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August 10, 1990

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Re: Moran Foundation Account 1-87-0022

The study of Epstein-Barr Virus and Primary Central Nervous System Lymphoma has been completed.

I am enclosing a copy of the manuscript which ensued and was published in Human Pathology.

Sincerely,

Emilie Rouah, M.D.

ER:lr

Enclosure

Demonstration of Epstein-Barr Virus in Primary Central Nervous System Lymphomas by the Polymerase Chain Reaction and In Situ Hybridization

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Primary lymphomas of the central nervous system (CNS) account for 0.3% to 1.5% of all intracranial neoplasms. Several reports have noted a coincidence between this neoplasm and serologic evidence of Epstein-Barr virus (EBV) infection, but in only a few instances has the EBV genome been demonstrated in these tumors. To further evaluate the frequency of this occurrence, we analyzed primary CNS lymphomas using nucleic acid hybridization methods and the polymerase chain reaction (PCR). In situ hybridization was used in selected cases. Sequences of EBV were found in two of nine cases by PCR and in situ hybridization. Southern blot hybridization of genomic DNA from these samples was negative for EBV. Both tumors arose in patients with conditions shown to produce secondary immunodeficiency, namely, chronic alcohol abuse and diabetes mellitus. We conclude that the association of EBV and CNS lymphoma is not restricted to patients with severe primary immune deficiency, and that PCR can be applied successfully to paraffin-embedded tissue for the detection of low-abundance viral sequences. HUM PATHOL 21:545–550. © 1990 by W.B. Saunders Company.

Primary central nervous system (CNS) lymphomas account for approximately 1% of primary intracranial neoplasms,[1] and recent epidemiologic studies suggest the incidence is increasing.[2] Since they occur more frequently in immunocompromised individuals,[1] the rising number of organ transplant recipients and patients with acquired immunodeficiency syndrome (AIDS) is partly responsible for this increase. The incidence is also rising in older individuals with no identifiable immune system deficiency.[2] While the etiology of this neoplasm is unknown, an association between Epstein-Barr virus (EBV) infection and primary CNS lymphoma has been demonstrated by serology, immunohistochemistry, Southern blot hybridization, and in situ hybridization in some cases.[3-10]

More recently, in vitro amplification of specific nucleic acid sequences by the polymerase chain reaction (PCR) has been applied to the identification of EBV DNA in one case of EBV-associated lymphoproliferation in an immune-compromised individual.[11] Major advantages of this technique are its potential use in the detection of DNA sequences present in a small subset of cells or in low abundance, and its potential application to the study of archival material.[12,13]

In this study, we analyzed nine cases of primary CNS lymphoma for EBV sequences by a variety of nucleic acid hybridization techniques depending on tissue availability. The techniques included Southern blot hybridization in six cases from which relatively high molecular weight DNA was extracted from two fresh-frozen or four formalin-fixed paraffin-embedded tumors. Amplification of PCR with subsequent Southern blot hybridization was applied to extracted genomic DNA and in selected cases, in situ hybridization was also done.

MATERIALS AND METHODS

Tumor Samples

A total of 25 intracranial lymphomas biopsied between 1981 and 1988 were retrieved from the Methodist Hospital, Houston, Texas pathology archives. Of these, only seven tumors were analyzed; the remaining 18 were excluded either because of insufficient material or because they were not primary lesions. Two additional cases were provided by Houston area community hospitals. Seven tumors had sufficient material available for DNA extraction. The remaining two cases were examined by in situ hybridization only (see below).

DNA Preparation

Genomic DNA from two fresh-frozen and six formalin-fixed paraffin-embedded tumors was extracted by the method of Goelz et al.[14] DNA from LA350 cells (an EBV-transformed lymphoblastoid cell line)[15] was isolated using a model 340A nucleic acid extractor (Applied Biosystems, Inc, Foster City, CA).

DNA Probes

The EBV probe. The EBV probe used in the Southern blot hybridization was a 3.3 kb (kilobase) EcoRI-BamHI fragment isolated from pACYC184 containing EBV fragment EcoRI B.[16] This fragment was recovered after electrophoresis through a 1% low melting temperature agarose gel and labeled using the oligolabeling kit from Pharmacia (Piscataway, NJ).

