Targeting Epstein–Barr virus nuclear antigen 1 (EBNA1) through the class II pathway restores immune recognition by EBNA1-specific cytotoxic T lymphocytes: evidence for HLA-DM-independent processing

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Abstract

Epstein–Barr virus (EBV) nuclear antigen 1 (EBNA1) is the only viral protein consistently expressed in all malignancies associated with EBV and there is now convincing evidence to suggest that EBNA1 is not recognized by MHC class I-restricted cytotoxic T lymphocytes (CTL). The lack of recognition of EBNA1 has been attributed to a cis-acting inhibitory effect of glycine–alanine repetitive (G-Ar) sequences on the endogenous processing of this antigen through the class I pathway. In the present study we have explored the possibility of targeting EBNA1 through an alternative mechanism using the MHC class II pathway. Using purified EBNA1 protein, we demonstrate here that CD4+ CTL can efficiently recognize EBV-transformed B cells and Burkitt’s lymphoma cells following exogenous sensitization with this antigen, and this immune recognition is not affected by the G-Ar domain within EBNA1. Analysis of the processing mechanism revealed that intracellular loading of class II molecules with an EBNA1 epitope occurs through an HLA-DM-independent pathway. These results highlight a novel mechanism for immune recognition of EBNA1 and also demonstrate that the G-Ar-mediated protection from processing can be overridden if this antigen is presented through the class II pathway.

Introduction

The question of whether or not Epstein–Barr virus (EBV) nuclear antigen 1 (EBNA1) is a target antigen for virus-specific cytotoxic T lymphocytes (CTL) has generated considerable scientific interest, primarily due to its important implications for the overall biology of EBV. Firstly, it has been proposed that the EBV-associated malignancies escape virus-specific immune surveillance by restricting latent gene expression to EBNA1 (1). This view is reinforced by earlier studies which showed that Burkitt’s lymphoma (BL) patients retain detectable EBV-specific T cell surveillance, indicating that CTL dysfunction is an unlikely cause of the outgrowth of these EBV-infected tumors in vivo (2). Secondly, an earlier study carried out by Trivedi and colleagues clearly showed that mammary carcinoma cells transfected with the EBNA1 gene were not rejected by histocompatible mice, whereas LMP-transfected cells were regularly rejected (3). It has recently been suggested that the poor immunogenicity of EBNA1 is due to glycine–alanine repeat (G-Ar) sequences within the EBNA1 protein which can generate a cis-acting inhibitory signal that interferes with antigen processing and presentation of MHC class I-restricted epitopes (4). Thus it is plausible that some virus-infected malignant cells can maintain a non-immuno-
genic phenotype by down-regulating the critical latent proteins needed for MHC class I-restricted CTL recognition.

On the other hand, there is no evidence to suggest that a similar effect might also be seen for EBNA1 epitopes processed through the class II pathway. In fact, we have recently shown that EBNA1 includes a sequence which can be presented by class II molecules and CTL specific for this epitope were isolated from a healthy seropositive donor (5). Interestingly, these CTL are unable to lyse EBV-infected B cells, suggesting that EBNA1 may not be endogenously processed and/or presented to the host CTL response. However, the presence of EBNA1-specific memory CTL in these EBV-seropositive individuals strongly suggests that this antigen might be processed through an alternative pathway and then presented on the cell surface in association with class II molecules. To explore this possibility we sensitized virus-infected cells with purified EBNA1 protein expressed either as a full-length protein or a truncated protein without the G-Ar sequence. Surprisingly, these target cells were efficiently recognized by the EBNA1-specific CTL and this immune recognition was not affected by the G-Ar sequence. Furthermore, analysis of the processing mechanism revealed that the EBNA1 epitope is processed through an HLA-DM-independent pathway. These results demonstrate for the first time that proteolytic enzymes within the class II processing compartment are capable of overriding the G-Ar-mediated protection of EBNA1 from immune recognition by virus-specific CD4+ CTL.

**Methods**

**Establishment and maintenance of cell lines**

EBV-transformed lymphoblastoid cell lines (LCL) were established from HLA-DR1-positive and EBV-seropositive donors by exogenous virus transformation of peripheral B cells using B95.8 or BL74 virus isolates. Three different BL cell lines (BL30, BL41 and BL54) derived by direct outgrowth from BL biopsies were used in this study (6). In addition, the B×T hybrid cell line 174×CEM.T2 (7) transfected with the HLA-DR1 (8), HLA-DR11 (T2.DR11), and both the HLA-DR11 and HLA-DM (T2.DR11/DM) genes (9), was also used in this study. All cell lines were routinely maintained in RPMI 1640 containing 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin plus 10% FCS (growth medium).

