DETECTION OF EPSTEIN-BARR VIRUS DNA BY POLYMERASE CHAIN REACTION IN BLOOD AND TISSUE BIOPSIES FROM PATIENTS WITH SJOGREN'S SYNDROME

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EBV is a double-stranded DNA virus of the herpes-virus family and is the causative agent of infectious mononucleosis, African Burkitt's lymphoma, and nasopharyngeal carcinoma (1). After primary infection involving the nasopharynx, EBV remains latent in epithelial cells of the normal salivary gland and periodically becomes reactivated (2, 3). The frequency of EBV reactivation, as evidenced by infectious virus in saliva, is elevated in patients with lymphoproliferative diseases and in transplant recipients receiving immunosuppressive medications (4–6). In these conditions, there is frequently an increased number of circulating EBV-transformed lymphocytes, as detected by their spontaneous outgrowth in vitro (7, 8). Since EBV-infected cells are controlled by T cell immune responses (9), accurate measurement of cells containing viral DNA in the circulation or at the site of latency (i.e., the salivary gland) may provide an important method to assess immune competence in vivo and early detection of EBV-related lymphomas/carcinomas that arise in these individuals (10). We have used polymerase chain reaction (PCR)[1] to provide a rapid, accurate method for detection of EBV DNA in PBMC and in tissue biopsies. We have chosen to study patients with Sjogren's syndrome (SS), an autoimmune disease involving lacrimal and salivary glands (11, 12), since these patients have increased frequency of lymphoma (13, 14) and frequently undergo salivary gland biopsies for diagnostic purposes (15). We found an increased level of EBV DNA in the salivary gland biopsies of these patients, suggesting that this virus is reactivated in vivo and may provide a target for the chronic "autoimmune" attack on the salivary gland.

Materials and Methods

Polymerase Chain Reaction. PCR is a technique based on the binding of specific oligonucleotide primers to defined DNA sequences and amplification of the DNA between these primers (16–18); PCR has been greatly simplified by automated procedures that use the thermostable

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[1] *Abbreviations used in this paper*: LN, lymph node; PCR, polymerase chain reaction; SG, salivary gland; SS, Sjogren's syndrome.

enzyme DNA polymerase (19). Specific primers and probes were synthesized based on the published DNA sequences of Baer et al. (20), corresponding to the Bam W and Bam M regions of the EBV genome (Table I). The Bam W region was chosen as a target gene since this DNA segment is reiterated 10 times (21) and thus should provide a more readily detected sequence than a single copy viral gene. PCR reaction mixtures contained 1 μg of peripheral blood or salivary gland genomic DNA, 50 mM KCL, 10 mM Tris (pH 8.4), 2.5 mM $MgCl_2$, each primer (Table I) at 100 pM, each dNTP (dATP, dCTP, dGTP, TTP) at 200 μM, gelatin at 200 μg/ml, and 2.5 U of thermostable Taq polymerase (Cetus Corp.). The amplification was carried out as previously described (19) with a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). After the amplification steps, one-tenth of the reaction mixture was removed, the NaCl concentration was adjusted to 0.15 M, and it was analyzed by Southern blot method with a ^{32}P-labeled oligonucleotide probe (Table I) at 42°C for 1 h. The filter was washed twice with 6 × SSPE (1× SSPE: 0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 0.1% SDS at room temperature, followed by wash for 10 min at 55°C (for Bam W) or 60°C (for BMRF-1 and BMLF-1) and autoradiography for 3 h at −80°C with a single intensifying screen. The intensity of the signal obtained with each oligonucleotide probe was determined by densitometry tracing of the autoradiograph.

All DNA samples were initially screened for EBV DNA using the Bam W primers and 35 cycles of PCR amplification. If the sample had detectable viral DNA, then 1 μg of the original tissue sample DNA was again amplified in the presence of other primers for EBV (as described above) plus primers for the single copy gene HLA-DQα (22). Aliquots were removed after 20, 25, 30, 35, and 40 cycles of amplification, separated on agarose gels, and analyzed by Southern blots with probes specific for EBV or HLA-DQα (22). Densitometry tracing was again performed to quantitate the relative intensity of the band. Standard mixtures of viral DNA plus uninfected cell DNA were run in parallel with the clinical samples. Tissue samples that did not contain detectable EBV were re-analyzed with the HLA-DQα primers and 35 cycles of PCR to insure that the DNA did not contain inhibitors of the PCR reaction.

