The CD8\(^+\) T-Cell Response to an Epstein-Barr Virus-Related Gammaherpesvirus Infecting Rhesus Macaques Provides Evidence for Immune Evasion by the EBNA-1 Homologue

Mark H. Fogg, Amitinder Kaur, Young-Gyu Cho, and Fred Wang

Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115, and Department of Immunology, New England Primate Research Center, Harvard Medical School, Southborough, Massachusetts 01771

Received 26 May 2005/Accepted 22 July 2005

Epstein-Barr virus (EBV) infection persists for life in humans, similar to other gammaherpesviruses in the same lymphocryptovirus (LCV) genus that naturally infect Old World nonhuman primates. The specific immune elements required for control of EBV infection and potential immune evasion strategies essential for persistent EBV infection are not well defined. We evaluated the cellular immune response to latent infection proteins in rhesus macaques with naturally and experimentally acquired rhesus LCV (rhLCV) infection. RhLCV EBNA-1 (rhEBNA-1) was the most frequently targeted latent infection protein and induced the most robust responses by peripheral blood mononuclear cells tested ex vivo using the gamma interferon ELISPOT assay. In contrast, although in vitro stimulation and expansion of rhLCV-specific T lymphocytes demonstrated cytotoxic T-lymphocyte (CTL) activity against autologous rhLCV-infected B cells, rhEBNA-1-specific CTL activity could not be detected. rhEBNA-1 CTL epitopes were identified and demonstrated that rhEBNA-1-specific CTL were stimulated and expanded in vitro but did not lyse targets expressing rhEBNA-1. Similarly, rhEBNA-1-specific CTL clones were able to lyse targets pulsed with rhEBNA-1 peptides or expressing rhEBNA-1 deleted for the glycine-alanine repeat (GAR) but not full-length rhEBNA-1 or rhLCV-infected B cells. These studies show that the rhLCV-specific immune response to latent infection proteins is similar to the EBV response in humans, and a potential immune evasion mechanism for EBNA-1 has been conserved in rhLCV. Thus, the rhLCV animal model can be used to analyze the immune responses important for control of persistent LCV infection and the role of the EBNA-1 GAR for immune evasion in vivo.

Epstein-Barr virus (EBV) is a gamma-1 herpesvirus that infects nearly all adult humans and maintains an asymptomatic, lifelong latent virus infection in peripheral blood B cells (24). EBV infection can immortalize the growth of peripheral blood B cells in tissue culture, but in vivo the proliferation of EBV-infected B cells is controlled by the host immune response. The cellular immune response is believed to be important for control of EBV infection in vivo because T lymphocytes can prevent the outgrowth of EBV-infected B cells in tissue culture (18), immunosuppression associated with transplant- or human immunodeficiency virus infection can result in EBV-driven lymphoproliferation (6), and adoptive immunotherapy with EBV-stimulated T lymphocytes can be effective treatment for EBV-associated posttransplant lymphoproliferative syndrome (28).

The specificity and restriction of the T lymphocytes important for control of EBV infection in vivo remains to be determined. CD8\(^+\) cytotoxic T lymphocytes (CTL) specific for epitopes in the six latent infection nuclear proteins (EBNA-1, -2, -3A, -3B, -3C, and -LP) and three latent infection membrane proteins (LMP1, -2A, and -2B) have been detected in EBV-infected individuals (4, 9, 11, 19, 30) and are presumed to be important for control of persistent, latent EBV infection in vivo. CTL specific for epitopes in EBNA-3A, -3B, and -3C frequently dominate the repertoire of CTL specific for EBV latent infection proteins, and it is hypothesized that the immunodominance of the EBNA-3 response may also correlate with protection against latent EBV infection. The success of adoptive immunotherapy with in vitro expanded populations of EBV-specific T lymphocytes (28) that are frequently enriched for EBNA-3-specific CTL is consistent with this hypothesis, but does not exclude other important immune components that might be present in these therapeutic cell populations.

It is unclear why the EBV immune response and the repertoire of CTL fail to completely eliminate EBV infection. Down-regulation of latent EBV gene expression to an EBNA-1-restricted pattern of expression, i.e., type III to type I latency, may be one mechanism EBV uses to evade the immunodominant EBNA-3-specific CTL response (29). A more restricted pattern of latent infection gene expression may also be advantageous for the virus if EBNA-1-specific CTL do not effectively kill EBV infected B cells. The glycine-alanine repeat (GAR) domain of EBNA-1 inhibits proteasomal degradation of GAR containing proteins, i.e., in cis, and thereby inhibits antigen processing and presentation of EBNA-1 peptides to CD8\(^+\) CTL (13, 14). Thus, EBNA-1-specific CTL typically do not
recognize and effectively kill EBV infected B cells endogenously expressing EBNA-1 in tissue culture, but this can be overcome by expressing EBNA-1 deleted for the GAR or coating EBV-infected B cells with EBNA-1 peptide. The potential importance for this EBNA-1 GAR-dependent mechanism of immune evasion has recently been challenged by three groups showing various degrees of EBNA-1-specific CTL recognition or killing of EBV-infected B cells in tissue culture, putatively resulting from presentation of EBNA-1 peptides from incompletely translated EBNA-1, or defective ribosomal products (12, 31, 32). Thus, it remains unclear to what extent persistent, latent EBV infection in humans is dependent on an EBNA-1 GAR-mediated mechanism for immune evasion in vivo.

EBV-related herpesviruses in the same lymphocryptovirus (LCV) genus infect most Old World nonhuman primate species and have a biology similar to EBV’s. They immortalize simian B cells in tissue culture, they infect most of the population by adulthood, virus infection persists for life as a latent infection in the peripheral blood and as a lytic infection in the oropharynx, and virus-positive B cell lymphomas occur in immunosuppressed hosts (reviewed in reference 33). Experimental rhesus LCV (rhLCV) infection in immunocompetent and immunosuppressed rhesus macaques can reproduce the acute, persistent, and lymphomagenic phenotypes of EBV infection in humans (17, 25). The identical repertoire of lytic and latent infection viral genes in the rhLCV and EBV genomes provides important genetic validation that rhLCV is an accurate animal model for EBV infection (26). Not only is there a rhLCV homologue for every EBV gene, but the rhLCV homologue has also been able to functionally substitute for a given EBV gene in virtually every in vitro assay tested to date (7, 8, 10, 16, 20, 27, 35). Thus, rhLCV can provide a highly accurate biologic and genetic animal model for studying EBV pathogenesis.

The only instance where a rhLCV homologue has failed to reproduce EBV gene function in vitro has been the inability of the rhLCV EBNA-1 (rhEBNA-1) GAR to inhibit antigen presentation (5). In these experiments, a simian immunodeficiency virus (SIV) Gag peptide sequence recognized by a SIV Gag-specific CTL clone was cloned into the rhEBNA-1 gene. A similar approach, insertion of an EBV EBNA-3B CTL epitope into EBV EBNA-1, was originally used by Levitskaya et al. (13) to demonstrate that the EBNA-1 GAR could inhibit the processing and presentation of the EBNA-1-encoded EBNA-3B epitope to EBNA-3B-specific CTL, i.e., the EBNA-1 GAR inhibited antigen processing and presentation in cis when the CTL epitope and GAR were in the same molecule.