**CTL clone and peptide epitopes**

The MHC class II-restricted EBNA1-specific CTL clone DM2 was used in this study. The specificity of this clone has been defined at the peptide epitope level: DM2 recognizes the minimal epitope sequence TSLYNLRGTAL (ORF BKRF1 residues 458–470) (5). This clone was maintained in growth medium containing highly purified recombinant human IL-2 from *Escherichia coli* (10,11).

**Expression and purification of recombinant EBNA1 protein**

EBNA1 protein was either expressed as a full-length protein or as a truncated antigen with the G-Ar sequence deleted. Full-length EBNA1 was expressed using a baculovirus construct AcEBNA1 (a kind gift from Dr Janet Hearing) (12,13). As a control, a baculovirus expressing an irrelevant protein, a human G protein subunit (AcGs), was also used in this study. Full-length EBNA1 was expressed and purified over a heparin–agarose column as previously described with minor modifications. AcEBNA1- or AcGs-infected High Five (BTI-TN-5B1–4) cells were washed and lysed in 1 M NaCl, 1% NP-40, 10% glycerol, 1 mM MgCl₂, 20 mM HEPES, pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were clarified by centrifugation at 38,000 g, 4°C, 30 min and diluted with Buffer A (20 mM HEPES, pH 7.5, 0.5 mM EDTA, 2 mM dithiothreitol, 1 mM PMSF, 20% glycerol) to a NaCl concentration of 250 mM. The lysates were then loaded onto a 1.5 ml bed volume heparin–agarose (BioRad, Hercules, CA) column that had been washed with Buffer A and equilibrated with Buffer A containing 250 mM NaCl. The column was washed with Buffer A containing 333 mM NaCl and the protein was eluted with Buffer A containing 1 M NaCl collected in 1 ml fractions. Fractions containing baculoEBNA1 were determined by spot Bradford assay, pooled, diluted with 20 mM HEPES (pH 7.5) to lower the NaCl concentration to 200 mM and concentrated with a Centriprep 50 (Amicon, Beverly, MA). The corresponding fractions from the AcGs lysate were treated identically. Protein preparations were analyzed by the Bradford assay and 10% SDS–PAGE (Fig. 1A).

The truncated EBNA1 antigen (E1NX) was constructed by digesting pBS:E1, containing full-length EBNA1 (4), with Ncol (genomic position 108,067) and XcmI (position 109,200), repairing the ends and re-ligating the vector to itself, resulting in a 379 amino acid deletion encompassing the G-Ar domain and flanking sequences. The E1NX fragment was excised using *RsaI* and inserted into the *Smal* site of the pMal-c expression vector (NEB, Beverly, MA). This results in an in-frame fusion of maltose binding protein with EBNA1 starting at amino acid position 8, extending to amino acid position 40 and concluding with amino acids positions 420–641, the C-terminus of EBNA1. This results in a 638 amino acid fusion protein with a predicted mol. wt. of 68.3 kDa. pMal-E1NX or pMal control plasmids were electroporated into *Escherichia coli* BL 21 strain, and expression of appropriately sized proteins (69 kDa for pMal-E1NX and 45 kDa for Mal) upon IPTG induction of several clones was confirmed by Coomassie staining and immunoblotting of 10% SDS–PAGE. Cultures of 100–200 ml of Rich media plus ampicillin were inoculated with pMal-E1NX or pMal control BL 21 clones, allowed to grow to an OD₅₅₀ ~0.5 and induced with 0.3 mM IPTG for 3 h. The cells were then pelleted and resuspended in 10–20 ml of lysis buffer (1 mg/ml lysozyme, 50 mM Tris, 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1 mM PMSF, pH 8.0), incubated on ice for 15 min and lysed by a freeze–thaw followed by sonication. The lysate was clarified by centrifugation at 38,000 g, 4°C, for 30 min, diluted 1.5 with column buffer (10 mM Tris, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, pH 7.4) and loaded onto a column containing a 3 ml bed volume of amylose resin (NEB) that had been washed with 8 column volumes of column buffer. After binding, the column was washed with 8 column volumes of column buffer and the protein was eluted with ~10 ml of elution buffer (10 mM maltose, 150 mM NaCl, 20 mM HEPES, pH 7.4) collected in 1 ml fractions. Fractions containing protein, as determined by...
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Fig. 1. Expression and purification of recombinant EBNA1 protein. EBNA1 was either expressed as a full-length protein (AcEBNA1; A) or as a truncated antigen with the G-Ar sequence deleted (E1NX; B). AcEBNA1 was expressed using a baculovirus expression system and purified over a heparin–agarose column. As a control, a baculovirus expressing an irrelevant protein, a human G protein subunit (AcGs), was also used in this study. Protein preparations were analyzed by the Bradford assay and 10% SDS–PAGE (A). SDS–PAGE lanes in (A) labeled as C refer to control protein (AcGs), while E1 refers to EBNA1 protein. For truncated EBNA1 protein, a bacterial expression system was used. For details see Methods. Purified fractions containing protein, as determined by the Bradford protein assay, were pooled and analyzed using 10% SDS–PAGE (B).

