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Epstein-Barr Virus and the Lacrimal Gland Pathology of Sjögren's Syndrome

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The lacrimal gland (LG) immunopathology of Sjögren's syndrome (SS) consists of a proliferation of B and CD4 lymphocytes surrounding epithelial structures (Pepose JS, et al: Ophthalmology 1990, 97:1599–1605). Based on the detection of EBV genomes in a greater percentage of SS than normal LG biopsies, we previously postulated that Epstein-Barr virus (EBV) is a risk factor for LG lymphoproliferation in SS (Pflugfelder SC, et al: Ophthalmology 1990, 97:976–984). The purpose of this study was to determine the cellular site(s) of infection, virus type, and antigen expression of EBV infecting normal and SS LGs. EBV DNA was detected by in situ hybridization in intraductal epithelia in 13–33% of lobules in 21% of normal LGs and in cells in areas of B lymphoproliferation as well as the majority of epithelia in 86% of SS LGs. EBV genomic sequences were amplified from 36% of normal and 88% of SS LG biopsies by polymerase chain reaction. Only type 1 EBV sequences were amplified in SS LGs; in contrast EBV nuclear antigen 2-deleted but not type 1 sequences were amplified in normal LGs. Immunohistochemistry with EBV-specific monoclonal antibodies was performed on normal and SS LGs. No EBV antigens were detected in normal LGs. In contrast, latent antigens (latent membrane protein, EBV nuclear antigen 2) were detected in lymphocytes in areas of B lymphoproliferation, and early and late lytic cycle antigens were observed in epithelia in SS LGs. These studies suggest that EBV may play a role in the LG B lymphoproliferation and epithelial pathologic changes observed in SS. (Am J Pathol 1993, 143:49–64)

Sjögren's syndrome (SS) is an immunological disease associated with lacrimal gland (LG) lymphocytic proliferation resulting in decreased aqueous tear production and severe ocular irritation. We previously reported that the lymphoproliferation in SS LGs consists of B and CD4 lymphocytes.[1] These lymphocytes frequently surround epithelial ducts or islands and replace secretory acini.

There is increasing evidence suggesting that Epstein-Barr virus (EBV) may play a role in the LG pathology of SS.[2] EBV is a human herpes virus capable of infecting B lymphocytes and mucosal epithelia *in vivo*.[3] One consequence of EBV infection of B cells is activation of intrinsic growth pathways, resulting in continuous cellular division.[3] There have been multiple case reports of primary SS developing immediately after serologically confirmed infectious mononucleosis.[4–6] We previously amplified EBV genomes using polymerase chain reaction (PCR) in a statistically significantly greater percentage of LG biopsies from SS patients than normal LGs from EBV seropositive controls and hypothesized that EBV is a risk factor for the LG pathology of SS.[7] Detection of EBV in the LG by PCR does not identify the infected cell type, nor does it determine if the amplified EBV DNA sequences are from latent EBV genomes or replicating virus.

The aims of this study were to: 1) evaluate LG biopsies from normal controls and primary SS patients to identify cellular sites of EBV infection using *in situ* DNA hybridization, 2) determine whether EBV infecting normal and SS LGs had intact EBV nuclear antigen (EBNA) 2 gene sequences characteristic of transforming type 1 EBV strains or EBNA 2 gene deletions similar to those found in nontransforming type 2 virus strains such as P3HR1, and 3) evaluate EBV genome expression in these glands using a panel of monoclonal antibodies specific for EBV latent and lytic infection cycle antigens.

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Patients and Methods

Lacrimal Gland Biopsies

This research was conducted by medically qualified personnel in strict accordance with the University of Miami School of Medicine Institutional Review Board. Fourteen main LG biopsies were obtained from 10 human cadavers ranging in age from 23 to 71 years (mean age, 48 years) within 12 hours postmortem. All of the cadaveric donors were human immunodeficiency virus (HIV) seronegative, and none of them had a history or physical evidence of systemic diseases associated with LG disease or recent EBV infection.

LG tissue was obtained from eight consecutive primary SS patients who underwent diagnostic transconjunctival biopsies of the palpebral lobe of the main LG from 1/90 through 12/91. Appropriate consent forms were signed by all donors. Clinical features of these patients are presented in Table 1. Diagnostic criteria for primary Sjögren's syndrome are modifications of those proposed by Fox and associates[8] and include all of the following: 1) 5-minute Schirmer test with anesthesia of ≤5-mm strip wetting, 2) interpalpebral conjunctival or conjunctival plus corneal rose bengal staining, 3) serum autoantibodies (rheumatoid factor ≥ 1:160 and/or ANA ≥ 1:160), and 4) squamous metaplasia of the bulbar conjunctival epithelium noted in impression cytology. The severity of ocular disease was graded based on a previously reported severity grading system.[9] None of the SS patients had received systemic immunosuppressive therapy, and none of them had any symptoms or physical signs of systemic lymphoma.

LG tissue from all normal controls and all but one SS patient (SS-6, Table 1) was fixed in buffered 4% paraformaldehyde for 24 hours and embedded in paraffin. A portion of the LG tissue obtained from all normal controls and from the last four SS patients biopsied (SS-5 to SS-8, Table 1), was embedded in OCT (TissueTek, Naperville, IL) and snap frozen in liquid nitrogen. Five-μm frozen sections were cut from these tissues within 24 hours and stored in sealed slide boxes at −70 C until they were processed for immunohistochemistry.

Whole blood was collected from five SS patients (SS-4 to SS-8, Table 1) in heparinized tubes, and peripheral blood mononuclear (PBMN) cells were separated on a Leukoprep gradient (Becton Dickinson, Mountain View, CA).

Cell Lines and Tissue

Control EBV-infected cell lines included MM-1, a lymphoblastoid cell line established from the peripheral blood of a primary SS patient; B95–8 (American Type Culture Collection (ATCC) catalogue no. 1612 CRL), a marmoset leukocyte cell line containing approximately 180 EBV genomes per cell;[10] and EBV-positive Burkitt's lymphoma cell lines Raji (ATCC catalogue no. CCL 86), containing 90 EBV genomes per cell,[11] Namalwa (ATCC catalogue no. CRL1432), which contains 1 or 2 EBV genomes per cell,[12] Akata (a gift of Dr. N. Miller, University of Miami School of Medicine, Miami, FL), and P3HR1 (ATCC catalogue no. HTB 62), a nontransforming EBV strain with a deletion in the region of the genome encoding EBNA-2.[13] BL-3 (a gift of Dr. B. Bloomberg, University of Miami School of Medicine) served as an EBV-negative Burkitt's lymphoma cell line.[14] A paraffin-embedded oral hairy leukoplakia biopsy from a patient with HIV infection was provided by Dr. M. Nadji (Jackson Memorial Hospital, Miami, FL).

Akata cells were induced into the lytic cycle by incubating cells in RPMI 1640 containing 10% fetal calf serum and goat anti-human IgG (100 μg/ml)

Table 1. *Clinical Data*

Patient	Age	Sex*	Duration of symptoms (years)	Parotid gland swelling	Severity of ocular disease†	Autoantibodies‡		
						ANA	RF	SS-A (Ro)
SS-1	36	F	6	No	III	40	1280	+
SS-2	65	F	2	No	III	640	0	−
SS-3	49	F	1	No	III	160	80	NP
SS-4	33	F	2	Yes	IV	320	640	+
SS-5	49	F	3	No	III	150	640	+
SS-6	40	F	2	No	III	320	640	+
SS-7	26	F	1	Yes	III	320	160	+
SS-8	40	F	1	No	IV	40	160	+

* F = female.
† Severity grading criteria previously published (Reference 9); grades III and IV are severe disease.
‡ ANA = antinuclear antibodies; RF = rheumatoid factors; SS-A = Sjögren's syndrome antibody A (anti-Ro); NP = not performed; (+) = positive; (−) = negative.

(Cappel, Westchester, PA) as previously described.[15] Cell preparations for immunohistochemical staining were prepared by placing a 40-μl drop of a suspension of 1×10^6 cells/ml in phosphate-buffered saline on a clean microscope slide and air drying for 1 hour at room temperature. Frozen sections of cell lines were prepared by pelleting 3×10^6 cells in 15-ml conical tubes and then resuspending them in 3 ml of phosphate-buffered saline (pH 7.4). Fifty μl of normal plasma were added to the tubes, they were gently agitated, and 50 μl of 0.025% $CaCl_2$ were added. One hundred μl of thrombin reagent (Baxter, Miami, FL) were added, and tubes were allowed to sit for 5 minutes. The clot was then gently lifted from the tube, embedded in OCT, frozen in liquid nitrogen, and sectioned.

