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Detection of Epstein-Barr Virus by Polymerase Chain Reaction

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The polymerase chain reaction (PCR) was used to study DNA extracted from the blood of 25 transplant patients, 5 patients with infectious mononucleosis, and 13 healthy subjects and autopsy or biopsy tissue from 29 patients with lymphoproliferative disorders. Primers were directed to conserved regions of the Epstein-Barr virus (EBV) genome encoding capsid protein gp220 and Epstein-Barr nuclear antigen 1. Specific EBV amplification was found in the blood of 11 of 25 transplant patients and all patients with infectious mononucleosis. All patients with lymphoproliferative disorders occurring in the presence of immunosuppression (eight organ transplant patients and two patients with acquired immunodeficiency syndrome) had biopsies positive for EBV by PCR. Only 1 of 19 samples from lymphomas or leukemias unrelated to immunosuppression contained EBV. PCR confirmed the very close association of EBV and lymphoproliferative disorders occurring in the presence of immunosuppression. The significance of detecting EBV sequences in the blood of transplant patients, particularly in relationship to lymphoproliferation, requires further study.

Studies of Epstein-Barr virus (EBV) infections are limited by the lack of routine culture techniques. Currently, laboratory diagnosis of primary or reactivated EBV infection depends on serologic tests (6). However, serology is an indirect marker of infection and is difficult to interpret in the presence of immunosuppression (8, 9, 14). Establishing the presence of EBV would have a definite impact on our understanding of the spectrum of EBV disease, pathogenesis, and management of the increasingly common EBV-related lymphoproliferative disorders (11, 13, 22). In addition, it could disclose associations of EBV to febrile illnesses and other poorly defined complications of immunosuppression (5, 14).

The polymerase chain reaction (PCR) allows specific and rapid identification of viral DNA sequences in blood and tissue samples (17, 20, 21; A. Telenti, A. J. Aksamit, J. Proper, and T. F. Smith, J. Infect. Dis., in press). PCR has been previously used to amplify EBV DNA in blood and tissue biopsies from patients with Sjogren's syndrome (19) and in limited studies of Hodgkin's lymphoma and EBV-related disorders occurring in immunosuppressed patients (2, 5, 17). Because of its sensitivity, PCR is ideally suited for studies of EBV-related phenomena.

MATERIALS AND METHODS

Clinical specimens. DNA was extracted (7) from leukocytes from the blood of 25 adult transplant recipients and 13 healthy adults. DNA was extracted directly from clotted blood from 5 patients with a clinical diagnosis of infectious mononucleosis, confirmed with a positive monospot test, and from frozen tissue from 27 patients with lymphoproliferative disorders and leukemias (autopsy or biopsy specimens of nodes, bone marrow, liver, brain, lung, pleura, parotid gland, and skin). Frozen tissue was not available from two additional patients with lymphoma; instead, paraffin-embedded material (liver and bone marrow specimens) was prepared as previously described (Telenti et al., in press). DNA extracted from a Burkitt's lymphoma cell line containing 118 to 224 copies of EBV per cell (1) (Daudi cells; American Type Culture Collection, Rockville, Md.) was the positive control for both EBV and human β-globin gene PCR.

Antibody titers to EBV capsid and nuclear antigens were determined by indirect and anticomplement immunofluorescence, respectively.

PCR procedure. Genomic DNA (0.1 to 0.5 μg) was added to 25 μl of PCR mix. The reaction mix contained a final concentration of 50 mM KCl, 10 mM Tris hydrochloride (pH 8.4), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μM each dNTP, 0.1 μM each primer, and 2.5 U of *Taq* polymerase. Primers were directed to conserved regions of the EBV genome encoding capsid protein gp220 (*Bam*HI L region) and Epstein-Barr nuclear antigen (EBNA) 1 (*Bam*HI K region) (Table 1).

After being denatured at 94°C for 10 min, samples were subjected to 30 cycles of amplification (1.5 min at 94°C, 45 s at 60°C, and 2 min at 70°C).

Amplification of the human β-globin gene was performed by using published primer sequences to assess the adequacy of each specimen (18). To avoid false-positive PCRs, we have instituted strict physical separation of the three main PCR steps (preparation of reaction mix, addition of target, and amplification and manipulation of PCR products).

Identification of viral sequences. PCR products were visualized with UV light as a single band by staining with ethidium bromide after agarose gel electrophoresis. Products were transferred to a nylon membrane by Southern blotting. Membranes underwent 5 h of prehybridization of 43°C with a solution containing 5× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]), 0.1% sodium dodecyl sulfate, and 100 μg of *Escherichia coli* RNA per ml (Calbiochem-Behring, San Diego, Calif.). The ^{32}P-end-labeled probes (100 pmol) (Table 1) were hybridized to the blots overnight. Southern blots were washed for 30 min at room temperature and for 30 min at 60°C (55°C for the human β-globin probe) with 2× SSPE and 0.1% sodium dodecyl sulfate prior to autoradiography.