Sample Preparation. Genomic DNA was prepared from PBMC of patients with SS and of age- and sex-matched normal controls (23). In addition to PBMC samples, DNA was extracted from salivary gland (SG) biopsy tissue samples from SS patients, from "normal" salivary gland removed at autopsy, and from the histologically normal margins of biopsies removed for SG adenomas. DNA was prepared from these tissues by lyophilization for 16 h, digested with RNAase (10 mg/ml) for 2 h, and proteinase K (1 mg/ml) for 16 h at 37°C, followed by phenol extraction and ethanol precipitation (24). This method of DNA preparation avoids use of tissue homogenizers, a possible source for cross-contamination of tissue

TABLE I
Sequence of Primers and Probes

Region	Sequence	Location in genome of EBV
I. Bam W		
TC60 Primer	CCAGAGGTAAGTGGACTT	1396–1416
TC61 Primer	GACCGGTGCCTTCTTAGG	1520–1503
TC62 Probe	TTCTGCTAAGCCCAAC	1424–1439
II. BMRF1		
TC67 Primer	CAGGCTTCCCTGCAATTTTACAAGCGG	80220–80246
TC69 Primer	CCCAGAAGTATACGTGGTGACGTAGA	80507–80482
TC68 Probe	GATGATAAGGTGTCCAA	80295–80311
III. BMLF1		
TC70 Primer	CTTGGAGACAGGCTTAACCAGACTCA	83520–83545
TC72 Primer	CCATGGCTGCACCGATGAAAGTTAT	83874–83850
TC71 Probe	TCTGGAGCCACGAGAT	83679–83694

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sample DNA. All buffers and pipettes used for PCR were carefully isolated from contact with bacteria that might contain plasmids with EBV DNA inserts.

Results

Detection of EBV DNA by PCR. Primers were synthesized corresponding to a reiterated gene (Bam W) and single copy genes (BMRF-1 and BMLF-1) of the EBV genome (Table I), and to the HLA-DQα gene (22). Gene segments corresponding to each of these primers were amplified using PCR (Fig. 1 *A*). The reiterated viral DNA sequence (Bam W) was detected at a lower number of cycles than the single copy viral genes or the single copy HLA-DQα gene (DQα).

The sensitivity of PCR to detect EBV-infected cells among uninfected cells was assessed by mixing DNA from an EBV-transformed cell with DNA from an uninfected cell DNA under conditions where the total amount of DNA remained constant (Fig. 1 *B*). Each curve represents a decreased amount of viral DNA diluted into uninfected cell DNA. Aliquots were removed after varying numbers of cycles of PCR, transferred to nylon membranes, probed with ^{32}P-labeled oligonucleotides specific for Bam W, and quantitated by densitometry tracing of the autoradiograph. An increased number of PCR cycles were required to obtain the same relative signal when a lower amount of EBV DNA was present in the original mixture (Fig. 1 *B*). Based on these reconstruction experiments, it was possible to detect approximately one EBV transformed cell per 100,000 uninfected cell DNA equivalents (Fig. 1 *B*). As an internal control, we also measured the content of a single copy human gene (DQα) in each sample. Since the total amount of human genomic DNA remained constant in each sample, an identical pattern of DQα amplification was noted for each dilution of viral DNA. This pattern of DQα amplification was identical to that described in Fig. 1 *A*, indicating that the presence of viral primers did not influence the amplification of DQα DNA (data not shown). Similarly, the presence of DQα primers did not influence the amplification of viral DNA. Thus, comparison of reactivity with viral-specific probes and single copy human gene probes makes it possible to approximate the content of viral DNA in an unknown sample (see below).

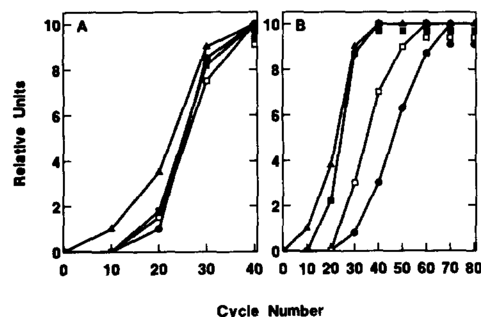

FIGURE 1. (*A*) DNA from an EBV-transformed cell line (Novako/EBV1, a diploid cell line that containing 2–3 copies of EBV DNA per cell) (25) was amplified by PCR methods using primers specific for the EBV-encoded genes Bam W (▲), BMRF-1 (■), or BMLF-1 (□) (Table I). Also, primers specific for the HLA-DQα (●) gene (22) were included as an internal control in each reaction mixture. 1 μg of Novako/EBV1 DNA was included with 100 pm of each primer and 2.5 U Taq polymerase per reaction mixture. After a varying number of PCR cycles, an aliquot of the reaction mixture was applied to agarose gels, transferred to nylon membrane and reacted with ^{32}P-labeled EBV or DQα-specific oligonucleotides. The intensity of reaction was quantitated using a densitometer. Peak areas are given as relative units. (*B*) Reconstruction experiments to detect EBV DNA. DNA from an uninfected T cell line (RPMI 8402) was mixed with DNA (1 μg) from an EBV-transformed cell line (Novako/EBV1) and was amplified by 10–80 cycles of the PCR with the Bam W primers. The amount of added viral DNA included 1 μg Novako/EBV1 DNA (▲), 100 ng (■), 1 ng (□), and 10 pg (●).

