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Detection of Epstein-Barr Virus DNA in Cerebrospinal Fluid for Diagnosis of AIDS-Related Central Nervous System Lymphoma

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The diagnostic utility of Epstein-Barr virus (EBV) DNA detection in cerebrospinal fluid for the diagnosis of central nervous system lymphoma was evaluated with two different PCR assays to test a collection of cerebrospinal fluid samples from 24 AIDS patients with central nervous system disorders. A PCR assay amplifying a fragment from the *Bam*HI-W region had the highest clinical and analytic sensitivity. The *Bam*HI-W PCR assay detected EBV DNA in cerebrospinal fluid from 83% (5 of 6) of patients with pathologically proven primary central nervous system lymphoma and 7% (1 of 16) of controls with autopsy-proven nonlymphomatous central nervous system disorders. EBV DNA was also detected in one patient with autopsy-proven systemic lymphoma involving the central nervous system and one patient with probable primary central nervous system lymphoma. EBV DNA was detected consistently when central nervous system lymphoma involved meningeal surfaces. PCR for EBV in cerebrospinal fluid appears to be useful for diagnosis of AIDS-related central nervous system lymphoma, but additional studies are required to better define the sensitivity of the assay and to understand the significance of a positive test in the absence of lymphoma.

The incidence of AIDS-related primary central nervous system lymphoma (P-CNSL) has increased dramatically in the last decade (1). At present, P-CNSL occurs in 2 to 5% of patients with AIDS and is the second most frequent space-occupying lesion after toxoplasmosis (1, 11). The definitive diagnosis of P-CNSL currently requires a brain biopsy. Brain biopsies are invasive, and lesions are sometimes inaccessible. In one study, only 30% of the patients had an antemortem biopsy (4). This low rate of diagnosis has had a detrimental impact on the ability to improve current therapeutic strategies, because it decreases the number of patients potentially eligible for new protocols (5). In a recent review, the overall length of survival of AIDS patients receiving treatment for P-CNSL was approximately 3 months (4). It is clear that there is an urgent need for better diagnostic and therapeutic methods for AIDS-related P-CNSL.

Epstein-Barr virus (EBV) DNA has been detected by in situ hybridization in the neoplastic tissue of virtually all AIDS-related P-CNSLs and in approximately 50% of patients with AIDS-related systemic lymphoma (7, 9, 10). Recently PCR performed on cerebrospinal fluid (CSF) to detect EBV DNA has been described as a sensitive and specific technique for diagnosis of P-CNSL (3). The use of PCR for diagnosis of P-CNSL in patients with AIDS is appealing because of the clinical need for a noninvasive diagnostic method. The objective of the present study was to evaluate the diagnostic utility of EBV PCR performed on CSF for diagnosis of P-CNSL in a series of AIDS patients. We present results obtained with the previously described PCR assay, which detects a segment of the EBNA-1 gene (3), and results obtained with a different PCR assay which had greater sensitivity.

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MATERIALS AND METHODS

Patients. Eight patients with CNSL were included in this study: six patients had definite P-CNSL, one had systemic lymphoma with CNS involvement, and one had probable P-CNSL. Seven of the eight patients were identified from the records of the Pathology Department, Infectious Diseases Division and Virology Laboratory at the Washington University Medical Center, and an additional patient was diagnosed at St. Louis University Medical Center. Of the eight patients, six had P-CNSL confirmed at autopsy (patients 1, 2, 5, 6, and 8) and/or biopsy (patients 1, 7, and 8). One patient (patient 4) had systemic lymphoma with CNS involvement confirmed at autopsy. Patient 3, who did not have a pathologically confirmed diagnosis, had an intracerebral mass which failed to respond to antitoxoplasma treatment and responded to irradiation. This patient was considered to have probable CNSL. Sixteen control subjects were identified in the same manner as that used to identify the patients with CNSL. These patients had AIDS and cytomegalovirus (CMV) encephalitis ($n = 4$), cryptococcal meningitis and CMV encephalitis ($n = 1$), amebic encephalitis ($n = 1$), progressive multifocal leukoencephalopathy ($n = 1$), CMV and human immunodeficiency virus encephalitis ($n = 1$), disseminated blastomycosis ($n = 1$), CMV and toxoplasmic encephalitis ($n = 1$), cryptococcal meningitis ($n = 1$), no pathologic diagnosis ($n = 1$), microglial nodule encephalitis ($n = 1$), diffuse laminar necrosis ($n = 1$), toxoplasmic encephalitis ($n = 1$), and human immunodeficiency virus encephalitis ($n = 1$). The study was approved by the Human Studies Committee at Washington University.

