

Antibodies to Lytic Infection Proteins in Lymphocryptovirus-Infected Rhesus Macaques: a Model for Humoral Immune Responses to Epstein-Barr Virus Infection[∇]

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Humoral immune responses to rhesus lymphocryptovirus (rhLCV) lytic infection proteins were evaluated in the rhesus macaque animal model for Epstein-Barr virus (EBV) infection. We found a hierarchy of humoral responses to 14 rhLCV lytic infection proteins in naturally infected rhesus macaques, with (i) widespread and robust responses to four glycoproteins expressed as late proteins, (ii) frequent but less robust responses to a subset of early proteins, and (iii) low-level responses to immediate-early proteins. This hierarchy of humoral responses was similar to that reported for EBV-infected humans, with the notable exception of the response to rhBARF1. Serum antibodies to rhBARF1 were frequently detected in healthy rhLCV-infected macaques, but in humans, anti-BARF1 antibodies have been reported primarily in patients with EBV-positive nasopharyngeal carcinoma (NPC). The macaque data accurately predicted that serum antibodies against BARF1 are a normal response to EBV infection when human serum samples are analyzed. The rhesus macaque animal provides a unique perspective on humoral responses to EBV infection in humans and can be a valuable model for EBV vaccine development.

Epstein-Barr virus (EBV) encodes over 60 different proteins during lytic virus replication, including (i) immediate-early (IE) proteins that act principally as transcriptional activators to initiate the cascade of lytic gene expression, (ii) early (E) proteins directed at a variety of functions, including gene regulation, immune evasion, nucleotide metabolism, and DNA replication, and (iii) late (L) proteins, most of which are virion proteins (26). Serum antibody responses to lytic infection proteins are commonly used to diagnose EBV infection.

Induction of humoral immune responses to EBV lytic infection proteins is also important for EBV vaccines. Antibodies against gp350, the major membrane glycoprotein (BLLF1), are capable of neutralizing EBV infection (24), and recent clinical trials showed that a gp350 subunit vaccine can induce EBV-neutralizing antibodies (7, 22) and protect humans from EBV-induced infectious mononucleosis (IM) (31). Protection was not complete, but this ground-breaking trial provided proof of principle for a vaccine strategy against EBV-induced disease. Since antibody responses are the foundation of most successful virus vaccines, it is not unreasonable to speculate that induction of better humoral immune responses against EBV gp350 or induction of antibody responses against other EBV lytic infection proteins may enhance efficacy of an EBV vaccine. However, testing these hypotheses in human studies can be prohibitive.

Rhesus macaques are naturally infected with an EBV-related herpesvirus, or lymphocryptovirus (LCV), that encodes a repertoire of viral proteins identical to that of EBV and which

biologically mimics EBV infection in humans with, e.g., oral transmission, asymptomatic persistent latent infections in peripheral blood B cells, lytic replication and viral shedding from the oral cavity, and association with malignant disease (38). EBV-related herpesviruses are found only in humans and non-human primates, and infection is tightly restricted to primate cells (19). Thus, models in other small laboratory animals require reconstitution of a human immune system for EBV infection (16), use of more distantly related gammaherpesviruses (32), or injection of EBV-infected B cells with tumor engraftment as the endpoint (21). Naive rhesus macaques can be experimentally infected by oral inoculation with rhesus lymphocryptovirus (rhLCV), providing a highly accurate experimental model for vaccine development and pathogenesis studies. The rhesus macaque model provides a distinct advantage due to a genome that bears a repertoire of viral genes identical to that of EBV (28), experimental infection of a natural host by the normal route of transmission (20), reproduction of a natural host-pathogen relationship resulting in persistent infection (20), and the potential for virus-induced malignancies (27).

We have previously demonstrated that the small viral capsid antigen (sVCA; rhBFRF3) is strongly immunogenic in rhLCV-infected macaques, as in EBV-infected humans, and can be used in serologic assays to distinguish between rhLCV-infected and -naive animals (25). In the current study, we evaluate a range of rhLCV lytic infection proteins in order to better understand the repertoire of immunogenic lytic infection proteins in rhLCV-infected macaques. These studies provide a comprehensive picture of humoral immune responses to lytic infection proteins in a natural, nonhuman primate host infected with an EBV-related herpesvirus. A comparison of humoral immune responses in LCV-infected macaques and humans provides insight into EBV serologic tests and the

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soundness of the rhesus macaque model for EBV vaccine development.

MATERIALS AND METHODS

Animals. Rhesus macaques were housed at the New England Primate Research Center (NEPRC). Serum samples were collected from 38 rhesus macaques between 1 and 18 years of age from a conventional colony and from 10 animals from a specific-pathogen-free colony. Stored serum samples from 3 rhLCV-naïve macaques experimentally infected by oral rhLCV inoculation 8 years prior to the study were also available. Results of experimental rhLCV infection have been previously reported (27). All animal work was performed with approval from the Committee on Animals for Harvard Medical School, and all animals were maintained in compliance with federal and institutional guidelines for animal care. Natural and experimental rhLCV infections were confirmed by serum immunoassay for positive IgG responses against the sVCA (rhBFRF3) (25).

Human sera. Deidentified serum samples were obtained from 15 EBV-seronegative and 35 EBV-seropositive human donors whose EBV statuses were determined by serologic assays for serum antibodies against EBNA and the viral capsid antigen in a clinical diagnostic laboratory. Serum samples were obtained from 47 patients with nasopharyngeal carcinoma (NPC) as part of an Institutional Review Board (IRB)-approved research study at the Dana Farber/Harvard Cancer Center. All NPC cases were confirmed to be EBV associated by detection of EBV-encoded small RNAs (EBERs) in tumor cells using *in situ* hybridization. Enzyme immunoassay (EIA) measurements of IgA antibodies to EBNA-1/early antigens (EA) in NPC sera were performed as described by the manufacturer (Bio-Check Laboratories Ltd., Taiwan).

