405*) were starved for 30 min in 1 ml of methionine/cysteine-free RPMI 1640 supplemented with 10% FCS. The cells were pelleted and resuspended in 200 μl of fresh methionine-cysteine-free medium and labeled with 150 μCi of [35S]methionine-cysteine for 10 min at 37°C. Cells were chased for 0, 5, 10, 30, 60, and 120 min in RPMI 1640 supplemented with 1 mM methionine-cysteine and 10% FCS and lysed in 1% digitonin-containing lysis mixture for 2 h on ice. After overnight preclearing with normal rabbit serum, the lysates were immunoprecipitated with W6/32 and the complexes were recovered with protein A-Sepharose. All samples were analyzed by one-dimensional isoelectric focusing (ID-IEF) (21).
consistently seen in multiple blood samples from these donors over a period of 5 years.

HLA B*4405 is more efficient in endogenous and exogenous presentation of the EENLLDVFRM epitope than HLA B*4402 and B*4403. To determine whether the disparity in CTLp frequency could be due to a difference in the presentation of the EENLLDVFRM epitope by different subtypes of HLA B44, we first compared the lysis of HLA B44+ LCLs by CTL...
clones from HLA B*4405 (DM D10 and CM13) and B*4403 (CS27) donors at different effector-to-target (E/T) ratios. The data shown in Fig. 2a and b clearly demonstrate that B*4405 LCLs are more efficiently recognized than B*4402 and B*4403 LCLs. Surprisingly, a CTL clone from a B*4403 donor (CS27) also recognized B*4405 LCLs more efficiently than other HLA B44 LCLs (Fig. 2b). This disparity in the level of lysis was not due to a difference in the expression of EBNA6 gene in these cells, since comparable levels of EBNA6 were detected when analyzed by immunoblotting (data not shown). Moreover, a similar contrast differential in CTL recognition was also seen with another CTL clone (CM13) from a B*4405 donor CM (Fig. 2c). Interestingly, although overexpression of EBNA6 in B*4402 and B*4403 LCLs, following infection with a recombinant vaccinia virus encoding this antigen (Vacc.EBNA6), increased the level of CTL recognition by the CM13 clone, the differential pattern of lysis was maintained (Fig. 2c).

In the next set of experiments, we examined the exogenous presentation efficiency of peptide EENLLDVFRM by the different subtypes of HLA B44. PHA blasts from donors DM (B*4405), CS (B*4403), and CF (B*4402) were presensitized with various concentrations of peptide EENLLDVFRM and then exposed to specific CTL clones or polyclonal CTLs. As illustrated in Fig. 3a to c, CTL clones from B*4405 (DM D10), B*4403 (CS27), and B*4402 (CF G12) donors recognized peptide-coated B*4405+ PHA blasts more efficiently than either B*4402+ or B*4403+ target cells. The concentration of peptide EENLLDVFRM required for half-maximal lysis of B*4405+ targets was approximately 0.003 μg/ml, while for B*4402 and B*4403 this concentration was 100-fold higher (0.1 to 0.3 μg/ml). To confirm that this difference in half-maximal lysis was not due to restricted recognition of these specific CTL clones, we repeated this analysis using an EENLLDVFRM-specific polyclonal CTL line from donor DM. As shown in Fig. 3d, the level of CTL recognition of peptide-sensitized B*4405+ PHA blasts was again significantly more efficient compared to B*4402+ and B*4403+ PHA blasts.

As a more stringent test to appraise the endogenous presentation efficiency of the HLA B*4405 subtype, B*4402+/B*4405+ and B*4403+/B*4405+ chimeric LCLs were generated, and the level of CTL lysis of these chimeric targets was compared to that of the B*4402+, B*4403+, and B*4405+ donors were exposed to EENLLDVFRM-specific CTL clones (DM D10, CS27, and CM13) at different E/T ratios (a and b) or at a fixed E/T ratio of 4:1 (c). In some experiments, B44+ LCLs were infected with recombinant vaccinia virus encoding EBNA6 antigen (which includes EENLLDVFRM epitope) and then used as targets in a standard 51Cr release assay (c).