In contrast to the EBV EBNA-1 studies, the rhEBNA-1 protein containing the SIV Gag epitope was processed and the Gag epitope was efficiently presented to a Gag-specific rhesus CTL clone after expression in rhesus B cells by infection with recombinant vaccinia virus or in fibroblasts by infection with recombinant adenovirus. Thus, there was no evidence of a cis-inhibitory effect of the rhEBNA-1 GAR on antigen presentation in these studies with a heterologous CTL epitope expressed within a recombinant rhEBNA-1. These tissue culture assays with heterologous epitopes and recombinant proteins suggested that an EBNA-1 GAR-mediated immune evasion strategy might not have been conserved in rhesus macaques.

In the current study, we examined the repertoire of latent infection LCV proteins recognized by CTL in naturally and experimentally infected rhesus macaques as a model for better understanding the immune components important for control of EBV infection. The presence or absence of effective killing by rhEBNA-1-specific CTL might provide more direct evidence for whether an EBNA-1 GAR-mediated immune evasion strategy is important or essential for persistent LCV infection in vivo.

MATERIALS AND METHODS

Animals. Rhesus macaques (Macaca mulatta) enrolled in the study were housed at the New England Primate Research Center in accordance with institutional and federal guidelines of animal care (1). Nineteen rhesus macaques were randomly selected from the out-bred conventional colony and naturally acquired rhLCV infection was confirmed by serologic testing for viral capsid antibodies as previously described (23). Four LCV-naı ¨ve rhesus macaques randomly selected from the specific-pathogen-free colony were experimentally infected by oral inoculation with rhLCV 24 to 36 months prior to study (25). Persistent rhLCV infection after experimental inoculation was confirmed in these animals by reverse transcription-PCR detection of rhLCV EBERs (EBNA-encoded RNAs) expression in peripheral blood mononuclear cells (PBMC) and persistent viral capsid antibody titers.

Recombinant vaccinia viruses. Recombinant vaccinia viruses expressing the rhLCV latent infection proteins rhEBNA-1,-2,-3A,-3B,-3C and -LP, truncated rhLMP2a (amino acids 212 to 580) and rhLMP2A (amino acids 1 to 309) were generated as described (15). A recombinant vaccinia virus expressing a mutant rhEBNA-1 deleted for amino acids 41 to 270 (vvrhEBNA-1dGAR) was generated using plasmid templates as previously described (5).

rhEBNA-1 peptides. We synthesized 69 peptides (15-mers overlapping by 10) spanning the N-terminal 100 and C-terminal 260 residues of the rhEBNA-1 protein, i.e., excluding the GAR, by F-moc chemistry at the Massachusetts General Hospital peptide core facility (Charlestown, MA) using an automated peptide synthesizer (MBS 396, Advanced Chemtech, Louisville, KY). Lyophilized peptides were dissolved in dimethyl sulfoxide at a concentration of 100 mg/ml.

Isolation of peripheral blood mononuclear cells. PBMC were isolated from heparinized blood by density gradient centrifugation over Ficoll-Hypaque and suspended at 2 × 10^9 cells per ml in RPMI 1640 medium containing 2 mM L-glutamine, supplemented with 10% fetal bovine serum, and penicillin and streptomycin, both at 50 IU/ml (R-10 medium).

Generation of LCV-immortalized B-lymphoblastoid cell lines. LCV-immortalized B lymphoblastoid cell lines (LCL) were generated by incubating PBMC with viral supernatants and culturing the infected PBMC in R-10 medium with 0.5 µg/ml cyclosporine A at 37°C in a 5% CO2 incubator for 4 to 6 weeks. LCL were expanded and maintained in R-10 medium. Viral supernatants containing the baboon LCV (baLCV; cercopithicine herpesvirus 12) were derived from S94 cells (21), and viral supernatants containing the rhesus LCV (rhLCV; cercopithecine herpesvirus 15) were derived from LCL 8664 cells (22).

Fractionation of T lymphocytes. CD4+ or CD8+ T lymphocytes were selectively depleted from PBMC by immunomagnetic selection (StemCell Technologies). Briefly, 10^7 PBMC in 1 ml of phosphate-buffered saline were incubated at room temperature for 15 min with 20 µl of either anti-dextran/anti-CD4 or anti-dextran/anti-CD8 tetrameric antibody cocktail followed by a 15-min incubation at room temperature with 60 µl of dextran coated magnetic nanoparticle colloid. The suspension was then passed through a 0.3-in.-high-gradient magnetic column of stainless steel mesh resulting in the depletion of either CD4+ or CD8+ T lymphocytes from the flow through fraction which was used in subsequent experiments. Flow cytometry analysis demonstrated the removal of >95% of CD4+ or CD8+ T lymphocytes.

In vitro stimulation of rhLCV-specific CTL. To expand rhLCV-specific CTL, PBMC were stimulated weekly for 3 weeks with autologous, γ-irradiated (100 Gy) rhLCV-LCL in R-10 medium at 37°C in a 5% CO2 incubator. Initial stimulation was at an effector-stimulator ratio of 50:1, followed by two restimulations at 30:1 in the presence of recombinant human interleukin-2 (kindly donated by M. Gately, Hoffmann-La Roche) at 10 IU/ml final concentration if required. Peptide-specific CTL were expanded by incubating one-third of PBMC with peptide for 1 hr at 37°C to generate stimulator cells. After washing once in R-10 medium, stimulator cells were added to the remaining two thirds of PBMC in a 24-well plate; 5 to 10 IU/ml of recombinant human interleukin-2 was added to the cultures 4 to 7 days after stimulation and twice a week thereafter. The
propotion of CD3+ CD4+ and CD8+ cells present following stimulation was determined by flow cytometry analysis. CTL assays were performed 14 days after antigen-specific stimulation.

rhEBNA1-specific CD8+ T-lymphocyte CTL clones. Clones were derived from rhLCV-LCL stimulated cultures by limiting dilution after 10-4 assays. rhLCV-LCL and 10-6/ml y-irradiated (30 Gy) autologous PBMC were generated. Concanaavalin A was added after 3 to 4 days, and clones were subsequently maintained in culture with R-10 medium containing 50 IU/ml interleukin-2 and 5 μg/ml concanaavalin A. A total of 96 replicates containing 5, 10, and 1 cells/well with 2 x 10^4/ml y-irradiated (100 Gy) autologous rhLCV-LCL and 10-6/ml y-irradiated (30 Gy) human feeder PBMC were generated. Concanaavalin A was removed after 3 to 4 days, and clones were subsequently maintained in culture with R-10 medium containing 50 IU/ml interleukin-2. Wells with growing cells were screened for antigen specificity by CTL assays using peptide-pulsed baLCV-LCL targets. rhEBNA1-specific CTL clones were restimulated with autologous rhLCV-LCL and feeder PBMC every 2 weeks.