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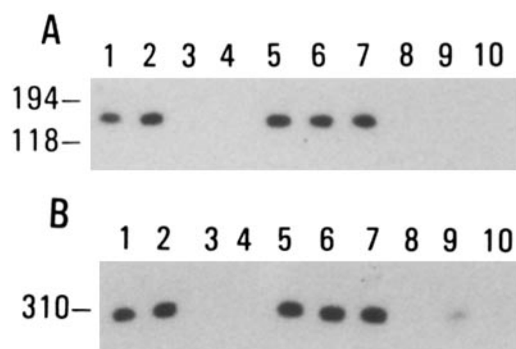

FIGURE 2. Southern analysis of PCR amplified genomic DNA with the EBV Bam W probe (*A*) and EBV BMRF-1 probe (*B*). PCR amplification performed as described below. (Lanes *1* and *2*) DNA from PBMC from patients with SS; (lanes *3* and *4*) DNA from normal PBMC; (lanes *5–7*) DNA from SS SG; (lanes *8–10*) DNA from normal SG.

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Increased Level of EBV DNA in Patient Samples. After 35 cycles of PCR amplification using the Bam W-encoded primers and ^{32}P-labeled Bam W oligonucleotide probe, we found significant reactivity in the PBMC DNA of 6/33 SS patients (Fig. 2 *A*, lanes *1* and *2*). Salivary gland biopsies from seven of nine SS patients contained EBV DNA using the Bam W primers and probes (examples shown in Fig. 2 *A*, lanes *5–7*). Specific amplification was also achieved using BMFR-1 primers in these DNA samples (Fig. 2 *B*, lanes *1–2*, and *5–7*). The specific reactivity with Bam W and BMRF-1 primers was confirmed using an additional set of primers/probes from the BMLF-1 region of EBV (Table I) (data not shown). In comparison, normal salivary gland biopsy DNA exhibited reactivity in only 2 of 15 cases when a similar number of cycles of amplification were used (Fig. 1, *A* and *B*, lanes *8–10*). Finally, EBV DNA was detected in only 3 in 50 normal PBMC and in 0 in 10 normal lymph node (LN) samples. These results are summarized in Table II. The frequency of SS salivary glands containing EBV DNA (78%) was higher than the frequency of SS PBMC containing EBV (18%) or from histologically normal salivary glands (13%) ($p <$ 0.001).

TABLE II
Summary of PCR Detection of EBV DNA in Clinical Samples

		Bam W primer					BMRF-1 primer				
Clinical samples*	*n*	+ + +	+ +	+	–	Percent positive	+ + +	+ +	+	–	Percent positive
SS SG	9	2	2	3	2	78	2	2	3	2	78
Normal SG	15	0	0	2	13	13	0	0	2	13	13
SS PBMC	33	3	0	3	27	18	1	2	1	29	12
Normal PBMC	50	0	0	3	47	6	0	0	5	45	10
Normal LN	10	0	0	0	10	0	0	0	0	10	0

The density of autoradiographic band after a limited number of PCR cycles corresponded to 10–20 copies of DNA per 10^5 cells equivalents indicated by + + + ; 5–10 copies EBV genome per 10^5 genomes (+ +); 1–5 copies EBV per 10^5 cells (+); or <1 copy EBV per 10^5 cells (–).

* SG refers to DNA from salivary gland, LN from lymph node, and PBMC from PBMC. SS refers to patients with Sjogren's Syndrome.