In Situ *DNA Hybridization*

Five-μm-thick paraffin-embedded histological sections were placed on sialinated slides (Probe-On Plus, Fisher, Atlanta, GA) and stored at room temperature. Paraffin-embedded sections were deparaffinized with three xylene washes and rehydrated by ethanol washes using 100%, 95%, 90%, 85%, 70%, and 50% solutions. The slides were then washed with phosphate-buffered saline three times and immersed in 0.2 N HCl for 10 minutes followed by a quick rinse in 2× SSC. The sections were digested with 10 μg/ml proteinase K (BRL, Bethesda, MD) at 37 C for 15 minutes, rinsed quickly in 2× SSC, and then acetylated using triethanolamine HCl for 10 minutes followed by the dehydration of the sections in 50%, 70%, and 100% ethanol. The slides were dried, prehybridized for 1 hour, and then hybridized under a siliconized coverslip for 20 hours at 37 C. The hybridization solution consisted of 20% dextran sulfate, 50% deionized formamide, 0.1 mol/L dithiothreitol, 10 mmol/L Tris-HCl, 0.6 mol/L NaCl, 1 mmol/L EDTA, 0.04% Ficoll, 0.04% polyvinylpyrolidone, 0.04% bovine serum albumin, and 1 ng/section ^{35}S-labeled EBV *Bam*HI W DNA probe. This probe is complementary to a sequence reiterated approximately 12 times in the major internal repeat region (IR1, Figure 1) of the EBV genome.[16] The probe was prepared by nick translation of a 3.1-kb EBV *Bam*HI W fragment cut from a pBR322 plasmid (generously provided by Claire Sample, St. Jude's Hospital, Memphis, TN).

Following hybridization, slides were washed for 1 hour in 50% deionized formamide, 0.1 mol/L dithiothreitol, and 2× SSC at 37 C; twice in 2× SSC, 0.1 mol/L dithiothreitol, and 0.1% sodium dodecyl sulfate at room temperature (15 minutes each); twice in 0.2× SSC, 0.1% sodium dodecyl sulfate at room temperature (15 minutes each); and twice in 0.13× SSC, 0.1% sodium dodecyl sulfate at room temperature for 15 minutes each. Slides were dehydrated and dried at room temperature overnight. Autoradiography was performed by dipping the slides into undiluted Kodak NTB-3 nuclear emulsion; the slides were dried vertically and placed in a dark box at 4 C for 4 to 11 days. Slides were developed in Kodak D19 developer, rinsed in tap water, and fixed. After rinsing the slides in tap water, the sections were stained with Mayer's hematoxylin, and coverslips were mounted using Accumount (Scientific Products, McGaw Park, IL). Grains were counted in 100 cells in three separate areas of acini, ducts, and lymphoproliferation in the LG sections. Clear accumulation of cell-associated grains in excess of the background level (5:1 or greater signal/noise ratio) was considered to represent a positive result. The sensitivity of EBV genome detection using a similar *in situ* hybridization protocol was estimated to be approximately 12 copies of EBV/cell.[17]

Polymerase Chain Reaction

Two 5-μm sections from paraffin blocks containing normal control and SS LG biopsies were cut and placed directly into sterile 1.5-ml Eppendorf tubes. A new sterile microtome blade was used to cut each specimen. The paraffin was removed by washing the sample with xylene and rehydrating the sample in decreasing concentrations of ethanol (100%, 100%, 90%, 70%, 50%, 25%) followed by two rinses with phosphate-buffered saline. Lysis buffer consisting of 50 mmol/L KCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0), and proteinase K (100 μg/ml) was added to the specimens, and they were incubated at 56 C for 1 hour and then boiled for 10 minutes. Samples were stored at −20 C until used for PCR.

DNA was prepared from control cell lines and PBMN cells by incubating approximately 2×10^6 cells in the lysis buffer described above at 56 C for 1 hour and then boiling for 10 minutes. Oligonucleotides used for primers and probes were synthesized by the methoxy-phosphoramidite method with a model 380B DNA synthesizer (Applied Biosystems) by Dr. R. Werner (Department of Biochemistry, University of Miami School of Medicine).

The strategy for PCR is presented in Figure 1. The first amplified segment is located in the *Bam*HI W region, which is reiterated approximately 12 times in the EBV genome[16] and is complementary

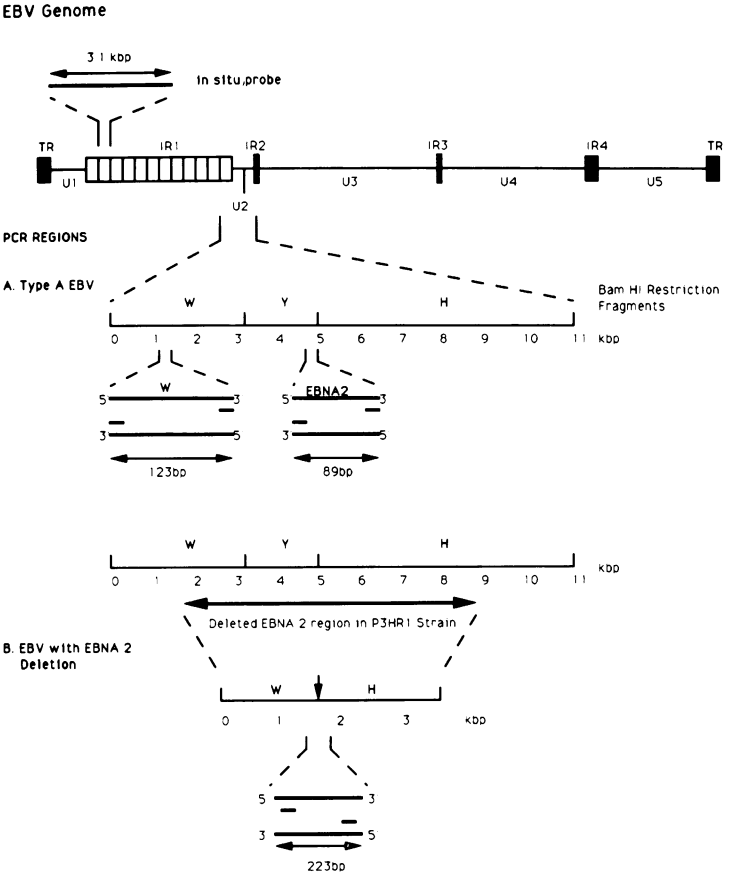

Figure 1. *Diagram of the EBV genome showing terminal repeats (TR), internal repeat regions (IR1–IR4), and unique regions (U1–U5). A 3.1-kb DNA probe complementary to the BamHI W region that is reiterated approximately 12 times in the major internal repeat region (IR1) was used for* in situ *hybridization. Three regions of the genome were chosen for PCR amplification: 1) a 123-bp segment in the* Bam*HI W region contained within the target sequence of the* in situ *hybridization probe. This segment was chosen because it may provide greater sensitivity than single-copy genes; 2) an 89-bp polymorphic region in the EBNA 2 gene which differs in type 1 (EBNA 2A) and type 2 (EBNA 2B) EBV strains; 3) the* Bam*HI WYH region, to determine if the infecting virus had a deletion in region encoding the EBNA 2 gene similar to that found in the type 2 nontransforming P3HR-1 EBV strain. The size of the amplified segment with these primers may vary between 60 and 600 bp depending on the size of the deletion.[19] The probe to this region is specific to the undeleted* Bam*HI W portion of the fused fragment.*

to the DNA probe used for *in situ* hybridization. This segment was chosen since it may provide greater sensitivity than single copy genes. The second amplified segment contains a polymorphic region in the EBNA 2 gene that differs in type 1 (EBNA 2A) and type 2 (EBNA 2B) virus strains.[18] The third region of amplification (*Bam*HI WYH) was chosen to determine if the infecting virus had a deletion in the *Bam*HI WYH region encoding the EBNA 2 gene similar to that found in the nontransforming P^3HR-1 EBV strain and recently described in mucosal secretions from healthy and HIV-infected adult donors.[19] The sequences of the oligonucleotide primers and the probe specific for each of the regions of the EBV genome amplified by PCR are noted in Table 2.

The PCR technique was a modification of that described by Saiki and associates.[20] All DNAs were first amplified for a conserved region of the human leukocyte antigen (HLA) DQ α gene using primers purchased from Synthetic Genetics (San Diego, CA) as an internal control to verify the integrity of the DNA sample and to demonstrate that there were no PCR inhibitors in the sample. Amplification was carried out using a thermal cycler (Perkin Elmer Cetus, Norwalk, CT). PCR reaction mixtures included: 30 µl LG DNA lysate and 70 µl PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.7 mmol/L $MgCl_2$], 1 µmol/L of each primer, 200 µmol/L deoxynucleotide triphosphates, 200 µg of gelatin/ml, and 2.5 units of AmpliTaq polymerase (Perkin Elmer Cetus). Forty PCR cycles were performed; each cycle consisted of 1) 95 C for 60 seconds, 2) 55 C for 60 seconds, and 3) 72 C for 60 seconds with a 1-second autoextension at 72 C each cycle.

