The B Subunit of Escherichia coli Heat-Labile Enterotoxin Enhances CD8+ Cytotoxic-T-Lymphocyte Killing of Epstein-Barr Virus-Infected Cell Lines

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Epstein-Barr virus (EBV) is associated with a number of important human cancers, including nasopharyngeal carcinoma, gastric carcinoma, and Hodgkin's lymphoma. These tumors express a viral nuclear antigen, EBV nuclear antigen 1 (EBNA1), which cannot be presented to T cells in a major histocompatibility complex class I context, and the viral latent membrane proteins (LMPs). Although the LMPs are expressed in these tumors, no effective immune response is made. We report here that exposure to the cholera-like enterotoxin B subunit (EtxB) in EBV-infected lymphoblastoid cell lines (LCLs) enhances their susceptibility to killing by LMP-specific CD8+ cytotoxic T lymphocytes (CTLs) in a HLA class I-restricted manner. CTL killing of LCLs is dramatically increased through both transporter-associated protein-dependent and -independent epitopes after EtxB treatment. The use of mutant B subunits revealed that the enhanced susceptibility of LCLs to CTL killing is dependent on the B subunit's interaction with GM1, but not its signaling properties. These important findings could underpin the development of novel approaches to treating EBV-associated malignancies and may offer a general approach to increasing the presentation of other tumor and viral antigens.

CD8+ cytotoxic T lymphocytes (CTLs) that recognize HLA class I-restricted virus peptide epitopes play an important role in the control of Epstein-Barr virus (EBV) infection in vivo (49). This is demonstrated by the regression of EBV-positive posttransplant lymphoma after either reduction of immunosuppression (47, 58) or adoptive transfer of EBV-reactive T lymphocytes generated ex vivo (18). Although EBV-associated nasopharyngeal carcinoma (NPC) and Hodgkin's disease (HD) express a number of EBV latent antigens, they are unable to trigger an immune response that eliminates disease (44). It is likely that the escape of these tumors from immune control is due to the limited repertoire and properties of the latent gene products that are expressed, namely, EBV nuclear antigen 1 (EBNA1) and latent membrane protein 1 (LMP1) and LMP2. The presence of an internal glycine-alanine repeat within EBNA1 prevents it from entering the HLA class I antigen-processing pathway (32). Nevertheless, LMP1 and LMP2 are potential target antigens for virus-specific CTL-based immunotherapy of NPC and HD since CD8+ T lymphocytes reacting to both LMP1 and LMP2 HLA class I-restricted peptide epitopes have been identified in healthy seropositive donors (37). Moreover, the HLA class I antigen processing and presentation pathway in both NPC and HD appears to be largely intact, since major pathway elements, including proteasome components, transporter-associated protein (TAP), and HLA class I molecules are detectable in these tumor cells (26, 40). Indeed, LMP2-specific CTLs generated from HD patients were able to lyse malignant Reed-Sternberg cells found in HD in vitro (54). However, LMP-specific CTLs are detectable at low frequencies in the peripheral blood of NPC and HD patients and are absent from the tumor infiltrate (8, 28). This could be due to inefficient processing and presentation of LMP1 and LMP2 expressed within these tumor cells, leading to little or no recognition by circulating LMP-specific CTLs.

LMP2 colocalizes with LMP1 in the plasma membrane, and both are concentrated within detergent-resistant, glycosphingolipid-enriched domains in the plasma membrane known as lipid rafts (10, 20, 24). Lipid rafts are ubiquitous in all mammalian cells and are involved in the sorting of proteins destined for the cell surface after exit from the Golgi complex, as well as in the organization of signaling molecules on the cell surface (5, 45, 60).

The B subunits of cholera toxin (CtxB) and Escherichia coli heat-labile enterotoxin (EtxB) are potent systemic and mucosal adjuvants (61). They bind avidly to their ganglioside receptor GM1 present in lipid rafts, which then undergo rapid cross-aggregation and internalization (31). This property of EtxB has led to its use as a carrier molecule for the intracellular delivery of exogenous peptides or antigen. Fusion proteins consisting of EtxB and peptide sequences derived from ribonucleotide reductase and DNA polymerase of herpes simplex virus type 1 have been successfully delivered to their intended intracellular compartments and remained functionally intact (35, 36). Peptides corresponding to known major histocompatibility complex (MHC) class I-restricted epitopes within ovalbumin or influenza nucleoprotein, when conjugated to EtxB, were also efficiently delivered into the MHC class I antigen processing and presentation pathway in murine dendritic cells (11).

