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Detection of Epstein-Barr virus in CNS lymphomas by in-situ hybridization

R.M. Bashir, MD; N.L. Harris, MD; F.H. Hochberg, MD; and R.M. Singer, PhD

Article abstract—We used an optimized in-situ hybridization technique employing a biotinylated Epstein-Barr (EB) virus sequence, *Bam*H1V (3.1 kb), to detect this sequence in 2 EB virus-infected cell lines (B95-8 and Namalwa) and 8 CNS lymphomas. We obtained a good hybridization signal from cytospins of B95-8 (EB virus productively infected) and Namalwa (EB virus latently infected, 1 copy per cell) cell lines. We were able to detect signal from both cell lines after overnight fixation in 10% formalin and paraffin embedding, but development time in the detection chromogen required longer incubation and the signal intensity was lower than in cytospin cells. We then used the technique to examine formalin-fixed, paraffin-embedded primary CNS lymphoma tissue from 4 patients who were immunocompromised (1 renal transplant, 3 acquired immune deficiency syndrome) and 4 patients who were not. All 4 CNS lymphomas from immunocompromised patients hybridized well with *Bam*H1V, exhibiting a pattern of staining similar to Namalwa cells and nonlytically infected B95-8 cells. There was no relationship between the intensity and degree of reaction and the patients' survival. None of the 4 CNS lymphomas in immunocompetent patients or uninvolved brain showed any reactivity with *Bam*H1V. We suggest that low-abundance targets are detectable in paraffin-embedded tissue by in-situ hybridization using biotinylated probes. Detection of EB viral sequences in CNS lymphomas in immunocompromised patients suggests a role for the virus in the pathogenesis of this tumor.

NEUROLOGY 1989;39:813-817

Primary CNS lymphoma accounts for about 1 to 3% of primary brain tumors.[1-3] Wider use of immunosuppressants and the acquired immune deficiency syndrome (AIDS) epidemic are the apparent causes of a gradual increase in incidence of primary CNS lymphomas over the past decade; tumor in nonimmunosuppressed patients has also increased.[4-6] Epstein-Barr (EB) virus, a human gamma herpesvirus, is endemic to humans and induces lymphoproliferative disorders in some animals.[7-10] It also induces human B cells to divide indefinitely.[11,12] EB virus is detectable in Burkitt's lymphoma cells and in a number of systemic lymphomas associated with immunosuppression, including both iatrogenic suppression for cardiac, renal, or liver allografts as well as congenital and acquired immunodeficiency diseases.[13-17] Some case reports have linked this virus to primary CNS lymphomas associated with both immunosuppression for renal allografts and AIDS.[18-20]