Recombinant vaccinia viruses. Open reading frames (ORFs) for rhLCV lytic infection proteins were amplified by PCR and cloned into a modified pAbt4587 plasmid vector containing an N-terminal Flag epitope. All plasmid clones were sequenced to confirm the integrity of the ORF. pAbt4587 clones containing individual rhLCV lytic infection genes were recombined into the vAbt33 vaccinia virus strain as previously described (18). Recombinant vaccinia viruses were cloned by plaque purification and analyzed for expression by immunoblot analysis of infected cells.

Immunoblotting. Protein samples were separated by 8%, 10%, or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The blots were blocked for 1 h at room temperature in 5% milk, phosphate-buffered saline (PBS), 0.1% Tween 20 and incubated for 1 h with a murine monoclonal antibody specific for the Flag epitope (M2; Sigma). The blots were washed, incubated for 1 h with a horseradish peroxidase-conjugated anti-mouse IgG antibody, and developed with Western Lightning chemiluminescence reagent (PerkinElmer).

Expression and purification of rhLCV proteins. BSC40 cells were infected with recombinant vaccinia viruses at a multiplicity of infection of 10. Cells were harvested 12 to 16 h after infection and resuspended in lysis buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% NP-40). The cell lysates were sonicated for 10 s, and insoluble debris was removed by centrifugation (12,000 × *g* for 10 min). The Flag-tagged proteins were purified overnight at 4°C using M2 antibody coupled to agarose beads (Sigma) and eluted with 3×Flag peptide (Sigma). Recombinant proteins were analyzed by SDS-PAGE separation and immunoblotting with M2 antibody. Protein concentrations were determined by spectrophotometry and Coomassie blue staining after SDS-PAGE separation.

Expression and purification of EBV BARF1. Full-length EBV BARF1 was cloned into a pcDNAneo plasmid with a C-terminal Flag epitope and confirmed by sequencing. The recombinant plasmid was transiently transfected into 293T cells (Effectene; Qiagen). Cell-free supernatants were collected 36 h posttransfection. Secreted recombinant BARF1 was immunopurified overnight at 4°C using M2 antibody coupled to agarose beads (Sigma) and eluted with 3×Flag peptide. The affinity-purified protein was quantified by comparison with albumin standards on SDS-PAGE separation and silver staining (Bio-Rad).

Enzyme-linked immunosorbent assays. Affinity-purified rhLCV lytic infection proteins and EBV BARF1 were diluted in bicarbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃; pH 9.6) at approximately 1 μg/ml, and 100 μl/well was incubated overnight at 4°C to coat 96-well polystyrene microtiter plates. Plates were washed 3 times with PBS containing 0.1% Tween 20 (PBS-T) and blocked with I-Block-PBS-T (PBS, 0.1% Tween 20, 0.3% I-Block [Applied Biosystems]) for 2 h at room temperature. Human and macaque serum samples were diluted 1/50 (for IgG detection) and 1/20 (for IgA detection) in I-Block-PBS-T, and 100 μl per well was incubated for 1 h at room temperature. Plates were then washed and incubated with horseradish peroxidase-conjugated goat anti-human IgG anti-

TABLE 1. LCV lytic infection proteins selected for study

rhLCV protein	% aa similarity to EBV homologue ^a	Function	Presence of serum antibody response in humans ^b
BZLF1	71	Transactivator	Yes
BRLF1	76	Transactivator	Rare
BALF2	90	DNA binding protein	Yes
BARF1	75	CSF1-R homologue	NPC
BMRF1	86	DNA processivity factor	Yes
BMLF1	80	mRNA export factor	Unknown
BALF5	95	DNA polymerase	Rare
BHRF1	73	Bcl2 homologue	Rare
BALF4	86	Glycoprotein B	Yes
BILF2	75	gp78	Yes
BLLF1	49	gp350	Yes
BDLF3	47	gp150	Yes
BBRF1	93	Capsid protein (portal protein)	Unknown
BVRF2	66	Capsid protein (protease)	Unknown

^a aa, amino acid.

^b “Yes” indicates that serum antibody responses to that protein are widespread and robust in humans. “Rare” indicates that serum antibody responses to that protein are frequent but less robust in humans. “Unknown” indicates that serum antibody responses in humans have not been described for that protein. “NPC” indicates that serum antibody responses are present primarily in NPC patients.

body (Jackson ImmunoResearch), diluted 1/1,000 in I-Block-PBS-T for detection of macaque IgG or diluted 1/7,500 for detection of human IgG. An anti-human IgA murine monoclonal antibody, coupled to horseradish peroxidase (Invitrogen) and diluted 1/500 in I-Block-PBS-T, was used for detection of human serum IgA antibodies. All enzyme-linked immunosorbent assays (ELISAs) were measured for peroxidase activity after 20 to 40 min of incubation by the addition of *O*-phenylenediamine dihydrochloride (Sigma) at 450 nm using a Bio-Rad microplate reader. All specimens were tested in duplicate, and LCV-negative sera, LCV-positive sera, and controls with no sera were included in all assays.

RESULTS

Expression of recombinant rhLCV lytic infection proteins.