RESULTS

On the basis of dilution experiments with DNA extracted from Daudi cells, the conditions described above optimally

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TABLE 1. EBV primer and probe sequences

EBV primer or probe	Sequence
gp220 (*Bam*HI L region)[a]	
Primer 1	5'-GGCTGGTGTCACCTGTGTTA
Primer 2	5'-CCTTAGGAGGAACAAGTCCC
Probe	5'-GGTGGAGGGGCTGAGTGTCTCTGGGTT TGAACTGGG
EBNA 1 (*Bam*HI K region)[b]	
Primer 1	5'-GTCATCATCATCCGGGTCTC
Primer 2	5'-TTCGGGTTGGAACCTCCTTG
Probe	5'-GGTGGCCCAGATGGTGAGCCTGACGTG CCCCCGGGAGCGA

[a] Product size, 239 base pairs.
[b] Product size, 269 base pairs.

amplified as few as 10 to 50 copies of the EBV genome (Fig. 1). The use of higher concentrations of genomic DNA (>1 μg) or of $MgCl_2$ (>2.5 mM) resulted in nonspecific amplification bands upon gel electrophoresis (although specificity was retained upon hybridization). Nonspecific bands were also seen with longer annealing time (≥1.5 min) or lower annealing temperature (≤55°C).

EBV-specific sequences were amplified from the blood of 11 of 25 transplant patients, all of whom were seropositive for EBV, and from clotted blood specimens of all 5 individuals with infectious mononucleosis. Sequential blood samples obtained over a 3-month-period were available from six of the EBV-positive transplant patients; five patients had multiple PCR-positive blood samples. EBV was not detected in the blood specimens of 13 healthy adults, 11 of whom were known to be seropositive (Table 2). EBV sequences were present in tissue from all 10 immunocompromised patients with lymphoproliferative disorders but only in 1 of 19 tissues from patients with other malignant B- or T-cell lymphomas or leukemias (Table 3). Blood samples available from two patients with EBV-positive lymphomas were also positive for EBV.

Frozen tissue specimens from transplant and acquired immunodeficiency syndrome (AIDS) patients with lymphoproliferative disorders were strongly positive for EBV, with bands of amplification readily visible upon gel electrophoresis (Fig. 2, lanes 1 and 2). The two paraffin-embedded tissue samples gave comparatively less amplification for both the human β-globin gene and EBV than frozen tissue samples (Fig. 2, lanes 3). In contrast, only 3 of 11 positive blood specimens from transplant patients had bands visible upon gel electrophoresis (Fig. 2, lanes 4 and 5). Samples of clotted blood from patients with infectious mononucleosis gave weak amplification of the β-globin gene, and EBV bands were detected only after Southern blotting and hybridization (Fig. 2, lanes 6).

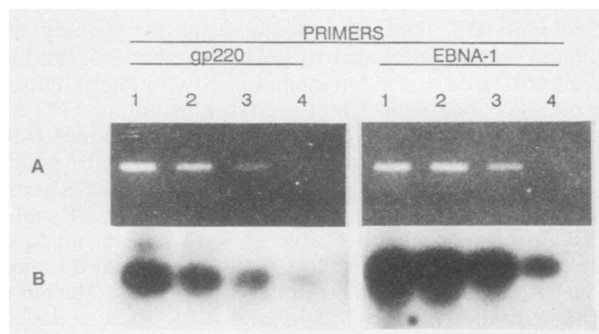

FIG. 1. Electrophoresis (A) and Southern blot (B) of EBV sequences amplified by gp220 or EBNA 1 primers. The source of DNA was an EBV-containing cell line (Daudi). Lanes 1 represent amplification of 10×10^3 to 50×10^3 viral copies. Lanes 2 to 4 are 10-fold dilutions to 10 to 50 EBV copies.

TABLE 2. Amplification of EBV DNA from blood

Subjects	No. positive by PCR/no. of patients
Healthy	0/13
Infectious mononucleosis	5/5
Transplant	11/25[a]
Liver	4/13
Heart	3/5
Kidney/kidney and pancreas	3/5
Bone marrow	1/2

[a] Two patients with EBV-positive blood had an EBV-related lymphoproliferative disorder.

The gp220 primer set amplified all 27 positive samples. EBNA 1 primers failed to detect EBV in two blood specimens positive by gp220 primers.

Amplification of human β-globin sequences rendered visible bands upon gel electrophoresis of all frozen biopsies from patients with lymphoproliferative disorders and all blood samples. The amplification products were not visible on the gel that was produced from paraffin-embedded tissue samples or from clotted blood samples (Fig. 2). The β-globin hybridization signal for sample 3 (Fig. 2) was visible upon prolonged radiographic exposure. In this sample, the stronger EBV signal suggests that there were more copies of EBV DNA than of genomic DNA.

