

Cloning of the Rhesus Lymphocryptovirus Viral Capsid Antigen and Epstein-Barr Virus-Encoded Small RNA Homologues and Use in Diagnosis of Acute and Persistent Infections

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Epstein-Barr virus (EBV) is the most common cause of infectious mononucleosis and is associated with the development of several human malignancies. A closely related herpesvirus in the same lymphocryptovirus (LCV) genera as EBV naturally infects rhesus monkeys and provides an important animal model for studying EBV pathogenesis. We cloned the small viral capsid antigen (sVCA) homologue from the rhesus LCV and developed a peptide enzyme-linked immunosorbent assay (ELISA) to determine whether epitopes in the rhesus LCV sVCA are a reliable indicator of rhesus LCV infection. In order to define a "gold standard" for rhesus LCV infection, we also cloned the EBV-encoded small RNA 1 (EBER1) and EBER2 homologues from rhesus LCV and developed a reverse transcription (RT)-PCR assay to detect persistent LCV infection in rhesus monkey peripheral blood lymphocytes. Animals from a conventional and a hand-reared colony were studied to compare the prevalence of rhesus LCV infection in the two groups. There was a 100% correlation between the peptide ELISA and EBER RT-PCR results for rhesus LCV infection. In addition, specificity for LCV infection and exclusion of potential cross-reactivity to the rhesus rhadinovirus sVCA homologue could be demonstrated using sera from experimentally infected animals. These studies establish two novel assays for reliable diagnosis of acute and persistent rhesus LCV infections. The rhesus LCV sVCA peptide ELISA provides a sensitive and reliable assay for routine screening, and these studies of the hand-reared colony confirm the feasibility of raising rhesus LCV-naive animals.

Epstein-Barr virus (EBV) is a human gammaherpesvirus in the lymphocryptovirus (LCV) genera which is associated with the development of several different malignancies, including Burkitt's lymphoma, B-cell lymphomas in immunosuppressed hosts, nasopharyngeal carcinomas, Hodgkin's disease, and gastric carcinomas (16). Closely related herpesviruses in the same LCV genera naturally infect a number of Old World primate species, and experimental infection of rhesus monkeys with the rhesus LCV is the only animal model which accurately reproduces the pathogenesis of acute and persistent EBV infections (24). Simian LCV infection is also associated with B-cell lymphomas in many Old World primate species and can cause lethal malignant disease in macaques experimentally infected and immunosuppressed with simian immunodeficiency virus (SIV) (8, 26). Thus, accurate diagnostic assays for LCV infection in Old World primates, and rhesus monkeys in particular, would be an important tool for primate care and for identifying LCV-naive animals for experimental studies.

The simian LCV and EBV genomes are colinear and appear to contain a similar repertoire of lytic and latent infection genes. The rhesus LCV latent infection genes have shown a surprising amount of sequence divergence from EBV, with only 20 to 50% amino acid identity (3, 4, 9, 10, 25, 28). The few lytic infection genes from simian LCV cloned to date have demonstrated better homology, with 50 to 90% amino acid identity (15a, 23, 46). Currently rhesus LCV diagnosis is made by detecting simian antibodies which are cross-reactive with EBV-lytic infection proteins. The cross-reactivity with EBV serologic assays can be useful for identifying animals with high antibody titers but can be difficult for excluding infection and

identifying truly seronegative, naive animals. Serologic screening for herpes B virus infection has been successfully used to establish specific-pathogen-free colonies (6, 13). A simple, sensitive, and specific serologic screening assay for LCV infection would be a valuable tool for breeding LCV-naive macaque colonies. LCV-naive animals would have less risk of LCV-induced malignancies associated with experimentally induced immunosuppression, e.g., transplant and SIV experiments, and would provide a reliable source of animals for experimental infection and pathogenesis studies.

EBV infection is associated with a lifelong antibody response to lytic infection viral capsid antigens (VCA) and EBV latent infection nuclear antigens (EBNA) (27). An immunoglobulin G (IgG) antibody response to either is a reliable indicator of previous infection in humans. There are six different EBNA expressed in EBV-immortalized cells which are recognized by antibodies in EBV-immune human sera. The highest antibody titers are usually generated against EBNA-1 (12, 21), but these are still quite low compared to titers of antibody against VCA (34, 41–43). In addition, antibodies to EBNA-1 may not appear for several weeks or months after acute infection and may be low or difficult to detect in patients with immunodeficiency (12).