Fifty µl of PCR product were placed into a vacuum centrifuge (Jouan, Winchester, VA) and concentrated to a volume of about 20 µl. PCR products were then loaded onto a 3% Nusieve/1% Seakem (FMC, Rockland, ME) agarose gel and separated by electrophoresis for 2 hours at 120 V. Low-molecular-weight digoxigenin-labeled DNA standards (Boehringer Mannheim, Indianapolis, IN; Bioventures, Murfreesboro, TN) were used to verify the size of the amplified fragment. An alkaline transfer of DNA to Hybond N+ (Amersham, Arlington Heights, IL) was performed.[21] Oligonucleotide probes were labeled with the DIG oligonucleotide 3′

Table 2. *PCR Primers and Probes*

Region of EBV genome	Primer sequences	Length of product	Probe sequence	References
*Bam*HI W	5′-CCA GAG GTA AGT GGA CTT T-3′	123 bp	5′-GAC CGG TGC CTT CTT AGG-3′	41
	5′-GAC CGG TGC CTT CTT AGG-3′			
EBNA 2	5′-CCA CCA GCA GCA CCA GCA CA-3′	89 bp	5′-TTA CAT CAT CTA CCC TCG-3′	18
	5′-GGT GGC CAC CAT GGT GGC CC-3′			
*Bam*HI WYH	5′-CAG AGG TAA GTG GAC TTT AA-3′	60–600 bp	5′-TTT CTG CTA AGC CCA ACA CT-3′*	19
	5′-TAT CCC AAT AGA ATA ACC TC-3′	(223 bp average)		

* The probe is specific for the undeleted *Bam*HI W portion of the fused fragment.

end labeling kit (Boehringer Mannheim). Prehybridization, hybridization, and chemiluminescent detection of amplified products with digoxigenin-labeled oligonucleotide probes were performed according to the protocols provided with the DIG nucleic acid detection and Lumiphos 530 kits from Boehringer Mannheim. X-ray films were exposed for 5 and 15 minutes, and hybridization signals were graded as strongly or weakly positive compared to controls.

Immunohistochemistry

Monoclonal Antibodies

Specificities of monoclonal antibodies reactive with EBV antigens, lymphocyte-associated antigens, epithelial cell-associated antigens, and isotype control antibodies used for immunohistochemical staining are provided in Table 3.

Antigen Detection

All frozen sections were fixed in cold methanol for 10 minutes [except for slides stained for EBV early restricted antigen (EA-R) that were fixed in in acetone]. Immunoperoxidase staining on frozen sections was performed using a four-step enhanced avidin-biotin-peroxidase complex technique (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA) as previously described.[22]

Paraffin-embedded sections were stained in a manner similar to that used for frozen sections after the slides were deparaffinized by incubating at 37 C overnight, followed by treatment with xylene, and rehydrating in decreasing concentrations of ethanol. The slides were examined and photographed with an Olympus BHT photomicroscope using Ektar 100 ASA film (Kodak, Rochester, NY).

Results

In Situ *Hybridization*

The specificity of the BamHI W EBV DNA probe used for *in situ* hybridization was verified on EBV-positive and -negative cell lines and paraffin-embedded tissue sections. In sectioned clots of MM-1, B95-8, and all EBV-positive Burkitt's lymphoma cell lines, an average of 7 cells/40× microscopic field were strongly positive (not shown). In contrast, more than 25 cells with a strongly positive signal were seen per 40× field in Akata cells induced into the lytic phase following immunoglobulin treatment. No signal above the background was seen in the EBV-negative BL-3 cell line (not shown). As previously reported,[23] strong hybridization signals were observed in the stratum spinosum of oral hairy leukoplakia, whereas the signal over the basal cells was similar to the background (Figure 2, upper left).

Hybridization signals for EBV DNA were observed in intralobular ductal epithelia in three of 14 (21%) normal human LG biopsies (Figure 2, upper right, middle left; Table 4). EBV DNA-positive ducts were observed in 12 to 33% of lobules in positive histological sections. No hybridization signals were seen in lymphocytes or acini in normal LGs (Figure 2, middle right).

In SS LGs, EBV-positive mononuclear cells were occasionally observed in the LG interstitium adjacent to residual LG acini; however, hybridization signals in acinar epithelial cells were similar to the background (Figure 2, bottom left and right). The majority of cells located in areas of B lymphoproliferation (identified by staining serial paraffin section with L26, an anti-CD20 antibody) showed positive hybridization signals (Figure 3). Additionally, intralobular ductal epithelia and epithelial islands (iden-

Table 3. *Antibody Specificities*

Specificity (Tissue)*	Type	Source	Clone	Dilution	(+) Control†	(−) Control
EBV-associated						
EBNA 2 (F)	IgG	‡	PE-2	1:20	MM1	BL3
LMP (F)	IgG	‡	CS 1–4	1:100	MM1	BL3
BZRF1	IgG	‡	BZ-1	1:10	Induced Akata	BL3
EA-R (F)	IgG2a	Dupont MEN	85K	1:50	Induced Akata	BL3
VCA (F)	IgG1	Seralab	F323	1:50	Induced Akata	BL3
CD21 (F)	IgG2a	Becton Dickinson	HB5	1:10	MM1	BL3
CD23 (F)	IgG1	Becton Dickinson	EBVCS-5	1:10	MM1	BL3
Lymphocyte-associated						
CD20 (P)	IgG2a	Dako	L26	1:150	Tonsil	Cornea
CD22 (F)	IgG1	Olympus	BL-9	1:250	Tonsil	Cornea
Class II MHC (F)	IgG2a	Becton Dickinson	L243	1:10	Skin LC	Cornea
CD3 (F)	IgG1	Becton Dickinson	SK7	1:20	Tonsil, LG	Cornea
CD4 (F)	IgG1	Becton Dickinson	SK3/SK4	neat	Tonsil, LG	Cornea
CD8 (F)	IgG2a	Ortho	OKT8	1:10	Tonsil, LG	Cornea
ICAM-1 (CD54) (F)	IgG	§	RR1/1	1:250	MM1	Cornea
LFA 1 (α chain, CD11a) (F)	IgG	Seralab	A9D764	neat	MM1	Cornea
Epithelial-associated						
Acidic keratins (nos. 10, 14, 15, 16–19) (F, P)	IgG	ICN	AE-1	1:50	Cornea, conj	Tonsil, BL3
Basic keratin (no. 3, 64 kD) (F)	IgG	ICN	AE-5	1:50	Cornea, conj	Tonsil, BL3
Basic keratin (no. 5, 58 kD) (F)	IgG	‖	AE-14	neat	Cornea, conj	Tonsil, BL3
Acidic and basic keratins (nos. 8, 18, 19) (F, P)	IgG1	Labsystems	PKK1	1:100	Cornea, conj	Tonsil, BL3
Secretory component (free and bound)	IgG1	Nordic	Hu/SC	1:100	LG, SG	Tonsil, BL3
Isotype controls						
IgG1 (F, P)	IgG1	Olympus		1:30		
IgG2a (F, P)	IgG2a	Olympus		1:30		
IgG2b (F, P)	IgG2b	Olympus		1:30		

* Type of tissue section, frozen (F) or paraffin (P), stained with each antibody is indicated in parenthesis. BZRF1 = immediate early antigen which induces lytic infection in B cells.

† LC = Langerhan's cells; LG = normal lacrimal gland; SG = normal lacrimal gland; conj = conjunctiva.

‡ Dr. Martin Rowe and Dr. L. Young, Cancer Research Campaign Laboratories, Department of Cancer Studies, University of Birmingham Medical School, Birmingham, England. (References 48–50)

§ Dr. T. Springer, Laboratory of Membrane Immunochemistry, Dana-Farber Institute, Boston, MA.

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tified with cytokeratin antibodies AE-1 and PKK-1 in serial sections) located in areas of B lymphoproliferation and adjacent interlobular ducts showed strong signals in SS LGs (Figure 3). This pattern of hybridization was observed in five of seven SS LGs following 4 days of autoradiographic development and in six of seven glands after 11 days (Table 4).

