Detection of Epstein-Barr Virus Genomes in Normal Human Lacrimal Glands

CECELIA A. CROUSE,^{1,2*} STEPHEN C. PFLUGFELDER,² TIMOTHY CLEARY,^{1,3}
STEPHEN M. DEMICK,² AND SALLY S. ATHERTON^{1,2}

Department of Microbiology and Immunology, University of Miami Medical School,¹ and Departments of Ophthalmology² and of Pathology and Microbiology,³ Jackson Memorial Hospital, Miami, Florida 33101

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Epstein-Barr virus (EBV) has been implicated in several ocular diseases; however, detection of the EBV genome in ocular tissues has not been documented. We report the detection of amplified EBV genomic sequences in 11 of 26 normal lacrimal gland DNA samples by using the polymerase chain reaction. Serum was available for 19 of the lacrimal gland donors. All 19 were EBV seropositive, although of the 19 lacrimal gland-seropositive patients, EBV sequences were detected in only 10 of the samples. Further, amplified EBV sequences were not detected in circulating lymphocyte DNA from normal seropositive volunteers, most likely because of the low frequency of circulating EBV-infected B cells. Amplification of EBV from cadaver lacrimal gland DNA was possible with minute quantities of DNA, whereas peripheral blood mononuclear cell DNA from normal volunteers did not amplify EBV sequences. Interestingly, the peripheral blood mononuclear cell polymerase chain reactions contained approximately 100 times more DNA than the lacrimal gland polymerase chain reactions. We conclude that the lacrimal gland may be a site for EBV persistence and that positive EBV serology is not an indicator of which individuals may have EBV harbored within their lacrimal glands.

Epstein-Barr virus (EBV) is a member of the gamma herpes group of viruses. The majority of adults worldwide have antibodies to EBV, indicating prior infection with this virus. EBV is the agent responsible for infectious mononucleosis, and this virus has been associated with several human malignancies, including nasopharyngeal carcinoma, Burkitt's lymphoma, thymoma, and B-cell lymphomas in immunosuppressed individuals (3, 8, 15, 17, 21, 30, 44). EBV nucleic acid sequences have been detected in these neoplastic tissues as well as in oropharyngeal epithelial cells, parotid gland ducts, cervical epithelium, and circulating B lymphocytes of previously infected individuals (7, 10, 14, 19, 35, 40).

The EBV genome within latently infected cells may be present as an extrachromosomal circular DNA molecule or may be integrated into the host genome (18, 26). Specific latency-associated proteins (EBV nuclear antigens [EBNAs]) are frequently detected in latently infected cells (16, 29). Serum antibodies to EBNA appear weeks to months after primary infection and are used as serologic markers of past infections (26).

Normal individuals periodically shed EBV into their saliva after primary infection (2, 27, 28, 37, 42). This virus most likely originates from epithelial and/or lymphoid cells lining the oropharynx (1, 12, 35, 40). The amount of viral reactivation in these cells appears to be influenced by the state of the immune system, because patients with acquired immunodeficiency syndrome or those receiving immunosuppressive medications shed more virus than immunocompetent individuals (6, 20, 45).

Other mucosal tissues are possible sites of EBV latency. The human conjunctival sac and oropharynx have many similarities. Both are epithelium-lined cavities lubricated by secretions from exocrine glands; secretions from the lacrimal glands (LG) lubricate the eyes, and secretions from the

salivary glands lubricate the mouth. Both the LG and salivary glands are components of the mucosal-associated lymphoid tissue (11). These tissues contain secretory immunoglobulin A (IgA)-producing plasma cells in close approximation to acinar epithelial cells (39). Furthermore, the literature cites several ocular diseases associated with acute or chronic EBV infections, such as bilateral uveitis, keratitis, and follicular conjunctivitis (23, 24, 36, 41) as well as autoimmune dysfunctions, such as Sjogren's syndrome (9). As a result of similar reports of LG and conjunctival inflammation during infectious mononucleosis and LG lymphoproliferative disorders in acquired immunodeficiency syndrome patients (4, 25), we hypothesized that the normal human LG may also be a site of EBV latency.

