Brief Definitive Report

Murine Cytotoxic T Lymphocytes Recognize an Epitope in an EBNA-1 Fragment, but Fail to Lyse EBNA-1-expressing Mouse Cells

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Summary

Major histocompatibility complex class I–restricted cytotoxic T lymphocytes (CTLs) specific for epitopes within eight of the nine Epstein Barr Virus (EBV)-encoded latency-associated proteins have been recovered from EBV-infected human subjects by restimulation of lymphocytes in vitro. However, human class I–restricted CTL responses capable of recognizing EBNA-1 expressing cells were not detected in these studies. We have raised a murine CTL line that recognizes an epitope within EBNA-1 by immunizing mice with a vaccinia virus encoding a COOH-terminal EBNA-1 fragment. This novel CTL line was used to investigate whether the epitope (positions 509–517 in EBNA-1, presented through Kd) was presented to CTL by mouse cells expressing full-length EBNA-1 or a deletion mutant of EBNA-1, lacking the Glycine-Alanine (Gly-Ala)–rich region. Cells expressing full-length EBNA-1 are not lysed by the CTL line, whereas cells expressing the Gly-Ala deletion mutant are recognized. These results suggest that epitopes from full-length EBNA-1 are poorly presented, and that the Gly-Ala-rich region is responsible for this phenomenon. The inefficient presentation of EBNA-1–derived epitopes may explain the absence or rarity of EBNA-1–specific CTLs in vivo, a strategy that may allow EBV to maintain persistence within the immunocompetent host without being eliminated by CTLs.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that establishes latency in B cells (1, 2). In B cells immortalized by EBV infection in vitro (lymphoblastoid cell lines; LCLs) a restricted repertoire of nine “latency-associated” viral genes are expressed: six EBNA1s (EBNA1s 1–6), LMP-1, LMP-2a, and LMP-2b (1).

CTLs eliminate virus-infected cells, which they recognize by the presence of peptides derived from viral proteins in association with class I molecules (3). To analyze the specificity of the human CTL response against EBV latency-associated proteins, lymphocytes recovered from EBV-infected individuals were restimulated in vitro with autologous LCLs, which express the nine latency-associated proteins (4, 5). The target antigens of the restimulated CTL were then identified by expressing the EBV latency-associated proteins individually through recombinant vaccinia viruses. By this protocol, polyclonal CTL responses, or individual CTL clones, capable of lysing autologous cells expressing most EBV latency-associated antigens were identified. Surprisingly, no CTL response capable of lysing autologous cells expressing EBNA-1 were ever found (4–6). Immunization of several strains of mice with syngeneic tumor-cell lines expressing EBNA-1 also failed to raise an EBNA-1–specific rejection response in vivo (7). Recently, MHC class II–restricted, CD-4 positive lymphocyte lines that recognize peptides within EBNA-1 have been identified; these lymphocytes were unable to lyse cells expressing full-length EBNA-1 (8).

Levitskaya et al. (9) created chimeric proteins containing regions of EBNA-1 inserted into another EBV latency-associated protein, EBNA-4, to investigate whether sequences within EBNA-1 affected the presentation of epitopes. Residues 39–498 of EBNA-1 were inserted, in frame, into the EBNA-4 protein. CTL specific for an EBNA-4 epitope, presented by HLA-A11, were unable to recognize cells expressing the EBNA-1–EBNA-4 chimera efficiently. Deletion of the Glycine-Alanine (Gly-Ala)–rich region (residues 93–325 in EBNA-1) from the chimera restored the presen-
tation of the EBNA-4 epitope, thus implicating this region as a ds-acting inhibitor of antigen processing and presenta-

tion (9).

Here, we examine the processing and presentation of EBNA-1–derived epitopes using a novel murine class I–restricted CTL line, raised against a fragment of EBNA-1. Using the same CTL line, we also evaluate the role of the Gly-Ala repeat on the presentation of the EBNA-1–derived epitope via class I MHC molecules.

Materials and Methods

Construction of Recombinant Vaccinia for Expression of a 78 Amino Acid, 160-residue fragment, residues 505–583, was PCR amplified from the plasmid pJ130, described in reference 7. The primers were 5’ CCAAGCCCTTACCATGG and 5’ AAGATCCGAGTCAAACAAGGTCCATTTAACAAGCTCAGTCATCATCCCTTA3’. The ATG within the NcoI sequence supplied the start Methionine. The product was cloned into the vaccinia virus vector pSC113OR.2 to generate pSC11/C-EB1 and a recombinant vaccinia virus VVΔEB1 was generated as previously described (10).

Cell Lines. The rabbit α–EBNA-1 polyclonal serum (11) was raised against a bacterially expressed EBNA-1 fragment (residues 7–37 and 420–617) and affinity purified against the recombinant protein. The 2C CTL line was a gift from Dr. Herman Eisen. The P-107 serum (12) is a human polyclonal serum from an EBV-infected EBNA-1–derived line, raised against a Gly-Ala fragment on the presentation of the EBNA-1–derived epitope. EBNA-1–derived epitopes using a novel murine class I–restricted CTL line, raised against a fragment of EBNA-1. Using the same CTL line, we also evaluate the role of the Gly-Ala repeat on the presentation of the EBNA-1–derived epitope via class I MHC molecules.