TABLE 1. Frequency of EENLLDVFRM-specific CTL clones from HLA B44+ individuals

<table>
<thead>
<tr>
<th>Donor</th>
<th>B44 subtype</th>
<th>Expn</th>
<th>No. of EBV-specific CTL clones</th>
<th>Total</th>
<th>EENLLDVFRM specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>B*4405</td>
<td>1</td>
<td>23</td>
<td>20</td>
<td>(86.69%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>19</td>
<td>17</td>
<td>(89.47%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>29</td>
<td>24</td>
<td>(82.75%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>27</td>
<td>24</td>
<td>(88.88%)</td>
</tr>
<tr>
<td>CS</td>
<td>B*4403</td>
<td>1</td>
<td>34</td>
<td>34</td>
<td>(100.00%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>24</td>
<td>24</td>
<td>(100.00%)</td>
</tr>
<tr>
<td>SE</td>
<td>B*4403</td>
<td>1</td>
<td>30</td>
<td>30</td>
<td>(100.00%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>22</td>
<td>22</td>
<td>(100.00%)</td>
</tr>
<tr>
<td>IT</td>
<td>B*4402</td>
<td>1</td>
<td>30</td>
<td>30</td>
<td>(100.00%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>27</td>
<td>27</td>
<td>(100.00%)</td>
</tr>
<tr>
<td>DY</td>
<td>B*4402</td>
<td>1</td>
<td>28</td>
<td>28</td>
<td>(100.00%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>22</td>
<td>22</td>
<td>(100.00%)</td>
</tr>
<tr>
<td>KE</td>
<td>B*4402</td>
<td>1</td>
<td>22</td>
<td>22</td>
<td>(100.00%)</td>
</tr>
</tbody>
</table>

* Each of the experiment data presented above refers to a separate blood sample collected from these donors between 1990 and 1995.

**EBV-specific CTL clones from HLA B44+ donors were isolated as described in Materials and Methods. The EBV specificity of these CTL clones was confirmed as described previously (12).

Percentage of EBV-specific CTL clones specific for the EENLLDVFRM epitope. EENLLDVFRM specificity of the clone was confirmed as described elsewhere (4, 18).
HLA B44 subtypes display significant variation in intracellular maturation and transport kinetics. Earlier studies have revealed that peptide loading of MHC molecules contributes directly to their intracellular transport and that many of the HLA class I alleles differ in efficiency of assembly (23, 27, 31). To explore the possibility that variation in CTL recognition of peptide bound to the various HLA B44 subtypes was also affected by the difference in the rate of maturation and intracellular transport of these subtypes, HLA B44 LCLs were studied by pulse-chase labeling and analyzed on 1D-IEF gels as described in Materials and Methods. Marked differences in the kinetics of assembly were noted between the different HLA class I alleles. Three representative gels showing pulse-chase analysis in HLA B*4405, B*4402, and B*4403 cell lines are illustrated in Fig. 5. One way of visualizing intracellular transport of newly assembled MHC class I molecules is by following the time-dependent disappearance of the nonsialylated form of class I heavy chains (indicating transport out of the ER lumen), which correlates with the appearance of the sialylated form upon arrival in the trans-Golgi. In Fig. 5, it is clearly evident that the sialylated forms (labeled S) of the B*4405 allele can be seen at the 0-min chase, whereas the sialylated forms of B*4402 and B*4403 do not appear until after 5- and 30-min chases, respectively. Moreover, an analysis of unsialylated forms (labeled O) of the B44 alleles indicates a significant reduction in the 35S-labeled B*4405 allele after 10 min of chase, while the unsialylated B*4402 and B*4403 alleles disappear after 30 min of chase. Thus, the B*4405 allele is assembled and transported more efficiently than the B*4402 and B*4403 alleles.

DISCUSSION

Hierarchies among MHC class I-restricted CTL responses to intracellular pathogens have attracted considerable attention, since CTL epitopes which elicit strong responses might be preferential targets for immunotherapy and vaccine design. Very limited information is available on the factors responsible for these hierarchies in the CTL response. In the present study, we have investigated this issue in the context of a herpesvirus, EBV, that is known to establish a persistent infection in healthy individuals and is associated with a number of human malignancies. It is now well established that EBV-specific memory T cells are responsible for controlling the level of EBV-positive B lymphocytes and that the majority of these T
ments, we noted that the specific CTL clones recognized
ing different subtypes of HLA B44. In the first set of experi-
were specific for this epitope. To understand the molecular
complexes with protein A-Sepharose were analyzed by 1D-IEF as described in Materials and Methods.
1
cells recognize epitopes included within the latent antigens of
EBV (4, 13, 19). The CTL response to the HLA B44-restricted
epitope EENLLDVFRM from EBNA6 in healthy individuals
is a classic example of this phenomenon (3, 12). We have
shown that healthy seropositive individuals carrying different
subtypes of the HLA B44 antigen exhibit a discrete hierarchy
in their CTL responses to the EENLLDVFRM epitope. This
phenomenon appears to be strongly influenced by the peptide-
HLA affinity and/or intracellular maturation and transport
kinetics of MHC molecules.