All lymphomas had been previously diagnosed on the basis of typical pathologic findings in 2- to 5-μm-thick paraffin sections stained with hematoxylin and eosin. All lymphomas were $L26^+$ by immunohistochemistry (monoclonal antibody against a CD-20-like B-cell antigen; Dako, Carpinteria, Calif.). Three patients (patients 1, 2, and 4) had leptomeningeal involvement by lymphomatous tissue apparent at autopsy. The three patients who did not have meningeal involvement by lymphoma included two patients (patients 5 and 6) with multiple lymphomatous masses in the brain and cerebellum and one patient with a solitary lesion (patient 8). For patient 7, who had a solitary lesion in the right parietal lobe, autopsy was not performed and the intracerebral extent of the lymphoma could not be evaluated.

For the purpose of this study, formalin-fixed samples from lymphomatous tissue were tested with four monoclonal antibodies (code M897; Dako) which recognize four distinct epitopes present on a 60-kDa latent membrane protein (LMP) encoded by the BNLF gene of EBV (12). LMP is a latent gene product which has been implicated in oncogenesis (15). LMP was detected by an indirect

TABLE 1. EBV DNA detection in the CSF of AIDS patients according to diagnosis, magnetic resonance imaging findings, and time of CSF sampling

Patient(s)	Diagnosis	Grade of LMP staining[a]	Lymphoma involving meninges at autopsy	No. of CNS lesions by MRI[b]	Time of sampling (no. of days before death)	No. of positive results by PCR assay[c]			
						EBNA-1		*Bam*HI-W	
						EB	SB	EB	SB
1	P-CNSL	+++	Yes	7	41	3	3	3	3
2	P-CNSL	+++	Yes	7	7	3	3	3	3
3	P-CNSL (probable)	ND[d]	Not done	1	26	3	3	3	3
4	SL[e]	++	Yes	9	9	1	1	3	3
5	P-CNSL	+++	No	Not done	155	0	0	0	0
				3	103	0	0	1	3
6	P-CNSL	+	No	0	163	0	0	0	0
				Not done	150	1	1	2	3
				Not done	135	0	0	1	3
				3	16	0	0	1	1
7	P-CNSL	++	Not done[f]	1	18	0	0	0	1
8	P-CNSL	+	No	1	18	0	0	0	0
9	Blastomycosis	ND	NA[g]	1	69	0	0	1	3
10–29	Various (other controls)	ND	NA	NA	1–574	0	0	0	0

[a] +++, strong; ++, medium; +, weak.
[b] Determined at the time of the CSF sampling. MRI, magnetic resonance imaging.
[c] Results represent three assays. EB, ethidium bromide; SB, Southern blotting.
[d] ND, not determined.
[e] SL, systemic lymphoma.
[f] Lymphoma diagnosed by brain biopsy (meningeal tissue not available).
[g] NA, not applicable.

immunoperoxidase technique with a Vectostatin kit according to the instructions of the manufacturer (Vector, Burlingame, Calif.). The staining was graded as follows: +++, strong; ++, medium; and +, weak. It has been shown previously that LMP antibodies do not have cross-reactivity with negative control tissues (13, 16). Therefore, they are useful probes for the diagnosis of latent EBV infection in tissue sections.

CSF specimens. CSF samples were collected from all patients prior to death and before antineoplastic treatment was started. Samples were stored frozen at −70°C for 3 to 48 months before testing.