Cytotoxicity assay
BL cells and EBV-transformed LCL were pre-sensitized with purified EBNA1 protein (AcEBNA1 or E1NX) or synthetic peptide epitope (TSLYNLRRGTALA) and then incubated with 51Cr for 90 min. Following incubation, these cells were washed in growth medium and used as targets in a standard 5 h 51Cr-release assay (14). mAb specific for the non-polymorphic determinants on MHC class I (W6/32) or class II (L243) antigens were added in the CTL assay to block CTL recognition. In some experiments, EBNA1-sensitized target cells were either pre-treated with chloroquine or Brefeldin A (BFA) to define the exact pathway used in the presentation of CTL epitopes (15).

To verify the endogenous presentation of the EBNA1 epitope, cells were fixed with 2% paraformaldehyde and then exposed to purified EBNA1 antigen or TSLYNLRRGTALA peptide. Following incubation, these target cells were mixed with DM2 CTL at an effector:target ratio of 5:1. After 20–24 h, culture supernatants were harvested and analyzed for IL-2 content by measuring the proliferation of the IL-2-dependent cell line CTLL-2 (16).

Analysis of HLA-DM gene expression in BL cells
To analyze HLA-DMB expression, BL cells and LCL were lysed by sonication in 10 mM Tris buffer, pH 7.0, and the protein concentration determined using a BioRad DC protein assay kit. Lysates were separated by SDS-PAGE, electroblotted onto nitrocellulose membrane and the DMB protein detected using a rabbit antiserum, R.DMB-C, prepared by immunizing rabbits with C-terminal peptide from HLA-DMB (9). DMA and/or DMB expression in BL cells and LCL were quantitated with a laser densitometer (Molecular Dynamics, Sunnyvale, CA) and analyzed with ImageQuant software.

Results
EBNA1-specific CTL efficiently recognize virus-infected cells following sensitization with purified EBNA1 protein
We have previously shown that EBNA1 includes sequences which can be presented by MHC class II molecules (5). CTL clones specific for one of these epitopes have been isolated and were shown to recognize an epitope, TSLYNLRRGTALA, in association with HLA-DR1. These CTL clones were unable to recognize EBV-infected B cells, suggesting that this EBNA1 epitope is not processed endogenously. To explore the possibility that this antigen might be processed through an alternative pathway and then presented on the cell surface in association with class II molecules, we sensitized virus-infected cells with purified EBNA1 protein expressed either as a full-length protein (AcEBNA1) or a truncated protein with the G-Ar sequences deleted (E1NX). The data presented in Fig. 2 clearly demonstrate that although EBV-infected, HLA-DR1-positive B cells were not recognized by the EBNA1-specific CTL clone DM2, exogenous sensitization of these cells with either the AcEBNA1 protein or the E1NX protein completely restored the immune recognition by these CTL. Target cells sensitized with either AcEBNA1 or E1NX showed similar levels of CTL lysis, suggesting that the G-Ar domain of EBNA1 does not inhibit class II processing of CTL epitopes. A significant reduction in the level of CTL lysis was seen
EBNA1-specific CTL efficiently recognize LCL following sensitization with purified EBNA1 protein. DM LCL were either sensitized with full-length EBNA1 protein (AcEBNA1) or a truncated antigen with the G-Ar sequence deleted (E1NX) and then exposed to the DM2 CTL clone at an effector:target ratio of 5:1. DM LCL sensitized with AcGs or pMal were used as controls for AcEBNA1 and E1NX respectively in the assay. To confirm whether the CTL recognition was restricted through an MHC class II antigen, EBNA1-sensitized target cells were pretreated with either HLA-DR-specific mAb (L243) or HLA-A,B,C-specific mAb (W6/32) and then exposed to the DM2 clone. Results are expressed as percent specific lysis.