We tested this hypothesis by examining LG tissue and peripheral blood mononuclear (PBMN) cells from asymptomatic normal donors for the presence of EBV genomes. Southern hybridization will detect approximately one EBV-infected cell per 10 noninfected cells (26). EBV genomes have been detected in Burkitt's lymphoma and nasopharyngeal carcinoma biopsies by using this technique; however, this technique lacks sufficient sensitivity to detect viral genomes in PBMN cells of infectious mononucleosis patients, in which there is approximately one EBV-infected cell per 10⁶ B cells (38). Because of the minute quantities of LG tissue typically available for analysis, we elected to use the polymerase chain reaction (PCR), a recently developed in vitro DNA amplification technique, to evaluate biopsies of LG cells from normal cadavers and PBMN cells from volunteers for EBV sequences. The PCR method, described by Saiki et al. (32), allows the logarithmic amplification of a specific gene sequence by a thermostable DNA polymerase by using designated primers flanking the desired gene sequence. The sensitivity of this technique is remarkable in that it is theoretically able to amplify a single-copy gene present in a sample containing the DNA from a single cell.

We report herein the detection of EBV genomes within the normal human LG. We conclude that the LG may be a site

* Corresponding author.

† Present address: Bascom Palmer Eye Institute, 1638 N.W. 10th Avenue, Miami, FL 33136.

of latency for EBV. EBV sequences were not detected in PBMN DNA from normal seropositive volunteers.

MATERIALS AND METHODS

DNA preparation. Positive control cell lines containing EBV genomes included three Burkitt's lymphoma cell lines (BL-8, P3HR-1, and Ly-67) and the marmoset cell line B95-8. The negative EBV control was the Burkitt's lymphoma cell line BL-3. Approximately 2×10^6 cells were placed in three volumes of lysis buffer (100 mM NaCl, 10 mM Tris chloride, pH 8.0, 25 mM EDTA, 0.5% sodium dodecyl sulfate [SDS], 0.1 mg of proteinase K per ml), briefly vortexed, and incubated at 37°C for 14 to 18 h. DNA was isolated by phenol-chloroform-isoamyl alcohol extractions followed by ethanol precipitations. Pellets were suspended in 10 mM Tris hydrochloride–1 mM EDTA, pH 7.5. Whole blood (5 ml) from normal volunteers from the University of Miami School of Medicine was placed on a Leukoprep gradient (Becton Dickinson, Mountainview, Calif.) and centrifuged as per the manufacturer's recommended protocol. The buffy coat was removed and frozen immediately at –70°C. Lymphocyte DNA preparation was as described above, with 0.5 ml of buffy coat cells (2×10^6 cells). LG tissues were obtained from cadavers shortly after death. Only donors serologically negative for human immunodeficiency virus and hepatitis B virus at the time of death were used in these studies. These donors ranged from 17 to 60 years old and did not have a history of ocular disorders or systemic diseases with LG involvement, i.e., sarcoidosis, lymphoma, or connective tissue disorders. Histological sections of the LG from most cadaver specimens showed a typical LG morphology, including discernible acini structures. Biopsies were placed in individual sterile foil packets and frozen at –76°C. For tissue preparation, the foil packets were placed in liquid nitrogen and pulverized with a hammer, and the powdered tissue was added to three volumes of lysis buffer. DNA extraction was as described for the cell lines. The DNA was quantitated by using a TKO Fluorometer (Hoeffer, San Francisco, Calif.)