DISCUSSION

Infection with EBV can be described as a low-to-moderate viral burden process that generates a prominent immune response. Infected B lymphocytes undergo cellular proliferation that is ultimately controlled by a T-lymphocyte response. Infectious mononucleosis is mainly the result of a prominent T-lymphocyte proliferation occurring in response to EBV-carrying B lymphocytes (4). In contrast, the lym-

TABLE 3. Amplification of EBV from lymphoproliferative disorders

Disorder	No. positive by PCR/no. of patients
Lymphoproliferative (in transplant patients)[a]	8/8
Lymphoproliferative (in AIDS patients)	2/2
Malignant B-cell lymphomas[b]-leukemias	1/8
Hodgkin's lymphoma	0/7
T-cell lymphocytic leukemia	0/4

[a] Liver (n = 2), heart (n = 2), kidney (n = 2), and pancreas (n = 2) transplants.
[b] None of these lymphomas occurred in the setting of immunosuppression. The EBV-positive patient had chronic lymphocytic leukemia.

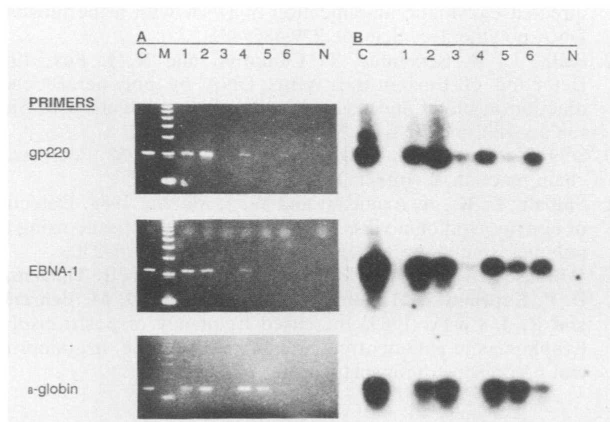

FIG. 2. Electrophoresis (A) and Southern blot (B) of amplified EBV sequences (gp220 or EBNA 1 primers) and the human β-globin gene from blood and tissue samples. Lanes 1, Transplant lymphoma (fresh frozen tissue); lanes 2, AIDS lymphoma (fresh frozen tissue); lanes 3, transplant lymphoma (paraffin-embedded tissue); lanes 4 and 5, blood from transplant patients; lanes 6, clotted blood from infectious mononucleosis. C, Positive control (Daudi cells); M, molecular weight markers; N, negative control (no target).

phoproliferative disorders following organ transplantation or AIDS represent an unchecked proliferation of EBV-infected B lymphocytes (3, 11, 12). Neither end of this spectrum of disease appears to be characterized by high viral burden and replication (8, 9). PCR is an attractive diagnostic tool in this setting because of its ability to detect reduced numbers of viral copies.

EBV was frequently and repeatedly detected in randomly collected blood samples from transplant patients and clotted blood samples from patients with infectious mononucleosis. We did not detect circulating EBV genome in normal EBV-seropositive subjects. The pool of EBV-infected lymphocytes in the blood of healthy EBV-seropositive individuals is very small. It has been estimated that there is only 1 EBNA-positive lymphocyte per 10^7 or 10^8 circulating cells in the peripheral blood of healthy EBV-seropositive adults and that 1 to 10 cells per million B cells are capable of developing into a continuous cell line in vitro because they are infected by EBV (3, 11, 16). The amount of sample used under our PCR conditions contained DNA extracted from only 15×10^3 to 75×10^3 leukocytes. Accordingly, the likelihood of detecting single EBV copies in our samples from healthy adults was greatly reduced.

EBV sequences were easily amplified from all the tissue samples from immunosuppressed patients with lymphoproliferative disorders. Therefore, PCR confirms prior Southern blot, EBNA immunostaining, and in situ hybridization data associating EBV to transplant- and AIDS-related lymphomas (10, 13, 15). Establishing the presence of EBV has prognostic importance, because EBV-associated tumors can be reversible upon reduction or discontinuation of immunosuppression (13).

EBV was not found in other malignant lymphoproliferative disorders not associated with immunosuppression except in one patient with chronic lymphocyte leukemia. The significance of this association is not clear at present.

We used paraffin-embedded tissue successfully on two occasions; however, the degree of amplification for the human β-globin gene and EBV was markedly reduced. Amplification from fixed material varies depending on source, length of Formalin fixation, and other less-studied factors (17; Telenti et al., in press). Successful amplification of the human β-globin gene should be a minimum requirement when paraffin-embedded tissue samples are used, and optimally, DNA extracted from frozen tissue should be used.

PCR will undoubtedly improve our understanding of the spectrum of EBV-related disease. Further study is necessary to determine the significance of finding EBV sequences in the blood of immunosuppressed patients, particularly in relationship to the development of lymphoproliferative disorders.

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