Three different EBV open reading frames are known to code for viral capsid proteins, BcLF1 (p135), BdRF1 (p40), and BFRF3 (p18 or p21) (31, 41, 43). Epitopes within EBV BFRF3, or small VCA (sVCA), are known to be immunodominant for the humoral response in EBV-infected humans and are routinely used in diagnostic serologic assays for EBV infection (34, 40–42). High sVCA antibody titers can be detected relatively early in acute primary EBV infection, i.e., infectious mononucleosis, and typically persist at high levels even in immunosuppressed hosts (20, 41). Therefore, we cloned the sVCA homologue from the rhesus LCV and developed a pep-

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enzyme-linked immunosorbent assay (ELISA) to determine whether antibody responses to the rhesus LCV sVCA are a sensitive and reliable indicator of rhesus LCV infection.

MATERIALS AND METHODS

Cell lines. LCL 8664 is a rhesus monkey LCV (cercopithecine herpesvirus 15)-infected B-cell line derived from retro-orbital B-cell lymphoma in a rhesus monkey (26). LCL 8664 was grown in RPMI medium supplemented with 10% fetal bovine serum. COS-7 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum.

Cloning of the rhesus LCV BFRF3 and EBER homologues. Genomic DNA from LCL 8664 cells was digested with a *SalI*-constructed cosmid library and screened as described previously (28). Rhesus LCV sVCA was cloned by PCR amplification from the rhesus LCV cosmid clone QA15, using EBV-specific primers (5' GAGGTAGAATTGCCACCTGG 3' and 5' TTCGTGAGCCAGC TTCGCCG 3') at reduced stringency. Multiple PCR products were cloned to derive the nucleotide sequence, and the open reading frame was cloned into pSG5 (Stratagene) for eukaryotic expression. The rhesus LCV EBV-encoded small RNA 1 (EBER1) and EBER2 homologues were identified by screening subclones of the rhesus LCV CCI cosmid by cross-hybridization with the EBV *EcoRI* J DNA fragment encoding the EBERs.

Expression of rhesus LCV sVCA and Western blot analysis. Recombinant sVCA expression constructs (30 µg) were transfected into COS-7 cells by electroporation. Twenty-four hours after transfection, cells were washed with phosphate-buffered saline (PBS) and lysed in 1× Laemmli buffer. Total cellular proteins were resolved by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis, transferred to nitrocellulose filters (Schleicher & Schuell), and immunoblotted with a 1:100 dilution of rhesus sera. A cassette Mini-protein II system (Bio-Rad) was used to screen multiple rhesus sera at one time. Filters were subsequently incubated with a goat anti-human IgG reagent coupled to horseradish peroxidase and detected with enhanced luminol and oxidizing reagents (NEN Life Science).

Peptide synthesis. Two peptides representing the carboxy-terminal domains of rhesus LCV sVCA were synthesized by standard fluorenylmethoxycarbonyl chemistry. Peptide 1, AASAPAATPAVSSSISLRAATSGAAAASSA, corresponds to amino acids (aa) 117 to 146 of the rhesus LCV sVCA. Peptide 2, AVDTGSGGGAQPQDSTRGARKKQ, corresponds to aa 147 to 170 of rhesus LCV sVCA. Peptides were purified to >80% purity by reverse-phase high-performance liquid chromatography.

Peptide ELISA. Peptides 1 and 2 were resuspended in bicarbonate buffer (50 mM; pH 9.6) and used (0.5 µg in 200 µl) for overnight coating of Immulon 1 microtiter wells (Dynatech Laboratories, Inc., Chantilly, Va.) at 4°C. Unbound peptides were washed with PBS containing 0.1% Tween 20 and blocked with PBS (0.1% Tween 20, 3% bovine serum albumin [BSA]) for 2 h at room temperature. Rhesus monkey serum was diluted 1:100 in PBS (0.1% Tween 20, 3% BSA) before incubation and added to 96-well plates. After 1 h at room temperature the wells were washed and incubated with horseradish peroxidase-conjugated anti-serum to human IgG diluted in PBS (0.1% Tween 20, 3% BSA). Peroxidase activity of bound conjugated antibodies was developed using *O*-phenylenediamine dihydrochloride tablets (Sigma Biosciences, St. Louis, Mo.). The absorbance at 450 nm was read after a 30-min incubation using a Bio-Rad microplate reader. Cutoff values were set at 3 standard deviations above the mean absorbance from triplicate wells with secondary antibody and no sera.