Polymerase Chain Reaction

To verify that the EBV primers were specific for EBV, PCR amplification of EBV *Bam*HI W, EBNA 2, and *Bam*HI WYH regions was performed with lysates of EBV-positive cell lines (MM-1, B95–8, Raji, Namalwa, P3HR-1), an EBV-negative Burkitt's lymphoma cell line (BL-3), and deparaffinized sections of an oral hairy leukoplakia biopsy. HLA DR α gene sequences were amplified in all control human cell lines and tissues as well as all LG specimens (data not shown). Following agarose gel electrophoresis of the reaction mixtures, a 123-bp band of amplification with the *Bam*HI W primers was seen with all five EBV-positive cell lines and the oral hairy leukoplakia biopsy (data not shown). When the EBNA 2 primers were used, an 89-bp band was observed with all EBV-positive cell lines except P3HR-1, which has a deletion of the EBNA 2 gene. The *Bam*HI WYH primers spanning the deletion in EBNA 2-deleted strains yielded several bands between 375 and 600 bp with P3HR-1 DNA and a weakly positive 350-bp band with the B95-8 strain (Figure 4). The other EBV-infected cell lines were negative with these primers. No amplification with any of the EBV primers was observed with BL-3, the EBV-negative cell line, or reaction mixtures without target DNA (water blanks). The separated PCR products

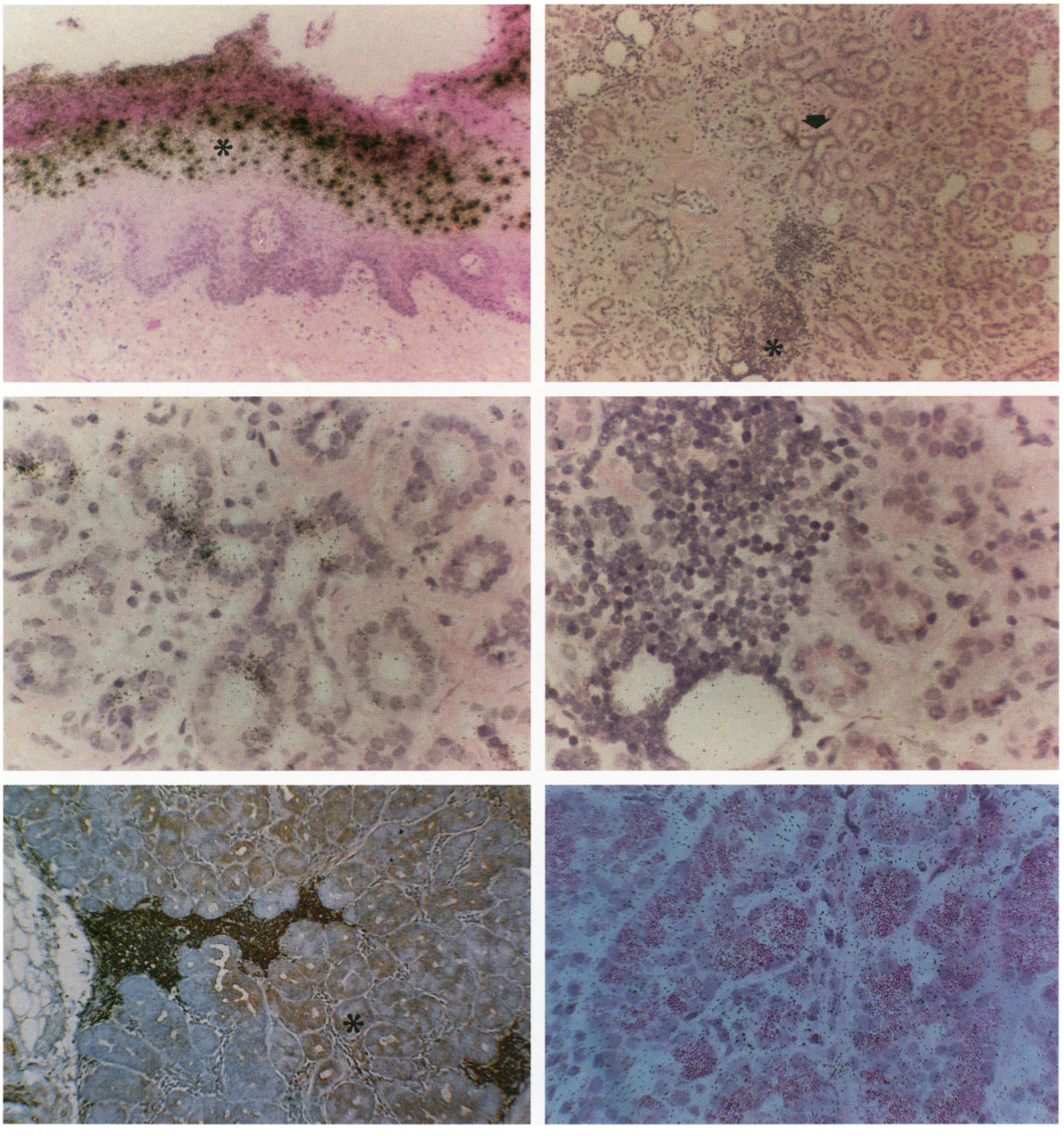

Figure 2. In situ *hybridization with* Bam*HI W*^{35}*S-labeled DNA probe.* **Upper left**, *positive tissue control: oral hairy leukoplakia biopsy from the lateral tongue of an HIV-infected patient. Strong hybridization signals are noted in the stratum spinosum (*, *×100 original magnification).* **Upper right**, *normal LG biopsy showing hybridization signals in intralobular duct (black arrow); no hybridization signals were seen in the acini or lymphocytes (*, *×100 original magnification).* **Middle left**, *strong hybridization signals in epithelia of intralobular duct indicated by black arrow in figure, upper right (×400 original magnification).* **Middle right**, *no hybridization signals were observed in lymphocytes in lymphoid aggregates in the center of the LG lobules (*, upper right) *or in acini or lymphocytes located in the interstitium of normal LGs (×400 original magnification).* **Lower left**, *paraffin section of LG biopsy from SS patient (SS-8, Table 4) stained with a B-cell-specific (CD20) antibody. B lymphoproliferation is present in the center of this lobule; the peripheral lobule (*) contains normal-appearing acinar structures (×100 original magnification).* **Lower right**, in situ *hybridization for EBV in area of asterisk in the* lower left *photomicrograph. Hybridization signals in acinar epithelia are similar to the background (×400 original magnification).*

were transferred to nylon membranes and hybridized to digoxigenin-labeled probes corresponding to the amplified segments. All EBV-positive controls amplified with *Bam*HI W primers hybridized with the *Bam*HI W probes. All EBV-positive cell lines, except P3HR-1, hybridized to EBNA 2A probes. Only P3HR-1 and B95–8 hybridized to the *Bam*HI WYH probes. These results indicate that the primers and