We postulated that EtxB might similarly act as a delivery molecule for raft-associated components, including LMP1 and LMP2. By triggering aggregation and internalization of raft-associated LMP1 and LMP2 after the binding of EtxB to GM1,
these antigens would traffic retrogradely to intracellular compartments, where they might be processed and presented in an efficient manner, leading to enhanced recognition and lysis by LMP-specific CTLs. We describe here a series of experiments that test this hypothesis.

MATERIALS AND METHODS

Blood donors and cell lines. LCLs were derived from healthy carriers of known HLA type and established by in vitro transformation of B cells with the B95.8 (type 1) virus isolate. EBV is an EBV-negative B-cell lymphoma cell line. These lines were maintained in complete RPMI medium supplemented with 10% fetal calf serum.

CtxB, EtxB, and mutants of EtxB. Recombinant CtxB and EtxB were expressed in a nontoxic marine vibrio, Vibrio sp. strain 60 and purified by using hydrophobic interaction and ion-exchange chromatography as reported earlier (1, 48). A non-GM1-binding mutant of EtxB, EtxB(G33D) containing a Gly→Asp substitution at position 33, and a mutant defective in EtxB-mediated signaling, EtxB(H57A) containing a His→Ala substitution at position 57 have been described and were expressed and purified as described above (13, 42). Purified preparations of CtxB, CtXB, EtxB(G33D), and EtxB(H57A) were depleted of lipopolysaccharide by using Detoxi-Gel columns (Pierce, Rockford, Ill.) and contained ≤5% endotoxin units per mg of protein, as determined by a Limulus amebocyte lysate assay (BioWittaker, Walkersville, Md.). The proteins were dialyzed against phosphate-buffered saline and stored frozen at −80°C until used.

Localization of LMP1 and EtxB on labeled cells was analyzed by using a scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) under a 63×1.40 NA immersion objective lens. In experiments in which EBNA1 was detected, 5 μg of anti-EBNA1 monoclonal antibody (DP15; Oncogene, Cambridge, Mass.)/ml was used.

Expression levels of HLA class I, CD95, and CD40. LCLs were treated for 4 h at 37°C with 10 μg of EBV DNA primer incubation with either culture supernatant containing monoclonal antibodies to HLA class I (1:10; Wb/32) or mouse anti-CD95 (1:50; ImmunoRecontact, West Grove, Pa.). Localization of LMP1 and EtxB on labeled cells was analyzed by using a scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) under a ×63 oil immersion objective lens. In experiments in which EBNA1 was detected, 5 μg of mouse anti-EBNA1 monoclonal antibody (DP15; Oncogene, Cambridge, Mass.)/ml was used in place of CS1 to CS4.

Generation of polyclonal CD8+ CTL lines. Peptide-specific polyclonal CD8+ CTL lines were generated by a modification of a protocol described elsewhere (3). CD8+ T lymphocytes were selected magnetically (MACS; Miltenyi Biotec, Bergisch, Germany) from peripheral blood mononuclear cells of healthy seronegative donors. The remaining cells were pulsed with a 50 μM concentration of a known peptide for 1 h at 37°C, washed, and pooled with the CD8+ T lymphocytes before being seeded at 105 cells/ml in RPMI 1640 medium supplemented with 10% autologous serum and 25 ng of interleukin-7 (IL-7; Sigma)/ml. Then, 10 μl of IL-2 (Sigma)/ml was added on day 3, and the cultures were fed twice weekly with growth medium containing IL-7 and IL-2. CD8+ T lymphocytes were again magnetically separated on day 12, with the remaining cells pulsed with 50 to 100 μM peptide for 1 h, and inactivated with 50 μg of mitomycin C/ml for another h at 37°C. Pulsed cells were then washed and added to the CD8+ T lymphocytes at a responder/stimulator ratio of 4:1. These lines were used in cytotoxicity assays from day 19.