Previously published studies on detection of EB virus in tumor tissue have used hybridization techiques to DNA extracted from these tumors and fixed to filters or Southern blots using [32]P- or ^{35}S-labeled probes.[14-20] Recently, Lawrence et al[21] used a high-resolution method, employing a biotin-labeled *Bam*H1V probe-
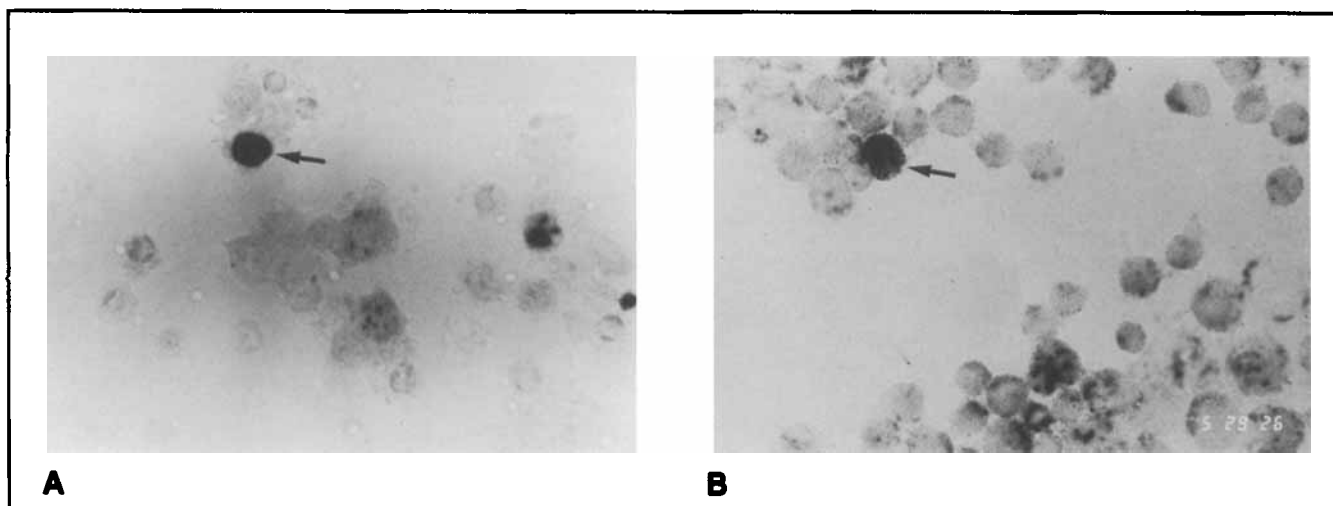
Figure 1. (A) Photomicrograph of cytospin, paraformaldehyde-fixed B95-8 cells hybridized with biotinylated BamH1V probe. Arrow shows focus of intense staining (×200 magnification before 54% reduction). (B) Photomicrograph of formalin-fixed, paraffin-embedded B95-8 cells hybridized with biotinylated BamH1V probe. Arrow shows focus of intense staining (×200 magnification before 54% reduction).

and an avidin fluorescence detection system to hybridize to Namalwa cells containing 1 integrated copy of EB virus per cell. A good signal was observed on chromosome 1, the site of EB virus integration in these cells.[21] These techniques have higher resolution than previously described methods and can localize sequences to specific cell types and specific intracellular regions.[21,22] Such localization is not possible with DNA extraction techniques, which involve cell destruction and DNA extraction, yielding an average number of viral sequences for a given number of cells. Moreover, biotinylated probes can be used on paraffin-embedded tissue, allowing greater safety when handling infectious material and access to old banked tissue, which is not possible with DNA extraction methods.[22]

In this report, we describe the optimization of an in-situ hybridization technique employing a biotin-labeled BamH1V sequence, and its use in examining a group of primary CNS lymphomas in patients with and without known immunosuppression. We detected EB viral sequences in the 4 CNS lymphomas arising in immunosuppressed patients, and none in lymphomas from 4 nonimmunosuppressed patients.

Materials and methods. *Preparation of cells.* B95-8, Namalwa, and EB virus-negative Ramos cells (gift from Dr. J. Sullivan) were maintained in minimum essential medium and 10% fetal calf serum (FCS) (GIBCO). Fifteen percent FCS was used for Ramos cells. The cells were cytospun at 1,800 rpm and washed twice in 0.3 M phosphate-buffered saline in 5 mM magnesium chloride (PBS-MgCl$_2$) and resuspended at a concentration of 10^6 cells/ml; 0.1 ml of this suspension was mixed with 0.1 ml FCS and cytospun on poly-L-lysine–coated (Sigma) coverslips. The coverslips were air dried and fixed in 4% paraformaldehyde for 10 minutes and stored in 70% ethyl alcohol at 4 °C until the time of the experiment, when the cells were washed twice in PBS-MgCl$_2$.

For formalin-fixed cells, the cells were again centrifuged at 1,800 rpm and washed twice in PBS-MgCl$_2$. The cell pellet was then fixed in 10% formalin overnight and processed for rou-

tine paraffin-embedding at the Massachusetts General Hospital pathology laboratory. Six-μm sections of the embedded cells were sectioned and mounted on poly-L-lysine–coated coverslips and left to dry overnight. The sections were dewaxed twice in xylene for 5 minutes each, then dipped twice in 100% ethyl alcohol and air dried.