PCR, agarose gel electrophoresis, slot blots, and hybridization of labeled probe. The PCR protocol was based on the method of Saiki et al. (32). The EBV primers and probe specific for a 240-base-pair region in the *Bam*HI-K region of the EBV genome (Fig. 1) were purchased from Synthetic Genetics (San Diego, Calif.). The EBV DNA sequences were 5'-GACGAGGGGCCAGGTACA-3' and 5'-GCAGCC AATGGCAACTTGGACGTTTTTGG-3' for the 5' and 3' primers, respectively, and 5'-CGTCCTCGTCTCTTCCCC GTCCTCGTCCATGGTTATCACC-3' for the probe. The β -globin primers were specific for the delta region of the β -globin gene (31, 32).

LG pre-PCR reactions contained various concentrations of DNA (5 ng to 1.0 μ g). Lymphocyte pre-PCRs contained 1 μ g of DNA. Samples were boiled for 5 min, and then 50 mM KCl, 10 mM Tris chloride (pH 8.3), 2.7 mM MgCl₂, 1 μ M of each primer, 200 μ M deoxynucleoside triphosphates, 200 μ g of gelatin per ml, and 2.5 U of *Taq* polymerase (Cetus, Emeryville, Calif.) were added. Amplifications were carried out by using the DNA Thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.) for 40 cycles. A single cycle consisted of (i) 94°C for 90 min, (ii) 45°C for 120 min, and (iii) 66°C for 120 min with a 1-min autoextension at 66°C. After amplification, 30 μ l of the sample was added to 170 μ l of Tris-EDTA and 20 μ l of 3 M NaOH and incubated at 60°C for 1 h followed by the addition of 220 μ l of 2 M ammonium acetate. Samples

FIG. 1. EBV genomic map (120×10^6 daltons) denoting the location of the 5.2×10^6 -dalton *Bam*HI K fragment. The synthetic oligonucleotides used for amplification in this region produce a 240-base-pair fragment. Terminal tandem direct repeats (RT), unique short (US), unique long (UL), and internal repeats (IR) are indicated.

were slot blotted onto Hybond (Amersham Corp., Arlington Heights, Ill.) filter paper as per the Schleicher & Schuell, Inc. (Keene, N.H.) slot blot protocol. Filters were vacuum baked at 80°C for 30 min, prewashed in 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS–0.5% sodium pyrophosphate–100 μ g of salmon testis DNA per ml for 4 h, and then hybridized (6 $\times$ SSC, 0.5% sodium pyrophosphate, 100 μ g of tRNA per ml) with γ -³²P-end-labeled oligonucleotide probe (specific activity, 2×10^5 cpm/ng) at 42°C for 18 h. The filters were washed with shaking four times at 37°C for 5 min each in 6 $\times$ SSC–0.5% SDS–0.5% sodium pyrophosphate and then for 30 min in 3 $\times$ SSC–0.5% SDS–0.5% sodium pyrophosphate at 60°C. Autoradiography was for 5 h at –76°C on Kodak XAR film with an intensifying screen. Three observers who did not have previous knowledge of the experimental details rated the signals by visually comparing the negative control background to the experimental samples. All DNA samples were initially amplified with primers to the delta β -globin gene region to ensure that the DNA was suitable for amplification. The remaining DNA sample was used for PCR amplification with EBV primers. Gel electrophoresis of the control samples was performed by adding 10 μ l of amplified sample and 2 μ l of running dye (22) to a 1.4% agarose gel, and the DNA was electrophoresed at 35 V for 15 h. Low-molecular-weight DNA (Bio-Rad Laboratories, Richmond, Calif.) standards were used to determine the size of the amplified fragment.

EBV serology. An indirect fluorescence assay (Gull Laboratories, Salt Lake City, Utah) was used for the detection of antibody to EBV viral capsid antigen (EBV-VCA) and EBV early antigen (EBV-EA). An anti-complement immunofluorescence test (Gull Laboratories) was used for the detection of antibody to EBNA.