Fourteen rhLCV lytic infection proteins were selected for analysis of serum antibody responses in rhLCV-infected macaques (Table 1). These included the rhLCV homologues for the two EBV immediate early (IE) proteins (rhBZLF1, rhBRLF1), 6 proteins homologous to EBV early (E) proteins (rhBALF2, rhBARF1, rhBMRF1, rhBMLF1, rhBALF5, and rhBHRF1), and 6 proteins homologous to EBV late (L) proteins (rhBALF4, rhBILF2, rhBLLF1, rhBDLF3, rhBBRF1, and rhBVRF2). This subset of lytic infection proteins includes (i) representatives from all three classes of LCV lytic infection proteins (IE, E, and L), (ii) viral proteins with different sizes, functions, and cell localization, and (iii) viral proteins that are both known and not known to induce antibody responses in EBV-infected humans.

Each of the selected rhLCV lytic infection proteins were expressed from recombinant vaccinia viruses with an N-terminal Flag tag to facilitate detection and purification. BSC40 cells were infected with individual recombinant vaccinia viruses, and cell lysates were analyzed for protein expression by immunoblotting with an anti-Flag antibody (Fig. 1). All of the recombinant proteins were expressed at relatively high levels, and all except 2 recombinant proteins migrated with relative molecular masses comparable to that of the EBV homologue, suggesting similar sizes and posttranslational modifications. Two of the recombinant rhLCV proteins (rhBDLF3 and rhBVRF2)

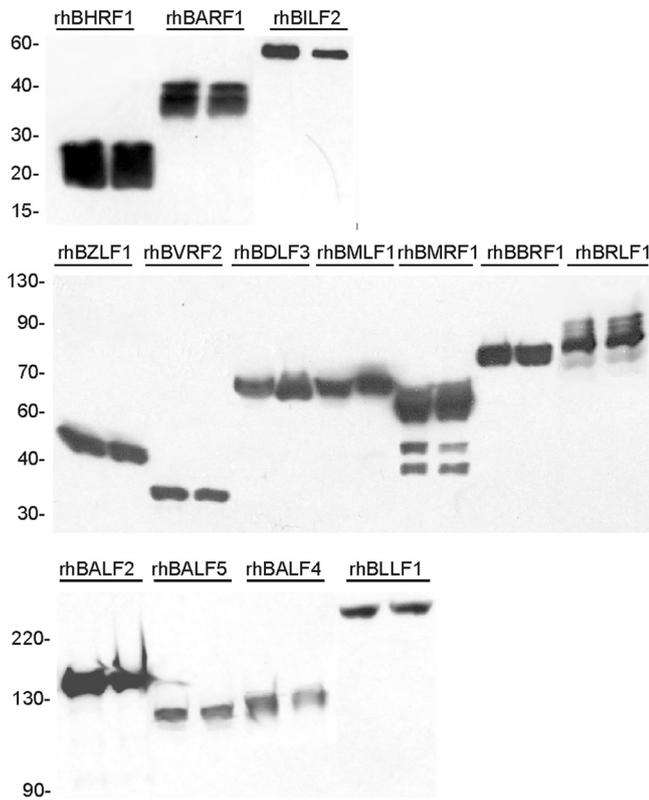


FIG. 1. Immunoblot analysis of epitope-tagged rhLCV lytic infection protein expression by recombinant vaccinia viruses. BSC40 cells were infected with independent, plaque-purified clones of recombinant vaccinia viruses expressing 14 rhLCV lytic infection proteins. Proteins from whole-cell lysates were separated by 8, 10, or 15% SDS-PAGE and probed by immunoblotting with an anti-Flag antibody. Results from samples from duplicate infections for each recombinant vaccinia virus are shown. Molecular mass markers in kilodaltons are shown.

migrated with smaller relative molecular masses than the EBV homologues. rhBDLF3 migrated at approximately 68 kDa, compared to 150 kDa for the EBV homologue. Partially glycosylated BDLF3 has been reported to migrate at approximately 68 kDa, and fully glycosylated BDLF3 migrates with an apparent molecular mass of approximately 150 kDa (23). The preferential detection of the partially glycosylated rhBDLF3 in our studies was most likely due to loss of the N-terminal Flag epitope upon cleavage of the signal peptide, i.e., the Flag epitope is mostly likely to be retained by intracellular rhBDLF3 which may not have fully progressed through the Golgi apparatus. In support of this interpretation, transient transfection of an rhBDLF3 expression plasmid with a C-terminal epitope tag revealed expression of both 150- and 68-kDa species (data not shown). We used the recombinant N-terminally tagged rhBDLF3, since previously reported studies also used a partially glycosylated BDLF3 as a target antigen for serologic studies in EBV-infected humans (23). Recombinant rhBVRF2 was detected migrating at approximately 32 kDa, compared to 80 kDa for the EBV homologue (2). EBV BVRF2 is a protease important for capsid formation that undergoes autoproteolytic cleavage (2). The full-length protein migrates with a relative molecular mass of 80 kDa, and detection of a 32-kDa rhBVRF2 containing an N-terminal epitope

