

Detection of Epstein-Barr Virus DNA in Mouthwashes by Hybridization

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An assay for the presence of Epstein-Barr virus (EBV) DNA was developed by using a cloned EBV DNA probe. After preliminary testing showed the assay to be sensitive and specific, it was applied to 135 mouthwashes from bone marrow transplant recipients, and 21 of these tests were positive. The concentration of EBV DNA in mouthwashes in some cases was as high as 10^8 genome equivalents per ml. When compared with the lymphocyte transformation assay on the same samples, the sensitivity was 75% and the specificity was 97%. In contrast to the lymphocyte transformation assay, the hybridization was semiquantitative and yielded results in 72 h. Potential applications include monitoring the effects of various interventions, such as immunosuppressive and antiviral chemotherapy, on EBV shedding.

Nucleic acid hybridization technology is being explored as a means of detecting the presence of pathogens not readily detected by classical techniques (5, 16, 20, 22). Epstein-Barr virus (EBV) is an example of an agent that has proven difficult to recover from clinical samples, despite the fact that this virus is ubiquitous in human populations. Serological studies have shown that most adults have been exposed to the virus (11), and EBV-transformed lymphocytes can regularly be recovered from the blood of seropositive individuals (8). The virus, which is transmitted via the saliva, is clinically important, being associated with mononucleosis, Burkitt's lymphoma, and nasopharyngeal carcinoma, as well as the polyclonal and monoclonal lymphoproliferative syndromes recently attracting attention in organ transplant recipients (3, 4, 9, 12-14, 18, 19, 21). An understanding of the relationship between viral excretion and the diseases with which EBV is associated has been hampered by the cumbersome cord blood lymphocyte transformation assay, which has been used to detect the presence of the virus in saliva. Neither the detection of viral antigen by immunological methods nor the detection of virions by electron microscopy has proven satisfactory. DNA-DNA hybridization provides an alternative approach. Spot blotting to detect EBV DNA with whole viral DNA probes was initially described as a technique to determine EBV genome copy number in EBV-transformed cell lines (2). However, until recently, EBV DNA was not available in a quantity sufficient to make this a technique applicable to clinical specimens. Now, however, fragments of viral DNA cloned into bacterial plasmids can be grown in quantity, and such cloned probes have been applied to the detection of EBV genomes in pathology specimens (1, 6). We report here our experience with the use of a cloned EBV probe to detect the presence of EBV DNA in mouthwash specimens from bone marrow transplant recipients. The hybridization assay is contrasted with the lymphocyte transformation method in bone marrow transplant recipients. The clinical and investigative value of this approach is illustrated in a repeatedly tested index case.

MATERIALS AND METHODS

EBV probe. In all experiments the EBV DNA probe used was the pSL76 plasmid which contains two copies of the *Bam*HI-W repeat fragment (10). Nick translation with [32 P]dCTP was carried out by standard procedures to achieve a specific activity of 5×10^7 cpm/ μ g (17). Material to be assayed by hybridization was treated with 1/10 volume of 5 M sodium hydroxide and allowed to stand on ice for 10 min. Each sample was neutralized with 1 volume of 3 M sodium acetate (pH 5.3). Samples (200 μ l) were applied under vacuum to wells of a 96-well apparatus (Minifold; Schleicher and Schuell) with a nylon filter (Pall biodyne A) prewetted in $4 \times$ SSC ($1 \times$ SSC = 0.15 M sodium chloride plus 0.015 M sodium citrate [pH 7.0]). The filter was baked at 80°C for 1 h, prehybridized for 2 h at 67°C in 5 ml of a solution containing $5 \times$ SSPE ($1 \times$ SSPE = 0.18 M sodium chloride, 10 mM sodium phosphate, and 1 M EDTA [pH 7.4]), $5 \times$ Denhardt ($20 \times$ Denhardt = 2% Ficoll, 2% polyvinylpyrrolidone, and 2% bovine serum albumin), 0.2% sodium dodecyl sulfate, and 100 μ g of salmon sperm DNA, and hybridized overnight at 67°C in a mixture of the same composition with the addition of 500 μ l of dextran sulfate and 100 ng of 32 P-labeled probe DNA. Filters were then washed three times for 45 min each with agitation at 37°C in a buffer containing 5 mM NaH_2PO_4 , 1 mM EDTA, and 0.2% sodium dodecyl sulfate (pH 7.0). Filters were air dried and exposed to Kodak X-AR5 film with intensifying screens for 12 to 36 h.