The assays for IgG and IgM antibodies against EBV-VCA were performed by using P3HR-1 infectious mononucleosis cells. Fluorescein isothiocyanate-labeled caprine anti-human IgM (heavy-chain specific) was used in the IgM assay. A caprine anti-human globulin conjugate was used for the detection of IgG antibody (Clinical Sciences Inc., Whappany, N.J.). An enzyme-linked immunosorbent assay (Clinical Sciences) was performed on five random normal human LG serum samples, as was the immunofluorescence assay. Both tests showed that all five samples were positive. The assays for antibodies against EBV-EA and EBNA used Raji

cells from a Burkitt's lymphoma. The cells were chemically induced in the presence of inhibitors to DNA synthesis to ensure expression of EBV-EA.

A screening dilution of 1:10 was used to detect IgM antibody to EBV-VCA and specific antibody to EBNA. All assays for EBV antibodies were read independently by two observers. Each test included a positive serum control of known titer and a negative serum control. The negative serum control for the IgM antibody assay contained IgG antibody to EBV-VCA.

RESULTS

Verification of EBV *Bam*HI-K primer specificity. The region of the EBV genome that was selected for PCR amplification was within the *Bam*HI-K restriction fragment which encodes an EBNA (EBNA-1) produced by the virus in the latently infected state (13). Sequences in this fragment are located within the unique long region of the genome (Fig. 1). In order to demonstrate that the region selected for amplification is relatively conserved, DNAs from four EBV-positive cell lines (B95-8, P3HR-1, Ly-67, and BL-8) and one EBV-negative Burkitt's lymphoma cell line (BL-3) were amplified with EBV-specific primers as described above. As an internal control to test the integrity of the DNA, the DNAs were initially amplified with β -globin-specific primers, blotted, and then hybridized to the β -globin probe specific for the amplified fragment. All human cell lines (P3HR-1, Ly-67, BL-3, and BL-8) were positive for β -globin, and the marmoset cell line (B95-8) was negative (data not shown).

The predicted 240-base-pair fragment for all EBV-positive controls was observed on an ethidium bromide-stained gel (Fig. 2A). After slot blot hybridization of 10% of the amplified product with the *Bam*HI-K-radiolabeled probe, a positive signal was detected for all EBV-positive cell lines (Fig. 2B). The 240-base-pair amplified fragment was not detected in BL-3-amplified DNA by either ethidium bromide staining or blotting and hybridization with the radiolabeled EBV probe. It is evident that even though BL-3 DNA does not amplify the EBV *Bam*HI-K fragment (Fig. 2A and B), pre-PCR DNA amounts of 500 and 1,000 ng followed by amplification, blotting, and hybridization with the radiolabeled EBV probe do elicit a very weak hybridization background signal after a 5-h exposure (Fig. 3). This signal is not evident at low BL-3 DNA amounts of 10 or 100 ng. In contrast, the B95-8 cell line has a very intense signal for EBV at 10 ng. This degree of background from the BL-3-negative EBV control was consistent and reproducible for all hybridizations. Rating experimental reactions, each of which contained a different pre-PCR DNA concentration, was based on the intensity of the sample signal compared with that of the BL-3 negative control at about the same pre-PCR DNA concentration in order to avoid rating a background signal as positive. The water blank reaction contained all reagents necessary for amplification, except a DNA template. After amplification and a 5-h autoradiograph exposure of the water blank reaction, no radioactive signal was detected. This result suggests that PCR reagents do not contribute to signal background under these conditions (Fig. 3).