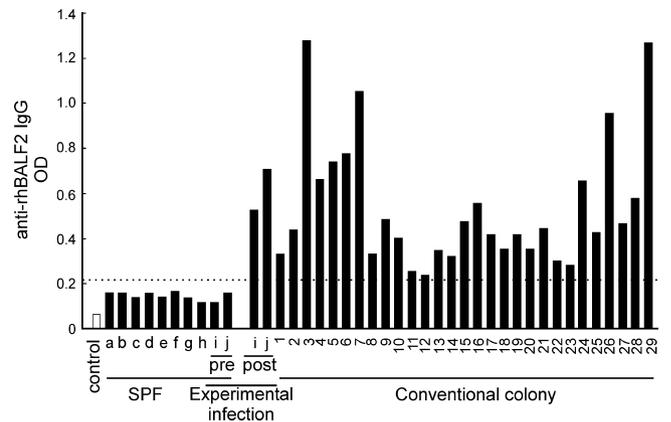


FIG. 2. ELISA detection of serum antibodies against recombinant rhBALF2. Ninety-six-well plates were coated with recombinant, immunopurified rhBALF2 protein and incubated with serum samples (black bars) from (i) 10 rhesus macaques raised in a superclean specific-pathogen-free (SPF) colony (a to j) and (ii) 29 rhesus macaques raised in a conventional colony (1 to 29). Results for serum samples from 2 SPF animals (i, j) before (pre) and after (post) experimental infection with oral inoculation are shown. Control wells (white bar) were treated in a similar manner but with no serum added. The average optical density (OD) from duplicate wells is shown. The dotted line indicates the cutoff value for determining positive results, defined as 3 times the value of the control wells.

tag was consistent with expression of a functional precursor, autocleavage, and detection of the N-terminal fragment.

ELISA for serum antibody responses in rhLCV-infected macaques. All 14 recombinant proteins were purified from cell lysates by anti-Flag immunoprecipitation, quantified by gel electrophoresis and spectrophotometry, and used as antigens for detection of serum antibody responses by ELISA. Sera were obtained from known rhLCV-naive and rhLCV-infected populations for analysis. Serum samples from rhLCV-naive animals were obtained from 10 rhesus macaques in the “super-clean” specific-pathogen-free (SPF) colony at the New England Primate Research Center. These animals were isolated shortly after birth and are screened regularly for the absence of several viral infections, including rhLCV. We have previously demonstrated that animals in the superclean SPF colony are free of rhLCV infection, as determined by serologic tests using an immunodominant peptide from the sVCA (rhBFRF3) and by reverse transcription-PCR (RT-PCR) of peripheral blood mononuclear cells for rhEBER expression (25).

Serum samples from rhLCV-positive animals were obtained from 38 rhesus macaques in the conventional colony and 3 superclean SPF animals after experimental infection with rhLCV by oral inoculation (27). All of these animals tested positive for serum antibodies to the sVCA peptide.

Figure 2 shows a representative ELISA to detect serum antibodies against rhBALF2. Microtiter wells were coated with affinity-purified, recombinant rhBALF2, incubated with or without serum, followed by secondary antibodies coupled to peroxidase and developed with substrate. Thresholds for determining positive results were set at three times the background levels of control wells with no serum (Fig. 2, dotted line). All 10 serum samples from rhLCV-naive macaques in the

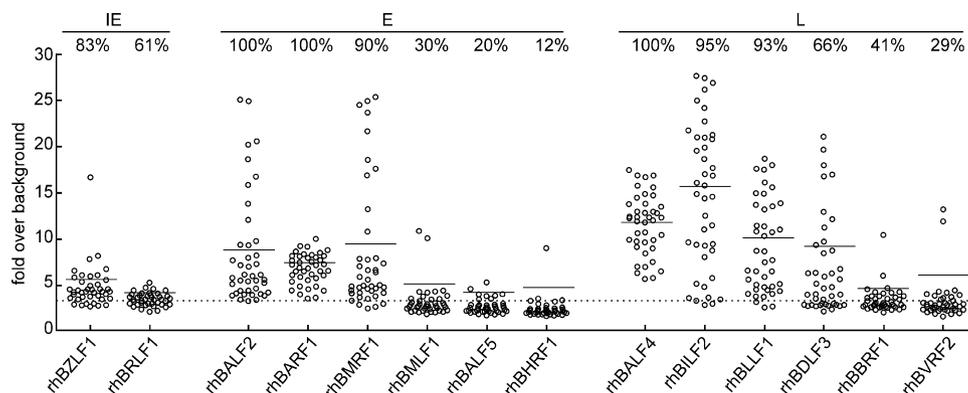


FIG. 3. Serum antibody responses to 14 rhLCV lytic infection proteins in experimentally and naturally infected rhesus macaques. Results from 41 rhLCV-infected macaques tested by ELISAs for serologic responses against immediate early (IE), early (E), and late (L) lytic infection proteins are shown. Each circle represents results for an individual animal, and the cutoff value, defined as 3 times background control levels, is shown as a dotted line. Results are expressed as the fold increase over background control values. The percentage of donors positive for each antigen is indicated at the top. The mean fold increase of all positive responses against a specific protein is indicated by the solid horizontal line.

superclean SPF colony (Fig. 2, animals a to j) were negative for antibodies to rhBALF2 with this cutoff level, whereas all of the serum samples from 29 naturally infected macaques in the conventional colony had levels greater than the threshold and tested positive for IgG antibodies against rhBALF2. Serum specimens from 2 superclean animals (Fig. 2, animals i and j) who were experimentally infected with rhLCV by oral inoculation tested negative before inoculation (pre) and showed evidence of seroconversion in specimens collected more than 1 year after experimental inoculation (post). These results indicated that the ELISA using the affinity-purified, vaccinia virus-expressed, recombinant rhBALF2 protein was specific for rhLCV infection and sufficiently sensitive to detect serum antibody responses in 100% of rhLCV-infected macaques.

ELISAs using the other 13 recombinant rhLCV lytic infection proteins were performed with serum samples from all 38 animals with natural rhLCV infection and from 3 animals with experimental infection. Serum samples from rhLCV-naive animals consistently tested below the cutoff (3-fold above background) for each protein, confirming specificity of the ELISAs for each rhLCV lytic infection protein (data not shown). Results of serologic testing against all 14 recombinant rhLCV lytic infection proteins in rhLCV-infected animals are shown in Fig. 3.