Enzyme linked immunospot assay for gamma interferon release. Gamma interferon (IFN-γ) ELISPOT assays were carried out using a commercially available kit (MAbtech). PBMC or T-lymphocyte-depleted populations in R-10 medium were infected with recombinant vaccinia viruses at a multiplicity of infection of 10 PFU for 90 min at 37°C, and vaccinia virus-infected cell populations were then placed at various concentrations into a 96-well microtiter plate coated with anti-human IFN-γ monoclonal antibody clone GZ-4. Alternatively, PBMC were incubated with rhEBNA1-1 peptides at 1 μg/ml final concentration directly in the microtiter plate. Following overnight incubation, cells were removed by extensive washing with phosphate-buffered saline. Wells were serially incubated with the biotinylated anti-human IFN-γ monoclonal antibody clone 7-B6-1 followed by streptavidin/alkaline phosphatase. Spots were visualized by the use of an alkaline phosphatase conjugate substrate kit (Bio-Rad, CA). Spots were counted by a KS ELISPOT Automated Reader (Carl Zeiss Inc., NY) using KS ELISPOT software 4.5 (Zeissnet, N.J.). Results for antigen-specific responses were calculated by subtracting background values of cells infected with control vaccinia virus or dimethyl sulfoxide and reported as spot-forming cells (SFC) per 10^6 cells. ELISPOT assays using in vitro-stimulated T-lymphocyte cultures were carried out essentially as described above with the exception that dilutions of effector cells began at 2 x 10^4 per well and target cells were 10^4 autologous baLCV-LCL infected overnight with recombinant vaccinia viruses.

Chromium release assays. Target cells consisted of autologous raLCV-LCL or autologous baLCV-LCL infected overnight with recombinant vaccinia virus expressing individual raLCV proteins (multiplicity of infection, 10 PFU) or pulsed with individual rhEBNA1-1 peptides. Target cells were labeled with 51 chromium (50 μCi) for 1 h at 37°C and were dispensed at 10^4 cells/well with effector cells at various effector-to-target cell (E:T) ratios in 96-well U-bottomed plates in R-10 medium containing 50 IU/ml human interleukin-2 and 5 μg/ml concanaavalin A. A total of 96 replicates containing 10, 3, and 1 cells/well with 2 x 10^4/ml y-irradiated (100 Gy) autologous rhLCV-LCL and 10-6/ml y-irradiated (30 Gy) human feeder PBMC were generated. Concanaavalin A was removed after 3 to 4 days, and clones were subsequently maintained in culture with R-10 medium containing 50 IU/ml interleukin-2. Wells with growing cells were screened for antigen specificity by CTL assays using peptide-pulsed baLCV-LCL targets. rhEBNA1-specific CTL clones were restimulated with autologous rhLCV-LCL and feeder PBMC every 2 weeks.

RESULTS

Repertoire of latent infection rHCV proteins recognized by ex vivo analysis of PBMC from naturally and experimentally infected rhesus macaques. Twenty-three healthy, raLCV-seropositive rhesus macaques at the New England Primate Research Center were studied. Nineteen randomly selected animals had been raised in the conventional colony and had acquired raLCV infection naturally. Four LCV-naive animals were raised in a specific-pathogen-free colony and had been experimentally infected by oral inoculation with raLCV 2 to 3 years prior to analysis (25).

Recombinant vaccinia viruses expressing full-length rhEBNA1-1, -2, -3A, -3B, -3C, and -LP were constructed. Full-length rhLMP1 and rhLMP2A could not be effectively expressed from recombinant vaccinia viruses, so vaccinia viruses expressing a truncated rHMLP1 (amino acids 212 to 589) and rHMLP2A (amino acids 1 to 309) were used for these studies. Ex vivo T-lymphocyte responses to specific raLCV proteins were detected by IFN-γ ELISPOT analysis of PBMC incubated overnight with different recombinant vaccinia viruses. Responses were calculated as the number of IFN-γ spot-forming cells per million PBMC (SFC/10^6 PBMC) minus background control values (PBMC infected with vector control vaccinia virus). Responses greater than 50 SFC/10^6 PBMC and at least twice the number of spots compared to those obtained with vector control vaccinia virus were considered positive. Six raLCV-seronegative animals had no significant ex vivo IFN-γ ELISPOT response to any of the raLCV latent infection proteins, consistent with their raLCV-naive status. In contrast, 17 of 23 raLCV-seropositive animals had a significant ex vivo IFN-γ ELISPOT response to at least one raLCV latent infection protein (Table 1), and each of the raLCV proteins was recognized by at least one animal among the cohort of raLCV-seropositive animals.

The repertoire of raLCV proteins recognized by a given animal varied. For example, rhEBNA1-1, -3B, and -3A stimulated a significant IFN-γ ELISPOT response in PBMC from Mm144-97, with the strongest responses to rhEBNA1-1 followed by rhEBNA3B and -3A (240, 145, and 96 SFC/10^6 PBMC, respectively) (Table 1). A negative response in ex vivo testing of PBMC was consistent with either an absent response or a precursor frequency of responding T lymphocytes below the level of detection without in vitro stimulation and expansion. The average number of raLCV latent proteins recognized among seropositive animals was 1.56 (range, 0 to 6; Table 1), and there was no significant difference between the responses in naturally and experimentally infected animals.

The raLCV latent infection protein most often recognized among seropositive animals was rhEBNA1-1 (11 of 23 seropositive animals, 48%; Fig. 1A). The rest of the raLCV latent proteins were recognized by less than a quarter of the animals (Fig. 1A). There was no obvious dominance of a single rhEBNA-3 protein in this cohort as rhEBNA-LP was the next most frequently recognized raLCV latent protein followed by rHMLP2A and rHMLP3C. rhEBNA1-1 responses were not only the most frequent, but also represented the T-lymphocyte response with the greatest magnitude. In every animal recognizing rhEBNA1-1, the rhEBNA1-1 response was greater than the response to any other latent infection protein (Table 1), and in the 11 rhEBNA1-1 responders the average number of spot-forming cells was 208 SFC/10^6 PBMC (Fig. 1B). The next most robust average response was to rhEBNA3C and...
In order to determine whether IFN-γ ELISPOT responses present in PBMC ex vivo were from CD4+ or CD8+ T lymphocytes, we selectively depleted these T-lymphocyte subsets from PBMC prior to analysis. As shown in Fig. 2, depletion of CD8+ T lymphocytes abrogated the IFN-γ ELISPOT response to rhEBNA-1 by PBMC from Mm141-97 and Mm144-97, whereas depletion of CD4+ T lymphocytes had little or no effect. In 5 additional animals, CD4+ T-lymphocyte depletion had no significant effect on the ex vivo IFN-γ ELISPOT response to rhEBNA-1 consistent with a CD8+ T-cell mediated response (data not shown). Similarly, CD8+ T-lymphocyte depletion eliminated the IFN-γ ELISPOT response to rhEBNA-2 observed with PBMC from Mm309-98 whereas CD4+ T-lymphocyte depletion had no effect (Fig. 2). Using recombinant vaccinia viruses to express rhLCV proteins should bias responses towards CD8+ T cells, and the depletion studies indicate that these ex vivo responses to rhLCV latent infection proteins were dominated by CD8+ T-cell responses. However, the presence of some CD4+ T-cell responses cannot be ruled out.