Mouthwash collection. Mouthwash samples were collected weekly from 43 bone marrow transplant recipients for lymphocyte transformation assay. Patients were instructed to swish and expectorate 10 ml of RPMI 1640 medium into a cup. The mouthwashes were passed through a filter (pore size, 0.45 μ m; Millipore), and 10% fetal calf serum was added. Specimens were coded and frozen at -80°C.

Lymphocyte transformation assay. The mononuclear layer of fetal cord blood was separated on a Histopaque (Sigma Chemical Co.) gradient. Cells were washed in RPMI 1640 and resuspended at 5×10^6 /ml in RPMI 1640 containing 20% fetal calf serum and 10% dimethyl sulfoxide. Cells were frozen to -50°C in a controlled-rate ethanol freezer (Vari-Rate 2; Virtis) and stored in the vapor phase of liquid nitrogen until use. Upon thawing, the cells were washed

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twice, and trypan blue-excluding cells were counted. Preliminary experiments demonstrated no significant difference between fresh and stored lymphocytes in capacity for EBV transformation. For mouthwash assay, 10^6 fetal cord blood lymphocytes were pelleted and resuspended in 1 ml of mouthwash. This was incubated for 1 h at 37°C. The lymphocytes were washed, resuspended in RPMI with 10% fetal calf serum, and aliquoted into five separate wells of 96-well round-bottom tissue culture plates. These were incubated at 37°C in 5% CO₂ for 3 to 8 weeks with weekly changes of culture media. The appearance of a continuously growing cell cluster accompanied by a decrease in the pH of the culture medium indicated that transformation had occurred.

Clinical sample hybridization. Frozen 300- μ l samples from specimens which had previously been tested in the cord blood lymphocyte transformation assay were available for hybridization assay. These were thawed, denatured, and neutralized as described above. Each treated sample was then divided into thirds, each of which was applied to a well in the 96-well apparatus. Raji cells and B95-8 cell culture supernatant fluids served as positive controls. Raji cells were applied in eight serial twofold dilutions to the filters such that the range from 7.8×10^2 to 1×10^5 Raji cells ($\times 60$ to 100 EBV genomes per cell = between 4.68×10^4 to 7.8×10^4 and 0.6×10^7 to 1×10^7 EBV genomes) was represented. It was possible to extrapolate from the mouthwash hybridization signal to a concentration of EBV genome equivalents in the mouthwash by visual comparison with the hybridization signal associated with the serial Raji dilutions. Autoradiograph exposures of various lengths of time (12 to 48 h) facilitated these visual comparisons. BJAB cells served as negative controls. To determine whether there was nonspecific adherence of probe to the filters, we used a strip wash procedure (7) to remove the pSL76 probe and reprobbed several filters with a pBR322-derived plasmid which did not contain EBV sequences.

Sensitivity and specificity. The sensitivity of the hybridization assay for EBV DNA was examined by suspending cells from EBV-carrying cell lines (Raji, Daudi, Namalwa, B95-8, P3HR1) in 0.9% sodium chloride, making successive twofold dilutions, and applying them to the filter as described above. Specificity was assessed by similarly applying cell lines known not to contain EBV DNA (BJAB, HL60) to filters. In addition, DNAs extracted from human placenta, human spleen, and cell cultures infected with viruses other than EBV were applied to filters. The proportions of viral DNA versus cell DNA in DNAs extracted from infected cell cultures were assessed by agarose gel electrophoresis such that 1 μ g each of the various viral DNAs was applied. These were then successively diluted fourfold, applied to the filter, and hybridized as described above.

RESULTS

Sensitivity and specificity. When serial dilutions of Raji cells were applied to filters, it was possible to distinguish the positive hybridization signal associated with as few as 200 Raji cells ($\times 60$ to 100 genomes per cell = 12,000 to 20,000 EBV genomes) from the background signal associated with 10^6 BJAB cells (data not shown). The EBV probe was tested against DNA from laboratory strains of cytomegalovirus, herpes simplex virus, herpes zoster virus, and adenovirus, and the hybridization signal was compared with the signal associated with extracted Raji cell DNA. The Raji cell DNA yielded a signal 100 times greater than that of any of the other samples, although the other viral samples contained