The most robust serum antibody responses were detected against a subset of L proteins. Antibody responses to rhBALF4, rhBILF2, rhBLLF1, and rhBDLF3 were detected in 100%, 95%, 93%, and 66% of rhLCV-infected macaques, respectively, with average magnitudes of 11.5-, 15.1-, 9.5-, and 8.8-fold above the background level, respectively (Fig. 3). Serum antibody responses against rhBHRF1 and rhBVRF2 were detected at lower frequencies (41% and 29%, respectively) and at lower magnitudes (4.3- and 5.2-fold above background, respectively). The development of serum antibodies against rhBVRF2 was strongly associated with age, since 6/18 animals between 7 and 18 years of age were positive, whereas serum antibodies against rhBVRF2 were not detected in any of the 23 animals between 1 and 3 years of age ($P = 0.0002$, Fisher's exact test), suggesting that serum antibodies to rhBVRF2 may arise in response to viral replication over time. The serum

antibody response to rhBHRF1 was not significantly associated with age.

Strong and frequent serum antibody responses were also detected against three E proteins (rhBALF2, rhBARF1, and rhBMRF1) (Fig. 3), whereas only weak and occasional antibody responses were detected against three other E proteins (rhBMLF1, rhBALF5, and rhBHRF1) (Fig. 3). The average serum antibody responses against rhBALF2, rhBARF1, and rhBMRF1 were 8.4-, 6.7-, and 9.1-fold above the background level, respectively, and antibody responses were detected in 100%, 100%, and 90% of rhLCV-infected animals, respectively. In contrast, antibody responses to rhBMLF1, rhBALF5, and rhBHRF1 were detected only in 30%, 20%, and 12% of infected animals, respectively, with average magnitudes of 4.8-, 4.0-, and 4.3-fold above background, respectively. The development of antibody responses against rhBHRF1 and rhBALF5 was also more associated with the higher age group (7 to 18 years old) than with the lower age group (1 to 3 years old) (rhBHRF1, 5/18 versus 0/23, $P = 0.01$; rhBALF5, 6/18 versus 2/23, $P = 0.05$; Fisher's exact test).

Serum antibody responses were frequently detected against both IE proteins (rhBZLF1, 83%, and rhBRLF1, 61%) (Fig. 3), but the magnitude of antibody responses to both IE proteins was generally modest (5.1- and 3.8-fold above the cutoff, respectively).

Kinetics of serum antibody responses to rhLCV lytic infection proteins in rhLCV-infected macaques. Serial serum samples were assayed from two rhLCV-naive rhesus macaques experimentally infected by oral inoculation (SPF animals i and j) (27). Serum antibody responses to rhBALF4 were detected in both animals at day 56 postinoculation and rapidly rose to high titers that persisted for as long as 8 years postinfection (Fig. 4A). The serum antibody response to rhBILF2 demonstrated a rapid rise and persistence in SPF animal i that were similar to those of the serum antibody response to rhBALF4. Anti-rhBILF2 responses in SPF animal j were more delayed and of lower magnitude than those in animal i but still showed persistence at easily detectable levels for 8 years postinfection (Fig. 4A).

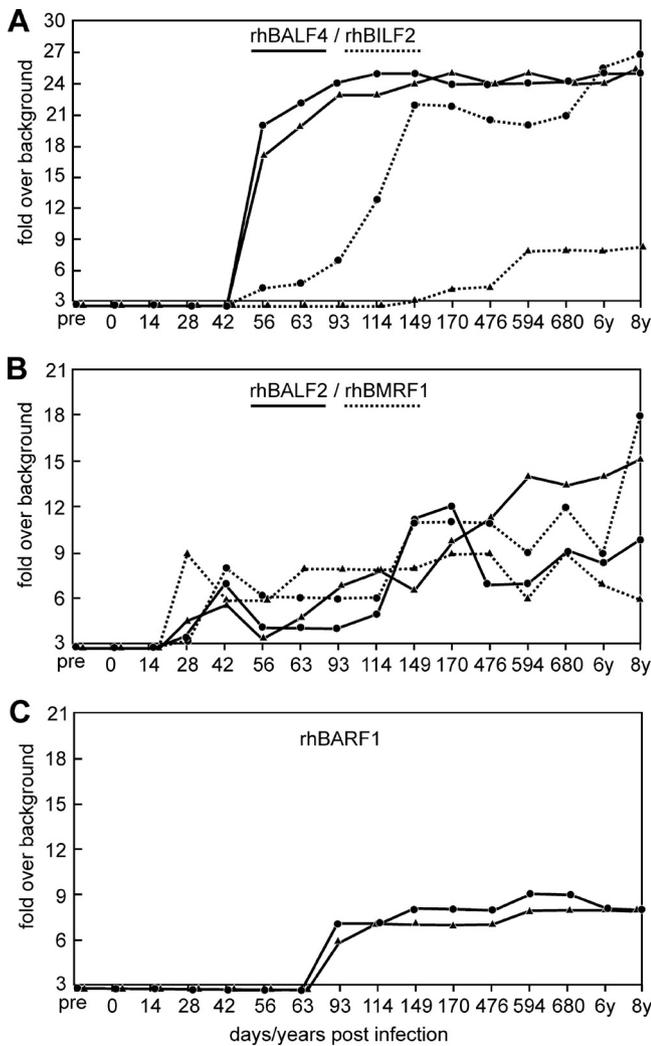


FIG. 4. Kinetic analysis of serum antibody responses against rhLCV E and L proteins. Two rhLCV-naive rhesus macaques (SPF animal i, ●; SPF animal j, ▲) were experimentally infected with rhLCV by oral inoculation. Results for serum samples from before inoculation (pre and day 0) and after inoculation (days 14 to 680, 6 years, and 8 years) are shown. Results from the averages of duplicate wells were plotted as the fold increase over background control values. (A) Serum antibody responses against L proteins rhBALF4 and rhBILF2; (B) serum antibody responses against E proteins rhBALF2 and rhBMRF1; (C) serum antibody responses against E protein rhBARF1.