Detection of EBV in normal lymphocyte DNA. It has been documented that normal patients who have been exposed to EBV have EBV-infected B cells in their circulating lymphocyte populations (34-36). To determine which normal patients would be likely candidates for detection of EBV genomes in PBMNs by PCR, EBV serological tests were

FIG. 2. DNAs from EBV-positive cell lines B95-8, P3HR-1, Ly67, and BL-8 and an EBV-negative cell line, BL-3, were amplified by PCR by using amplimers specific for the *Bam*HI-K region of the genome, and 10 μ l of the reaction mixture was electrophoresed on a 1.4% agarose gel and stained with ethidium bromide (A). All PCRs contained 1 μ g of DNA. Molecular weight standards (in thousands of base pairs [bp]) are indicated on the left, and the predicted molecular weight of the amplified EBV sequence is shown on the right of the autoradiogram. Amplified products (10 μ l) from the cell line DNAs were blotted onto a nylon filter and probed with a radiolabeled oligonucleotide specific for the *Bam*HI-K region of the EBV genome (B). The autoradiogram was exposed for 1 h. The DNA sample in each blot is indicated on the diagram at the bottom.

performed on 17 normal volunteers from the University of Miami Medical School. This information was used to determine whether the patients had serologic evidence of past, acute, or chronic EBV infection.

The serum from each normal lymphocyte sample identified in Fig. 4 was evaluated by immunofluorescence for antibodies to EBNA and EBV-EA as well as for IgG and IgM antibodies to viral capsid antigens. The serological results for each patient are listed to the right of the autoradiograph (Fig. 4, columns 2, 3, and 4). Antibodies to EBNA appear weeks to months after primary infection and remain persistently detectable (14). All of the 17 normal PBMN volunteers were positive for one or more of the antibodies tested. Serum samples from these 17 donors were negative for the viral capsid antigen IgM, which is indicative of a

FIG. 3. Various concentrations of DNA from the EBV-negative cell line BL-3 and 10 ng of the EBV-positive cell line B95-8 were amplified with primers specific for the *Bam*HI-K region of EBV (30 μ l), blotted onto nylon membranes, and probed with an EBV-K-radiolabeled oligonucleotide. The autoradiogram was exposed for 5 h. The H₂O blank amplified reaction contained all PCR reagents except a DNA template.

current infection (data not shown). These serological results indicate that all 17 normal PBMN samples had EBV antibodies consistent with past or present infection and therefore should have circulating EBV-infected lymphocytes in their blood.

The hallmark of the PCR is its sensitivity. Theoretically, the presence of a single-copy gene would allow amplification of a desired sequence within that gene nearly a million times. Saito et al., by using PCR, estimated that there is one EBV-transformed B cell in 100,000 uninfected-cell DNA equivalents in PBMN DNA from normal subjects with an active EBV infection. We predicted that it would be possible to amplify EBV in 1 μ g of PBMN DNA from EBV-seropositive donors, since this concentration of DNA represents approximately 10^5 cells and lymphocytes are a known site of EBV latency. To test this hypothesis, whole blood was withdrawn from the 17 EBV-seropositive normal volunteers mentioned above. For each sample, 1 μ g of lymphocyte DNA was amplified with the EBV *Bam*HI-K primers (Fig. 4). All lymphocyte DNAs were positive for amplified β -globin fragments (data not shown); however, amplified EBV DNA was not detectable in any of the lymphocyte DNA samples (Fig. 4, lane 1).

EBV sequences were not detected by PCR in 1 μ g of DNA from circulating lymphocytes of these 17 samples, a known site of EBV infection or latency. This finding indicates that >1 μ g of PBMN DNA would need to be screened by PCR in order to detect EBV sequences.

Detection of EBV in normal LG DNA. Due to the paucity of DNA from normal LG samples and the necessity to optimize the total template DNA to be amplified, the DNA concentration within the PCR mixtures varied from 5 ng to 1.0 μ g (Fig. 5). However, all reactions were otherwise treated identically by using the described PCR protocol.

DNA from normal LG biopsies was extracted and amplified with EBV *Bam*HI-K primers and amplified independently with β -globin primers. Thirty percent of each amplified DNA sample was blotted and hybridized to the EBV-radiolabeled probe, and the autoradiograph was exposed for 5 h. Hybridization signal intensities on autoradiographs of experimental samples were visually compared with the negative EBV BL-3 cell line amplified DNA (Fig. 3) and were then rated as a positive or negative for EBV amplification on the basis of the concentration of DNA, as determined fluorometrically, in the sample before amplification.