Earlier studies have shown that conventional MHC class II-restricted antigen presentation requires assembly of the peptide epitopes with class II molecules in an acidified vacuolar compartment and is therefore sensitive to lysosomotropic agents that disrupt acidification of endosomes. We therefore tested whether the presentation of the EBNA1 epitope was dependent on this pathway by preventing acidification of the endosomal compartment with chloroquine treatment. Treatment of EBNA1-sensitized LCL with chloroquine significantly reduced the level of CTL lysis by clone DM2, whilst the CTL recognition was not affected by BFA (Fig. 3). Furthermore, pre-treatment of synthetic peptide-sensitized target cells with chloroquine had a minimal effect on this recognition (Fig. 3).

To exclude the possibility that the observed CTL recognition was due to EBNA1 peptides present in the protein stocks, DM/B95.8 LCL were initially fixed with 2% paraformaldehyde and then incubated with either purified EBNA1 protein or the TSLYNLRRGTLA peptide. Following incubation, these target cells were exposed to the DM2 clone for 20–24 h and the level of stimulation was analyzed by assaying IL-2 production by these T cells. Paraformaldehyde-fixed target cells fed with the EBNA1 antigen were unable to stimulate CTL and showed very low levels of IL-2 production, while target cells sensitized with peptide TSLYNLRRGTLA induced strong stimulation of DM2 cells (Fig. 4).

HLA-DM-negative cells efficiently process an EBNA1 epitope
It is now well established that peptide loading of MHC class II molecules is facilitated by a family of genes referred to as HLA-DM. DM is a transmembrane glycoprotein composed of an α (DMA) and a β (DMB) chain (17,18). Mutant cells carrying deletions within the DMA and/or DMB gene consistently express class II molecules complexed with a peptide from the invariant chain, which is designated as αβCLIP (19,20).

To further characterize the class II processing pathway for EBNA1 presentation, DM-positive (CM LCL) and DM-negative (T2.DR1) cells were either sensitized with EBNA1 protein or the TSLYNLRRGTLA peptide at a concentration of 1.0 µM and then exposed to the DM2 clone at a responder:stimulator ratio of 5:1. After 20–24 h, culture supernatants were harvested and analyzed for IL-2 content by measuring proliferation of the IL-2-dependent cell line CTLL-2.
EBNA1-sensitized BL cells are efficiently recognized by the CTL clone DM2

It is now well established that BL cells consistently display a defect in the presentation of class I-restricted CTL epitopes (21). This defect is reflected in the inability of EBV-specific CTL to recognize HLA-matched BL cells, and correlates with transcriptional down-regulation of TAP-1 and TAP-2 transporters and surface MHC allele expression. To explore the possibility that class II-restricted CTL epitopes can be processed by BL cells, we tested two different BL cell lines, positive for HLA-DR1, for the exogenous processing of TSLYNLRGTA. The data presented in Fig. 6 clearly demonstrate that not only were these cells recognized by clone DM2 in the presence of the peptide epitope, but they were also recognized with equal efficiency following exogenous sensitization with EBNA1 protein. Significant inhibition in the level of CTL lysis was seen in the presence of HLA-DR-specific mAb (Fig. 6) and following treatment of target cells with chloroquine (data not shown).

To further characterize the class II processing phenotype of BL cells, we analyzed the expression of HLA-DM in these tumor cells by immunoblotting. Data from one such analysis is illustrated in Fig. 7. Both BL41 and BL54 cell lines consistently showed normal expression of the DMB gene product, and levels were comparable to two different LCL and T2.DR11/DM cells used as positive controls in this analysis. On the other hand, DM-negative T2.DR11 cells showed no reactivity with the HLA-DMB-specific antibody.