**In vitro stimulation and expansion of rhLCV latent protein-specific CTL.** In order to test for cytolytic activity, rhLCV-specific T lymphocytes were expanded in vitro by stimulation with irradiated, autologous rhLCV immortalized B cells (rhLCV-LCL) for three weeks. The expanded cell lines were predominantly T lymphocytes (>85% of cells were CD3+) of which between 87 to 95% were CD8+ and 3 to 11% were CD4+ (data not shown). The expanded polyclonal T-lymphocyte lines were used as effectors against rhLCV-LCL targets in chromium release assays. In vitro-stimulated T-lymphocyte

| Animal   | rhEBNA-1 | rhEBNA-2 | rhEBNA-3A | rhEBNA-3B | rhEBNA-3C | rhEBNA-LP | rhLMP1 | rhLMP2 |
|----------|----------|----------|-----------|-----------|-----------|-----------|--------|--------|
| Mm466-78 | — (0)    | —        | —         | —         | —         | —         | —      | —      |
| Mm140-83 | — (0)    | —        | —         | —         | —         | —         | —      | —      |
| Mm161-85 | 423 (100)| —        | —         | —         | —         | —         | —      | —      |
| Mm127-86 | 358 (100)| —        | —         | —         | —         | —         | —      | —      |
| Mm138-86 | — (0)    | —        | —         | —         | —         | —         | —      | —      |
| Mm468-87 | — (0)    | —        | —         | —         | —         | —         | —      | —      |
| Mm63-90  | 119 (100)| —        | —         | —         | —         | —         | —      | —      |
| Mm543-91 | 97 (61)  | —        | —         | —         | —         | —         | —      | —      |
| Mm207-93 | — (0)    | —        | —         | —         | —         | —         | —      | —      |
| Mm244-93 | — (0)    | —        | —         | —         | —         | —         | —      | —      |
| Mm395-93 | — (0)    | —        | —         | —         | —         | —         | —      | —      |
| Mm218-95 | 156 (48) | —        | —         | —         | —         | —         | —      | —      |
| Mm287-95 | 140 (100)| —        | —         | —         | —         | —         | —      | —      |
| Mm339-95 | 114 (100)| —        | —         | —         | —         | —         | —      | —      |
| Mm382-96 | 379 (86) | —        | —         | —         | —         | —         | —      | —      |
| Mm257-97 | — (0)    | —        | —         | —         | —         | —         | —      | —      |
| Mm400-97 | 93 (100) | —        | —         | —         | —         | —         | —      | —      |
| Mm406-97 | — (0)    | —        | —         | —         | —         | —         | —      | —      |
| *Mm141-97| 165 (100)| —        | —         | —         | —         | —         | —      | —      |
| *Mm144-97| 240 (41)| —        | —         | —         | —         | —         | —      | —      |
| *Mm211-98| — (0)    | —        | —         | —         | —         | —         | —      | —      |
| *Mm309-98| — (0)    | 97       | —         | —         | —         | —         | —      | —      |

* * *, experimentally infected animals.

—, undetectable response, fewer than 50 spot-forming cells per 10^6 PBMC. The numbers in parentheses are the percentages of the total response represented by rhEBNA-1-specific responses.

**TABLE 1. IFN-γ ELISPOT responses (SFC/10^6 PBMC) to rhLCV latent infection proteins by ex vivo analysis of PBMC from 23 rhLCV-seropositive macaques.**

**FIG. 1. IFN-γ ELISPOT responses to individual rhLCV latent proteins by ex vivo analysis of PBMC from rhLCV-seropositive rhesus macaques. (A) Number of animals with a positive IFN-γ ELISPOT response to a specific rhLCV latent infection protein by ex vivo PBMC analysis from a total of 23 rhLCV-seropositive macaques. (B) Average magnitude of IFN-γ ELISPOT responses to a specific rhLCV latent infection protein by ex vivo analysis of PBMC. Values represent averages of positive responses to a given latent infection protein.**
lines effectively lysed autologous rhLCV-LCL targets (Fig. 3, solid symbols). In order to test whether the effector cells were specific for rhLCV antigens and not cell-derived antigens, the T-lymphocyte lines were tested against B cells from the same animal immortalized with the baboon LCV (baLCV) as a rhLCV negative, major histocompatibility complex class I identical target.

BaLCV is useful for generating autologous negative control target cells because baLCV is able to immortalize rhesus macaque B cells and rhLCV-specific CTL are unlikely to recognize baLCV latent infection proteins because of significant sequence divergence between rhLCV and baLCV latent infection genes. The effectiveness of autologous baLCV-LCL as negative control targets was demonstrated by the low-level or absent lysis of baLCV-LCL targets incubated with rhLCV-LCL-stimulated T-lymphocyte lines from four rhesus macaques (Fig. 3, open symbols). These results suggested that polyclonal T-lymphocyte lines expanded in vitro by stimulation with autologous rhLCV-LCL contained CTL specific for rhLCV latent infection proteins.

In order to determine which rhLCV latent infection proteins were recognized by the CTL lines, autologous baLCV-LCL infected with vaccinia viruses expressing either vector control or single rhLCV latent infection proteins were used as targets in chromium release assays. Polyclonal CTL lines were derived from eight rhesus macaques (Table 2), and lines from all animals effectively lysed autologous rhLCV-LCL targets (Table 2, rhLCV-LCL). CTL lines from five animals lysed baLCV-LCL targets expressing rhEBNA-3C, four lines lysed targets expressing rhEBNA-2, and three lines lysed targets expressing rhEBNA-3A or 3B. None of the CTL lines lysed targets expressing rhEBNA-1, rhEBNA-LP, rhLMP1, or rhLMP2.

In the case of rhLMP1 and rhLMP2, none of the animals studied had a detectable IFN-γ ELISPOT response by ex vivo analysis of PBMC, and only one animal had an rhEBNA-LP response based on ex vivo IFN-γ ELISPOT assays (Mm309-98, Tables 1 and 2). In the case of rhEBNA-2 and the rhEBNA-3s, there were a total of seven ex vivo IFN-γ ELISPOT responses.

Table 2. Cytolytic activity of rhLCV-LCL-stimulated T-lymphocyte lines against autologous rhLCV-LCL targets or autologous baLCV-LCL targets expressing individual rhLCV latent infection proteins

| Animal     | % Lysis* | rhLCV-LCL | rhEBNA-1 | rhEBNA-2 | rhEBNA-3A | rhEBNA-3B | rhEBNA-3C | rhEBNA-LP | rhLMP1 | rhLMP2 |
|------------|----------|-----------|---------|---------|----------|----------|----------|----------|--------|--------|
| Mm161-85  | 24       | —         | —       | —       | 24       | 15       | —        | —        | —      | —      |
| Mm127-86  | 24       | —         | —       | —       | 12       | 14       | —        | —        | —      | —      |
| Mm468-87  | 24       | —         | —       | —       | 24       | 50       | 10       | —        | —      | —      |
| Mm543-91  | 24       | —         | —       | —       | 14       | —        | —        | —        | —      | —      |
| *Mm141-97 | 24       | —         | —       | —       | 10       | 13       | —        | —        | —      | —      |
| *Mm144-97 | 24       | —         | —       | —       | 24       | —        | —        | —        | —      | —      |
| *Mm211-98 | 24       | —         | —       | —       | 10       | —        | —        | —        | —      | —      |
| *Mm309-98 | 24       | —         | —       | —       | 15       | 14       | —        | —        | —      | —      |