Real-time RT-PCR. A two-step reverse transcription (RT)-PCR was optimized for the quantitation of rhesus LCV EBER1 using TaqMan technology (Roche Molecular Systems, Inc.) and a GeneAmp sequence detector (model 5700) during the PCR. The EBER1 forward and reverse primer sequences were 5' G GAGGAGATGAGTGTGACTTAAATCA 3' and 5' TGAACCGAAGAGAG CAGAAACC 3', and the probe sequence was labeled with a fluorescent reporter (FAM) at the 5' end and a fluorescent quencher (TAMRA) at the 3' end (5' CCCGTCTTACCACCCGGGA 3').

Total RNA was isolated from 5 million Ficoll-Hypaque-purified peripheral blood mononuclear cells (PBMC) using TRIzol reagent (GIBCO BRL). The first step of the RT reaction was carried out using random hexamers and 400 ng of total RNA in a volume of 50 µl according to the manufacturer's protocol (PE Applied Biosystems). The PCR mixture was prepared as follows: 2× TaqMan universal PCR master mix, 10 µl of cDNA, 200 nM EBER1 probe, and a 75 nM concentration of each primer were mixed in a final volume of 50 µl and PCR amplified for 40 cycles (15 s at 94°C, 30 s at 60°C, 30 s at 72°C). Serial dilutions of a recombinant rhesus LCV EBER1 plasmid of known concentration were used as a PCR standard. The limit of detection for the real-time RT-PCR assay was determined to be 50 copies. Rhesus EBER1 RT-PCR products were also analyzed by electrophoresis through a 3% agarose gel. The DNA was transferred to a nylon membrane and probed with a ³²P-labeled EBER1 oligonucleotide at 65°C. The blots were washed with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (with 0.5% sodium dodecyl sulfate) at 65°C three times for 15 min.

Nucleotide sequence accession numbers. The nucleotide sequences for the rhesus LCV sVCA, EBER1, and EBER2 homologues have been submitted to GenBank (accession no. AF227123, AF227124, and AF227125).

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ATGGCACCGCGGCTGCCAAGCCACCCCTCCAGGGAGGCTGGAGGCGGATTTTCATGAC
M A R R R L P K P T L Q G R L E A D F H D
AGCCCCCTGCTTCTAAAATTCAAGAGCTGCAGCAGGGCAATCTCCCAACGACGTTTTC
S P L L P K F Q E L Q Q G N L P N D V F
CGGGAGGCTCAGAGGAGCTACCTGGCCTTTCTGACGGCCAGTCTGTACGAGGATAC
R E A Q R S Y L A F L T A Q S C Y E E Y
GTGCAGCGGACCTTTGGGGTGCCTCGGGCCAGCGCGCCCTGGACAGAGGAGCGGGCC
V Q R T F G V P R R Q R G L D K R Q R A
GGCGGTGCGCCAGGTTAGTCACTCTCACC CGCGCGCGCCCTCGGCCCGCCAGGGC
G G A A Q V S H S H P A G A A S A A Q G
CAGCAGCGCAGCCCTGCGCAGGGGTGGCTTAGCGGGGGCAGCAGCGGCTGCCTGTGCC
Q Q Q A Q P V A G G G L A G A A A A A S T
CCGGCTGCGACCCCGCGCTCTCCTCGTATAGTAGCTGCGGGCCCGCCATCTGGA
P A A T P A V S S S I S S L R A A T S G
CGGCAGCCTCTCGGGCGCGTTGACACCGGCTCCGGCGCGGGCCCGCCCAAGAC
A A A S S A A V D T G S G G G A Q P Q D
ACCTCCAGCAGGGCGCGCGCAAGACGATAG
T S T R G A R K K Q *

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B

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RhBFRF3  MARRLPKPTLQGRLEADPHDPSLLPKFQELQGNLNDVREARQSYLAF 50
EBVBFRF3 MARRLPKPTLQGRLEADFPDPSLLPKFQELQGNLNDVREARQSYLVP 50
*****