PCR. Twenty-eight CSF samples from 24 patients were tested three times each with two different PCR assays to detect the presence of EBV DNA. Both assays were chosen because of their high level of sensitivity (3, 14). The EBNA-1 PCR assay amplifies a 297-bp segment of the EBNA-1 gene with a reported level of sensitivity of four EBV genomes and has been reported previously to be highly sensitive and specific for the diagnosis of P-CNSL in patients with AIDS (3). A second PCR assay employed primers which amplify a 296-bp segment of the *Bam*HI-W region that is repeated up to 11 times within the EBV genome (2). This assay has been previously reported (14) to be capable of detecting EBV DNA in peripheral blood mononuclear cells from healthy EBV-seropositive subjects. The reported level of sensitivity of the *Bam*HI-W PCR assay is one EBV genome (14). For both PCR assays, a 10-μl aliquot of CSF was used after being heated at 95°C for 10 min. The amplification reactions were performed as described previously (3, 14). PCR products were analyzed by electrophoresis through 3% 3:1 Nusieve-agarose gels containing 0.5 μg of ethidium bromide per ml and photographed under UV illumination. DNA was transferred to nylon membranes (Hybond-N_2; Amersham, Arlington Heights, Ill.) with 0.5 M NaOH–1.5 M NaCl as the transfer buffer. After blotting for 18 h, the membranes were cross-linked by UV irradiation for 0.5 min at 12,000 μJ (Stratalinker UV Crosslinker; Stratagene, La Jolla, Calif.) and incubated in blocking solution (Lightsmith II system, Promega, Madison, Wis.) at 56°C (EBNA-1 products) or 46°C (*Bam*HI-W products) for 60 min. A 39-bp probe (3) homologous to an internal segment of the EBNA-1 gene and a 24-bp probe (14) homologous to an internal segment of the *Bam*HI-W fragment were conjugated with alkaline phosphatase with an oligonucleotide-alkaline phosphatase conjugation kit according to the instructions of the manufacturer (Lightsmith II system). Hybridization was done at 56°C (EBNA-1 probe) or 46°C (*Bam*HI-W probe) for 45 min with 6 ml of hybridization solution per 100 cm^2 of membrane and 200 fmol of the alkaline phosphatase-conjugated probe per ml. After hybridization, the nylon membrane was washed three times in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate at 56°C (EBNA-1 probe) or 46°C (*Bam*HI-W probe) for 5 min and once in 1× SSC at room temperature for 5 min. Bound probes were detected with Kodak X-Omat AR film and the chemiluminescent detection reagents provided with the Lightsmith II system.

Lysates from the EBV-infected cell line X50-7 served as positive controls. (The cell line was a gift from S. Speck, Department of Pathology, Washington University School of Medicine.) This cell line is known to contain approximately five copies of the EBV genome per cell (13a). Aliquots of 200,000 X50-7 cells were transferred to a microcentrifuge tube and pelleted by a brief centrifugation. After the supernatant was discarded, 27 μl of 0.04 M NaOH was added to the dried cell pellet. Specimen tubes were boiled for 10 min, after which 53 μl of 0.1 M Tris-HCl (pH 7.6) was added. Serial 10-fold dilutions of the lysate were made in water and tested by PCR.

To avoid false-positive amplifications, procedures recommended for preventing contamination were strictly observed (8). Additionally, before use, all tubes, pipette tips, and reagents, except for primers and *Taq* polymerase, were exposed to 254 nm of UV irradiation with a UV nucleic acid linker oven (Stratalinker UV Crosslinker; Stratagene).

To rule out the presence of inhibitors of PCR in CSF (6), 8-μl aliquots of CSF samples from patients with P-CNSL and negative EBV PCR results were mixed with 8-μl aliquots of serial 10-fold dilutions of the X50-7 cell line lysate containing 1, 10, and 100 EBV DNA copies and subjected to PCR.

To try to further improve the clinical sensitivity of the *Bam*HI-W assay, DNA was purified (QIAamp blood kit; QIAGEN, Chatsworth, Calif.) and concentrated by ultrafiltration (Centrex UF, Keene, N.H.) from 200-μl aliquots of the CSF samples.

Clinicopathologic correlation. The clinical and autopsy records of each patient were reviewed. PCR results for patients with CNSLs were correlated with CSF parameters (cell count and chemistry), meningeal involvement and number of mass lesions detected at autopsy, grade of staining for LMP, and number of mass lesions detected by a magnetic resonance imaging performed closest to the day of CSF sampling.