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Key words: Epstein-Barr virus, central nervous system, brain, lymphoma.

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The human albumin cDNA probe. The human albumin cDNA probe was a 1.1 kb fragment isolated by PstI digestion of F47.[17] Recovery and labeling were as described above. This probe was used as a control in Southern blot hybridizations to evaluate the integrity of the extracted DNA.

Oligonucleotide primers and probes. Two pairs of oligonucleotide primers were synthesized by Genetic Designs, Inc (Houston, TX) using an Applied Biosystems DNA Synthesizer (Foster City, CA).[18] Primer sequences used for the amplification of EBV were homologous to the *Eco*RI B fragment and spanned a 375-nucleotide sequence. A 20 bp (base pair) internal oligonucleotide (EBV2) was labeled to a specific activity of 10^9 cpm/μg using a 3′ end labeling kit from Boehringer Mannheim (Indianapolis, IN). The sequences were as follows:

EBV 3 (primer) GTGTGCGTCGTGCCGGGGCAGCCAC
EBV 4 (primer) ACCTGGGAGGGCCATCGCAAGCTCC
EBV 2 (probe) ACCTCAACCTGGAGACAATT

Primers for c-K-*ras* amplification were taken from the first exon and spanned a 135-nucleotide sequence that includes the 12th codon.[19-21] A 20 bp internal oligonucleotide (K-*ras* 4) was labeled as above. This sequence, which is present in all cells, should amplify in all samples. It was used to ensure that the conditions used in the PCR were optimal. The sequences were as follows:

K-*ras* 2 (primer)
TATTATAAGGCCTGCTGAAAATGACTGAAT
K-*ras* 3 (primer)
TTACCTCTATTGTTGGATCATATTCGTCCA
K-*ras* 4 (probe)
CCTACGCCACCAGCTCCAAC

Polymerase Chain Reaction on Extracted DNA

Reagents were added to microcentrifuge tubes containing 1 or 5 μg of extracted DNA. The reaction mixture contained 50 mM KCl, 10 mmol/L Tris-Cl, pH 8.3, 1.5 mmol/L magnesium chloride, 0.01% (w/v) gelatin, 200 μm each dNTP and water to 99.5 μL. This was overlaid with 100 μL of heavy mineral oil (Sigma, St Louis, MO) and the tubes were boiled for 6 minutes. One-half μL of Taq polymerase (activity 4 U/μL) was added to each tube immediately prior to amplification which was performed using the DNA Thermal Cycler (Perkin/Elmer Cetus, Norwalk, CT/Emeryville, CA). Each cycle included a 94°C denaturation step (2 minutes), a 60°C annealing step (1.5 minutes) and a 72°C extension step which was 60 seconds for the first cycle and extended by 10 seconds for each additional cycle to a total of 40 cycles.

Controls

Positive control for the presence of EBV sequences. DNA extracted from the EBV-transformed lymphoblastoid cell line LA350[15] was used as positive control in the genomic DNA Southern blot analysis. The same DNA was used as a positive control for the PCR.

Negative controls. DNA extracted from LA350 cells and genomic DNA from leukocytes of known cytomegalovirus-(CMV) infected individuals were amplified with CMV primers LA1 and LA6[22] and used as negative controls in hybridizations with the internal oligonucleotide probe EBV2 to exclude any cross-reactivity between these two viruses of the Herpes group. Genomic DNA extracted from leukocytes of a serologically EBV-negative individual amplified with EBV primers was also used as a negative control.

Southern Blot Hybridizations

Genomic DNA. Ten micrograms of DNA from each sample were digested with the restriction endonucleases *Eco*RI and *Bam*HI, fractionated by electrophoresis through an 0.8% agarose gel, blotted, and hybridized as described previously.[17] Hybridizations using the 3.3 kb EBV and the 1.1 kb F47 probes were carried out simultaneously.