RhBFRF3  LTAQSCYEEYVQRTFGVPRRQRGLDKRQRAGGAQVSHSHPAGAASAAQ 100
EBVBFRF3 LTSQFCYEEYVQRTFGVPRRQRGLDKRQRASVAGAGAH-LGSSATPV 99
*.* ***** *.* *.*

PEPTIDE-1

RhBFRF3  QQAQPVAGGGLAGAAAAS---APAATPAVSSSISLRAATSG---AAA 143
EBVBFRF3 QQAQAAASAGTGALASSAPSTAVASATPVSSSSISLRAATSGATAAS 149
**** * * * * * * * * * * * * * * * * * * * * * * * * * * *

PEPTIDE-2

RhBFRF3  SAAAVDTGSGGGAQPQDSTRGARKKQ 170
EBVBFRF3 AAAAVDTGSGGGQPHDTPRGRARKKQ 176
..***** *.* *.*

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FIG. 1. Sequence analysis of the rhesus LCV sVCA. (A) Nucleotide and predicted amino acid sequence of the rhesus LCV sVCA. (B) Amino acid comparison between rhesus LCV sVCA and EBV sVCA p18 (p21). Identical amino acids are represented by an asterisk, similar amino acids are represented by a period, and gaps are indicated with a hyphen.

RESULTS

Cloning of rhesus LCV sVCA. The rhesus LCV homologue for the EBV sVCA was cloned by PCR amplification using EBV-specific primers at reduced stringency from a rhesus LCV cosmid clone, QA15. Three PCR clones were sequenced to derive the nucleotide and amino acid sequence shown in Fig. 1A. Overall the rhesus LCV sVCA shows 69% amino acid identity with the EBV sVCA (Fig. 1B). To determine whether the antibody response in rhesus LCV-immune animals is directed at the sVCA gene product, the rhesus LCV sVCA was expressed after transfection of COS-7 cells, and lysates of vector control- or rhesus LCV sVCA-transfected cells were used for Western blotting with sera from five randomly selected rhesus monkeys in the conventional colony at the New England Regional Primate Research Center (NERPRC). As shown in Fig. 2, a unique 18- to 21-kDa band is detected by all five sera in rhesus LCV sVCA-transfected cells (Fig. 2A) but not in vector control-transfected cells (Fig. 2B). Thus, the rhesus LCV sVCA appears to be immunogenic in rhesus monkeys. However, the high background levels relative to the specific signal in these Western blots were emblematic of the difficulties that may arise in distinguishing between naive animals and those with lower antibody responses.

Rhesus LCV sVCA ELISA. In order to develop a more rapid, less labor-intensive, and more discriminating assay, we tested the potential utility of a rhesus LCV peptide ELISA. EBV sVCA is known to be an important target for the human antibody response to EBV infection, and the immunodominant epitopes are known to reside in the carboxy terminus (aa