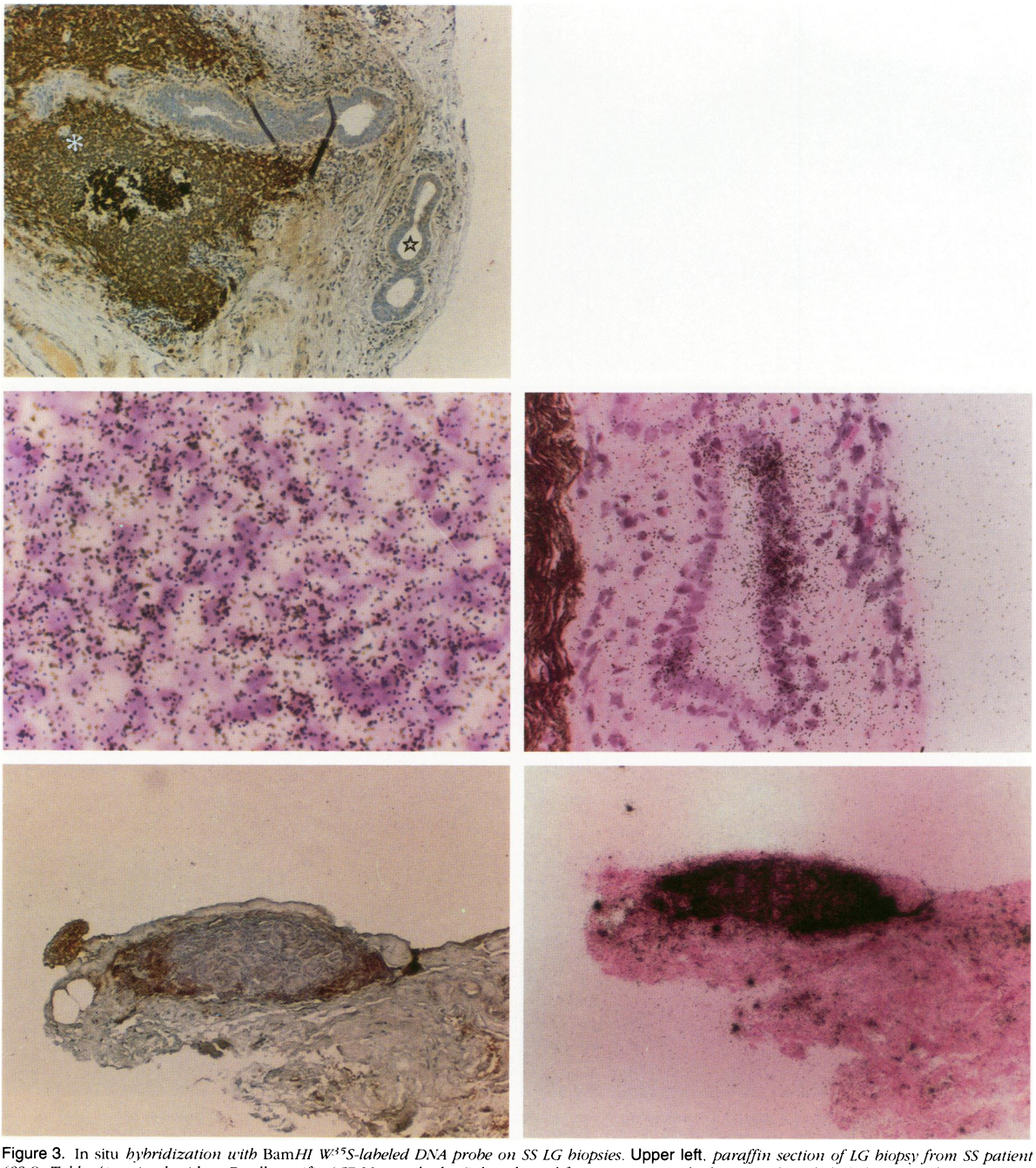

Figure 3. In situ *hybridization with* Bam*HI W*^{35}*S-labeled DNA probe on SS LG biopsies.* **Upper left**, *paraffin section of LG biopsy from SS patient (SS-8, Table 4) stained with a B-cell-specific (CD20) antibody. B lymphoproliferation surrounds the central epithelial duct and fills the lobule (*, ×100 original magnification).* **Middle left**, *intense hybridization signal noted in the majority of mononuclear cells in the area of B lymphoproliferation* (*, upper left) *(×1000 original magnification).* **Middle right**, *Strong hybridization signals for EBV present in ductal epithelia* (★, upper left), *particularly the inner cell layer (×400 original magnification).* **Bottom left**, *paraffin section from LG biopsy from SS patient (SS-2, Table 4) stained with a CD20 antibody. B lymphoproliferation was noted at both ends of LG section (×100 original magnification).* **Bottom right**, *strong hybridization signals for EBV in areas of B lymphoproliferation and epithelial structures in serial section of figure.* **lower left** *(×100 original magnification).*

probes used to detect EBV genomic sequences by PCR are specific for type 1 EBV or EBNA 2-deleted type 2 strains of EBV.

LG biopsies were initially amplified using primers to the *Bam*HI W reiterated sequence in the EBV genome to confirm the presence of EBV. EBV genomic

Table 4. *Results*

LG specimen*	*In situ* hybridization†	PCR			Immunohistochemistry				
		Bam W	EBNA 2A	Bam WYH	EBNA 2	LMP	BZ-1	EA-R	VCA
Normal LGs (n = 14)	scattered ductal epithelia in 3/14 (21%)	5/14 (36%)	–	5/14 (36%)	–	E‡	–	–	–
SS-1	L, E	+	–	–	NP	NP	NP	NP	NP
SS-2	L, E	+	+	–	NP	NP	NP	NP	NP
SS-3	–	+	+	–	NP	NP	NP	NP	NP
SS-4	L, E	+	+	–	NP	NP	NP	NP	NP
SS-5	L, E	+	+	–	–	L, E‡	L, E	L, E	E
SS-6	NP§	–	–	–	–	L	L	L	E
SS-7	L, E	+	–	–	–	L, E‡	–	–	–
SS-8	L, E	+	–	–	L	L, E‡	–	L, E	E

* LG = lacrimal gland.
† 11-day exposure results. L = positive cells in areas of lymphoproliferation; E = positive cells in epithelial ducts or islands; NP = not performed.
‡ Nonspecific nuclear staining in ductal epithelia.
§ Sufficient tissue available only for immunohistochemistry and PCR.

sequences were amplified from LG sections cut from paraffin blocks from five of 14 normal human LGs (Figure 4A; Table 4). In contrast, the *Bam*HI W region was amplified from LG sections cut from seven of eight SS LG biopsies (Figure 5A; Table 4).

The five EBV-positive normal control LG specimens and all SS LG biopsies were also amplified with EBNA 2 and *Bam*HI WYH primers to determine whether the infecting virus had an intact EBNA 2A gene or an EBNA 2 gene deletion. None of the five normal LG specimens positive for EBV DNA with the *Bam*HI W primers showed amplification products with the EBNA 2A primers (Figure 4B; Table 4); however, all five showed deletions of the region of the genome encoding EBNA 2 with the Bam WYH primers (Figure 4C; Table 4). Four of seven *Bam*HI W-positive SS LG biopsies hybridized with EBNA 2A probes (Figure 5B, Table 4), whereas none hybridized with the *Bam*HI WYH probes (Figure 5C; Table 4).

PBMN specimens from five SS patients were also evaluated for the presence of EBV DNA by PCR.

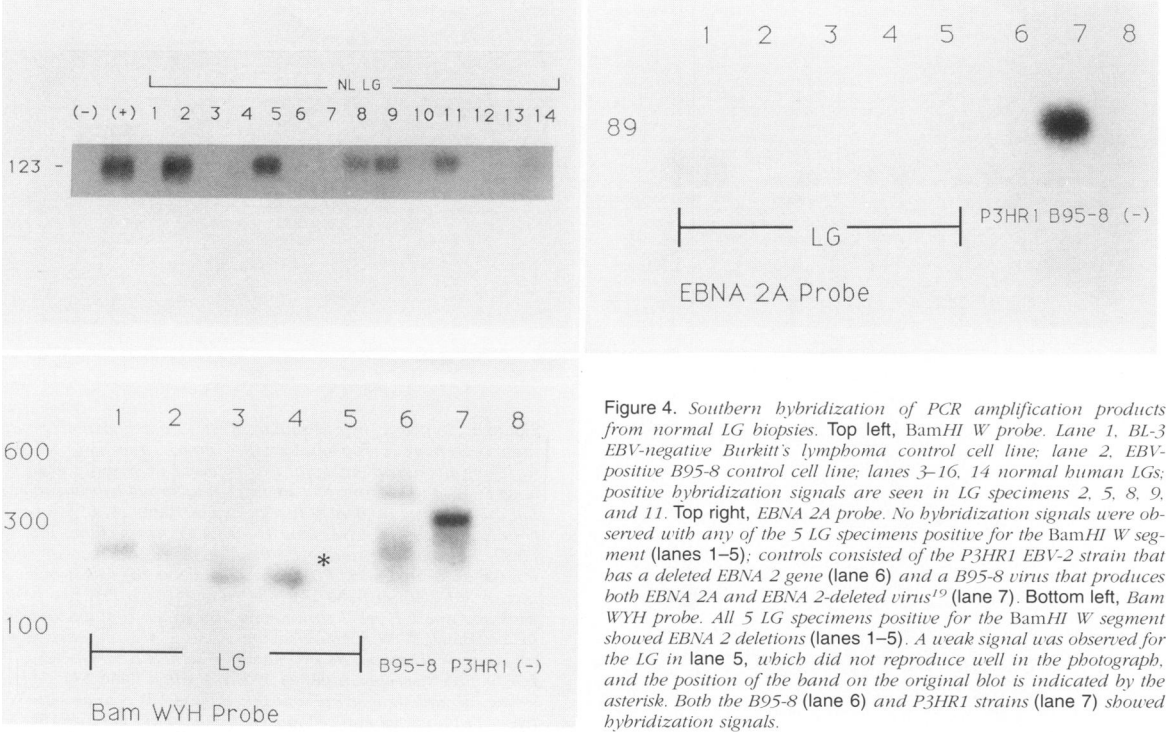

Figure 4. *Southern hybridization of PCR amplification products from normal LG biopsies.* Top left, Bam*HI W probe. Lane 1, BL-3 EBV-negative Burkitt's lymphoma control cell line; lane 2, EBV-positive B95-8 control cell line; lanes 3–16, 14 normal human LGs; positive hybridization signals are seen in LG specimens 2, 5, 8, 9, and 11.* Top right, *EBNA 2A probe. No hybridization signals were observed with any of the 5 LG specimens positive for the* Bam*HI W segment* (lanes 1–5)*; controls consisted of the P3HR1 EBV-2 strain that has a deleted EBNA 2 gene* (lane 6) *and a B95-8 virus that produces both EBNA 2A and EBNA 2-deleted virus*[19] (lane 7). Bottom left, *Bam WYH probe. All 5 LG specimens positive for the* Bam*HI W segment showed EBNA 2 deletions* (lanes 1–5). *A weak signal was observed for the LG in* lane 5, *which did not reproduce well in the photograph, and the position of the band on the original blot is indicated by the asterisk. Both the B95-8* (lane 6) *and P3HR1 strains* (lane 7) *showed hybridization signals.*

Three of five SS PBMN specimens amplified the *Bam*HI W segment. One of three *Bam*HI W-positive PBMN specimens was positive for EBNA 2A, and none of the three were positive for *Bam*HI WYH (Figure 5). All PCR experiments were repeated, and identical results were obtained each time.