	1	2	3	4
	EBV-K	EBNA	VCA G	VCA EA
P3H-R1	+	NA	NA	NA
B95-8	+	NA	NA	NA
NL-3	-	-	++++	+
NL-4	-	-	-	+
NL-5	-	+	+	-
NL-6	-	+	++	+
NL-7	-	+	ND	ND
NL-8	-	-	+	-
NL-9	-	+	++	+
NL-10	-	+	++++	+
NL-11	-	+	++++	+
NL-12	-	+	++++	+
NL-13	-	-	++++	-
NL-14	-	+	++++	-
NL-15	-	+	++++	-
NL-16	-	-	+++	-
NL-17	-	+	++++	-
NL-18	-	+	++++	-
NL-19	-	+	ND	ND

FIG. 4. Normal lymphocyte DNA was amplified with primers specific for EBV *Bam*HI-K primers (lane 1). The initial template concentration before amplification for all samples was 1 μ g. After PCR, 30 μ l of amplified product was blotted onto nylon and probed with a radiolabeled oligonucleotide specific for the EBV *Bam*HI-K-amplified fragment. Serum from each sample was tested serologically by using immunofluorescence for IgG (lane 3) or EBV-EA (lane 4). EBNA serology was positive at a 1:10 dilution. Viral capsid antigen IgG was rated as + at 1:10, ++ at 1:40, +++ at 1:80 to 1:160, ++++ at $>1:320$, or negative (-). The EBV-EA EA ratings were + at 1:10 to 1:40 or negative (-). NA, Not applicable; ND, not done.

Therefore, a hybridization signal detected in a sample with 5 ng of DNA pre-PCR that was as intense as the BL-3 (1,000 ng of DNA pre-PCR) background was considered positive for EBV amplification. The PCR reagents and BL-3 at amounts of 100 ng and lower did not contribute to background signal; therefore, amplification of EBV must be the source of the positive signal.

Samples amplified for EBV sequences were electrophoresed on an agarose gel, and extension products were visible for all reactions (data not shown). The products from β -globin amplification were hybridized with a β -globin probe (data not shown). Seven (21%) of the thirty-three normal LG DNAs amplified with EBV primers did not amplify for the β -globin fragment and were not rated as having amplified EBV even when an EBV signal was detected (Fig. 5). Of the remaining 26 LG samples, 15 (61.5%) were negative for EBV

FIG. 5. DNA from normal LG was amplified with primers specific for the *Bam*HI-K region of the EBV genome, and 30 μ l of the reaction was blotted onto nylon and probed with the EBV-K-radiolabeled oligonucleotide. Interpretation of this blot is provided on the right. Signal intensity ratings, positive (+) or negative (-), were based on visual comparisons of the BL-3 negative control for background (Fig. 3). Samples labeled NA were not rated for EBV positivity, since they were negative for β -globin amplification. The amount of DNA in the reaction before amplification is shown to the right of the rating (in nanograms).

and 11 (42.3%) were positive. Although quantitation of EBV copy number within a sample was impossible, we observed an intense hybridization signal for EBV when amplifying only 30 ng of LG DNA and a moderate signal from as little as 5 ng of LG DNA.

EBV serology was performed on serum samples from 19 of the 26 cadaver LG donors. All of these donors were seropositive for viral capsid antigen IgG, EBV-EA, and/or

EBNA antibodies (Table 1), and all were negative for IgM. Positive EBV serology does not appear to be a reliable indicator for the presence of EBV sequences in LG tissues, since EBV sequences could be amplified in only 10 of the 19 seropositive LG donors. Unfortunately, only serum samples were available from these normal cadaver LG donors, and as a consequence PCR analysis for EBV genomes of DNA from cadaver lymphocytes could not be performed. Our results indicate that there is a significantly higher probability of detecting EBV genomes in the DNA of normal LG tissues than in blood cells ($P < 0.002$; Fisher two-tailed exact test).