Statistical analysis. Statistical analysis of the data was performed with the Mann-Whitney U test. A P value of <0.05 was considered significant.

RESULTS

Detection of EBV LMP. Epstein-Barr virus LMP was detected by immunocytochemistry in the six P-CNSLs tested and in the systemic lymphoma with CNS metastasis. Immunoperoxidase stained infiltrates of atypical lymphoid cells with elongated, often hyperchromatic nuclei and prominent nucleoli. These infiltrates were most often perivascular; however, parenchymal staining and leptomeningeal staining were occasionally seen. Staining for LMP was present both on the surface membrane and in the cytoplasmic granules. Not all atypical lymphoid cells stained positively, and the intensity of the reaction product on atypical lymphoid cells varied among patients (Table 1). Smaller, benign-appearing cells were negative.

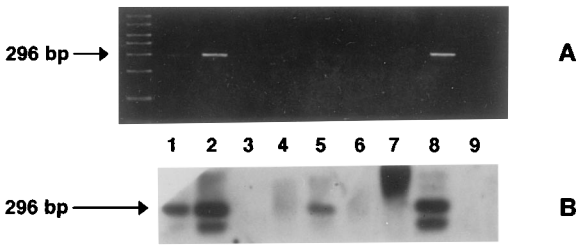

FIG. 1. Agarose gel electrophoresis and ethidium bromide staining (A) and Southern Blot hybridization (B) of amplification products after the *Bam*HI-W PCR assay. Lanes: 1 and 2, DNA extracted from X50-7 cells (approximately 1 and 10 EBV DNA molecules, respectively); 3, DNA-free control; 4 and 5, CSF samples from the same patient with P-CNSL (patient 5) obtained 155 and 103 days before death, respectively; 6, CSF sample from a patient with P-CNSL and a solitary lesion (patient 7); 7, CSF sample from a patient with CMV radiculomyelitis (note strong nonspecific hybridization at a different location from the amplified product of the PCR; 8, CSF sample from a patient with P-CNSL involving leptomeninges (patient 2); 9, CSF sample from a patient with amebic encephalitis.

PCR. PCR testing of serial 10-fold dilutions of the alkaline lysate prepared from X50-7 cells showed that the EBNA-1 and *Bam*HI-W PCR assays were able to detect approximately 10 genomes when ethidium bromide staining was used to detect the amplified products. When Southern blotting was performed, the *Bam*HI-W PCR assay but not the EBNA-1 PCR assay increased in sensitivity to approximately one EBV genome.

With the *Bam*HI-W PCR assay, EBV DNA was detected in CSF from seven of eight patients with CNSL, including five of six patients (83%) with P-CNSL, the patient with presumed P-CNSL, and the patient with systemic lymphoma and CNS metastasis (Fig. 1 and Table 1). EBV DNA was also detected in a CSF sample from one of 16 (7%) controls with nonlymphomatous CNS disorders. The EBNA-1 PCR assay detected EBV DNA in CSF samples from five of eight patients with CNSL and none of the 16 control subjects. With the *Bam*HI-W PCR assay, the increased sensitivity afforded by Southern blotting of the PCR product increased the rate of detection of EBV DNA in CSF of patients with CNSL (Table 1).

Two patients (patients 5 and 6) had sequential CSF samples available for testing. On the basis of the results from the *Bam*HI-W PCR assay, patient 5 converted from negative to positive on samples obtained 155 and 103 days before death and patient 6 converted from negative to positive on samples obtained 163 and 150 days before death.

Inhibition studies showed that the ability to detect EBV DNA in positive control material containing 1, 10, or 100 EBV DNA copies was not affected by mixing with aliquots of CSF from the two patients with P-CNSL and consistently negative PCR results (patients 7 and 8), suggesting that the negative PCR results in these samples were not caused by PCR inhibitors in the CSF.

No increase in the clinical sensitivity of the *Bam*HI-W PCR was found after purification and concentration of the CSF samples (data not shown).