**Discussion**

There is now convincing evidence that EBV-specific memory T cells are responsible for controlling the level of EBV-positive B lymphocytes which all healthy seropositive individuals carry for life following primary infection with the virus (22,23). Earlier studies have clearly shown that BL cell lines are not killed by HLA-matched EBV-specific CTL, thus raising the question of the role of this effector mechanism in the control of EBV-associated tumors (21,24). Several possible mechanisms have been advanced to explain this lack of recognition of BL cells by EBV-specific CTL. These include down-regulation of MHC class I processing genes (21), loss of HLA class I expression (25) and restricted viral gene expression to EBNA1 (1) by BL cells. Although previous attempts to identify potential MHC class I-restricted CTL epitopes within EBNA1 have been futile (26), recent studies from our laboratory have shown that EBNA1 includes a potential epitope for MHC class II-restricted presentation (5). Interestingly, CTL clones specific for this epitope were unable to recognize EBV-infected B cells, suggesting that EBNA1 may have evolved a mechanism to avoid endogenous processing and/or presentation through the class II pathway. The presence of memory CTL specific for this EBNA1 peptide epitope and their activation in vitro following LCL stimulation, however, suggests that EBNA1 protein from EBV-infected cells might be exogenously endocytosed by professional antigen-presenting cells and presented in associ-
ation with MHC class II molecules. In the present study we have explored this possibility to determine whether such a mechanism can prime virus-infected cells for immune recognition by EBNA1-specific CTL. Indeed, data presented in this study has clearly shown that LCL presensitized with purified EBNA1 protein can efficiently process the relevant CTL epitope and are recognized by specific CTL. It is unlikely that CTL recognition of these target cells is due to the EBNA1 peptides present in the protein stocks, since paraformaldehyde-fixed, EBNA1-sensitized LCL were unable to stimulate specific CTL. It is therefore likely that in situ degradation of the EBNA1 protein in the prelysosomal/lysosomal compartment generates peptides which are capable of binding freshly synthesized MHC class II molecules. Thus contrary to the inhibitory effect of internal G-Ar domain of EBNA1 on HLA class I processing pathway (4), efficient processing of full-length EBNA1 was seen through the MHC class II pathway. It therefore appears that the G-Ar domain does not protect EBNA1 from class II-restricted processing.

An unusual feature of the class II-restricted processing pathway utilized for the TSYLNLRRGTALA epitope was that it was apparently independent of the HLA-DM gene products. Earlier studies have shown that HLA-DM mutant cells, either with mutations (27,28) or deletions (19,29) of DM genes, in addition to being defective in the presentation of class II-restricted protein antigens, their class II molecules are conformationally altered and unstable suggesting that DM molecules play an important role in the loading of class II molecules. Efficient processing of EBNA1 protein by DM-negative cells suggests that under certain circumstances native antigen presentation can occur in the absence of DM molecules. It is possible that class II loading of EBNA1 may be occurring in a non-endosomal compartment (30) or through a recycling pathway, as described for an influenza hemagglutinin epitope (31). Alternatively, the leakiness of class II loading in DM-negative cells may result from poor binding of CLIP with HLA-DR1 molecules. Since CLIP prevents peptide loading and formation of stable class II molecules, efficient dissociation of the HLA-DR1–CLIP complex might allow DM-independent presentation of the EBNA1 epitope.

Another important aspect of the present study was to determine whether EBV-associated malignancies such as BL cells are also capable of processing EBNA1 epitopes through the class II pathway. Since previous studies from our laboratory and others have shown that BL cells consistently display defective class II processing function (21,25), an alternative strategy to target these tumor cells might involve MHC class II-restricted, EBV-specific T cells. Indeed, evidence from other tumor models has accumulated that CD4+ T cells play a critical role in immune surveillance against human cancers (32,33). To examine this possibility, we tested the class II-restricted processing function in HLA-DR1-positive BL cell lines using EBNA1 as a target antigen. This analysis revealed that EBNA1-specific CD4+ CTL can efficiently recognize tumor cells following sensitization with purified antigen. Interestingly, BL cells also showed normal levels of the HLA-DMB gene product, which is an important component for the processing of most class II-restricted epitopes. It is important to mention here that recent studies from our laboratory have also shown normal class II processing function in BL cells for a CTL epitope derived from another latent antigen of EBV (34). Since the importance of CD4+ T cells in the priming and effector phases of the anti-tumor immune response has been shown in animal models (35–37), the results of the present study raises a possibility of immune targeting class I processing defective EBV-associated malignancies (such as BL) using class II-restricted epitopes from EBV.

Taken together, the data presented herein demonstrate a novel mechanism for immune recognition of EBNA1 which is not affected by the G-Ar domain within this antigen. In future studies it will be important to delineate the precise mechanism of proteolytic processing of EBNA1 through the class II pathway which allows it to override the G-Ar-mediated protection seen for class I-restricted processing. Such information could be exploited to induce the expression of the relevant proteases in virus-infected malignant cells to allow endogenous processing of EBNA1 CTL epitopes.

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Abbreviations
BFA Brefeldin A
BL Burkitt’s lymphoma
CTL cytotoxic T lymphocyte
EBNA1 Epstein–Barr virus nuclear antigen 1
EBV Epstein–Barr virus
G-Ar glycine–arginine repeat
LCL lymphoblastoid cell line
PMSF phenylmethylsulfonyl fluoride

References