Results

The CTL Line V9L Recognizes an Epitope in the COOH-terminal Region of EBNA-1. CTL against potential epitopes within EBNA-1 were raised using the protocol described in Fig. 1 a. “Candidate” epitopes, octa-, nona-, and deca-mer peptides that matched the previously characterized consensus motifs for peptides eluted from common mouse MHC alleles (for Kβ, Kκ, Lβ, and Dβ) were identified (13). For instance, residues 509–517 (VYGGSKTSLL), which contain a Tyr in position 2, and a Leucine in position 9, matched the consensus motif for Kd-binding peptides (see Fig. 1 b).

Although candidate epitopes were interspersed throughout EBNA-1, the largest number of such epitopes for common mouse alleles was found between residues 505 and 583. The length of the immunogen was minimized to this 78-amino acid region, to avoid any potential ds-acting inhibitory sequences. All the candidate peptides were synthesized chemically (see Materials and Methods). By DNA sequencing and PCR from the virus, we confirmed that the recombinant virus generated carried the appropriate fragment. Three strains of mice were immunized with a vaccinia virus encoding the EBNA-1 fragment, corresponding to residues 505–583 of EBNA-1 (of the prototypic B-95-8 sequence) (see Fig. 1 b for mouse genotypes and strains). To recover CTLs specific for EBNA-1–derived epitopes, splenocytes derived from the immunized mice (effectors) were cocultured with stimulators, autologous splenocytes pulsed with candidate peptide epitopes (as shown in Fig. 1 a) to restimulate effector cells that were specific for the candidate peptide.

To determine whether CTLs had been generated upon secondary restimulation, a 51Cr-release assay was used after 5 d of coculture of effectors with stimulators. 51Cr-labeled target cells expressing the relevant class I MHC molecule (Fig. 1 b) were pulsed with 100 nM of the candidate peptide and the effector cultures tested for the ability to lyse these peptide-pulsed targets. The level of lysis was compared against control targets, in which no peptide was used. For eight of the nine candidates, no cytotoxicity above background on peptide-pulsed targets was observed (Fig. 1 b,
The ninth candidate, the peptide VYGGSKTL (V9L), containing a putative K<sub>d</sub>-binding motif, showed lysis above background (Fig. 1b, column 3). All lines of CTLs were then grown for another 7 d on peptide-pulsed stimulators with IL-2 (100 U/ml) and tested again for cytotoxicity. Again, only the V9L line showed lysis above background. This CTL line grew continuously in culture with weekly restimulation with peptide-pulsed spleen cells supplemented with IL-2. In total, three BALB/c mice immunized at separate times with VV<sub>Δ</sub>EB1 yielded a V9L-specific CTL response.

The V9L CTL line was unable to lyse K<sub>d</sub>-expressing cells unless the V9L peptide was added exogenously (i.e., it was peptide specific) and was unable to lyse cells that lacked the K<sub>d</sub> MHC molecule even in the presence of peptide (i.e., MHC class I restricted) (Fig. 1c). The dose of peptide required for the recognition of target cells by the V9L CTL line was determined. P815 cells were pulsed with varying doses of peptide (as indicated in Fig. 1d), washed extensively to remove peptide, and used as targets in a <sup>51</sup>Cr-release assay. The V9L CTL line was capable of recognizing cells pulsed with low doses of peptides (up to 0.005 nM).

Expression of EBNA-1 in Murine Cells. To determine whether the V9L epitope was presented from murine cells expressing full-length EBNA-1, or EBNA-1 lacking the Gly-Ala region, K<sub>d</sub>-expressing murine cells expressing full-length and Gly-Ala-deleted EBNA-1 were generated.

P815 (H2-d, mouse mammary mastocytoma cells) were transfected with an expression vector containing full-length EBNA-1, previously described in reference 7. P815 cells were also transfected with a vector containing an EBNA-1 construct, with the Glycine-Alanine rich region deleted, called <sub>Δ</sub>EBNA-1, in which residues 93–325 have been deleted (Fig. 2a and reference 9).

Expression of EBNA-1 and the Gly-Ala-deleted EBNA-1 (<sub>Δ</sub>EBNA-1) in transfected murine cells were determined by Western blots and immunocytochemistry (Fig. 2c).
Cytotoxic T Lymphocyte Recognition of EBNA-1–derived Epitopes

A rabbit (Rbt) polyclonal serum (11), Rbt-α-EBNA-1, raised against a fragment of EBNA-1 and an affinity-purified human serum, P-107, with reactivity against the Gly-Ala repeat only (12) were used in two separate Western Blots (Fig. 2, b i and ii). With the Rbt-α-EBNA-1 serum, a 72-kD band was detected in P815/EBNA-1 cells, which comigrated with a 72-kD band detected in the EBV-transformed human 721 LCL line, used as a positive control. The 72-kD band was not present in P815 untransfected cells. In P815 cells transfected with EBNA-1 lacking the Gly-Ala repeat (P815/ΔEBNA-1), the size of the mutant protein is expected to be between 40 and 43 kD. Indeed, a band of appropriate size (43 kD) was detected (Fig. 2 b, i). With the P-107 serum, which had been affinity purified to retain reactivity against the Gly-Ala repeat alone, a 72-kD band was detected in P815/EBNA-1 cells, but no 43-kD band was detected in cells transfected with the Gly-Ala deleted construct.