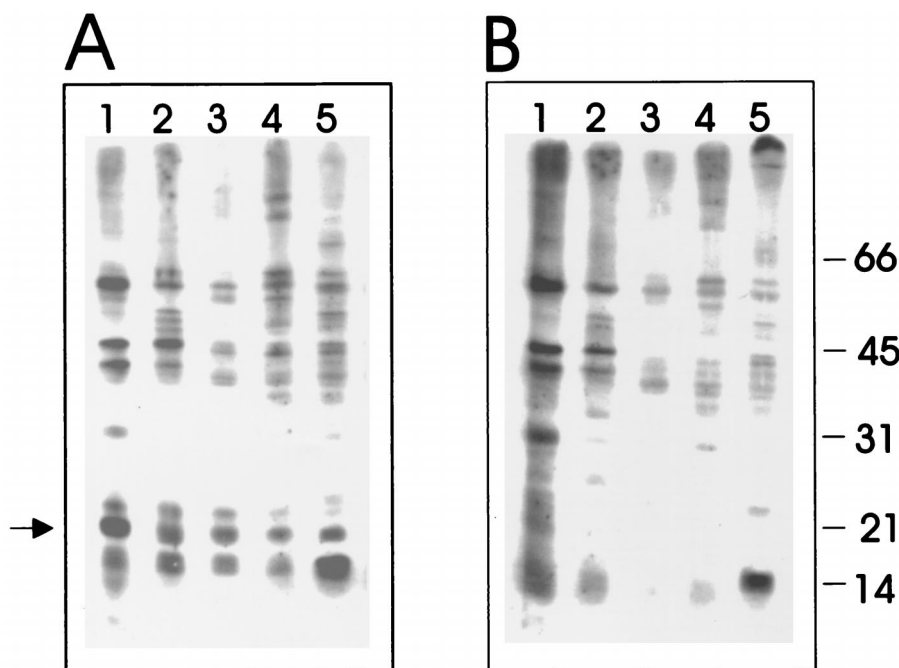


FIG. 2. Expression of rhesus LCV sVCA in COS-7 cells. (A) Immunoblot strips with cell lysates of pSG5 rhesus LCV sVCA-transfected cells; (B) cell lysates of pSG5-transfected cells. Immunoblots containing cell lysates positive (A) and negative (B) for rhesus LCV sVCA were probed with five different LCV-immune rhesus sera (lanes 1 to 5) using a cassette Mini-protein II system (Bio-Rad).

119 to 176) (42). Therefore, we synthesized two peptides from similar regions of the rhesus LCV sVCA carboxy terminus (peptide 1, aa 117 to 146, and peptide 2, aa 147 to 170) (Fig. 1B). We used these peptides individually and in combination to test serologic responses in two populations of rhesus monkeys. The first population consisted of 20 randomly selected animals from the conventional colony at the NERPRC. Rhesus LCV infection was known to be highly prevalent in this colony (4). The second population was selected from a colony of hand-reared animals (6). These animals were separated early from their mother, reared by hand, and then raised in isolated colonies. This colony was serologically screened to exclude herpes B and D retrovirus infection. These animals were not specifically screened for LCV infection, but we hypothesized that these handling procedures were likely to result in a low prevalence of rhesus LCV infection. The average age of these animals was 1.5 years.

As shown in Fig. 3, none of the hand-reared animals had a detectable rhesus LCV sVCA serologic response by ELISA using peptides 1 and 2 alone or in combination. These animals were also tested for sVCA antibodies by Western blotting using sVCA protein expressed in COS-7 cells, and the results were negative (data not shown). In the conventional colony, sera from 14 of 20 animals reacted to peptide 1 with an optical density greater than 3 standard deviations above background levels. Sera from all 20 animals from the conventional colony had stronger reactivity to peptide 2 and could be easily distinguished from those from the hand-reared colony. The combination of peptides 1 and 2 did not provide any significant advantage over the results with peptide 2 alone. Previous studies of the rhesus LCV strains present in the oropharyngeal washes of the animals from the conventional colony showed an equal prevalence of type 1 and type 2 rhesus LCV (4). Thus, the carboxy terminus of the rhesus LCV sVCA contains immunodominant antibody epitopes independent of viral type.

Currently, there is no "gold standard" for identifying rhesus

LCV infection, making it difficult to validate the sensitivity and specificity of these peptide ELISA results. LCV infection persists for life. However, oropharyngeal shedding of virus is episodic, and PCR detection from oropharyngeal washes is positive for only a fraction of adult, EBV-seropositive humans (45). EBV infection also persists in 1 per 10^5 to 10^6 peripheral blood B cells, a level which is difficult to detect by DNA PCR of PBMC (22). However, the EBERs are expressed at a high copy number, 10^6 to 10^7 copies per infected cell, and could potentially increase the sensitivity of detecting persistent viral infection in the peripheral blood (37). Therefore, we cloned the rhesus LCV EBER homologues and tested whether an EBER RT-PCR could effectively detect persistent LCV infection in rhesus PBMC as an independent test for rhesus LCV infection.

RT-PCR for rhesus LCV EBER expression in rhesus monkey PBMC. The rhesus LCV EBER1 has 73 and 68% nucleotide identity with the EBV and the baboon LCV EBER1 genes, respectively. The EBER2 genes are less well conserved, with only 42 and 38% identity with the EBV and baboon LCV EBER2 (Fig. 4). EBER1 was targeted since EBER1 is more abundant than EBER2 in EBV- and baboon LCV-infected cells (5, 14, 17) and better conserved. PCR primers were designed from EBER1 sequences conserved among all three species to minimize the potential effect of strain-dependent sequence variation.