* Values are % lysis at effector-target ratio of either 30:1 or 20:1. —, less than 10% lysis (following subtraction of vector control vaccinia virus); nd, not done. Italic values represent cytolytic activity that correlated with a positive ex vivo IFN-γ response detectable in PBMC (Table 1). Also see Table 1, footnote a.
in PBMC from these animals, and five out of seven targets were recapitulated by the in vitro-stimulated CTL lines (Table 2, italic values). Nine of the cytotoxic responses to rhEBNA-2 and rhEBNA-3-expressing targets by in vitro-stimulated CTL lines in chromium release assays was in marked contrast to the dominant rhEBNA-1 ex vivo response detected by IFN-γ ELISPOT analysis. In order to determine whether the lack of rhEBNA-1-specific cytotoxic responses was due to the lack of in vitro expansion of the rhEBNA-1-specific T lymphocytes, a defect in cytolytic activity, or a defect in rhEBNA-1 presentation by the target cell, IFN-γ ELISPOT analysis was performed on the in vitro-stimulated T-lymphocyte lines from three animals using baLCV-LCL infected with recombinant vaccinia viruses as targets. In the case of rhEBNA-1, there was neither significant cytolytic activity nor IFN-γ ELISPOT response in any of the three CTL lines (Fig. 4A, B, and C) even though all three animals had significant rhEBNA-1 responses in PBMC ex vivo. This was in contrast to the other latent infection proteins, where strong cytolytic activity was associated with a significant IFN-γ ELISPOT response in most instances (e.g., rhEBNA-2 in Fig. 4A, rhEBNA-3B in Fig. 4B, and rhEBNA-3A and -3B in Fig. 4C). These results suggested that rhEBNA-1-specific T lymphocytes either had not been expanded in vitro by stimulation with autologous rhLCV-LCL or were present but unable to generate an effective cytotoxic or IFN-γ response against rhEBNA-1-expressing target cells.

Identification of CD8+ T-lymphocyte epitopes within the rhEBNA-1 protein. In order to develop a more sensitive means of detecting and stimulating rhEBNA-1-specific CD8+ T lymphocytes, we identified regions containing rhEBNA-1 epitopes recognized by rhesus CD8+ T lymphocytes in two animals. We synthesized a series of overlapping 15-mer peptides spanning the entire unique sequence of the rhEBNA-1 protein and assembled the individual peptides into 17 pools containing seven, eight, or nine peptides each. The pools were constructed such that each peptide was uniquely represented in two different pools, thereby allowing easy identification of individual peptides, i.e., positive results from two pools would identify the target since only one peptide would be common to both pools. PBMC from one experimentally (Mm211-98) and one naturally infected (Mm543-91) rhesus macaque, who were known to have an ex vivo IFN-γ rhEBNA-1 response, were incubated overnight with the peptide pools in IFN-γ ELISPOT assays. Both animals had a predominant positive response to two peptide pools each (data not shown), and the identity of the pools suggested that PBMC from Mm141-97 recognized an epitope contained within peptide 67 (amino acids 331 to 345, GGRGFKKFENMAKNL) and that PBMC from Mm543 to 91 recognized an epitope contained within peptide 82 (amino acids 406 to 420, PGPQPGPMRESTDCY).

CD4+ and CD8+ T-lymphocyte-depleted PBMC were then screened by IFN-γ ELISPOT assays with these individual peptides. Unfractionated PBMC from Mm141-97 and Mm543-91 recognized peptides 67 and 82, respectively, confirming that these rhEBNA-1 peptides contained the epitopes recognized in the rhEBNA-1 peptide pools (Fig. 5). In addition, the IFN-γ ELISPOT response to the respective peptides was ablated from both animals when CD8+ T lymphocytes were depleted and increased slightly when CD8+ T lymphocytes were enriched (Fig. 5). When the IFN-γ ELISPOT response in PBMC from Mm141-97 to peptide 67 was compared with the response to a pool containing all of the rhEBNA-1 peptides, the number of spot-forming cells per 10^6 T lymphocytes following subtraction of ELISPOT responses with baLCV-LCL targets infected with control vaccinia viruses is shown.

![FIG. 4. Comparison of cytolytic and IFN-γ-secreting activity of in vitro-stimulated T-lymphocyte lines.](http://jvi.asm.org/Downloaded from http://jvi.asm.org)
5A). There was no CD4⁺ T-lymphocyte response to peptide 67 or the whole pool of rhEBNA-1 peptides, suggesting that a CD8⁺ T-lymphocyte epitope within peptide 67 was the predominant target of the rhEBNA-1 response in this animal.

In Mm543-91 peptide 82 was a dominant target of the CD8⁺ T-lymphocyte response, but there was also a significant response using the whole peptide pool with CD4⁺-enriched T lymphocytes (Fig. 5B), suggesting the presence of CD4⁺ T-lymphocyte epitopes within the subdominant, positive peptide pools. In summary, epitope containing peptides representing the dominant rhEBNA-1-specific CD8⁺ T-lymphocyte response from two rhLCV-infected animals were defined.

In vitro expansion of rhEBNA-1-specific CD8⁺ T lymphocytes using rhEBNA-1 peptides. The rhEBNA-1 peptides 67 and 82 were used to stimulate and expand T lymphocytes from PBMC of Mm141-97 and Mm543-91, respectively, in order to determine whether rhEBNA-1-specific CD8⁺ T lymphocytes were capable of recognizing and killing rhLCV-LCL. Peptide 67- and 82-specific T-lymphocyte lines were generated by a 2-week in vitro stimulation with the respective rhEBNA-1 peptide. The lines were then tested for their ability to lyse autologous rhLCV-LCL targets in a chromium release assay. As shown in Fig. 6, neither peptide 67 (Fig. 6A) nor peptide 82 (Fig. 6B)-specific T-lymphocyte lines lysed autologous rhLCV-LCL (Fig. 6, open triangles) even though these T-lymphocyte populations had cytolytic activity against peptide-pulsed, autologous baLCV-LCL (Fig. 6, solid squares) or peptide-pulsed autologous rhLCV-LCL (Fig. 6, solid triangles) targets. The ability of the peptide-stimulated rhEBNA-1-specific CTL lines to lyse peptide-loaded targets but not rhLCV-LCL endogenously expressing rhEBNA-1 suggested a defect in antigen presentation of endogenous rhEBNA-1 by the rhLCV-LCL targets.

rhEBNA-1 peptide-specific CTL are present in polyclonal T-lymphocyte lines stimulated in vitro with autologous rhLCV-LCL. We used the rhEBNA-1 epitope containing peptides to revisit the question of whether rhEBNA-1-specific T lymphocytes were absent versus present, but undetectable in rhLCV-LCL-stimulated T-lymphocyte lines. As previously seen, the CTL line established from Mm141-97 PBMC by in vitro stimulation for 3 weeks with autologous rhLCV-LCL had no cytolytic activity against autologous baLCV-LCL targets alone (Fig. 7, open square), infected with control vaccinia virus (Fig. 7, open circle), or infected with rhEBNA-1-expressing vaccinia virus (Fig. 7, solid circle). However, when autologous baLCV-LCL targets were pulsed with peptide 67, the rhLCV-LCL-stimulated CTL line efficiently lysed peptide-pulsed targets (Fig. 7, solid square) indicating that rhEBNA-1-specific CTL were present in the T-lymphocyte line and were capable of cytotoxic activity. Thus, the lack of cytotoxic activity against rhEBNA-1-expressing targets was most likely due to a defect in antigen presentation of endogenously expressed rhEBNA-1 in the target cells.