Immunohistochemistry

All control LG biopsies evaluated showed normal histology and an immunohistochemical staining pattern with lymphocyte- and epithelial cell-associated monoclonal antibodies.

The typical staining pattern observed in SS LG lobules was a proliferation of CD21-, CD22-, CD23-, and intercellular adhesion molecule 1 (ICAM 1)-positive B cells surrounding epithelial ducts or islands of epithelium (Figure 6). Epithelial ducts in LG lobules showing intense lymphoproliferation had an abnormal morphology with intraductal rosettes (Figure 6). The pattern of immunoreactivity of SS LGs with cytokeratin antibodies was found to differ from that of normal LGs. In the normal human LGs, the basal ductal epithelial cells stain intensely with an antibody specific for keratin no. 5 (AE-14), and the suprabasal ductal epithelia stain with an antibody specific for keratin no. 3 (AE-5).[22] In contrast, all layers of ductal epithelia in SS LGs react with both keratin no. 3- and no. 5-specific antibodies (Figure 6, bottom center).

The specificity of EBV antibodies was confirmed on EBV-infected cell lines (data not shown). EBNA 2 and latent membrane protein (LMP) were detected in every cell of the EBV-positive lymphoblastoid cell line MM-1. BZ-1, EA-R, and viral capsid antigens (VCAs) were detected in more than 50% of Akata cells induced into the lytic phase. As expected, BL-3 cells were negative for EBV latent and lytic infection cycle antigens.

After the optimal dilution was determined for EBV-specific monoclonal antibodies on cell lines, they were used to stain LG biopsies. The results of immunohistochemical staining of LG biopsies with EBV-specific monoclonal antibodies are summarized in Table 4. A specific staining pattern for EBV-specific antigens was not observed in any of the 14 normal human LG biopsies evaluated. Weak nonspecific punctate nuclear staining of ductal epithelia but not lymphocytes with anti-LMP was noted in approximately half of the normal LG biopsies. Similar nonspecific staining of salivary gland epithelia with anti-LMP has previously been reported.[24]

EBV-associated antigens were observed in all four SS LG biopsies evaluated, and the results are summarized in Table 4. EBNA 2 was observed in large CD23-positive lymphocytes located in the center of areas of B lymphoproliferation in one SS LG biopsy (Figure 7, middle right). A specific pattern of cell membrane and cytoplasmic staining with anti-LMP was observed in lymphocytes in areas of

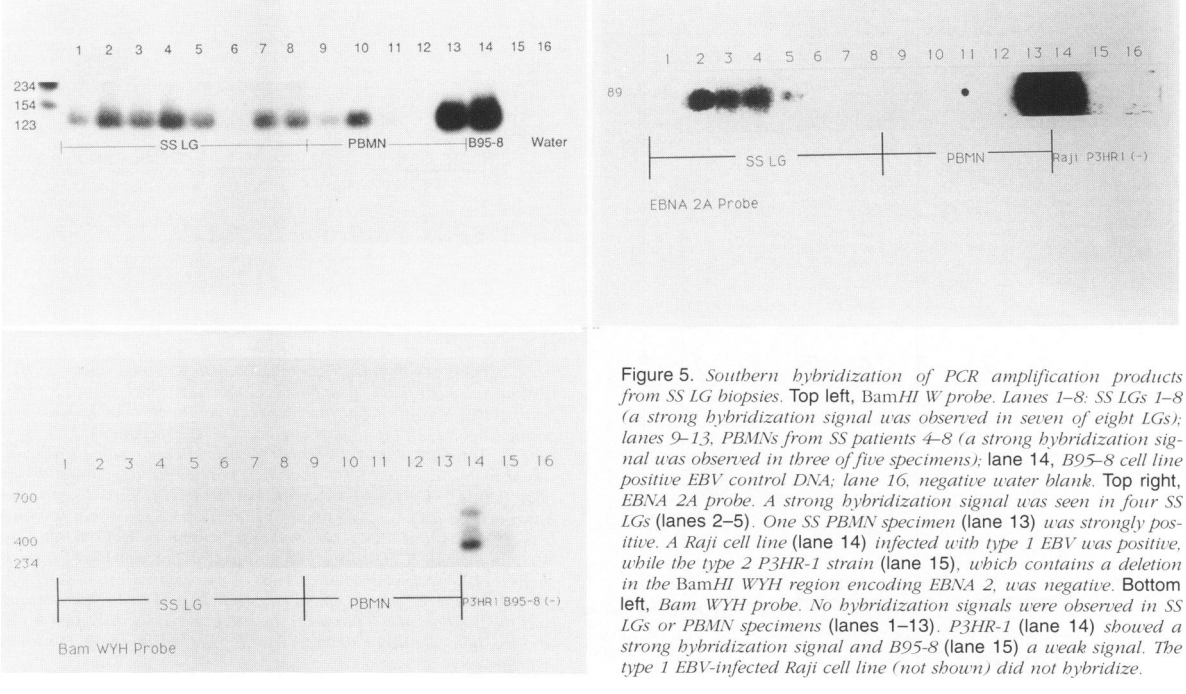

Figure 5. *Southern hybridization of PCR amplification products from SS LG biopsies.* Top left, Bam*HI W probe. Lanes 1–8: SS LGs 1–8 (a strong hybridization signal was observed in seven of eight LGs); lanes 9–13, PBMNs from SS patients 4–8 (a strong hybridization signal was observed in three of five specimens);* lane 14, *B95–8 cell line positive EBV control DNA; lane 16, negative water blank.* Top right, *EBNA 2A probe. A strong hybridization signal was seen in four SS LGs* (lanes 2–5). *One SS PBMN specimen* (lane 13) *was strongly positive. A Raji cell line* (lane 14) *infected with type 1 EBV was positive, while the type 2 P3HR-1 strain* (lane 15), *which contains a deletion in the* Bam*HI WYH region encoding EBNA 2, was negative.* Bottom left, *Bam WYH probe. No hybridization signals were observed in SS LGs or PBMN specimens* (lanes 1–13). *P3HR-1* (lane 14) *showed a strong hybridization signal and B95-8* (lane 15) *a weak signal. The type 1 EBV-infected Raji cell line (not shown) did not hybridize.*