PCR products. Twenty-five microliters of PCR-reaction mixture were analyzed by Southern blot hybridization following electrophoresis on 1.5% Seakem Agarose gels and transfer to 0.45 μm Nytran (Schleicher and Schuell, Keene, NH) using 10X SSC (20X SSC = 3.0 mol/L sodium chloride, 0.3 mol/L sodium citrate). The gel-purified oligonucleotide probe was labeled as described above. The filters were prehybridized for at least 4 hours in 6X SSPE (20X SSPE = 3.0 mol/L sodium chloride, 0.2 mol/L sodium phosphate, 0.02 mol/L sodium ethylenediaminetetraacetic acid), 0.2% polyvinylpyrrolidone, 0.2% Ficoll and 50 μg/mL sheared, denatured salmon sperm DNA.

Hybridizations were done in 6X SSPE and 1% SDS overnight, at high stringency (Tm-5°C). Filters were washed in 6X SSPE and 1% SDS at room temperature for 21 minutes and in 1X SSPE, 1% SDS at Tm-5°C for 3 minutes, and developed by autoradiography to Kodak XAR film.

To further confirm that the amplified sequence was EBV, 25 μL of PCR product of positive samples were digested with the restriction endonuclease *Hind*III and analyzed by Southern blot hybridization. *Hind*III cuts the 375 bp product into 214 and 161 bp fragments; only the former should hybridize to the internal oligonucleotide probe, EBV2.

In situ hybridization. Three-micron sections from five formalin-fixed, paraffin-embedded tumor blocks were mounted on silane coated slides and dried in an 80°C oven overnight. The sections were then deparaffinized in xylene, hydrated to water through graded alcohols, digested in 0.3% pepsin for 8 minutes at 37°C, dehydrated to absolute alcohol, and air-dried. The EBV probe obtained from Enzo Diagnostics, Inc (New York, NY) was diluted in the hybridization solution (50% formamide, 10% dextran sulfate, 5X SSC, 10X Denhardt's and 250 μg/uL denatured salmon sperm DNA) to a final dilution of 1:15. The sections were covered with 20 μL of the probe, heated to 100°C for 8 minutes, and hybridized overnight at 37°C. They were rinsed in 4X SSC, developed using the nickel chloride-enhanced diaminobenzidine method described by Hsu and Soban,[23] and counterstained with nuclear fast red.

The positive control, provided with the probe, consisted of EBV-infected cells (ATCC # B95-8) shown to have up to 200 to 400 copies of the EBV genome. Two negative controls included uninfected cells provided with the probe as well as adjacent tissue sections treated as described above except for omission of probe.

RESULTS

Six tumors which yielded moderately high molecular weight DNA were analyzed by Southern blot hybridization. Hybridization using the F47 albumin insert showed the expected 1.6, 3.5, and 10.8 kb hybridization bands in the LA350 control and in four of six tumors. The expected 3.3 kb EBV hybridization

band was seen in the LA350 control DNA. Sequences of EBV were not detected in these four tumors by this method (Table 1). No definite hybridization bands were detected with either the F47 or the EBV probe in the remaining two cases, suggesting that the DNA was too degraded to be examined by genomic Southern blot hybridization.

Southern blot hybridization using the internal oligomer EBV2 on the EBV-amplified PCR products showed the expected 375 bp hybridization band, indicative of the presence of EBV sequences, in two of seven tumors analyzed. No hybridization band was detected in a DNA template-minus control. Southern blot hybridizations to *Hind*III restriction digests of these same EBV-amplified products showed hybridization of EBV2 to the 214 bp fragment, in both cases, confirming the specificity of the amplified product (Fig 1). No cross-hybridization to CMV was detected.

Southern blot hybridization of K-*ras*–amplified PCR products with the internal oligomer K-*ras* 4 showed the expected 135 bp hybridization band in all seven samples but not in a DNA template-minus negative control.

Five cases were analyzed for EB viral sequences by in situ hybridization including four cases also analyzed by PCR. Two of five cases, which were shown to have EBV sequences by the PCR, demonstrated EB viral sequences in several tumor cells (Fig 2). The remaining two cases, found to be negative by PCR, and an additional case, were also negative by in situ hybridization.

The results are summarized in Table 1.