Cytotoxicity assays. Autologous LCLs used as targets in these assays were incubated for 4 h or overnight with 10 μg of EtxB/ml or one of its mutants. Target cells were labeled with 70 to 100 μCi of 51Cr for 90 min at 37°C. Some targets were pulsed with 5 μM peptide in the final 1 h. Target cells were then used in a standard 5-h chromium release assay. To inhibit HLA class I antigen presentation, labeled targets were incubated with Wb/32 culture supernatant (1:10) for 1 h at room temperature (RT) before addition of the effector cells. Control targets were treated with culture supernatant A (200 μg/ml) containing monoclonal antibodies to HLA class II (DA6.231). To demonstrate the proteasome or lysosome dependence, targets were pulsed with 100 μM lactacystin (Calbiochem, San Diego, Calif.) for 2 h, washed, and either incubated overnight or treated with 0.2 mM chloroquine (Calbiochem) for 4 h prior to 51Cr labeling.

Western blotting. LCLs were treated with 10 μg of EtxB/ml, harvested, and lysed in 50 mM Tris buffer (1% CA-630, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA) for 20 min on ice. Samples were centrifuged at 5,000 × g for 5 min, with the resultant supernatant mixed 1:1 with 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and heated at 70°C for 5 min. Protein separation was done by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% gel before transfer to a polyvinylidene difluoride membrane. The membrane was incubated with 1:50 rat anti-LMP2 antibody (1B7; F. Grasser, Abteilung Virologie, Universitatsklinikum, Homburg/Saar, Germany) overnight at 4°C. The blot was then incubated with horseradish peroxidase (1:2,000)-conjugated anti-rat immunoglobulin (Amersham, Piscataway, N.J.) for 1 h at RT, placed in a Luminol-based chemiluminescent medium, and exposed to X-ray film. The same blot was stripped and reprobed for LMP1 and tubulin by using mouse anti-LMP1 antibodies CS1 to CS4 (1:50) overnight at 4°C or mouse anti-tubulin (1:5,000; Sigma) for 1 h at RT, respectively. In both cases, horseradish peroxidase-conjugated anti-mouse immunoglobulin (1:10,000; Sigma) was used for 1 h at RT.

RESULTS

EtxB colocalizes with LMP1 on the cell surface and within intracellular compartments of LCLs. Although the presence of GM1 and LMPs within lipid rafts has been previously reported (20, 56), there is some evidence that not all raft-associated components are randomly distributed in all rafts and that specialized rafts concentrate subsets of raft proteins (38, 51). We assessed the distribution of LMP1 and EtxB on LCLs at 4 and 37°C by using confocal microscopy. Incubation of LCLs at 4°C did not interfere with the binding of EtxB but prevented the formation of large aggregates, patching, and internalization of lipid rafts. EtxB was distributed in a punctate fashion over the cell surface and colocalized with LMP1 (Fig. 1A). Given the degree of colocalization observed and that GM1 is the principle receptor for EtxB, it is highly likely that GM1 and LMP1 are found within the same domains on the surface of LCLs. After incubation at 37°C, both EtxB and LMP1 were redistributed by cocapping to one pole of the cell. Furthermore, the majority were also observed to have subsequently accumulated in the cytoplasm or other extranuclear compartments. In contrast, no redistribution of EBNA1 was seen after binding of EtxB (Fig. 1B), and no colocalization with internalized EtxB was observed. These findings suggest that the coaggregation and cointernalization of EtxB and LMPs may have occurred. As a result, binding by EtxB to EBV-infected B cells may be able to influence the endocytic pathways through which LMP1 is processed.