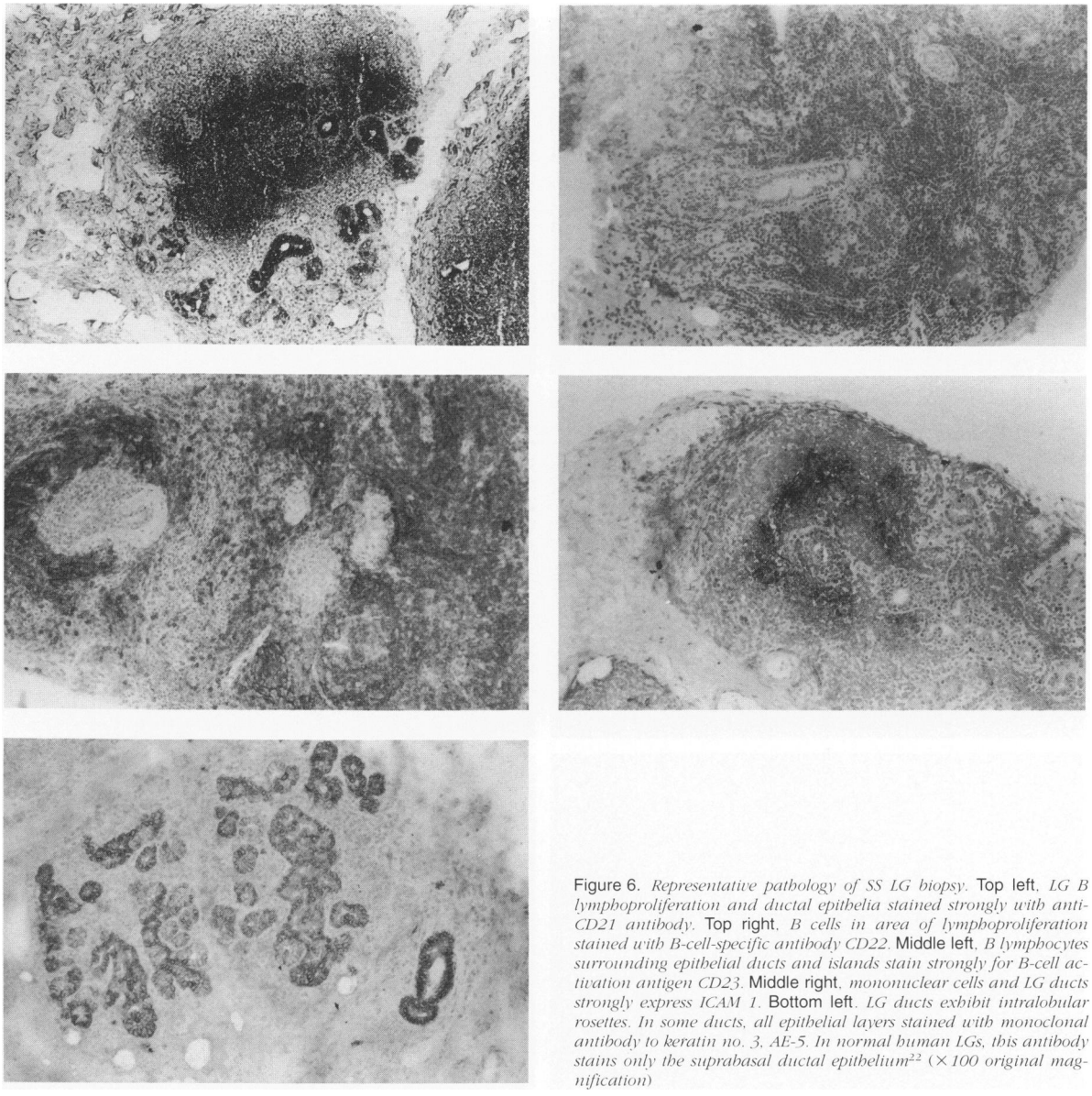

Figure 6. *Representative pathology of SS LG biopsy.* Top left, *LG B lymphoproliferation and ductal epithelia stained strongly with anti-CD21 antibody.* Top right, *B cells in area of lymphoproliferation stained with B-cell-specific antibody CD22.* Middle left, *B lymphocytes surrounding epithelial ducts and islands stain strongly for B-cell activation antigen CD23.* Middle right, *mononuclear cells and LG ducts strongly express ICAM 1.* Bottom left. *LG ducts exhibit intralobular rosettes. In some ducts, all epithelial layers stained with monoclonal antibody to keratin no. 3, AE-5. In normal human LGs, this antibody stains only the suprabasal ductal epithelium*[22] (×100 *original magnification*)

B lymphoproliferation in all 4 SS LGs, and similar to EBNA 2, the strongest LMP staining was noted in areas of CD23-positive B cells (Figure 7, middle left). Anti-LMP produced nonspecific punctate nuclear staining in ductal epithelia in two of four SS LGs similar to that observed in normal LGs. EA-R-positive cells were scattered throughout areas of B lymphoproliferation and were also occasionally observed in epithelial cells in SS LGs. VCA staining was not observed in mononuclear cells, but epithelia in areas of lymphoproliferation were often VCA-positive (Figure 7, bottom right), suggesting that these cells may be lytically infected. BZ-1 staining was observed in the ductal epithelium in two biopsies, and rare BZ-1-positive mononuclear cells were seen in two biopsies (not shown).

Discussion

In our study, EBV DNA was detected in a small percentage of intralobular ductal epithelia in 21% of normal LGs. Lymphocytes and acinar epithelia in normal LGs were negative for EBV DNA. We failed to detect any EBV antigens using a panel of monoclonal antibodies reactive to EBV latent and lytic infection cycle antigens. These findings suggest that EBV may persist in the normal human LG in a latent

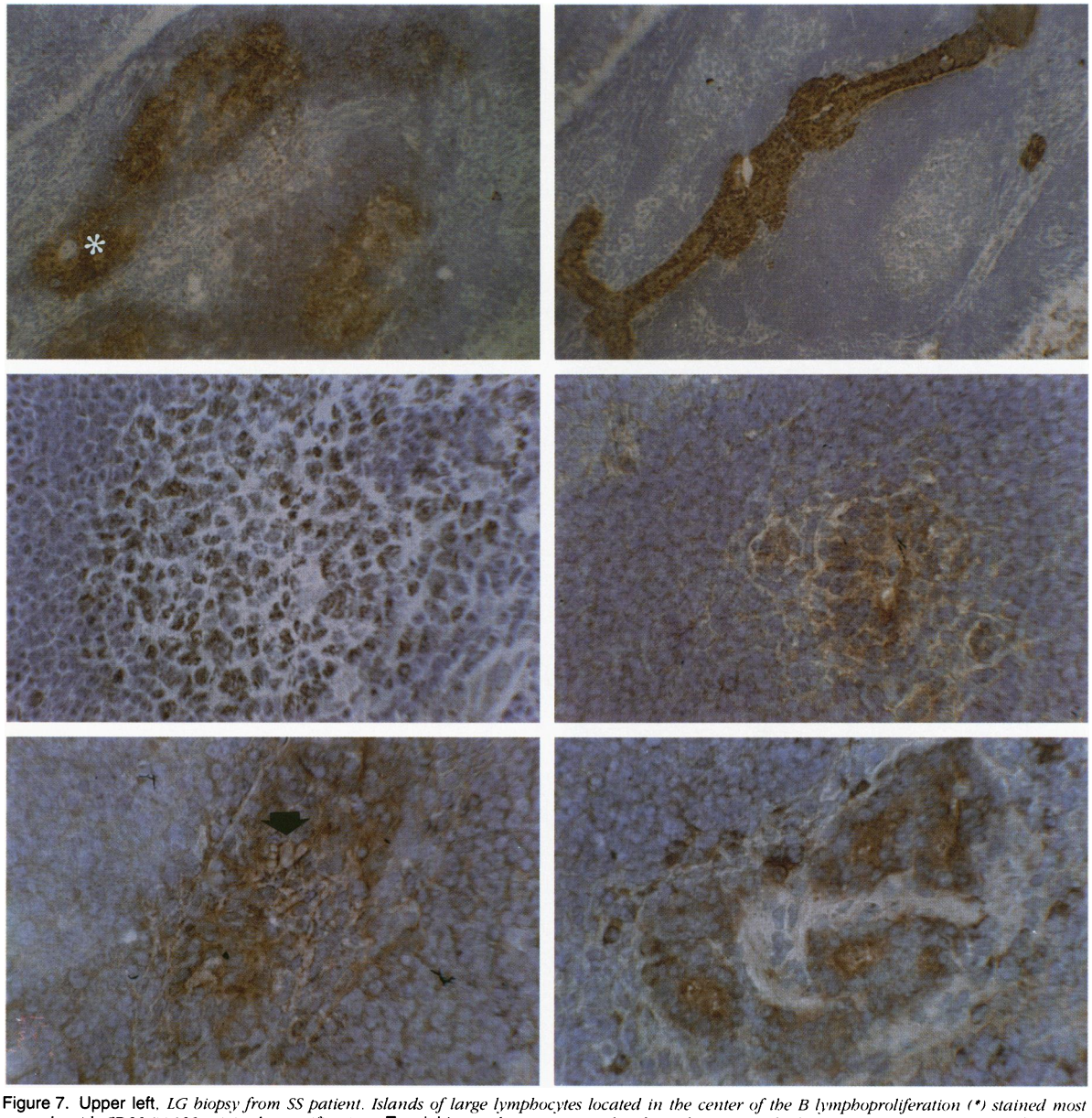

Figure 7. Upper left, *LG biopsy from SS patient. Islands of large lymphocytes located in the center of the B lymphoproliferation (*) stained most strongly with CD23 (×100 original magnification).* Top right, *serial section stained with cytokeratin antibody AE-1 (×100 original magnification). Large CD23-positive lymphocytes in area of asterisk in* top right *section stained intensely with antibodies specific for LMP* (middle left) *and EBNA 2* (middle right) *(×400 original magnification). Slender epithelial cells intercalated with lymphocytes in a serial LG section stained with monoclonal antibody to secretory component* (bottom left) *and anti-VCA* (bottom right) *antibody (×400 original magnification).*

nonpathologic state. The cellular site and state of genome expression of persistent EBV infection in the normal human LG appears to be similar to that reported to occur in normal labial and parotid salivary glands.[25,26]

In contrast, the results of studies using *in situ* DNA hybridization and immunohistochemical techniques to evaluate SS LGs for EBV indicate that there is a more extensive infection of ductal epithelia than of normal LGs, as well as infection of lymphocytes in areas of B lymphoproliferation. EBV antigens were detected in lymphocytes and epithelial cells in SS LG biopsies; however, the pattern of antigen expression appeared to differ in these two types of LG cells. LMP was expressed in the majority of lymphocytes in areas of lymphoproliferation. Additionally, EA-R staining was noted in approximately 20% of lymphocytes. EBNA 2, which has been implicated as one of the EBV proteins responsible for activation of intrinsic B cell growth pathways in transformed cells,[27] was noted in lymphocytes in an area of B lymphoproliferation in only one

SS LG. Lymphocytes staining positively for the zebra switch protein were rarely observed in areas of lymphoproliferation. Based on these findings, it appears that there is an active latent EBV infection of B lymphocytes in SS LGs that may be responsible for the B lymphoproliferation observed in these glands.