RT-PCR amplification for EBER1 RNA followed by Southern blot hybridization with an internal oligonucleotide probe was positive for 20 of 20 animals from the conventional colony (results from 16 animals shown in Fig. 5A, lanes 1 to 16). Signal intensity varied among these samples, and the signal was weakly detected in one animal on repeated assays (Fig. 5A, lane 6). All hand-reared animals were negative for EBER1 expression by RT-PCR amplification from peripheral blood lymphocytes (results from 16 animals shown in Fig. 5B, lanes 1 to 16). These results were identical to the rhesus LCV sVCA peptide ELISA results and suggested a nearly 100% preva-

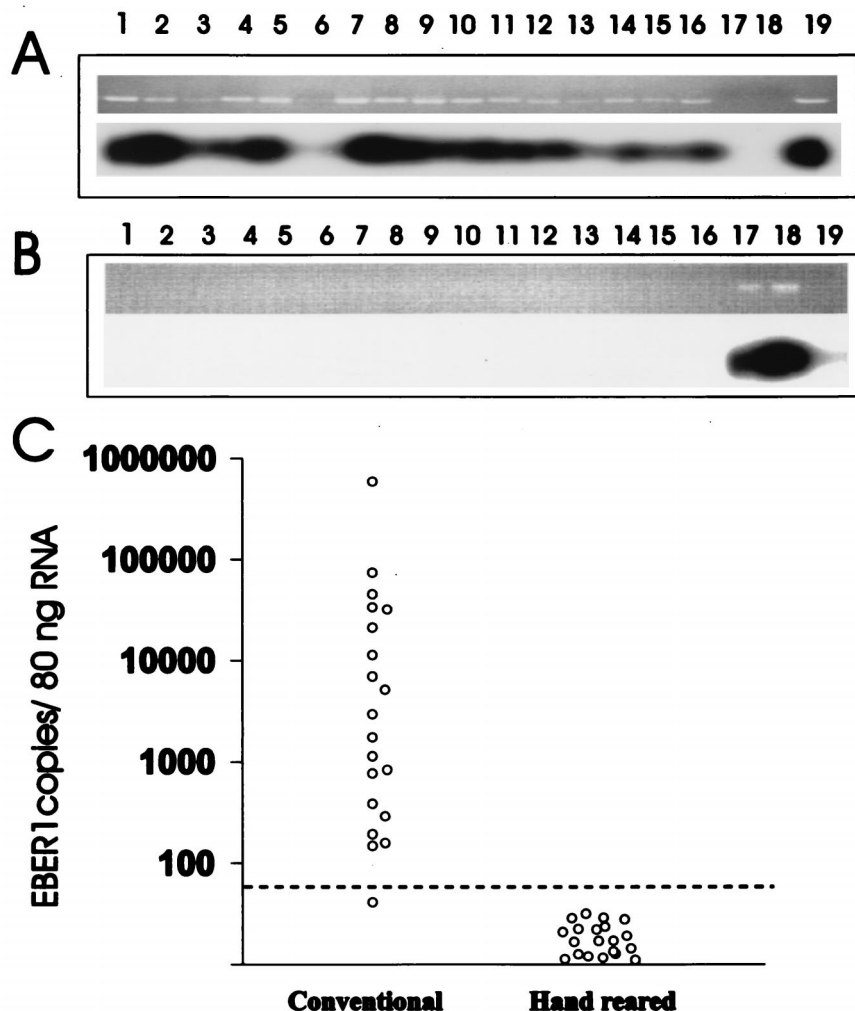


FIG. 5. RT-PCR for EBER1 expression in rhesus monkey PBMC. (A) Southern blot analysis of RT-PCR-amplified products from conventional colony animals. Lanes 1 to 16 represent 16 animals randomly selected from the conventional colony. Positive (lane 19) and negative (lanes 17 and 18) controls are shown. (B) Southern blot analysis of RT-PCR-amplified products from hand-reared colony animals. Lanes 1 to 16 represent 16 randomly selected hand-reared animals. Positive (lanes 17 and 18) and negative (lane 19) controls are shown. (C) Semiquantitation of rhesus LCV EBER1 by real-time RT-PCR. The EBER1 copy number per 80 ng of total cellular RNA is shown. The limit of detection (50 copies/80 ng of total RNA) is shown by the dotted line.