Patients. We studied 4 patients with primary CNS lymphoma who were immunocompromised and 4 patients with primary CNS lymphoma who were not immunocompromised. All patients were seen at the Massachusetts General Hospital; all were men, aged 26 to 41 years. The 1st patient had received a kidney transplant 6 months earlier and was taking cyclophosphamide following an episode of graft rejection. This patient's clinical course has been reported previously.[20] The other 3 patients had AIDS. Three of the patients died within 3 months of diagnosis and only 1 survived more than 6 months. We also studied 4 patients with primary CNS lymphoma and no documented immunocompromise, 2 men and 2 women aged 36 to 72 years.

Lymphoma tissue. Formalin-fixed, paraffin-embedded sections of CNS lymphoma tissue were cut at 6 μm and mounted on poly-L-lysine–coated coverslips. The coverslips were dewaxed in xylene as described earlier.

Preparation of probes. EB virus sequence BamH1V (3.1 kb) (gift from Dr. J. Sullivan) was grown in PBR 322 vector. BamH1V and PBR 322 control probe were nick translated using biotinylated 11-dUTP nucleotide (BRL) according to standard methods.[23] The probes were separated from unincorporated nucleotides on a Sephadex G-50 column.

Hybridization protocol. Tissue preparation. Cytospins of cultured cells were washed twice in PBS-MgCl$_2$ and dewaxed paraffin-embedded sections were hydrated in 70% ethyl alcohol, then deproteinated in 0.2 N HCl for 10 minutes. This was followed by proteinase K (Sigma) (4 μg/ml) digestion at 37 °C for 10 minutes. The coverslips were next incubated in 0.1 M TRIS-glycine for 10 minutes, then 0.1 M triethanolamine for 10 minutes, and finally kept in 50% formamide (Sigma) in 2× sodium citrated saline (0.15 M SSC) until the probes were ready for hybridization.

Probe preparation. Thirty ng of labeled probe were added to 4 μl of carrier DNA-RNA (equal mix of E coli RNA [10 mg/ml] [Boehringer] and sheared salmon sperm DNA [10 mg/ml] [Sigma]) and lyophilized. Next, 11 μl formamide were
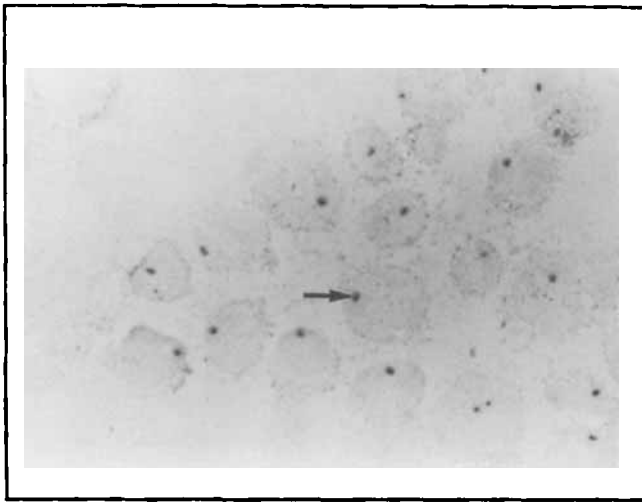
Figure 2. Photomicrograph of cytospin, paraformaldehyde-fixed Namalwa cells hybridized with biotinylated BamH1V *probe. Arrow shows a typical area of signal (×300 magnification before 54% reduction).*

added and the tubes were heated to 90 °C for 2 minutes. After it was removed from the heat, 11 µl of hybridization buffer (2 × SSC, 50% dextran sulfate, 2% bovine serum albumin [BSA; Boehringer], 0.1 M vanadyl sulfate [BRL]) were added to the probe, mixed well, and pipetted to Parafilm laid over a glass plate. The coverslips containing the specimens were turned over the probes (specimens facing the probes) and covered with Parafilm. The glass plates carrying the coverslips were heated to 100 °C for 2 minutes, then incubated at 37 °C overnight.