Epithelial cells located in areas of lymphoproliferation in SS LGs stained strongly for early and late (VCA) lytic-cycle antigens. Similar to LG lymphocytes, only a small percentage of LG ductal epithelia expressed the zebra protein. These findings suggest that a lytic EBV infection may occur in epithelial cells in SS LGs. Because we have previously reported the detection of EBV genomes in the majority (80%) of tear specimens obtained from primary SS patients,[7] it is possible that these ductal epithelia may release infectious virus.

Similar to several other EBV-associated neoplasias, a lymphoepithelial pathology is frequently observed in SS LG biopsies.[28] The lymphoepithelial pathology in SS differs from nasopharyngeal carcinoma in that the lymphoproliferation surrounding ducts and islands in SS LGs consists predominantly of B cells, whereas T cells typically surround epithelia in nasopharyngeal carcinoma.[29] In SS LG lobules with mild inflammation, the B lymphoproliferation is observed surrounding ducts in the center of the lobule, and normal-appearing acini are present in the peripheral lobule. In more severely affected glands, the lymphoproliferation replaces all secretory acini, and the ducts in areas of B proliferation often appear abnormal and have an altered pattern of cytokeratin expression. B cells surrounding epithelial ducts in SS LGs express ICAM 1 and the B cell activation antigen CD23, both of which are upregulated by EBV infection and are typically observed in EBV growth transformed B cells.[30]

As in previously reported studies,[7,31] EBV DNA was amplified from the majority of LG and PBMN specimens from SS patients. PCR genotype analysis indicated that approximately 60% of the EBV-positive SS LG specimens are infected with type 1 EBV. Type 1 EBV strains efficiently transform B lymphocytes into continuous cell lines,[32] and our detection of this virus strain in SS LGs is consistent with the B lymphoproliferation occurring in these glands. This contrasts with normal LGs, from which we were unable to amplify type 1-specific sequences and which were found to be infected exclusively by EBV with EBNA 2 deletions typically found in nontransforming type 2 EBV strains. Although the sample size in our studies is small and additional studies are needed to confirm our results, the difference in virus strain between the normal and SS LG may be important in the pathogenesis of this disease.

The predominant EBV-specific cytotoxic lymphocytes (CTLs) in humans are HLA class I restricted (CD8) T cells.[33,34] EBV-specific CTLs have been reported to efficiently lyse HLA-restricted B cells infected with type 1 EBV and poorly recognize cells infected with type 2 EBV strains.[33,34] CD8 T cells are the predominant population surrounding acini and proximal ducts in normal human LGs.[35] One potential role of CD8 cells in the normal LG may be to recognize and destroy cells within the LG infected with type 1 EBV. These cells could include EBV-infected ductal epithelia or B cells, which continuously flow into the gland. LG cells infected with type 2 EBV strains may be able to elude recognition by resident CD8 CTLs. This hypothesis may explain the fact that we found only EBNA 2-deleted type 2 EBV DNA in normal LGs.

The higher frequency of EBV infection in the blood and LGs of SS patients may result from an inability of CTLs from SS patients to recognize and destroy cells infected with certain strains of type 1 EBV. Misko and associates recently studied paternal EBV-specific CTL activity against EBV-infected lymphoblastoid cell lines (LCLs) established by infecting peripheral blood B cells obtained from five children from one family with either the B95-8 or the BL 74 EBV strain.[36] The paternal HLA type was A1,11; B51,8; DR3,7. Paternal EBV-specific CTLs efficiently lysed haploidentical EBV LCLs infected with the B95-8 strain expressing the HLA A11, B51, DR7 paternal haplotype but failed to lyse haploidentical LCLs infected with the B95-8 strain expressing the HLA A1, B8, DR3 paternal haplotype. LCLs expressing either of the paternal HLA haplotypes infected with the BL 74 strain were efficiently lysed by paternal LCLs. The authors found that the failure to lyse the HLA B8-restricted LCLs infected with the B95-8 strain was not due to T-cell dysfunction, and they concluded that the failure to lyse was probably due to an inability of HLA B8 antigen to present the immunodominant B95-8 epitope to HLA class I-restricted CTLs. Interestingly, the HLA B8, DR3, DW52a, DQw2 haplotype is strongly associated with primary SS (relative risk of 8).[37] As suggested by Misko and associates, the HLA B8 haplotype association in SS patients may be one of the principal risk factors for their abnormal EBV infection. Alternatively, the EBV-induced LG B lymphoproliferation in SS may be related to other cellular immune derangements previously reported to occur in SS patients with severely dry eyes.[9]

SS patients have an increased frequency of B-lymphoproliferative disorders.[38] Recently, monocytoid B-cell lymphomas in major salivary glands with lymphoepithelial histology similar to that of the SS lacrimal glands presented in our series were reported.[39,40] Although it is possible that the B lymphoproliferation observed in our SS LG biopsies might be a lymphoma, we feel that the B lymphoproliferation probably represents the normal immunopathology of SS. None of our patients had evidence of lymphoma on systemic evaluation. Futhermore, we have followed primary SS patients with similar LG pathology for up to 6 years without enlargement of the LG or development of B lymphoproliferation at other sites.

Previous studies evaluating parotid and minor salivary gland biopsies from SS patients for EBV infection have reported conflicting results. Saito and associates amplified EBV genomic sequences in 78% of SS salivary gland biopsies as compared to 13% of biopsies from normal controls.[41] Using PCR, Mariette and associates detected EBV DNA in 86% of minor salivary gland biopsies from primary SS patients, 60% in secondary SS patients, and 29% of normal controls.[26] Also using PCR, Deacon and colleagues reported in 1991 that 90% of SS minor salivary gland biopsies and 70% of normal salivary gland biopsies harbored EBV DNA.[24] Using *in situ* hybridization with probes reactive with the *Bam*HI W region of the EBV genome, Mariette et al detected EBV DNA in ductal epithelia and lymphocytes in 50% of primary SS biopsies and only 8% of controls.[26] In contrast, Venables and associates found EBV in 17% of primary SS, 33% of secondary SS, and 71% of normal controls using *in situ* DNA hybridization.[42]

Salivary gland biopsies from normal controls and SS patients have also been evaluated for the presence of EBV antigens. Fox and associates reported ductal epithelial staining with anti-EA-D antibodies (clone R3) in 8 of 14 and gp350/220-specific antibodies in 2 of 14 salivary gland biopsies from SS patients, and 0 of 10 control glands.[43] In contrast, Venables and associates found anti-EA-D (clone R3) staining of ductal epithelia in 30% of primary SS, 66% of secondary SS, and 86% of controls, and anti-membrane antigen staining in 17% of primary SS, 20% of secondary SS, and 66% of controls.[42] Recently, Deacon and associates[24] reported a lack of specific immunoreactivity of salivary gland biopsies from primary and secondary SS patients and normal controls with monoclonal antibodies specific for EBV latent (EBNA 2, LMP) or lytic (EA-D, EA-R, VCA) infection antigens, although anti-EA-D antibodies (clone R3) showed nonspecific immunohistochemical staining with EBV-negative cell lines and a variety of cell types including the luminal border of acinar and ductal epithelia in normal salivary gland biopsies when used at the same concentrations previously used by Venables and associates.[42]

There are several possible explanations for the differences in the type of EBV infection observed between LG and salivary gland biopsies from SS patients. First, the immunoarchitecture, phenotype of resident lymphocytes, and inflammatory response of the minor salivary gland might not be equivalent to the LG. The predominant infiltrating cell type in SS minor salivary gland biopsies has been reported to be CD4 T cells rather than B cells.[44,45] In SS patients undergoing simultaneous LG and salivary gland biopsies for diagnosis of SS, more inflammation is observed in the LG than in the salivary gland.[46] In most of the previous studies evaluating SS salivary gland biopsies for EBV infection, EBV antigens and DNA were detected primarily in ductal epithelia, and an EBV-associated B lymphoproliferation surrounding infected ducts was not noted. Furthermore, the lymphocytic infiltration in published photomicrographs of SS salivary glands generally appears to be less severe than that typically observed in SS LG biopsies. A recent study by Tsubota and colleagues using quantitative PCR suggests that the amount of EBV DNA in the LG is tenfold greater than in the salivary gland when both tissues were obtained from the same SS patient.[31]

The results of the study reported herein have definite therapeutic implications for SS. Since there appears to be a lytic infection in LG ductal epithelial cells, antiviral therapy might prove effective in limiting virus-induced LG destruction. Additionally, therapies such as soluble CR2 (CD21)[47] or stimulation of the defective EBV immune surveillance in SS to prevent or reverse EBV-induced B lymphoproliferation may prove useful.

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