EtxB-induced susceptibility to CTL killing. If EtxB mediates or at least accelerates the internalization of LMP1 and LMP2, these antigens could be exposed to one or more of the proteolytic systems present within the cell, resulting in antigen processing and increased presentation of LMP-derived HLA class I-restricted peptide epitopes. To test this hypothesis, peptide-specific polyclonal CD8+ CTL lines from three seropositive adults were generated and used in cytotoxicity assays (Table 1). CTL lines specific for the HLA class I-restricted LMP2 epitope CLG, TYG, and IED were used against EtxB-treated autologous LCL targets (Fig. 2A to C). Untreated LCLs were not recognized and lysed by the CTL lines used despite expressing the full spectrum of EBV latent antigens. This phenomenon has been reported elsewhere, especially with EBV-specific CTLs generated by using autologous LCLs as antigen-
presenting cells (21, 53). One explanation offered was that the level of peptide epitopes produced after processing of these EBV latent antigens in LCLs is insufficient to trigger CTL recognition. In contrast, there was significant killing of EtxB-treated LCLs by all of the CTLs, amounting to approximately half the level seen against targets pulsed with peptides corresponding to the specificity of the CTL used. However, CTL recognition and lysis of LMP2 peptide-pulsed LCL targets was not further enhanced after EtxB treatment (data not shown). A likely explanation is that the concentration of peptide used was sufficient to saturate available peptide binding sites on MHC class I molecules and results in maximal CTL killing. Likewise, a CTL line specific for the HLA-A2.01-restricted LMP1 epitope, YLL, was also able to recognize and kill EtxB-treated but not control LCLs (Fig. 2D). Enhanced susceptibility to CTL killing of EtxB-treated target cells was evident after 4 h and was maintained at a similar level even after an overnight incubation (Fig. 2A and D). The release of $^{51}$Cr from LCLs was not due to EtxB-induced cell death and lysis, since the viability of LCLs after EtxB treatment for up to 72 h was

![Confocal immunofluorescence analysis showing colocalization and internalization of EtxB and LMP1. LCLs were incubated with 10 μg of EtxB/ml for 4 h at either 4 or 37°C. (A) EtxB is shown in red, with LMP1 labeled green. The merged image demonstrates an overlap of staining (yellow). (B) EtxB is red, whereas the EBV nuclear antigen, EBNA1, is green.](image)

**FIG. 1.** Treatment of target LCLs with EtxB renders them more susceptible to killing by LMP-specific CTLs. The polyclonal, peptide-specific CD8$^+$ CTLs listed in Table 1 were used in the experiments shown here. Cytotoxicity assays with CTLs specific for LMP2 epitopes CLG (A), TYG (B), and IED (C) against autologous LCL targets. (D) The same experiment as for panels A to C but with CTLs specific for the LMP1 epitope YLL. (E) Cytotoxicity assay with CTLs specific for the EBNA3A epitope RYS. Symbols: ○, percent specific lysis of untreated LCLs; ◊, percent specific lysis of LCLs pulsed with 5 μM peptide specific for the CTL used; □, percent specific lysis of LCL treated for 4 h with 10 μg of EtxB/ml; △, percent specific lysis of LCLs treated overnight with 10 μg of EtxB/ml. Effector/target ratios of 10:1, 5:1, and 2.5:1 were used for all of the experiments shown in this figure, and each line represents the mean of three separate experiments ± the standard error of the mean (SEM).

**TABLE 1.** CD8$^+$ CTL lines specific for LMP1 and LMP2 peptide epitopes

<table>
<thead>
<tr>
<th>Donor</th>
<th>HLA type(s)</th>
<th>Epitope</th>
<th>Antigen</th>
<th>Peptide (amino acid numbers, sequence)</th>
<th>HLA restriction type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A2.01, A23, B7, B49, C7</td>
<td>YLL</td>
<td>LMP1</td>
<td>125–133, YLLEMLWRL</td>
<td>A2.01</td>
<td>25</td>
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<td></td>
<td></td>
<td>CLG</td>
<td>LMP2</td>
<td>426–434, CLGGLLTMV</td>
<td>A2.01</td>
<td>29</td>
</tr>
<tr>
<td>2</td>
<td>A1, A24, B7, B57, C6, C7</td>
<td>TYG</td>
<td>LMP2</td>
<td>419–427, TYGPVFMLCL</td>
<td>A24</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RYS</td>
<td>EBNA3A</td>
<td>246–253, RYSFFDY</td>
<td>A24</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
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<td>IED</td>
<td>LMP2</td>
<td>200–208, IEDPPFNSL</td>
<td>B60</td>
<td>30</td>
</tr>
</tbody>
</table>
similar to that of untreated cells (data not shown). Importantly, a CTL line specific for the HLA-A24-restricted epitope, RYS, within an EBV nuclear antigen was highly effective at killing peptide-pulsed target cells but showed no increased killing of target LCLs treated with EtxB (Fig. 2E). These data demonstrate that EtxB treatment may not be exerting a generalized effect on the susceptibility of LCLs to killing by CTLs; instead, the EtxB-mediated abrogation of resistance to killing by EBV-specific CTL is, perhaps, mechanistically linked to EBV membrane-associated antigens found within lipid rafts.