Washing and development. Parafilm, which hardens in the form of small envelopes around coverslips, was cut with sharp razor blades. The glass plates were submerged in 2× SSC, and the coverslips floated a few minutes later (this technique minimizes tissue loss). The coverslips were placed in Coplin jars and washed in 50% formamide in 2× SSC for 30 minutes at 37 °C followed by 50% formamide 1× SSC at 37 °C for another 30 minutes. This was followed by 2 10-minute washes in 1× SSC and 0.5× SSC at room temperature. The coverslips were next washed twice in 4× SSC-0.05% Triton for 10 minutes with shaking. For target detection, a 30-minute incubation in streptavidin alkaline phosphatase (Dako) (1:250 dilution in 4× SSC-1% BSA) was carried out at 37 °C. Color development was carried out by incubation of the coverslips in nitro blue tetrazolium—5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) (BRL) in buffer 3 (0.2 M TRIS, 0.2 M sodium chloride, 50 mm $MgCl_2$) with monitoring at frequent intervals. When an adequate signal was obtained or the background became too high, the reaction was stopped by fixation in 4% paraformaldehyde for 2 minutes, washing in PBS, then mounting in aqueous crystal medium (BRL).

Results. Cytospin preparations of B95-8 cells showed foci of intense nuclear staining in 5 to 10% of the cells. Other scattered cells showed some nuclear granules and many cells showed none (figure 1A). Formalin-fixed, paraffin-embedded B95-8 cells exhibited a similar signal pattern to cytospin preparations (figure 1B), but the signal was slightly weaker and took much longer to develop in NBT-BCIP (20 minutes for cytospins and 2 hours for paraffin-embedded cells). In contrast, Namalwa cells showed 1 or 2 discrete areas of definite

signal uniformly seen in most cells (figure 2). Formalin-fixed, paraffin-embedded Namalwa cells showed signal only from 25 to 30% of the cells. The development time in NBT-BCIP was again considerably longer than for cytospins of Namalwa cells (1 hour for cytospins and 12 hours for paraffin-embedded cells).

We next examined formalin-fixed, paraffin-embedded tumor tissue from the 4 immunocompromised patients with primary CNS lymphoma. All these tumors had cells that hybridized with the BamH1V probe but not the control probe PBR 322. Tumor cells from patients 1 and 4 had a similar pattern of hybridization, with most cells in the section showing some nuclear hybridization (figure 3, A and D). No foci of intense hybridization similar to the ones noted with B95-8 cells were seen in any of the tumors. Tumor cells from patients 2 and 3 showed fewer areas of nuclear hybridization seen in about 1% of the cells (figure 3, B and C). Areas of uninvolved brain seen in patient 2 showed no hybridization to BamH1V or pbR 322 control probe. None of the tumors from the 4 CNS lymphomas from nonimmunosuppressed patients hybridized with either BamH1V probe or control probe.

Discussion. Using a biotinylated BamH1V probe, we detected EB virus sequences on cytospin preparations of B95-8 and Namalwa cells. We chose BamH1V sequence (3.1 kb) because it encodes for the internal repeat unit of EB virus, which is repeated 11 times, giving about 35 kb coverage. To maximize the signal, we did not attempt to eliminate hybridization to RNA.

Formalin-fixed, paraffin-embedded cells showed loss of signal intensity and required a much longer development time in NBT-BCIP, which increased the background significantly on Namalwa cells. Extensive cross-linking of DNA and proteins due to fixation in formalin and processing of the tissue may have made the hybridizing targets less accessible, accounting for the reduced signal.[22] In addition, only about one-third of the paraffin-embedded cells showed signal compared with nearly 100% of cytospin Namalwa cells. Since the diameter of the nucleus is 8 to 10 µm, and the section thickness is 6 µm, the target DNA could easily be missed in many cells, depending on the plane of sectioning. This factor may explain why only a third of paraffin-embedded Namalwa cells showed signal. This loss of signal due to the plane of sectioning must be considered when interpreting results from paraffin-embedded tumor tissue.