the rhesus LCV sVCA, with a peak response developing by day 10. Rhesus macaques are also commonly infected with rhesus rhadinovirus (RRV), a gammaherpesvirus in the rhadinovirus subgroup (19, 44). Therefore, to rule out the remote possibility that serologic responses detected with the rhesus LCV sVCA might be due to cross-reactive antibodies specific for the RRV capsid protein, we tested sera from macaques experimentally inoculated with RRV. We obtained acute- and convalescent-phase sera from four animals with documented RRV seroconversion after experimental RRV inoculation (19) and tested these samples in the rhesus LCV sVCA peptide ELISA. Two animals were seropositive for rhesus LCV sVCA antibodies prior to infection, and the antibody titers did not change after RRV inoculation (Fig. 6B). Two animals were rhesus LCV seronegative prior to RRV inoculation and remained negative as shown by the rhesus LCV sVCA peptide ELISA. The previously documented RRV seroconversion in these specimens (19) confirmed that antibodies to the RRV VCA do not cross-react with the rhesus LCV sVCA.

DISCUSSION

We have developed highly sensitive and reproducible assays for acute and persistent rhesus LCV infections. As in humans, LCV infection is highly prevalent in nonhuman primates and is associated with B-cell malignancies in immunosuppressed hosts (8, 26). In addition, experimental infection of rhesus monkeys with the rhesus LCV provides the only animal model which accurately reproduces many aspects of acute and persistent EBV infections in humans (24). As these studies demonstrate, it is possible to breed a rhesus LCV-naive colony which would reduce the risk of LCV-induced malignancies in immunosuppressed animals and provide naive animals for pathogenesis and vaccine studies after experimental rhesus LCV infection.

The rhesus LCV sVCA is moderately well conserved with the EBV sVCA (69% amino acid identity), falling approximately halfway between the most and least well conserved lytic genes studied to date (90 to 50% amino acid identity) (15a, 23). The greatest sequence divergence is in the middle of the

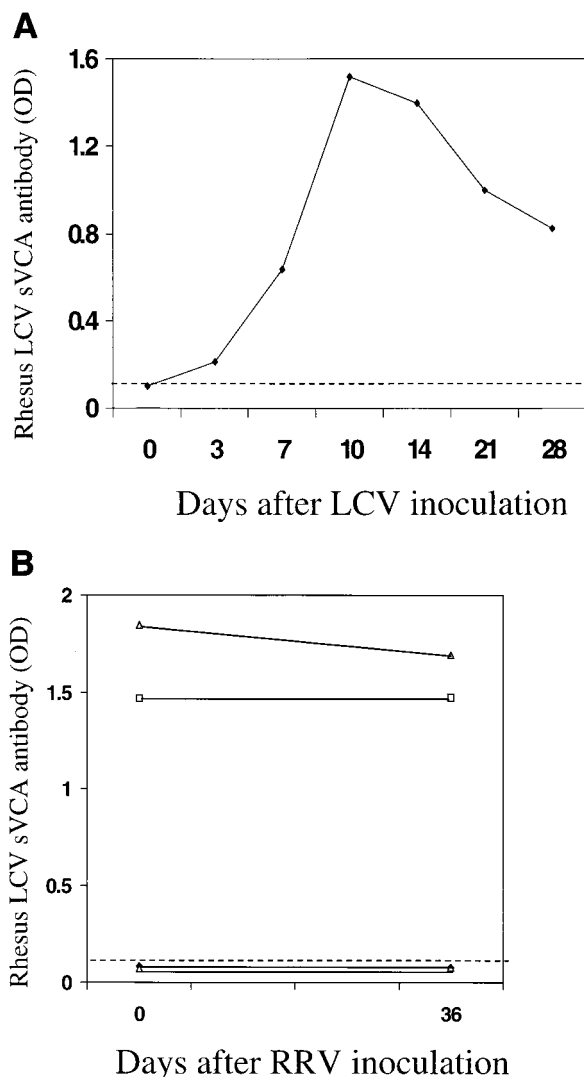


FIG. 6. Rhesus LCV sVCA peptide ELISA results from an animal experimentally inoculated with rhesus LCV (A) and from four animals experimentally inoculated with RRV (B). The dotted line represents the ELISA cutoff value.