DISCUSSION

Primary CNS lymphomas are rare and account for approximately 1% of intracranial neoplasms.[1] They frequently occur in patients who have a well-defined underlying primary immune deficiency, congenital or acquired.[1] The incidence of this neoplasm is rising, partly due to the increasing number of organ transplant recipients and patients with AIDS. Increasing numbers of older patients with no identifiable immune deficiency are also being reported.[2] Several immunohistochemical studies have shown that the majority of these lymphomas are of B-cell origin.[24-27] Although their etiology remains unknown, EBV infection has been implicated in the pathogenesis of some cases.[3-10] An association between the inability to mount an effective immune response to EBV and B-cell lymphoproliferation, involving primarily the CNS, was first demonstrated in a patient with the X-linked lymphoproliferative syndrome.[3] A number of studies have shown an association between EBV and polyclonal as well as monoclonal B-cell lymphoproliferative diseases occurring in renal transplant recipients.[1,5,28] Epstein-Barr virus DNA has also been identified in CNS lymphomas of both immunocompromised individuals[1,4,7] as well as a single individual with no identifiable immune deficiency.[4] Some of these patients also had demonstrable serologic evidence of EBV infection.[4,28] A recent study of eight CNS lymphomas by in situ hybridization demonstrated EB viral sequences in four out of four tumors arising in immunocompromised and none of four tumors arising in nonimmunocompromised hosts.[10] The authors suggest EBV is unlikely to play a role in the pathogenesis of CNS lymphoma in the nonimmunocompromised individual.

Our patients were between 43 and 76 years of age, with six being 65 years or older. A summary of pertinent clinical data is presented in Table 2. A review of medical records confirmed that all cases examined were true primary CNS lymphomas originating in the cerebrum or cerebellum with no evidence of extracranial disease. None of our patients had a documented immune deficiency. Three had associated conditions (chronic alcohol abuse, and diabetes mellitus)[29-32] known to alter humoral or cell-mediated immunity and/or neutrophil function, and considered to be secondary immune deficiency states. The radiologic features, outlined in Table 2, included single and multiple intraaxial mass lesions, and one bifrontal mass radiographically interpreted to be a high-grade glioma. Histologically, the tumors were classified as malignant lymphoma, not otherwise specified (four cases) or large-cell lymphoma (five

TABLE 1. Hybridization Results

	Southern Analysis				
	Genomic DNA				
Sample No.	F47	EBV	EBV Ampl DNA	K-*ras* Ampl DNA	In Situ Hybridization
81-90 TA	—	—	—	—	Neg
81-20 JMR	—	—	Neg	Pos	—
83-16 JAH	—	—	—	—	Neg
83-21 MM	Neg	Neg	Neg	Pos	Neg
86-11 EB	Neg	Neg	Neg	Pos	—
86-22 WS	Pos	Neg	Pos	Pos	Pos
87-11 MLG	Pos	Neg	Pos	Pos	Pos
87-17* MCR	Pos	Neg	Neg	Pos	—
87-18* LWS	Pos	Neg	Neg	Pos	—

Abbreviations: Ampl, amplified; Neg, negative hybridization; Pos, positive hybridization; PCR, polymerase chain reaction.
* Fresh frozen samples.