Serum antibody responses to the E proteins, rhBMRF1 and rhBALF2, were detected early in both animals (Fig. 4B, day 28) and demonstrated relatively low and fluctuating levels. Serum antibody responses to rhBARF1 arose later (Fig. 4C, day 93 postinoculation) and persisted at lower levels with less fluctuation than that observed for antibodies against rhBMRF1 and rhBALF2.

Anti-BARF1 antibody responses in healthy EBV-infected donors and patients with EBV-associated NPC. Our data showing anti-rhBARF1 antibodies as a natural and frequent response to LCV infection in rhesus macaques was in contrast to reports that anti-BARF1 serum antibodies are found primarily in patients with EBV-associated NPC (12, 33).

Interest in anti-BARF1 antibodies as a potential biomarker for NPC was also stimulated by the detection of BARF1 mRNA in NPC tumor tissue (1). We developed an ELISA to detect anti-BARF1 antibodies in sera from healthy EBV-positive donors and NPC patients to determine whether humoral immune responses to BARF1 were different in EBV-infected humans than in rhLCV-infected macaques. EBV BARF1 was purified from the supernatant of 293T cells transiently transfected with a recombinant C-terminal Flag-tagged EBV BARF1 expression vector as the target antigen in the ELISA. Recombinant vaccinia virus protein was not used because of the potential for cross-reactivity in humans who had received the smallpox vaccination. In addition, the use of recombinant protein purified from transfected 293T cells was similar to the approach used in a previous study (12).

A total of 97 human serum specimens from three different donor groups were analyzed for serum antibodies against BARF1: 15 EBV-seronegative individuals, 35 healthy EBV-seropositive individuals, and 47 patients with EBV-associated NPC. As shown in Fig. 5A, anti-BARF1 serum IgG antibodies were not detected in any of the EBV-negative donors but were detected in 66% of the healthy EBV-seropositive individuals and in 70% of the NPC patients. The magnitude of the response was slightly higher in NPC patients than in healthy EBV-seropositive individuals (mean, 11.2- versus 8.9-fold over background), but neither the frequency nor the magnitude of anti-BARF1 antibody responses in NPC patients was significantly different from those in healthy donors with persistent EBV infection. These results suggest that humoral responses against BARF1 can frequently be detected in healthy EBV-infected humans, as in LCV-infected macaques.

Since the detection of serum IgA antibodies against certain EBV antigens can be a useful biomarker for NPC (9), we asked whether the development of serum IgA antibodies against BARF1 might be uniquely associated with NPC patients. Seven EBV-negative donors and 8 NPC patients known to have positive serum IgA responses against a combination of EBNA-1 and early antigen by testing with a commercial assay are shown in Fig. 5B. IgA responses to recombinant BARF1 were not detected in any of the sera (Fig. 5B), even though 6 of the 8 NPC patients had a positive IgG response to BARF1 (data not shown). As a positive control for the detection of serum IgA antibodies, we could detect IgA responses to recombinant EBNA-1 in the sera of these NPC patients by using immunopurified EBNA-1 expressed from transfected EBV-negative BJAB cells as the target antigen in an ELISA (Fig. 5C). In total, we screened sera from 41 NPC patients, and there was no serum IgA response against BARF1 detected in any of the NPC patients, even though an IgG response against BARF1 was detectable in 32 of the NPC patients and 15/32 had a positive IgA response against other EBV antigens by commercial assay (data not shown). Thus, serum IgG antibodies against BARF1 were frequent in both LCV-infected humans and macaques, and we found no association of IgA or IgG antibodies against BARF1 with NPC patients.