**Action of EtxB on LMP1 and LMP2 is HLA class I restricted and proteasome dependent.** Blocking antibody against HLA class I was used to confirm that the CTL killing after EtxB treatment is mediated through interaction between the T-cell receptor and the peptide-HLA class I complex. The lysis of both EtxB-treated and peptide-pulsed targets was clearly inhibited when these cells were preincubated with blocking pan-HLA class I antibodies prior to exposure to CTLs but remained unaffected when anti-HLA class II antibodies were used (Fig. 3A and B). This demonstrates that EtxB treatment of LCLs leads to the enhanced presentation of the EBV-derived epitopes in association with HLA class I molecules on the cell surface of target cells.

Endocytosed proteins are generally degraded within lysosomes (16), whereas most HLA class I-restricted peptide epitopes are produced after proteasomal processing of cytoplasmic antigens, especially by a subset known as immunoproteasomes (50). It has also been reported that the production of both TAP-independent and TAP-dependent LMP2 epitopes require proteasomal processing (27). Hence, we sought to determine whether one or both of these proteolytic organelles are responsible for the production of TAP-independent and TAP-dependent epitope peptides from endocytosed raft-associated LMPs. EtxB-treated LCLs were treated with lactacystin, a proteasome inhibitor, or chloroquine, which prevents acidification of lysosomes and the activation of lysosomal proteases. Neither inhibitor prevented CTL-mediated killing of LCLs pulsed with an exogenous TAP-independent epitope, TYG, or a TAP-dependent epitope, IED, since additional intracellular processing was not required (Fig. 3C and D). In contrast, lactacystin but not chloroquine abrogated the recognition and lysis of EtxB-treated targets for both TAP-independent and TAP-dependent epitopes. Hence, the increased susceptibility of EtxB-treated LCLs to CTL killing through both TAP-independent and TAP-dependent LMP2 epitopes is dependent on proteasomal processing but not enzymatic degradation within lysosomes.

Given the wide-ranging effects that EtxB has on the various cell types in the immune system (61), it might be possible that the effects of EtxB seen could be due to increased surface HLA class I expression, upregulation of other mediators of cell-cell killing such as CD95-CD95 ligand (Fas-Fas ligand) interac-

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**FIG. 3.** Killing of EtxB-treated LCLs by LMP1 and LMP2 is HLA class I restricted and proteasome dependent. (A) Cytotoxicity assay with the LMP2 epitope CLG-specific CTLs against LCL targets blocked by antibodies to HLA class I (W6/32) but not by antibodies to HLA class II (DA6.231). (B) The same experiment as in panel A but with CTLs specific for the LMP1 epitope YLL. (C) Cytotoxicity assay with CTLs specific for the TAP-dependent LMP2 epitope TYG in which LCL targets were treated with the proteasome inhibitor lactacystin or the lysosome inhibitor chloroquine. (D) Repeat of the experiment in panel C but with CTLs against the TAP-independent LMP2 epitope IED. Bars: □, untreated LCLs; ■, LCLs incubated with 10 μg of EtxB/ml for 4 h; □, LCLs pulsed with 5 μM peptide specific for the CTLs used. For all of the experiments shown in this figure, the effector/target ratio used was 10:1. Each bar represents the mean of three separate experiments ± the SEM.
tions, or a generalized activation of B cells. However, the levels of HLA class I, CD95, and CD40, as well as LMP1 and LMP2, remain similar before and after EtxB exposure. (A) Levels of HLA class I, CD95, and CD40 were measured by flow cytometry of untreated LCLs (continuous lines) and compared to cells treated with EtxB for 4 h (broken lines). (B) Western blots with antibodies to LMP1 and LMP2 showing the levels of both LMP1 and LMP2 expression at various times after exposure to EtxB. α-Tubulin was used as a marker protein to show equal protein loading between lanes.