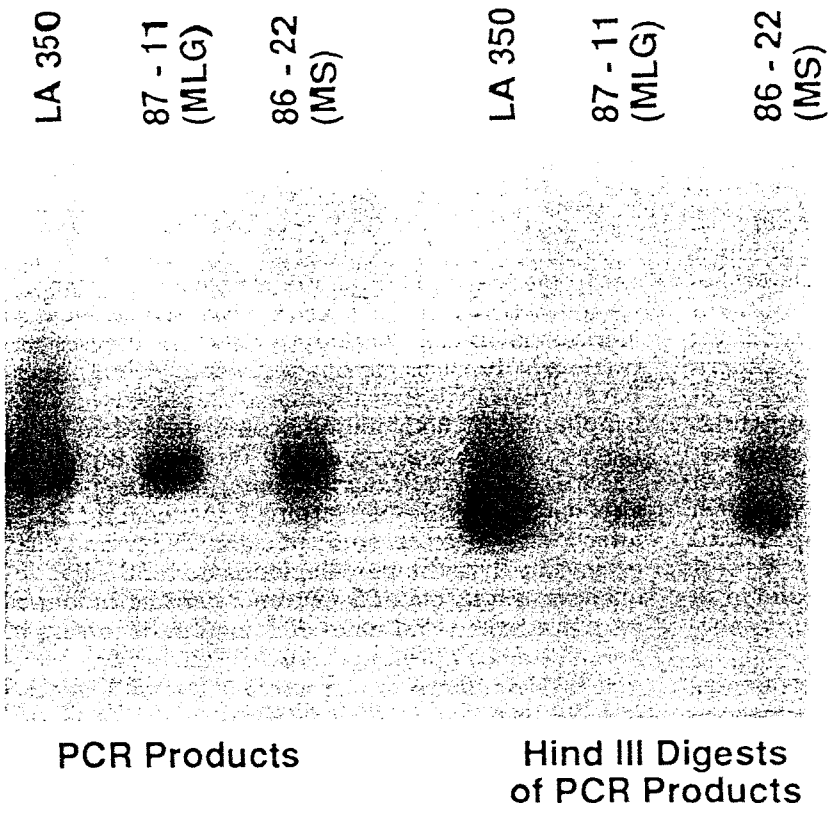

FIGURE 1. Southern blot analysis of EBV-amplified sequences using EBV2 as a probe shows the expected 375 bp hybridization band. Hybridization of EBV2 to the 214 bp band of *Hind*III restriction digests confirms the identity of the amplified sequence. The 375 bp band is also seen due to incomplete digestion of the PCR product with *Hind*III.

cases). Two tumors were further characterized as B-cell type by immunohistochemistry.

We were able to demonstrate EBV sequences in two cases (86-22, 87-11) by using PCR to amplify a 375 bp fragment of the EBV genome and by in situ hybridization. The latter demonstrated the presence of EB viral sequences in a subset of cells which cytologically appeared immunoblastic, having large vesicular nuclei and prominent nucleoli (Fig 2). We were unable to demonstrate EBV sequences by Southern blot hybridization of genomic DNA extracted from these tumors. This was most likely due to the relatively low concentration of EBV DNA in these samples as well as to some degradation of the extracted DNA in at least two samples. Southern blot hybridization of K-*ras*–amplified PCR products with the internal oligomer K-*ras* 4 confirmed that PCR could be applied successfully to the DNA extracted from par-

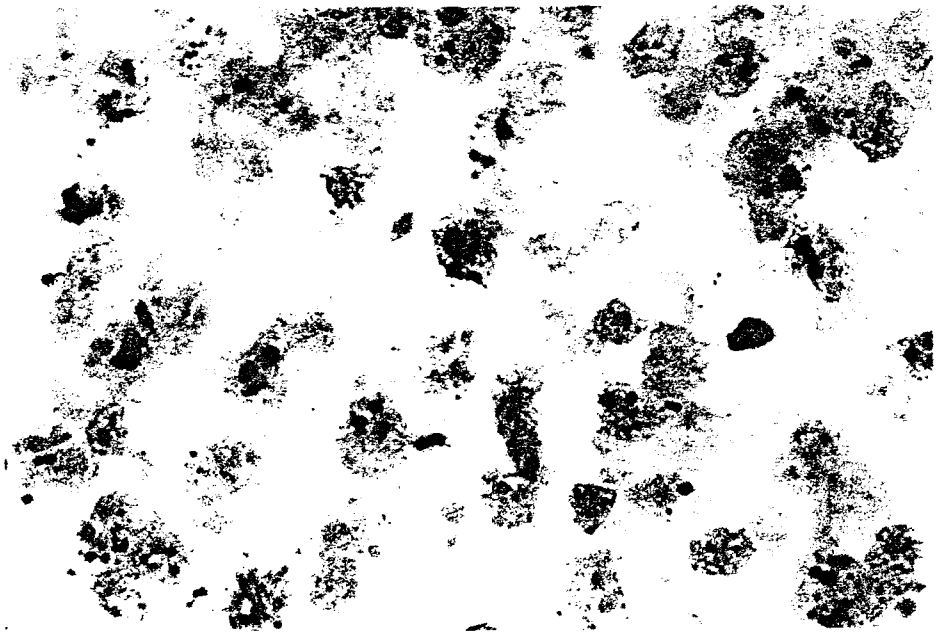

FIGURE 2. In situ hybridization of tumor 86-22 showing an area of the tumor in which EB viral sequences were most abundant (magnification × 1000). In other areas, fewer cells demonstrated the presence of EBV. Tumor 87-11 showed a similar distribution of staining.