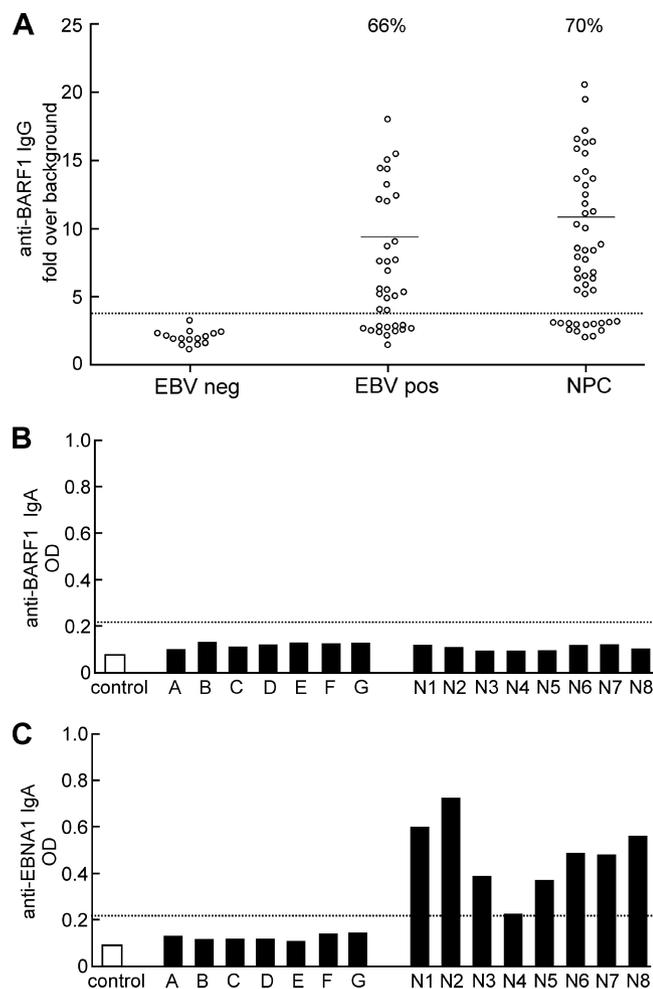


FIG. 5. Serum antibodies against BАРF1 in healthy humans and NPC patients. The dotted line represents the cutoff value of 3 times the background control levels. (A) IgG responses against BАРF1 in sera from 15 EBV-negative donors (EBV neg), 35 healthy EBV-positive donors (EBV pos), and 47 patients with EBV-associated NPC (NPC). Results are plotted as the fold increase over background control values (no serum added). Each circle represents an individual serum donor. Solid horizontal lines represent the mean values of positive responses against BАРF1 in healthy EBV-positive individuals (8.9) and NPC patients (11.2) ($P = 2.046$, t test). The percentage of positive responses is shown at the top ($P = 0.75$, Fisher's exact test). (B) Serum IgA responses against BАРF1 from 7 EBV-negative donors (A to G) and 8 NPC patients (N1 to N8) who tested positive for IgA antibodies to EBV antigens by a commercial assay. Results are plotted as the average optical density (OD) of duplicate wells. (C) Serum IgA responses against EBNA-1 were detected in the same group of NPC patients (N1 to N8) but not in the EBV-negative donors (A to G).

DISCUSSION

We found a hierarchy of humoral responses to 14 rhLCV lytic infection proteins in naturally infected rhesus macaques, with (i) widespread and robust responses to four glycoproteins expressed as L proteins, (ii) frequent but less robust responses to a subset of E proteins, and (iii) low-level responses to IE proteins. Expression and purification of recombinant proteins from mammalian, LCV-negative cells ensured that the antigens were pure, free from contamination with other rhLCV

proteins, and likely to have natural conformation and post-translational modifications. Serum samples from populations of known rhLCV-naive and -infected animals were used to confirm assay specificity.

How similar are the humoral immune responses in rhLCV-infected macaques to those in EBV-infected humans? A comprehensive analysis of serum antibody responses using a consistent assay with the same set of EBV-infected humans is not available for comparison. Thus, a single snapshot for the repertoire of serum antibody responses against lytic infection proteins in EBV-infected humans must be compiled from different studies using (i) a variety of antigens, including infected cells, peptides, bacterial fusion proteins, and recombinant proteins, (ii) multiple assay methods, including immunofluorescence, immunoblotting, and ELISA, and (iii) separate patient populations.

It is clear from published studies that serum antibody responses to L proteins in EBV-infected humans are similar to those in rhLCV-infected macaques. Humans have strong and almost universal serum antibody responses against three L proteins, with response rates as high as 100% reported for BALF4, BLLF1, and BILF2 (17, 29, 39), and these three L proteins were also strongly immunogenic in macaques. Less frequent responses to BDLF3 were seen in both humans (40 to 50% [23]) and macaques with similar, partially glycosylated antigens. Serum antibody responses in humans have not been described against two L proteins, rhBBERF1 and rhBVERF2, which had low immunogenicity in macaques.

We have previously shown that nearly all rhLCV-infected macaques develop serum antibody responses against the sVCA (rhBFRF3) (25). Clinical EBV serologic tests routinely use viral capsid proteins, i.e., L proteins forming the structure of the nucleocapsid, as opposed to viral glycoproteins embedded in the virus lipid envelope or membrane of infected cells producing infectious virus. Antibody responses against the major VCA (BcLF1) and sVCA (BFRF3) are known to be immunodominant in EBV-infected humans (30, 35–37), and recombinant versions for either of these proteins are commonly used in commercial assays. Thus, the pattern of humoral responses against various L proteins, including both capsid proteins and glycoproteins, appears well conserved in LCV-infected macaques and humans.

Comparing serum antibody responses to IE and E proteins in healthy humans to those in macaques is more complicated. Serum antibodies against BZLF1 have been reported to occur in 29 to 74% of EBV-infected humans (34, 36), but anti-BRLF1 antibodies have been detected in only 2% of healthy human donors (4). Anti-BZLF1 antibody titers were typically low in healthy human donors (36), and immunoblotting may have contributed to the low detection rate for anti-BRLF1 antibodies (4). We found low-magnitude rhBZLF1 and rhBRLF1 responses in macaques, with significant numbers of specimens near the assay cutoff, so small differences in assay sensitivity may result in relatively large differences in the apparent positivity rate. Thus, it is likely that LCV-infected humans and macaques share low-level humoral responses to IE proteins. Measuring antibodies to either IE protein is not typically used for clinical diagnostic tests, even though expression of IE proteins is sufficient to initiate lytic viral replication and they are the earliest indicators for viral reactivation.

E proteins are easily confused, and often interchanged, with early antigens (EA). There are more than 30 different E proteins that are defined by expression kinetics, i.e., requiring protein synthesis after viral infection but not viral DNA replication for expression. In contrast, EA were originally defined by distinctive staining patterns, diffuse or restricted (EA-D or EA-R), in immunofluorescence assays using immune sera on EBV-infected cells undergoing lytic replication (11). Many immunoassays for EA antibodies now use recombinant E proteins, e.g., BMRF1 (an EA-D), a processivity factor essential for EBV DNA polymerase activity, or BALF2, the major DNA binding protein.