The possibility that the host MHC genotype can influence
the strength of the CTL response to an individual CTL epitope
has previously been proposed based on preliminary experi-
ments with polyclonal CTL cultures from HLA B44+ EBV-
immune donors. In the present study, we have substantiated
this assumption by demonstrating that CTLp specific for
EENLLDVFRM are seen more frequently in B*4405 indi-
viduals than in B*4402+ virus carriers. Furthermore, a similar pattern of response was also seen in the number
of EENLLDVFRM-specific CTL clones isolated from B*4402+, B*4403+, and B*4405+ donors over a period of 5
years. More than 60 to 80% of the clones isolated from
B*4405+ individuals were EENLLDVFRM specific, while 0 to
5% of the CTL clones from B*4402+ and B*4403+ donors
were specific for this epitope. To understand the molecular
basis of this hierarchy, we analyzed the endogenous and exog-
aneous presentation of EENLLDVFRM by target cells express-
ning different subtypes of HLA B44. In the first set of exper-
iments, we noted that the specific CTL clones recognized
B*4405+ LCLs more efficiently than B*4402+ or B*4403+ EBV-infected cells. This phenomenon was seen not only with
CTL clones from B*4405+ individuals; surprisingly, CTLs spe-
cific for this epitope from a B*4403+ donor also preferentially
recognized B*4405+ virus-infected cells. Similarly B*4405+ PHA blasts, exogenously sensitized with EENLLDVFRM pep-
tide, were consistently recognized more efficiently than
B*4402+ or B*4403+ targets by all CTL clones irrespective
of the HLA B44 subtype expressed by the donors from whom
these effectors were isolated. Thus, it is unlikely that the dif-
ference in T-cell repertoire in these individuals is responsible for
a strong or a weak response to EENLLDVFRM. On the other
hand, the disparate CTL recognition of the EENLLDVFRM
peptide in association with the different subtypes of HLA B44
likely reflects the relative abundance of corresponding class I-
peptide complexes on target cells, and this may be influenced by
two distinct factors. First, it is possible that the three subtypes
of HLA B44 have different affinities for this peptide. Indeed, the
data shown in Fig. 3 strongly argue in favor of this notion. Com-
parison of the amino acid sequences of the three different sub-
types of HLA B44 shows that they differ by a single amino acid at
position 116 or 156 (9, 35). It is possible that the difference
between B*4405 and the other subtypes at position 116, which
forms part of pocket F of the peptide binding cleft for MHC,
influences the binding of EENLLDVFRM. Indeed, the tyrosine
residue at position 116 of the B*4405 subtype is likely to interact
more favorably with the methionine residue at the carboxy ter-
minus of the peptide, while aspartic acid, a negatively charged
residue, at the same position in B*4402 and B*4403 might reduce
the efficiency of binding of the EENLLDVFRM peptide.

It is possible that, in addition to differences in peptide bind-
ing efficiencies, the differential rates of intracellular assembly
and transport of HLA B44 subtypes also contribute to the
disparate efficiency in the presentation of the EENLLDVFRM
epitope. Although earlier studies have shown that class I mol-
ecules display marked differences in the efficiency of assembly
and transport (32), the biological significance of this observa-
tion is not understood. Data presented in this study argue in
favor of a model in which efficient peptide binding and rapid
intracellular assembly and transport kinetics by the MHC al-
leles contribute to a strong CTL response. It is important to
mention here that since a single peptide is unlikely to induce an
effect on the overall rate of transport of an individual class I allele,
the specific rate in transport of each HLA B44 subtype is probably an intrinsic property of that allele. Thus, both
efficient peptide binding and rapid transport kinetics have a
synergistic effect on the presentation of the EENLLDVFRM
epitope. Preliminary studies carried out in our laboratory have
shown a similar strong correlation between other rapid-assembly
alleles and immunodominant responses. HLA B8 and HLA
B35 alleles, which are rapidly assembled and transported (32),
consistently display strong responses, while weaker responses
are seen for slowly assembling alleles such as HLA A3 and
HLA B51 (unpublished observations).

Taken together, the data presented in this study strongly
suggest that class I alleles that efficiently bind peptides in the
ER and are rapidly assembled and transported to the cell
surface for presentation to CTL may provide a selective ad-
vantage in their ability to present peptide epitopes and confer
a protective advantage against EBV infection. More impor-
tantly, these observations have significant implications for any
future EBV vaccine designed to control acute or persistent
viral infection. It is clear from the data presented here that
HLA subtype distribution within different individuals might
influence the long-term efficacy of CTL epitope-based vaccines.
ACKNOWLEDGMENTS

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