The results of using the PCR to detect EBV in normal lacrimal tissue are summarized in Fig. 5. Using the most extreme case as an example, when amplifying EBV in 5 ng of normal LG DNA (approximately 10^4 cells), or 99.5% less DNA (5 ng versus 1 μ g) than was used in the lymphocyte amplification study, moderate EBV positivity was evident in the DNA sample. The inability to amplify EBV sequences from 1 μ g of PBMN DNA from normal seropositive patients suggests that the detection of EBV sequences in the normal LG results from cells containing latent EBV permanently residing within the gland rather than the amplification of the EBV sequences in an incidental transient EBV-infected B cell circulating through this gland.

DISCUSSION

We have presented evidence for the presence of EBV sequences in normal LG DNA with PCR. The studies reported herein describe the detection of PCR-amplified EBV sequences in normal LG DNA in 11 (42.3%) of 26 biopsies. By slot blot hybridization and ethidium bromide-stained agarose gels, it was possible to detect amplified EBV sequences from three samples containing less than 10 ng (pre-PCR) of LG DNA and seven samples with 10 to 100 ng of DNA. In contrast, amplification of 1 μ g of PBMN DNA from 17 EBV-seropositive donors was negative for EBV sequences. Serological analysis of LG donors for EBV antibodies did not provide a means to predict whether EBV sequences in LG DNA could be detected by PCR. We have previously been unsuccessful in detecting EBV genomic sequences or EBV antigens in LG tissues of normal individuals by using techniques less sensitive than PCR. Detection of EBV sequences within the LG by PCR does not clarify the infected cell type nor determine whether the amplified EBV sequences are latent EBV genomes or replicating EBV particles, although the inability to detect the virus by less-sensitive techniques would favor the hypothesis that the virus is latent in the LG.

It was hypothesized that the sensitivity of the PCR technique could detect EBV sequences in circulating lymphocyte DNA from EBV-seropositive patients, since circulating B cells are a known site of latency. A previous report of PCR results estimated that approximately 1 in 10^5 circulating B cells is infected with EBV in seropositive individuals (32). The PCR amplification for EBV sequences in each PBMN DNA contained about 10^5 B cells, whereas in the LG DNA PCR the average number of cells was approximately 10^4 . Our inability to detect EBV genomes in 1 μ g of PBMN DNA is most likely due to the low frequency of EBV-infected cells in our samples. Saito et al., using PCR to estimate the number of infected B cells in normal PBMN DNA (1 EBV-infected cell per 10^5 uninfected cells), could detect EBV in 1 μ g of normal PBMN DNA in only 3 of 50 samples (33). The report did not state whether the donors were EBV seropositive. Amplification of B-cell-enriched samples or

TABLE 1. EBV amplification in LG DNA and EBV serological data for normal LG donors

Sample ^a	PCR result for LG EBV	Reaction with antibody to ^b :		
		VCA	EBV-EA	EBNA
A1	-	+++	-	+
A2	+	++	-	+
A3	+	+++	-	+
A4	-	++	-	-
A5	+	++	-	+
A6	-	+++	+	+
A7	+	++	-	+
A10	+	+	-	-
B1	-	+++	-	+
B2	+	+++	-	+
B3	-	+++	-	+
B4	+	++	+	+
B5	-	++	-	+
B6	-	+++	+	+
B7	-	++	-	+
C1	+	+++	-	+
C3	+	++	-	-
C5	-	++	-	+
C6	+	+++	-	+

^a Letters and numbers correspond to columns and rows, respectively, of Fig. 5.

^b Grading of EBV antibody titers is as described in the legend to Fig. 4.

increasing the PCR template concentration may increase the probability of detecting the EBV genome in seropositive individuals.