The intracellular location of EBNA-1 in transfected cells was determined by immunohistochemistry, using the Rbt-EBNA-1 antibody. Cell staining demonstrated that both EBNA-1 and the Gly-Ala–deleted EBNA-1 was located primarily in the nucleus (Fig. 2 c).

The V9L Epitope Is Not Presented to CTLs by Cells Expressing Full-length EBNA-1, whereas Cells Expressing ΔEBNA-1 Are Lysed. 51Cr-release assays were performed to determine whether the V9L epitope was being presented by murine cells expressing either the full-length EBNA-1, or EBNA-1 lacking the Gly-Ala-rich region. As targets, the transfected P815 cell lines expressing full-length EBNA-1 or ΔEBNA-1 were used. The presence of the MHC restriction element of the V9L CTLs, Kd, in the target cells, had been previously confirmed by using these cells as targets for other Kd-restricted CTLs (not shown).

The results of a representative 51Cr-release assay using the murine cells appears in Fig. 3. Untransfected P815 cells, used as negative control, were not lysed by V9L CTLs. P815 cells pulsed with the V9L peptide were efficiently lysed. In three separate instances, the P815/EBNA-1 cells were not lysed above background. In contrast, P815/ΔEBNA-1 cells, which express EBNA-1 lacking the Gly-Ala-rich region, were lysed efficiently.

To determine whether EBNA-1 expression altered the ability of cells to present endogenous antigens, P815/EBNA-1 cells were used as targets in 51Cr-release assays with the 2C CTLs line as effectors. The 2C line recognizes the intracellular location of EBNA-1 and the Gly-Ala–deleted EBNA-1.
an endogenous mitochondrial protein presented in association with Ld molecules (14). In a 51Cr-release assay, both P815 and P815/EBNA-1 cells showed no impairment in lysis by 2C CTLs, whereas L/Db cells, which do not express Ld molecules (14), were not lysed (not shown). Thus, cells lysis by 2C CTLs, whereas L/Db cells, which do not express Ld molecules (14), were not lysed (not shown). The presentation of EBNA-1-derived epitopes has recently been examined using class I-restricted human CTL clones that recognize peptides from EBNA-1. Like the murine CTL described here, these CTLs were unable to lyse human cells expressing the full-length protein.

To investigate the means by which EBNA-1 is able to resist antigen processing and presentation, the intracellular locations of EBNA-1 and Gly-Ala-deleted EBNA-1 (ΔEBNA-1) was examined by staining transfected P815 cells with the Rbt-EBNA-1 antiserum. P815/EBNA-1 cells and P815/ΔEBNA-1 cells were both stained prominently in the nucleus (Fig. 2 d). Previously, the nuclear localization sequence in EBNA-1 had been mapped to residues 379–387, outside the region deleted in ΔEBNA-1 (17). Since EBNA-1 and EBNA-1 are both found in the nucleus, it is unlikely that the deletion of the Gly-Ala repeat exposes the protein to more efficient cytosolic proteolysis (18) and thus restores the presentation of the V9L epitope.

Interestingly, the Gly-Ala-rich region is not required for the replication function of EBNA-1, since vectors carrying the EBV-OriP can be maintained episomally in cells expressing the Gly-Ala-deleted EBNA-1. We speculate that this region may have evolved due to the selective advantage it confers on latently infected cells that are known to express EBNA-1 (but not EBNA-2–6) by protecting them from CTL-mediated rejection.

Since only EBNA-1 is required for the maintenance of the EBV episome in latently infected cells (19), the ability of the Gly-Ala repeat to inhibit the presentation of epitopes derived from this protein may be related to the ability of EBV to maintain a persistent infection of B cells, in the face of a host immune response (2, 9). A subpopulation of latently infected B cells expressing only EBNA-1 (or EBNA-1 in conjunction with LMP-2) have been found in vivo (20). If epitopes derived from EBNA-1 are not presented efficiently, latently infected B cells, in which EBNA-1 is expressed, may be partially protected from elimination by host CTLs. This may allow the reservoir of latently infected cells to seed other cellular compartments, such as epithelial cells, where EBV can replicate and be transmitted. The fact that epitopes from EBNA-1 are not presented effectively to CTLs may also explain why EBV-positive Burkitt’s lymphoma cells, which appear to express only EBNA-1 in vivo (21), can survive in immunocompetent hosts, although several other features in these cells may contribute to their lack of immunogenicity for CTLs (20, 22).

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