All 4 CNS lymphomas from immunosuppressed patients showed evidence of EB viral sequences. The signal observed was mostly nuclear and was similar in appearance to the pattern observed in Namalwa cells. That is, most positive cells had only a few granules of staining in the nucleus. This pattern was also similar to that seen in the scattered, weakly positive B95-8 cells and much different from the intense staining of the rare lytically infected B95-8 cells. Thus, the virus in the lymphomas may exist in a latent state, possibly integrated to host DNA, and be passed on with cell division instead of being propagated by lytic infection. In patient 1, the presence of EB virus DNA was confirmed by
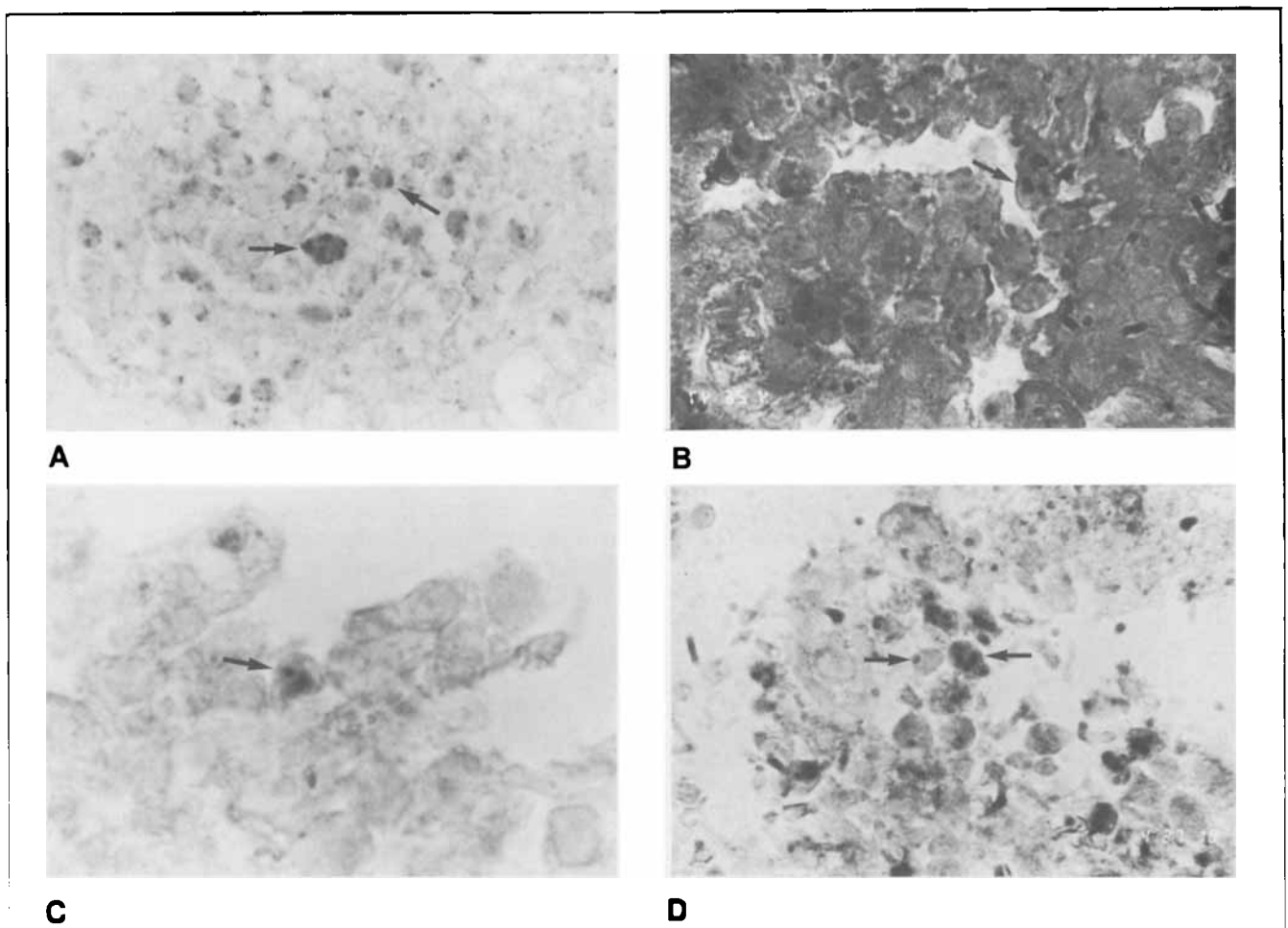
Figure 3. Photomicrograph of formalin-fixed, paraffin-embedded CNS lymphomas hybridized with biotinylated BamH1V probe. The arrow indicates hybridization signal. (A) Patient 1, renal transplant, shows numerous positive cells. (B) Patient 2, AIDS, shows rare positive cells. (C) Patient 3, AIDS, shows few positive cells. (D) Patient 4, AIDS, shows many positive cells. (×200 magnification before 53% reduction.)