TABLE 2. Clinical and Pathologic Features of Patients With Central Nervous System Lymphoma

Sample No.	Age/Sex	Location	Pathology	Other Studies	Clinical Features
81-90 TA	77/F	R cerebellar and L thalamic masses	Large-cell lymphoma	Abd CT—neg, Bone scan—neg	Difficulty with balance, incoordination and difficulty swallowing for 10 days
81-20 JMR	43/M	L parietal mass and multifocal periventricular lesions, R cerebral hemisphere	Malignant lymphoma, NOS	No clinical evidence of systemic disease	Difficulty writing and irritability for 6 months; diplopia, loss of balance, ataxia, dystonia, nystagmus, and incoordination for 1 month
83-16 JAH	49/M	R parietal mass	Malignant lymphoma, NOS	Bone scan—neg, Abd CT—neg, CXR—neg	Severe headache for 1 week, followed by loss of consciousness and tonic seizure
83-21 MM	76/F	R cerebellar and L thalamus masses	Large-cell lymphoma	Abd CT—neg	Nausea, vomiting, headache, and incoordination for 10 days
86-11 EB	56/F	Bifrontal enhancing lesion, "butterfly glioma"	Large-cell lymphoma	Unavailable	Unavailable
86-22 WS	65/M	R cerebellar mass with obstructive hydrocephalus	Malignant lymphoma, large-cell (B-cell type)	Abd CT—neg, Bone scan—neg	Headache, diplopia, dizziness, confusion, depression, nausea, vomiting for 1 month; history of alcohol abuse
87-11 MLG	65/F	R temporal mass with marked edema mass effect	Malignant lymphoma, large-cell (B-cell type)	Abd CT—neg, Bone scan—neg	Headache R parietal region, confusion, lethargy; L hemiparesis, history of diabetes mellitus
87-17 MCR	66/F	R medial temporal mass	Malignant lymphoma, NOS	CXR—TB, Abd CT—neg, BM bx—neg	Sudden onset L hemiparesis, diabetes mellitus; pulmonary TB
87-18 LWS	76/F	R medial temporal mass	Primary lymphoma	CXR—neg	R orbital temporal-headache for 5 weeks; history of hypertension

Abbreviations: Abd, abdomen; BM, bone marrow; bx, biopsy; CT, computed tomography; CXR, chest x-ray; DX, diagnosis; L, left; MS, multiple sclerosis; Neg, negative; NOS, not otherwise specified; R, right; TB, tuberculosis.

affin-embedded samples, validating its usefulness in assaying for the presence of EBV DNA.

This study is, to our knowledge, the first demonstration of the application of PCR to the detection of EB viral sequences in CNS lymphoma. This method appears to be as sensitive as in situ hybridization, and more sensitive than standard Southern blot hybridization, for analysis of archival DNA specimens. Our study brings the number of primary CNS lymphomas examined for EBV sequences by methods other than standard Southern blot analysis to a total of 17. Bashir et al[10] demonstrated EBV sequences by in situ hybridization in four out of four immunocompromised and in none of four nonimmunocompromised individuals. Our finding of EB viral sequences in tumors arising in patients without a primary immune deficiency state broadens the spectrum of conditions in which this association occurs. Furthermore, EBV has been previously demonstrated in a tumor found in a nonimmunocompromised individual.[7] It is therefore unlikely that EBV alone is responsible for the induction of these neoplasms and suggestive that additional, as yet unidentified mechanisms, predispose to B-cell lymphoproliferation. As suggested by Hochberg et al,[4] the development of an animal model using transgenic mice will be helpful in elucidating the role of EBV and other factors involved in the pathogenesis of tumors arising in both immunocompromised and nonimmunocompromised hosts.

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