Clinicopathologic correlation. EBV DNA was detected repeatedly by either PCR assay in CSF samples from two patients (patients 1 and 2) with multiple lymphomatous masses involving meningeal surfaces. Of the two patients with solitary lesions not involving the ventricles (patients 7 and 8), one was consistently negative by both assays and the other was positive only once with the *Bam*HI-W PCR assay and Southern blot detection. Two patients with strong staining for LMP (patients 1 and 2) had EBV DNA detected consistently in their CSF. In the remaining patients, no correlation between the grade of staining for LMP and the results of PCR was found.

Patients in whom EBV DNA was detected repeatedly (patients 1 to 4) by the *Bam*HI-W PCR assay had a higher median number of CNS lesions detected on the autopsy than those in whom EBV DNA was detected in some but not all replicate runs of the assay (7 versus 2; $P = 0.03$). There was also a trend towards higher levels of protein in the CSF of patients in which EBV DNA was detected repeatedly (121 versus 65 mg/dl; $P = 0.09$). There was no correlation between the number of erythrocytes or leukocytes and glucose levels in CSF with the results of the EBV PCR.

DISCUSSION

In our series of AIDS patients, EBV DNA was detected in the CSF of 87.5% (seven of eight) of patients with CNSLs. The detection of EBV DNA in the CSF was affected by the extent and location of the CNSL and by the limit of detection of the PCR assay used. Our results suggest that EBV DNA is more likely to be detected in the CSF of AIDS patients with CNSLs who have widespread disease or meningeal involvement.

Using the EBNA-1 PCR assay described by Cinque et al. (3), we detected EBV DNA in CSF from three of six patients with P-CNSL. This result differs from those of Cinque's study (3), in which EBV DNA was detected in CSF from 16 of 16 patients with P-CNSL. Decreased sensitivity of the EBNA-1 PCR assay in our laboratory is a possible explanation, but the ability of our assay to detect 10 EBV genome equivalents is comparable to the 4 EBV genome equivalents reported in the previous study (3). An alternative explanation could be a difference in the extent of disease in the patients studied. Two of the patients in our series for whom the EBNA-1 PCR assay was negative had solitary lesions at the time of CSF sampling. Cinque et al. (3) did not report the extent of disease in their series, and therefore a direct comparison is not possible. In our study, the *Bam*HI-W PCR assay had a lower limit of detection (approximately one EBV genome) and was positive for two patients with P-CNSL whose CSF specimens were negative by the EBNA-1 PCR assay. This finding suggests that the level of EBV DNA in the CSF of some patients with P-CNSL is very low. This low level of EBV DNA in CSF might also explain the inconsistent results among replicate runs obtained with the *Bam*HI-W PCR assay for approximately half of the patients with CNSLs.

Although the results of our study suggest that EBV DNA detection in CSF might be useful for diagnosis of AIDS-related CNSL, our data indicate that EBV CSF PCR must be refined before it can achieve optimal clinical utility. In particular, the PCR assay producing the best trade-off between sensitivity and specificity for diagnosis remains to be established. A more sensitive PCR assay might detect EBV DNA in cases of AIDS-related P-CNSL not involving meningeal or ventricular surfaces and might improve the reproducibility of results but might also be positive for more patients without clinically evident CNSL. Both the present study and that of Cinque et al. (3) detected EBV DNA in one patient in whom CNSL was not identified at autopsy. At present, it is unknown whether these are false-positive results or true detection of EBV DNA, possibly identifying patients at high risk for future development of P-CNSL.

The present study had two main limitations: first, the number of patients was small; second, autopsy findings might not have reflected brain pathology present at the time of CSF sampling, which was performed at various intervals before death. However, in two patients (patients 2 and 4) whose

autopsies showed meningeal involvement by lymphoma, CSF samples were obtained shortly before death (7 and 9 days). This suggests that meningeal involvement was already present when these CSF samples were obtained.

In summary, our study suggests that PCR detection of EBV DNA in the CSF may be useful in the diagnosis of AIDS-related P-CNSL. It is encouraging that EBV DNA can be detected in the CSF of most AIDS patients with P-CNSL. In our study, the sensitivity, specificity, and reproducibility of EBV PCR performed on CSF samples varied, depending on the extent and location of the tumor and the limit of detection of the PCR assay used. Further studies will be required to establish the diagnostic utility of EBV PCR performed with CSF samples. Until additional data are available, EBV PCR performed with CSF should be carried out in an investigational setting.

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