hybridization to EB virus sequences on Southern blots of DNA extracted from the tumor.[20] The concentration of EB virus in the tumor by this method was 30 to 50 copies per tumor cell. In-situ hybridization showed a large percentage of tumor cells exhibiting signal.

In this limited number of patients we could not find a relationship between the degree of EB virus content of tumor cells and patient survival. Patient 4, whose tumor exhibited the intense staining and the largest number of positive cells, had the longest survival time (more than 6 months). Patient 1, who also had a large percentage of positive cells in his tumor, survived 1 week after diagnosis. Patients 2 and 3, who had only scattered positive cells, had intermediate survival times.

Our results are compatible with earlier observations that primary CNS lymphomas in immunocompromised patients contain EB virus genomes.[18-20] The earlier studies used hybridization to host DNA extracted and fixed to a filter or to Southern blots of DNA from these tumors.[18-20] Our study shows that the technique of in-situ hybridization is sensitive enough for detection of low abundance EB virus sequence in paraffin-embedded tissue. This technique yields additional information on the type of cells involved as well as about the pattern of

viral persistence in these tumors. Whether the virus gives growth advantage to already transformed tumor cells or is simply a passenger in neoplastic B cells is not clear at this time.[24] The absence of detectable EB virus sequences in CNS lymphomas in nonimmunosuppressed patients argues against a causative role for this agent in sporadic CNS lymphomas.

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Agenesis of the corpus callosum and gyral malformations are frequent manifestations of nonketotic hyperglycinemia

William B. Dobyns, MD

Article abstract—A 2-week-old infant who presented with myoclonic encephalopathy had biochemical abnormalities consistent with nonketotic hyperglycinemia. Cranial CT showed agenesis of the corpus callosum, gyral malformation, and ventricular enlargement. Similar brain abnormalities occurred in 9 of 15 previously reported patients. Including this patient, agenesis of the corpus callosum appeared in 6 of 15 patients, gyral malformation in 6 of 14, ventricular enlargement in 5 of 15, and cerebellar hypoplasia in 2 of 16. Nonketotic hyperglycinemia thus joins a growing list of inborn errors of metabolism associated with brain malformations.

Nonketotic hyperglycinemia is an autosomal recessive disorder of glycine metabolism. Clinical symptoms usually begin early in life and consist of myoclonic seizures, progressive neurologic deterioration, and often neonatal death. Survivors have continued myoclonic and other seizures, profound mental retardation, and severe neurologic impairment. Biochemical changes consist of elevated glycine levels in plasma, urine, CSF, and body tissues, especially the brain.[1-8] The disorder is caused by deficient activity of various components of the glycine cleavage system (H, L, P, and T proteins).[6,7]

Diagnosis requires quantitative amino acid analysis