Since this phenotype was similar to the inhibition of antigen presentation associated with the EBV EBNA-1 GAR, we
tested whether expression of a GAR-deleted rhEBNA-1 would result in lysis of target cells expressing the mutant rhEBNA-1. The rhLCV-stimulated CTL line showed modest but significantly more lysis of baLCV-LCL targets infected with the GAR-deleted rhEBNA-1 vaccinia virus (Fig. 7, shaded circle) versus wild-type rhEBNA-1 vaccinia virus (Fig. 7, solid circle), indicating that a domain within the rhEBNA-1 deletion was capable of inhibiting processing and presentation of rhEBNA-1 peptide epitopes in cis. The functional similarity to the EBV EBNA-1 GAR strongly implicates the rhEBNA-1 GAR as the domain responsible for the cis-acting inhibition of antigen presentation.

rhEBNA-1-specific CD8\(^+\) T-lymphocyte clones do not lyse rhEBNA-1 expressing cells but can lyse cells expressing GAR-deleted rhEBNA-1. Since the rhLCV-LCL-stimulated CTL lines were polyclonal, we could not test the activity of the rhEBNA-1-specific CTL against the autologous rhLCV-LCL without first generating rhEBNA-1-specific CTL clones, i.e., CTL specific for other latent infection antigens such as rhEBNA-2 and rhEBNA-3s would lyse the targets. rhEBNA1-specific CTL clones were generated by CD4\(^+\) T-lymphocyte depletion and limiting dilution of rhLCV-LCL-stimulated T-lymphocyte lines from Mm141-97. These clones were generated using only rhLCV-LCL for stimulation and were not stimulated with rhEBNA-1 peptides. CTL clones were screened for cytolytic activity against rhEBNA-1 peptide 67-pulsed baLCV-LCL, and eight rhEBNA-1-specific clones were identified (two representative clones shown in Fig. 8, solid triangle).

All rhEBNA-1-specific clones were unable to lyse autologous rhLCV-LCL (Fig. 8, open square), but were able to recognize and lyse peptide-pulsed rhLCV-LCL (data not shown). Similarly, all clones failed to lyse autologous baLCV-LCL expressing rhEBNA-1 peptide 67-pulsed baLCV-LCL, and eight rhEBNA-1-specific clones were identified (two representative clones shown in Fig. 8, solid triangle).

All rhEBNA-1-specific clones were unable to lyse autologous rhLCV-LCL (Fig. 8, open square), but were able to recognize and lyse peptide-pulsed rhLCV-LCL (data not shown). Similarly, all clones failed to lyse autologous baLCV-LCL expressing rhEBNA-1 peptide 67-pulsed baLCV-LCL, and eight rhEBNA-1-specific clones were identified (two representative clones shown in Fig. 8, solid triangle).

All rhEBNA-1-specific clones were unable to lyse autologous rhLCV-LCL (Fig. 8, open square), but were able to recognize and lyse peptide-pulsed rhLCV-LCL (data not shown). Similarly, all clones failed to lyse autologous baLCV-LCL expressing rhEBNA-1 peptide 67-pulsed baLCV-LCL, and eight rhEBNA-1-specific clones were identified (two representative clones shown in Fig. 8, solid triangle).

We tested the rhEBNA-1 CTL clones for increased IFN-\(\gamma\) production in the supernatants, since Lee et al. (12) recently described EBV EBNA-1-specific CTL that could respond to full-length EBNA-1 expressing targets by increasing IFN-\(\gamma\) production in the absence of cytolytic activity. Negative control supernatants from two rhEBNA-1-specific CTL clones cocultured with baLCV-LCL contained no detectable IFN-\(\gamma\), loaded targets was a uniform property of the rhEBNA-1-specific CTL. Thus, the autologous rhLCV-LCL can stimulate the expansion of rhEBNA-1-specific T lymphocytes in vitro, but these rhEBNA-1-specific T lymphocytes are unable to lyse the autologous rhLCV-LCL in vitro, consistent with immune evasion through a rhEBNA-1 GAR-mediated inhibition of antigen presentation in cis.
whereas positive control supernatants from CTL clones cultured with peptide 67-loaded baLCV-LCL contained 600 pg/ml IFN-γ (Fig. 8b). Supernatants from wells containing baLCV-LCL infected with GAR-deleted rhEBNA-1 expressing vaccinia viruses contained detectable, but 10-fold lower levels of IFN-γ (50 pg/ml), consistent with the intermediate cytotoxic activity against GAR-deleted rhEBNA-1 versus peptide-loaded target cells. However, there was no detectable IFN-γ in supernatants from wells containing rhLCV-LCL or baLCV-LCL expressing wild-type rhEBNA-1, suggesting that in these studies, the rhEBNA-1 GAR inhibition of antigen presentation extends to IFN-γ production as well as cytotoxic responses of rhEBNA-1-specific CTL.

**DISCUSSION**

We set out to characterize the CD8+ T-lymphocyte response to rhLCV infection in rhesus macaques to better understand the repertoire of immune responses important for controlling LCV infection and the immune evasion mechanisms that might contribute to persistent LCV infection. We analyzed the immune responses to individual rhLCV latent infection proteins using two different, but complementary assays. The IFN-γ ELISPOT assay provides direct quantitation of CD8+ T-lymphocyte responses in the peripheral blood without bias from in vitro stimulation and expansion. However, low-frequency responses may not be detected, functional killing of virus-infected cells is not demonstrated, and without depletion or blocking studies, the contribution of CD4+ T lymphocytes to the response cannot be excluded. The presence of a vaccinia virus-encoded soluble IFN-γ receptor could also potentially reduce the ELISPOT response by competing for the detection of IFN-γ. In vitro stimulation and expansion of LCV-specific T lymphocytes provides an opportunity to assess killing of virus infected cells using the chromium release assay and can enhance detection of CTL present at a lower precursor frequency, but may introduce bias by selecting for cells that most successfully expand in tissue culture. Therefore, we felt that the use of both approaches were complementary and important for this initial evaluation of the rhLCV cellular immune response.

The repertoire of CD8+ T lymphocytes to rhLCV latent infection proteins appeared broader compared to the more focused human CD8+ T-lymphocyte response to EBV EBNA-3 latent proteins (9, 11, 19, 30), although the use of slightly different assays for rhesus and human responses makes direct comparisons difficult. Ex vivo IFN-γ ELISPOT analysis of rhesus PBMC demonstrated a broad response directed towards all latent infection rhLCV proteins, with rhEBNA-1, rhEBNA-3C, rhEBNA-LP, and rhLMP2 being recognized most frequently by this cohort of animals. In vitro stimulation of rhLCV-specific T lymphocytes resulted in an enrichment for rhEBNA-3 responses, with rhEBNA-3C/rhEBNA-2/rhEBNA-3A, and -3B becoming the most frequently recognized latent infection proteins. Thus, the type of assay used to assess CD8+ T-lymphocyte activity can have a significant impact on the interpretation of which targets “dominate” the response.