Discussion

We found increased levels of EBV DNA in SG biopsies from patients with SS, in comparison to biopsies from normal salivary glands (autopsy), patients with other autoimmune disorders, or patients with benign SG adenomas. We estimate that normal SG contained <1 copy of EBV DNA per 100,000 cell DNA equivalents; in contrast, the SS biopsies contained 20 or more EBV copies per 100,000 cell DNA equivalents. These results suggest that EBV latency in the normal SG is maintained through infection of a small number of epithelial cells and that EBV is reactivated in the SG of SS patients (3, 25). The increased content of EBV DNA in the SS salivary gland may represent a primary defect (i.e., an inherited inability to eliminate EBV-infected cells, as occurs in some immunodeficiency diseases) (4) or a secondary defect (i.e., the decreased ability to respond effectively to EBV as an acquired consequence of immune dysfunction) (6). Further characterization of the salivary and lacrimal gland T cells in vitro will be required to help evaluate these possibilities. Since EBV antigens are known to induce very strong T cell immune responses (26, 27) and epithelial cells of SS patients are intensively HLR-DR$^+$ (in contrast to normal SG cells that lack HLA-DR) (28), it is likely that immune responses directed against EBV-infected epithelial cells play a role in the perpetuation of SG destruction in SS patients. In this regard, SS may serve as a prototype human disease for studying the interaction of genetic and environmental factors in chronic autoimmune disease.

To analyze the small tissue biopsies from these patients, we used PCR methods. This study extends the use of PCR for clinical diagnosis in several regards. First, we were able to estimate the content of EBV DNA in comparison to cellular DNA. This was done by including primers for both viral DNA and for a single copy human gene (i.e., HLA- DQα). This approach is in contrast to previous studies where PCR has been used to detect the presence/absence of viruses such as human immunodeficiency virus, hepatitis virus, or rhinovirus (29–32), where viral presence is sufficient to allow disease diagnosis. In the case of EBV, virtually all adults harbor the virus at low levels and only increased levels are likely to be associated with disease (4, 6, 10). Therefore, it is necessary to quantitate the amount of EBV DNA and we have used a method for comparison to a single copy gene (i.e., HLA-DQα). This method also ensures that the DNA sample does not contain inhibitors of PCR that could lead to artefactually negative results of viral detection. Second, a potential problem in the use of a sensitive technique such as PCR is possible contamination of the tissue sample by small amounts of viral DNA during sample preparation. We and others (33) have noted that contamination of samples may occur at the step of tissue homogenization to make DNA, perhaps due to the need to "handle" tissues or due to "carryover" of trace amounts of DNA from prior samples. This problem has been minimized by placing the sterile tissue sample immediately into a sterile plastic tube where it remains during lyophilization to dryness, digestion with RNAase and proteinase K, and phenol extraction. This modification has significantly decreased the frequency of artefactual false positives, as well as allowing more rapid processing of a larger number of clinical samples. These methods of sample preparation also may prove useful in analyzing tissue biopsies from other autoimmune disease (i.e., diabetes, thyroiditis, polymyositis, systemic lupus erythematosus) where only small tissue samples are accessible early in the course of disease.

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Several different methods have been used to quantitate EBV DNA in patient samples. Tosato et al. (7) used the method of spontaneous outgrowth of lymphoblastoid cell lines in the presence of cyclosporin A to estimate the frequency of EBV-transformed lymphocytes in vivo. Other studies have used cocultivation with newborn lymphocytes (34) or in situ hybridization with labeled EBV-specific probes (35). PCR offers several advantages over detection of EBV by spontaneous outgrowth (7) or co-cultivation methods (34). First, PCR detection of viral DNA is not influenced by the endogenous immune response that may inhibit outgrowth of viral transformed cells in vitro (27). Second, DNA amplification will detect infected cells that are not actively shedding virus (i.e., a requirement for co-cultivation assays). Third, PCR is not dependent on cell suspensions required for in vitro assays and is suitable for small tissue biopsies (37) such as SG. Other methods such as in situ hybridization (35) or immunohistology (36) also may be used for viral detection in tissue samples, but the enumeration of rare infected cells (i.e., 1 in 10^5) is quite difficult and is often limited by nonspecific cross-reactions of antibodies or DNA probes.

Summary

Polymerase chain reaction has been used to detect increased levels of EBV DNA in salivary gland (SG) biopsies and PBL from patients with Sjogren's syndrome (SS). These results suggest that EBV, which has a normal site of latency in a small number of SG epithelial cells, may be reactivated in SS patients and provide a target for immune attack. The great sensitivity of polymerase chain reaction (PCR) and the ability to analyze very small tissue biopsies (37) make this technique well suited for clinical diagnosis. Specific methods to prevent artefactual contamination of tissue biopsy DNA with viral DNA of other samples (i.e., lyophilization of samples before DNA extraction) and the use of an internal positive control (i.e., inclusion of primers for a single copy human gene) during PCR amplification are presented. Since EBV reactivation occurs with markedly increased frequency in patients with lymphoproliferative and immunodeficiency diseases, as well as transplant recipients receiving cyclosporin A (10), rapid methods of viral detection such as PCR may allow better monitoring of medications and early detection of EBV-related lymphomas that may arise in these patients.

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