We found frequent humoral responses to rhBMRF1 and rhBALF2 in healthy macaques, with a few robust responders but with a larger population having more modest responses that were closer to the cutoff. Heterogeneous responses to E proteins have also been reported in healthy EBV-infected humans based on the target antigen and assay. IgG antibody responses to BMRF1 using the BioPlex platform were detected in 79% of patients determined to have past EBV infection as confirmed by medical record review (15). In contrast, IgG antibodies to BMRF1 and BALF2 were reported to be present in approximately 10% and 30% of healthy blood donors, respectively, based on different ELISAs (6), or in only 8% of healthy donors, based on a combined BMRF1/BALF2 ELISA (3). Differences may be due to assay sensitivity, since EA antibody titers were reported to fall over time, with 51% of patients having detectable EA antibodies by immunofluorescence 40 to 104 months after acute IM and with most at low titers (between 1:10 and 1:40) (13). Thus, assay sensitivity may again be an important determinant of seropositivity rates for these antigens, but BMRF1 and BALF2 do appear to be some of the most immunogenic E proteins in both LCV-infected macaques and humans.

What do antibody responses to E proteins mean? Tests for EA antibodies are typically positive in patients with acute primary EBV infection, although the presence of positive IgM antibodies against VCA and the absence of antibodies against EBNA are more reliable indicators of acute infection (11). After acute primary infection, positive EA antibodies in the presence of IgG antibodies to VCA and EBNA are often interpreted to indicate reactivation of EBV infection (13). However, there is no clinical syndrome associated with reactivation of EBV lytic replication in healthy humans. Studies show that healthy humans continuously replicate and shed EBV from the oral cavity (8), so a high prevalence of low-level antibodies to E proteins may not be unexpected when lytic infection proteins must be continuously expressed. Thus, the value of antibodies to E proteins as a routine diagnostic marker for the level of EBV lytic viral replication in otherwise healthy humans appears to be marginal. Elevated antibody responses against E proteins and other lytic infection proteins have been associated with development of EBV-associated NPC (10). However, the etiology of this NPC biomarker is curious, since the tumor cells are latently infected and do not express most lytic infection proteins.

Why do L proteins stimulate a more frequent and robust antibody response than IE or E proteins in LCV-infected macaques and humans? Responses to IE and E proteins may be muted by viral immune evasion genes that inhibit the inflam-

matory response around infected cells undergoing lytic replication, e.g., viral interleukin-10 (IL-10) and CSF-1R homologues. L proteins in the virion may be capable of inducing more potent antibody responses because they are intrinsically more antigenic or because they deliver antigen away from the muted immune environment surrounding cells undergoing lytic replication, e.g., release from a replicating cell or during superinfection of a host. The L protein BBRF1 is a capsid protein that forms the portal for packaging nucleic acids, but since there is only a single portal per capsid, BBRF1 is present in much lower quantities than the other structural capsid proteins that are highly immunogenic, e.g., BcLF1 and BFRF3 (14). BVRF2 is a protease that associates with and is necessary for capsid formation, but only minute quantities remain in mature virions (14). This model may explain the pattern of low-level antibodies to IE and E proteins and high-level antibodies to virion proteins that is shared by both LCV-infected macaques and humans.

Several lines of evidence suggest that LCV lytic replication in healthy macaques may be slightly more robust or immunogenic than in humans, e.g., a slightly higher detection rate for antibodies to E proteins (rhBMRF1, rhBALF2, and rhBARF1), increased responses to less immunogenic E and L proteins in older macaques, higher numbers of rhBZLF1-specific cytotoxic T cells in older macaques (5), and increasing rhBMRF1 and rhBALF2 titers in experimentally infected macaques over time. Macaques may experience more frequent LCV superinfection than humans because they are constantly being challenged with LCV from other animals due to close proximity in captive group housing. Endogenous virus may also reactivate more frequently in macaques than in humans, since rising antibody titers to rhBMRF1 and rhBALF2 were observed over time in experimentally infected macaques housed in single units, i.e., not exposed to superinfection.

We found that antibodies to BARF1 are a common result of LCV infection in macaques and humans and found no association in patients with EBV-associated NPC. The explanation for the differences from previous studies is not clear. Tanner et al. detected anti-BARF1 antibodies in patients with NPC and IM using either a bacterial fusion protein or an assay for antibody-dependent cellular cytotoxicity (ADCC) (33). The inability to detect anti-BARF1 antibodies in healthy EBV-infected donors may have been due to decreased sensitivity associated with functional ADCC assays or with nonglycosylated target antigens produced in bacteria (33). Hoebe et al. prepared their BARF1 antigen from transfected 293T cells in an approach similar to ours (12). They detected antibody responses in healthy donors but did not establish a cutoff or describe a positivity rate; their NPC patients were reported to have significantly higher anti-BARF1 antibody levels than the healthy donors. There may be differences associated with different patient populations, since Hoebe et al. studied a population of NPC patients primarily from Indonesia, whereas our population of NPC patients was derived from a mixture of ethnic and geographic backgrounds.

Our studies indicate that the humoral immune responses to LCV lytic infection proteins in rhesus macaques and humans are similar, i.e., those proteins that were strongly immunogenic in macaques were also strongly immunogenic in humans and

vice versa. The analysis of humoral immune responses in rhesus macaques provides a unique perspective for understanding serum antibody responses in EBV-infected humans. Rhesus macaques provide an accurate model for humoral immunity to EBV lytic infection proteins in humans and can be a valuable animal model for developing a more effective EBV vaccine.

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