protein and overlaps positionally with our peptide 1 (aa 117 to 146), whereas the region represented by peptide 2 was more well conserved. In rhesus macaques the antibody responses to peptide 2 were clearly more prevalent in rhesus LCV-infected animals, whereas human antibody responses to the regions represented by peptides 1 and 2 are equally prevalent (42). The observed sequence divergence and potential differences in immunodominant epitopes may contribute to a loss of sensitivity when using the EBV sVCA as a cross-reactive antigen for detecting rhesus LCV-immune sera. The rhesus LCV VCA peptide 2 epitope was particularly useful since it not only identified all positive animals but also was associated with an extremely robust signal, clearly separating positive and negative results.

The sensitivity and specificity of the VCA peptide ELISA were also tested using sera from experimentally infected animals. First, a brisk antibody response was detected in an animal experimentally infected with rhesus LCV, similar to that previously detected by immunoblotting (24). Second, the specificity of the assay was checked using sera from animals experimentally infected with RRV. Rhadinoviruses are the herpes-

viruses most closely related to the LCV subgroup, and RRV infection is also highly prevalent in rhesus monkeys (19, 30, 44). The RRV VCA homologue shares only 24% amino acid identity (30), and the absence of any cross-reactivity could be confirmed by studying two animals who remained rhesus LCV seronegative after experimental RRV infection and documented RRV seroconversion (19). This specificity is similar to the lack of serologic cross-reactivity reported between the sVCAs of Kaposi's sarcoma-associated herpesvirus and EBV (18).

Since we had no gold standard for rhesus LCV infection, we also cloned the rhesus LCV EBER1 and EBER2 homologues and developed an EBER1 RT-PCR assay as an independent parameter for rhesus LCV infection. These two assays measuring different aspects of rhesus LCV lytic and latent infections gave identical results which further validate the accuracy of these assays. The ability to directly measure rhesus LCV RNA in the peripheral blood will also be an important tool for studying LCV pathogenesis in experimental infections. The RT-PCR assay is a sensitive indicator for persistent infection since EBER RNA expression could be detected in at least 95% of the animals. This rapid assay will be an important tool for experimental pathogenesis studies in which viral mutants with specific genetic lesions will be tested for their ability to establish persistent infection. The relatively broad range of EBER RNA expression levels was somewhat surprising and probably reflects a combination of different precursor frequencies of LCV-infected cells in the peripheral blood and different amounts of EBER RNA per quantity of infected cells. Therefore, it may be difficult to identify quantitative effects on viral persistence using simple quantitation of EBER RNA levels in the peripheral blood, given this broad range of EBER expression levels. Detecting more subtle quantitative effects on viral persistence will likely require limiting dilution analysis scored by an EBER RT-PCR assay to determine the precursor frequency of virus-infected cells (22).

Conservation of the EBER genes also suggests that they play an important role for the pathogenesis of LCV infection in vivo. The EBERs are expressed in LCV-immortalized B-cell lines in vitro, but genetic studies demonstrate that they can be deleted from the EBV genome with no detectable effect on virus replication or B-cell immortalization in tissue culture (36). Thus, one predicts that the EBERs must provide a function which is important for successful LCV infection in vivo but not necessarily in vitro. The EBERs have sequence similarity and can functionally replace the adenovirus VA RNAs (29), which block interferon-induced responses by inhibiting activation of an interferon-induced protein kinase and phosphorylation of the protein synthesis initiation factor eIF2 alpha (1, 2). The EBERs have also been reported to bind the interferon-inducible protein kinase PKR (33), the ribosomal protein L22 (7, 38, 39), cellular La protein (11, 15, 17), and (2'-5') oligoadenylate synthetase (32). However, there is no obvious differential phenotype observed when B cells infected with wild-type EBV or EBER deletion mutant EBV are challenged with interferon in vitro (35, 36). The cloning and identification of the EBER genes from rhesus LCV are the first step towards generating an EBER deletion mutant of rhesus LCV and using the rhesus animal model to test the hypothesis that the EBERs are essential for successful LCV infection in vivo.

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