Both assays suggest that rhEBNA-3C is frequently targeted by CD8+ T lymphocytes in infected rhesus macaques, but individually the responses to rhEBNA-3A, -B, and -3C are not strikingly greater than responses to other latent infection proteins such as rhLMP2 or rhEBNA-LP. Thus, we conclude that all three rhEBNA-3s are immunogenic in LCV-infected rhesus macaques, and rhEBNA-3C-specific CTL are frequently present at various precursor frequencies in infected animals. However, a variety of other latent infection proteins were also recognized in many animals so that there was no obvious immunodominance by the rhEBNA-3 proteins.

The current results also show that targets expressing full-length rhEBNA-1 are not effectively killed by rhEBNA-1-specific CTL, but expression of a GAR-deleted rhEBNA-1 allows more efficient processing and presentation of rhEBNA-1 peptide epitopes. rhLCV-LCL-stimulated T-lymphocyte lines failed to elicit a cytotoxic or IFN-γ ELISPOT response in assays using targets expressing full-length rhEBNA-1. The mapping of specific rhEBNA-1 epitopes from two animals showed that rhEBNA-1-specific T lymphocytes were present in the rhLCV-stimulated cultures and that these rhEBNA-1-specific T lymphocytes had cytotoxic activity. Similarly, rhEBNA-1-specific T-lymphocyte clones retained cytotoxic activity against peptide-loaded targets, but failed to lyse targets expressing full-length rhEBNA-1 and, most importantly rhLCV-infected targets. The ability to lyse targets expressing a GAR-deleted rhEBNA-1 indicated that a cis-acting block in rhEBNA-1 antigen presentation was operative in rhLCV-infected B cells, similar to EBV-infected B cells.

It is not clear why rhEBNA-1 can block presentation of native rhEBNA-1 peptide epitopes, but failed to block presentation of a heterologous SIV epitope inserted into a recombinant rhEBNA-1 (5). It is possible that overexpression of the SIV Gag/rhEBNA-1 chimera by recombinant vaccinia virus or adenovirus infection overwhelmed the inhibitory activity of the rhEBNA-1 GAR. Since the rhEBNA-1 GAR is much shorter than the EBNA-1 GAR, seven glycine/alanine-rich repeats over 47 amino acids versus 84 G1–3A repeats over 252 amino acids, it may not be as potent an inhibitor of antigen presentation as the EBNA-1 GAR. In any case, the rhEBNA-1 expression level in an rhLCV-LCL is probably the most relevant in vitro scenario, and the current studies indicate that a cis-acting block in antigen presentation has been conserved in rhLCV.

The high precursor frequency of rhEBNA-1-specific T lymphocytes detected by ex vivo analysis of PBMC was also striking: 48% of seropositive animals had IFN-γ ELISPOT responses in PBMC infected with rhEBNA-1 vaccinia virus, and rhEBNA-1 also elicited the most potent IFN-γ response in PBMC among all latent infection proteins. In contrast, no IFN-γ ELISPOT response to rhEBNA-1 vaccinia virus-infected targets was detected in rhLCV-stimulated CTL lines, despite the presence of rhEBNA-1-specific CTL documented by the use of rhEBNA-1 peptides. This difference may be explained by the presence of professional antigen-presenting cells in PBMC that were subsequently lost in the stimulated CTL lines. The professional antigen-presenting cells in PBMC may lead to the detection of rhEBNA-1-specific IFN-γ responses through the uptake of exogenous antigen, i.e., a cross-presentation mechanism similar to the cross-priming mechanism proposed for generating EBV EBNA-1-specific CTL (3).

The high precursor frequency of rhEBNA-1-specific T lymphocytes in PBMC is not inconsistent with a biologically sig-
nificant GAR-mediated inhibition of antigen presentation. The high precursor frequency indicates efficient in vivo priming of naïve T lymphocytes and their subsequent differentiation into effector cells. This priming in vivo probably occurs by dendritic cells processing and presenting exogenously derived rhEBNA-1 from dead or dying rhLCV-infected cells. The rhEBNA-1 GAR may not significantly influence the priming of rhEBNA-1-specific T lymphocytes in vivo because the inhibitory activity of the GAR is not believed to have a major effect on the ability of dendritic cells to process and present exogenous EBV EBNA-1 to both naïve T lymphocytes (2) and EBNA-1-specific CTL clones (3).

The high precursor frequency of rhEBNA-1-specific T lymphocytes in persistently infected macaques may also be due in part to expansion and maintenance of memory CTL. This may be due to continued stimulation from cross-presentation of exogenous rhEBNA-1 from dying cells, where the GAR may exert little influence. Alternatively, there could be direct peptide presentation by rhLCV-infected B cells, where the GAR could be inhibiting efficient processing of rhEBNA-1. In this case, small amounts of rhEBNA-1 peptides sufficient for T-cell stimulation may be derived from full-length rhEBNA-1 or come from defective ribosomal products (28). Whether from cross- or direct presentation, the maintenance of a high precursor frequency in persistently infected animals suggests the presence of continued stimulation of rhEBNA-1-specific T lymphocytes in vivo.

The key question is whether there is sufficient rhEBNA-1 peptide presentation by target cells in vivo to activate effector function, i.e., killing of the rhLCV-infected cell. The GAR may be important for preventing sufficient peptide presentation to activate CTL killing in vivo, but sufficient peptide presentation for T-lymphocyte stimulation may still occur by the mechanisms described above. The ability to present sufficient peptides for stimulation but not killing was reproduced in these studies by the rhLCV-LCL-stimulated CTL lines and clones, where rhEBNA-1 CTL were expanded and capable of lysing peptide-loaded targets but were unable to lyse targets expressing full-length rhEBNA-1. Functional conservation of this potential immune evasion mechanism in rhLCV suggests that GAR-mediated inhibition of antigen presentation is biologically important for the virus.

How does this scenario for the rhEBNA-1 GAR and rhEBNA-1 CTL compare to EBV infection in humans? The rhEBNA-1 block for antigen presentation in cis is comparable to the classic paradigm for EBNA-1 GAR-mediated immune evasion. However, this model has recently been challenged by three different observations suggesting that EBV EBNA-1-specific CTL may recognize EBV-infected B cells more effectively than previously believed. Voo et al. (32) identified an EBNA-1-specific CTL clone that had modest killing activity. Lee et al. (12) found that even though their EBNA-1-specific CTL clones failed to lyse EBV-infected B cells, they responded with increased IFN-γ secretion and could control the outgrowth of EBV-infected cells. Finally, Tellam et al. (31) reported that they were able to frequently and reproducibly stimulate EBNA-1-specific CTL that could lyse EBV-infected B cells. Thus, the degree of EBNA-1 immune evasion evidenced in vitro remains an evolving story.