FIG. 4. Levels of HLA class I, CD95, and CD40, as well as LMP1 and LMP2, remain similar before and after EtxB exposure. (A) Levels of HLA class I, CD95, and CD40 were measured by flow cytometry of untreated LCLs (continuous lines) and compared to cells treated with EtxB for 4 h (broken lines). (B) Western blots with antibodies to LMP1 and LMP2 showing the levels of both LMP1 and LMP2 expression at various times after exposure to EtxB. α-Tubulin was used as a marker protein to show equal protein loading between lanes.

Effect of EtxB requires GM1 binding but not EtxB-mediated signal transduction. Some of the immunomodulatory effects of EtxB include activation of B cells (41), alteration of CD4+ T-cell differentiation (42), modulation of cytokine production in monocytes (59), and induction of apoptosis of CD8+ T lymphocytes via a c-myc- and NF-κB-dependent pathway (52, 55). The effect of EtxB on the susceptibility of EBV-infected target cells to CTLs could rely on its ability to bind to GM1, to activate signaling pathways, or both. Two mutant derivatives of EtxB were used to discriminate between these possibilities: EtxB(G33D), which does not bind to GM1 (42), and EtxB(H57A), which binds to GM1 and undergoes retrograde vesicular trafficking to the Golgi compartment but lacks the ability to trigger the signaling events associated with the wild-type molecule (13). LCLs treated with EtxB(G33D) failed to increase their susceptibility to either LMP2- or LMP1-specific CTL lines, whereas treatment with EtxB(H57A) was as effective as wild-type EtxB at triggering susceptibility to CTL killing (Fig. 5A and B). We conclude that the GM1 binding by EtxB but not its known signaling properties is essential for enhancing the presentation of LMPs. The importance for GM1 binding is further supported by the observation that CtxB, which is a close homologue of EtxB but binds with greater specificity to GM1 (14, 22), also enhances the susceptibility of LCLs to LMP-specific CTLs (Fig. 5C).

FIG. 5. Modification of the LMP epitope presentation pathway by EtxB is dependent on the GM1 binding, but not the signaling capacity, of EtxB. (A) LMP2 epitope CLG-specific LMP1 CTLs were used in cytotoxicity assays after treatment of autologous LCL targets for 4 h in the absence of any additions (LCL); in the presence of 10 μg of EtxB, EtxB(G33D), or EtxB(H57A)/ml; or with 5 μM peptide specific for the CTLs used. (B) The same experiment as in panel A but with LMP1 epitope YLL-specific CTLs. (C) Cytotoxicity assay of LMP2 epitope TYG-specific CTL on autologous LCL targets treated with the GM1-binding B subunits of CtxB (○) or EtxB (●). Untreated controls (□) and peptide-pulsed controls by (□) are indicated. All figures are the mean of three separate experiments ± the SEM. An effector/target ratio of 10:1 was used in all cases.