EBV serology was available for 19 of the 26 cadaver LG donors. All 19 samples were positive for at least one of the EBV antibodies tested, although only 10 of the patients amplified EBV sequences from their LG DNA. It is possible that this may be due to a sampling error. It is not clear why LG may be a site of EBV persistence in some individuals and not in others. It may be necessary to evaluate several EBV-susceptible tissue sites within an individual to determine whether there may be tissues or individual cell types that are preferential sites for EBV persistence. Furthermore, some individuals may be capable of clearing EBV from certain tissue sites. Since we were able to detect EBV in normal cadaver LG DNA but not circulating PBMN DNA from EBV-seropositive donors, we conclude that the LG may be a site of EBV persistence. Alternatively, it is possible that sampling error may account for the inability to amplify EBV sequences in LG DNA from normal EBV-seropositive individuals.

EBV has been shown to replicate in pharyngeal epithelial cells during acute infectious mononucleosis, and viral particles are easily detected in the pharyngeal secretions (7, 8, 14, 19). It has been suggested that normal B cells trafficking through the lymphoid tissue in the regions shedding virus may be transiently infected with EBV and then enter the circulation, where they are readily detected (1). An EBV receptor molecule similar to the CR2 EBV receptor on B cells has been identified on the surfaces of pharyngeal and cervical epithelia (34, 43). After primary infection, periodic shedding of EBV from these cells has been demonstrated, and this may occur when the fine balance between the host and the virus is altered. Patients who are immunosuppressed by disease or receiving immunosuppressive drugs have an elevation of EBV-infected circulating B cells and EBV particles shed in their saliva (14, 20, 27, 35, 39, 42). Evidence suggests that this might be due to a loss of host-specific EBV suppression, leading to increased EBV replication in the blood and nasopharynx. Once the disease is arrested, EBV-infected B cells and EBV shedding in the saliva decrease.

Morphologically, histologically, and physiologically, the parotid gland and LG are very similar (39). The primary function of the LG is lubrication of the eye surface, which is thought to be accomplished by the same mechanism of exocytosis as that used by the parotid glands. Additionally, both contain a large number of IgA-producing plasma cells in close proximity to the secretory acinar cells. Since EBV particles are readily detected in the pharyngeal epithelium and secretions of seropositive individuals (1, 35, 40), it is not surprising that EBV sequences were detected in a tissue similar in function and morphology, i.e., the LG of EBV-seropositive patients. In a disease such as Sjogren's syndrome, in which EBV genomes and extensive B-cell proliferation have been detected in both the parotid and LG (which are eventually destroyed), serious consideration should be given to EBV as a potential risk factor. EBV has also been associated with other ocular diseases, such as follicular conjunctivitis, stromal keratitis, and dendritic epithelial keratitis (23, 24, 36, 41). The potential destruction and loss of function in ocular tissues due to reactivation of latent EBV may be of considerable clinical significance.

The importance of the role of the LG in the maintenance of normal ocular function is unquestionable. The evidence presented here of an infectious agent harbored by the LG in normal individuals defines a potential site for opportunistic

EBV infection not only of the LG but of the entire ocular surface. The pathogenesis of EBV from within the LG would most likely be dictated by the immunological status of the host. The etiology of some ocular disorders, especially for those with depressed cellular immunity or diseases with loss of EBV-specific suppression, may be related to the reactivation of persisting EBV within the LG.

We are presently extending these studies to determine not only the cell type harboring the EBV within the LG but also whether EBV is shed in the tears of normal and immunosuppressed patients. These data will be important when assessing the potential role of EBV in the pathogenesis of LG disorders.

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ADDENDUM IN PROOF

Since the submission of the manuscript, we have tested by PCR an additional eight normal LG specimens from EBV-seropositive individuals. All of these specimens were PCR EBV negative. This makes a total of 34 normal LG samples that have been tested for EBV sequences by PCR, of which 11, or 32%, were EBV positive.

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