All three groups suggested that newly synthesized protein was required for EBNA-1 recognition through defective ribosomal products and not stable, long-lived EBNA-1 protein. This model still implicates an inhibitory effect of the EBNA-1 GAR on antigen presentation of wild-type EBNA-1 protein, and the critical question is what mechanisms are operative in vivo. Defective ribosomal products may be a source for presentation of EBNA-1 peptides to various degrees in actively growing EBV- or rhLCV-infected B cells in tissue culture, but whether this mechanism is limited to tissue culture and whether EBNA-1 GAR-mediated immune evasion is required for persistent EBV infection in vivo remains to be determined.

The precursor frequency of EBNA-1-specific CTL in EBV-infected humans is also not well established, since ex vivo responses have not been extensively studied. Subklewe et al. (30) reported that only 2 of 13 random donors had a significant IFN-γ ELISPOT response when PBMC were exposed to a recombinant vaccinia virus expressing EBNA-1-deleted for the GAR, suggesting that the frequency of EBNA-1 recognition in a random population is low. However, HLA type can have a strong influence, as shown by Blake et al. (3), where PBMC from 15 out of 15 HLA-B*3501 individuals had an ex vivo IFN-γ ELISPOT response to an HLA-B*3501 restricted EBNA-1 epitope. Thus, it remains to be determined whether the frequency and prevalence of EBNA-1-specific CD8+ T lymphocytes in the peripheral blood is truly higher in macaques than that reported for randomly selected humans.

The conservation of a potential immune evasion mechanism in the rhEBNA-1 GAR resurrects the question of whether the ability to evade cytolysis by EBNA-1-specific CTL is fundamentally important for persistent LCV infection in human and non-human primate hosts. The fact that rhEBNA-1 peptides are not efficiently presented by rhLCV-infected cells provides a rationale for constructing a mutant rhLCV with an rhEBNA-1 deleted for the GAR to determine its effect on LCV infection in vivo. If GAR-mediated immune evasion is required for persistent infection, the host immune response may be able to successfully eliminate this mutant virus after experimental infection. On the other hand, the GAR may be important for providing stable, long-lived EBNA-1 in latently infected cells. Potential effects of the GAR on EBNA-1 stability and translation may impact episomal maintenance in vivo despite the lack of such an effect in vitro, thereby complicating the interpretation of why a mutant virus containing a GAR-deleted rhEBNA-1 may not persist. Determining whether the mutant virus is able to establish persistent infection in the setting of immunosuppression may be useful for differentiating a dominant effect of the GAR on immune evasion versus episomal maintenance in vivo.

ACKNOWLEDGMENTS

This work was supported by grants from the U.S. Public Health Service (DE14388 and CA68051). Services from the New England Primate Research Center were supported by a base grant to the institution (USPHS P51RR00168).

We thank Ashok Khatri for assistance with peptide synthesis, The- rion Inc. for providing the recombinant vaccinia virus vector and assistance with the construction of recombinant vaccinia viruses, Angela Carville for expert assistance with animal resources, and Corrina Hale and Deirdre Garry for technical assistance.
REFERENCES

1. Anonymous. 1996. Guide for the Care and Use of Laboratory Animals. p. 86–123. The Institute of Laboratory Animal Resources, National Research Council, Washington, D.C.

2. Bickham, K., K. Goodman, C. Paludan, S. Nikiforow, M. L. Tsang, R. M. Steinman, and C. Munz. 2003. Dendritic cells initiate immune control of Epstein-Barr virus transformation of B lymphocytes in vitro. J. Exp. Med. 198:1633–1636.

3. Blake, N., T. Haigh, G. Shaka’a, D. Croom-Carter, and A. Rickinson. 2000. The importance of exogenous antigen in priming the human CD8+ T-cell response: lessons from the EBV nuclear antigen EBNA1. J. Immunol. 165: 7078–7087.

4. Blake, N., S. Lee, I. Redchenko, W. Thomas, N. Steven, A. Leese, P. Steigerwald-Mullen, M. G. Kurilla, L. Frappier, and A. Rickinson. 1997. Hum. CD8+ T-cell responses to EBV EBNA1: HLA class I presentation of the (Gly-Ala)-containing protein requires exogenous processing. Immunity 7:791–802.

5. Blake, N. W., A. Moghaddam, P. Rao, A. Kaur, R. Glickman, Y. G. Cho, A. Marchini, T. Haigh, R. P. Johnson, A. B. Rickinson, and F. Wang. 1999. Inhibition of antigen presentation by the glycine/alanine repeat domain is not conserved in simian homologues of Epstein-Barr virus nuclear antigen 1. J. Virol. 73:7381–7386.

6. Blake, N., T. A. Haigh, M. A. Epstein, and J. S. Sklar. 1986. Failure in immunological control of the virus infection: post-transplant lymphomas, p. 163–181. In M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus: recent advances. Heinemann Medical Books, London, England.

7. Franken, M., O. Devergne, M. Rosenzweig, B. Annis, E. Kieff, and F. Wang. 2000. Further characterization of a herpesvirus-positive orangutan cell line and comparative aspects of in vitro transformation with lymphotropic old world primate herpesviruses. Int. J. Cancer 83:425–432.

8. Rabin, H., R. H. Neubauer, R. F. Hopkins, 3rd, and M. Nonoyama. 1978. Inhibition of antigen presentation by the glycine/alanine repeat domain of the Epstein-Barr virus nuclear antigen LMP1. J. Virol. 45:141–146.

9. Moreno, I., J. M. Brooks, H. Al-Jarrah, W. A. Thomas, M. Rowe, E. Kieff, and A. B. Rickinson. 1992. Identification of target antigens for the human cytotoxic T-cell response to Epstein-Barr virus (EBV): implications for the immune control of EBV-positive malignancies. J. Exp. Med. 176:157–168.

10. Wang, R., A. V. Gordienko, M. A. Epstein, J. Zong, J. Zong, G. S. Murray, R. J., M. G. Kurilla, J. M. Brooks, W. A. Thomas, M. Rowe, E. Kieff, and A. B. Rickinson. 1999. Strong selective pressure for EBNA-LP and EBNA2 proteins from nonhuman primate lymphocryptoviruses. J. Virol. 74:379–389.

11. Anonymous. 1997. Epstein-Barr virus-related herpesvirus from a rhesus monkey (Macaca mulatta) with malignant lymphoma. Int. J. Cancer 58:3219–3225.

12. Yewdell, J. W., L. C. Anton, and J. R. Bennink. 1995. Presentation of Epstein-Barr virus latent gene expression in Burkitt’s lymphoma cells. Int. J. Cancer 61:1145–1151.

13. Kieff, E., and D. J. Moss. 1992. Localization of Epstein-Barr virus cytotoxic T-cell epitopes using recombinant vaccinia: implications for vaccine development. J. Exp. Med. 176:169–176.

14. Bhardwaj, N., and B. G. Achong (ed.). 1998. Control of the virus infection: post-transplant lymphomas, p. 163–181. In M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus: recent advances. Heinemann Medical Books, London, England.

15. Kieff, E., and D. J. Moss. 1992. Localization of Epstein-Barr virus cytotoxic T-cell epitopes using recombinant vaccinia: implications for vaccine development. J. Exp. Med. 176:119–176.

16. Bhardwaj, N., and B. G. Achong (ed.). 1998. Control of the virus infection: post-transplant lymphomas, p. 163–181. In M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus: recent advances. Heinemann Medical Books, London, England.