DISCUSSION
EtxB has been shown to undergo rapid internalization upon pentavalent binding to GM1. After internalization, EtxB is believed to traffic via a vesicular pathway in a retrograde manner to reach the trans-Golgi network, Golgi apparatus, and the endoplasmic reticulum (39). CtxB taken up via this route is transported in transferrin-negative endosomes, implying that this might represent an alternative, clathrin-independent endocytic pathway (43). LMP1 has previously been found to colocalize with GM1-bound CtxB on the surfaces of EBV-infected B cells (24), and this finding is consistent with the results with EtxB reported here. In addition, we demonstrate that internalized EtxB can be found within the same cytoplasmic or intracellular compartments as LMP1. We hypothesize that the internalization of EtxB after GM1 binding might result in the “capture” and cotransportation of raft-associated anti-
It could be that the EtxB effect does not occur with EBNA3A because the levels of this antigen are much lower. However, the EBNA3 family is the immunodominant group of EBV antigens in human CTL responses to EBV, and it has been suggested that their relatively high representation as MHC class I-peptide complexes contributes to their immunodominance (34). This is in marked contrast to LMP1, which is a relatively abundant EBV protein but is a very poor CTL target (25). EBNA1 is expressed in all EBV-infected cells with the possible exception of the latency 0 phenotype of circulating memory B cells, yet it is not a CTL target because it cannot access the proteasome degradation pathway (25, 25). We have also found that a fivefold upregulation of LMP2 expression in LCLs containing inducible LMP2 expression vectors does not enhance susceptibility to CTL killing compared to LCLs with normal or uninduced levels of LMP2 (G. Patsos and A. Morgan, unpublished data). Collectively, these findings imply that the availability of EBV latent antigens as CTL targets cannot be explained on the basis of the expression levels of these antigens. It is more likely that access to proteasome degradation pathways is more important (49). Indeed, here we have shown that the EtxB-mediated enhancement of susceptibility to specific CTLs is proteasome dependent.

LMP1 is degraded by a ubiquitin-proteasome pathway (2), whereas LMP2 also possesses amino-terminal PY motifs that interact with the Nedd4 family of ubiquitin ligases and hence is a potential substrate antigen for proteasomal degradation (23). However, in the absence of EtxB treatment, peptide epitopes are not efficiently presented to CTLs. More specifically, the manner in which raft-associated LMPs located on the plasma membrane are processed remains unknown. Although the exact degradation pathway after EtxB treatment has yet to be defined, the simplest explanation remains that EtxB acts to accelerate proteasome-dependent degradation of raft-associated LMPs with concomitant increased production and presentation of peptide epitopes. However, we were unable to demonstrate a reduction of the total cellular LMPs after EtxB treatment (Fig. 4B), indicating that the overall rate of degradation has not increased. Rather, we believe that EtxB-induced endocytosis diverts raft-associated LMPs away from their normal degradation pathway. Internalized LMPs, either within these endosomes or released by as-yet-undefined mechanisms into the cytoplasm, could provide an additional source of substrate antigens for immunoproteasomes that are concentrated predominantly around the endoplasmic reticulum (4), favoring the generation of immunogenic peptide epitopes. A possible model for the action of EtxB on LMP1 and LMP2 processing and presentation is shown in Fig. 6.

Various toxoid derivatives conjugated to peptides or antigens have been used successfully in delivering exogenous peptides to the intracellular HLA class I pathway (12). The use of EtxB for such a purpose has also recently been described (11). In these cases, the exogenous peptides conjugated to the toxin derivative resulted in efficient intracellular delivery into the endogenous HLA class I pathway. We believe that the results shown here differ from these examples in three important aspects. First, the antigens in question are expressed endogenously rather than administered exogenously. Second, raft-associated LMP1 and LMP2 are functional, full-length viral antigens that require highly complex processing. This is in contrast to earlier studies wherein the antigens delivered were synthetic peptide epitopes that require little or no processing prior to entry into the HLA class I pathway, except when full-length human immunodeficiency virus proteins were used with derivatives of anthrax toxin (7, 17). Finally, fusion to EtxB was not required for efficient delivery. We postulate that the localization of LMPs within GM1-rich lipid rafts allows them to enter the same endocytic pathway as EtxB, thus avoiding the need for chemical coupling to EtxB.

For CTL therapy in EBV-associated malignancies such as NPC and HD to be effective, the failure to efficiently present EBV antigens expressed by tumor cells needs to be overcome. The use of EtxB by intranasal/oral administration could form the basis of a novel immunotherapeutic strategy by rendering EBV antigens expressed by tumor cells needs to be overcome. The use of EtxB by intranasal/oral administration could form the basis of a novel immunotherapeutic strategy by rendering EBV antigens expressed by tumor cells needs to be overcome.
from CTL killing could be used simultaneously alongside ex vivo CTL expansion protocols currently being developed (18). The alteration of raft-associated antigen processing by EtxB may also have broader therapeutic applications in other tumors or viral infections in which raft-associated candidate antigens can be identified.

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