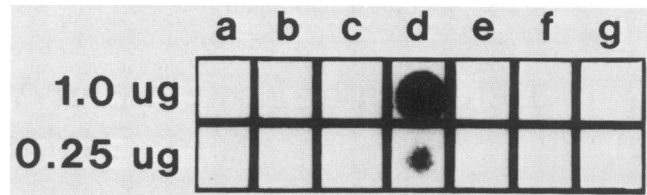


FIG. 1. Dot blot hybridization to determine specificity for EBV DNA. The pSL76 probe was hybridized against DNA from herpes simplex 1 (a), adenovirus (b), herpes zoster (c), Raji cells from a cell line carrying 60 to 100 EBV genomes per cell (d), human placenta (e), cytomegalovirus Towne strain (f), and cytomegalovirus Colburn strain (g). Only the Raji cell DNA yielded a detectable signal.

400 to 1,600 times as many viral genomes per spot (Fig. 1a through d, f, and g). The signal from human placental DNA was less than 1% of that of the same quantity of Raji cell DNA (Fig. 1e). Similarly, the signal from human spleen and non-EBV-containing cell lines was less than 1% that of the same quantity of Raji cell DNA (not shown). Thus the DNA probe used for these studies was free of nonspecific cross-reactivity with human DNA and other viral DNAs present in the patient population under study.

Clinical samples. Of a total of 483 mouthwashes collected and tested in the lymphocyte transformation assay, samples from 135 were available for hybridization assay (Fig. 2), and 24 samples were positive for the presence of EBV DNA. The remaining 111 samples were negative for EBV DNA. The positive samples came from eight patients. The remaining 35 patients had exclusively negative samples. The range of concentration of EBV in the mouthwash samples was from less than 10^5 to 10^8 EBV genomes per ml as determined by extrapolation from the signal associated with the serial Raji cell dilutions. To prove that the EBV probe employed was specific for EBV in clinical material, we used a non-EBV-containing pBR322-derived plasmid to reprobe several of the filters. There was no hybridization signal from the spots which had previously bound the pSL76 probe.

The results of the hybridization assay and the lymphocyte transformation assay on the same specimens are compared in Fig. 3. The sensitivity of hybridization relative to lymphocyte transformation (i.e., the percentage of samples which were positive in the lymphocyte transformation assay which were also positive in the hybridization assay) was 75%. The specificity of hybridization relative to lymphocyte transformation (i.e., the percentage of samples which were negative in the lymphocyte transformation assay which were also negative in the hybridization assay) was 97%.

On the basis of the large number of samples assayed in the lymphocyte transformation assay, it was possible to subdivide patients into intermittent excretors of EBV (24 patients, each of whom had at least one mouthwash specimen positive and one mouthwash specimen negative by transformation) and nonexcretors (19 patients, among whom there were no mouthwash specimens which were positive by transformation). There were no patients identified as shedding transforming virus in every assay. In the nonexcretor group, a total of 175 lymphocyte transformation assays were performed. Samples from 57 of these latter samples were tested by hybridization assays, and all were negative. The three mouthwash samples which were positive by hybridization but not by lymphocyte transformation came from two patients, both of whom were intermittent excretors. The results of serial assays on mouthwash samples from one of these patients are shown in Fig. 4.

Case analysis. An index patient with acute myelogenous leukemia undergoing marrow transplantation was evaluated to assess the value of the EBV probe in monitoring EBV shedding under different therapeutic conditions. The results of serial lymphocyte transformation and hybridization assays are illustrated in Fig. 4. The initial mouthwash of the patient was negative by lymphocyte transformation assay. During marrow ablative therapy with cyclophosphamide and total body irradiation, lymphocyte transformation and hybridization assays became positive. Hybridization showed 10^5 EBV genome equivalents per ml. Beginning on day 1 after marrow transplantation, cyclosporine and steroids were administered in an attempt to prevent graft-versus-host disease, and treatment with acyclovir was begun to prevent reactivation of herpes simplex infection. During the 97 days of acyclovir administration, with the exception of the first day, there was no further shedding of EBV in the saliva as detected either by hybridization or by lymphocyte transformation. On day 106, 9 days after acyclovir was discontinued, EBV was again detected in the mouthwash by lymphocyte transformation assay. Samples obtained on days 113 and 121

LYMPHOCYTE TRANSFORMATION

		Pos	Neg
HYBRIDIZATION	Pos	21	3
	Neg	7	104

FIG. 3. Comparison of the results of the hybridization assay with the results of the lymphocyte transformation assay on 135 mouthwash samples.

were negative by the lymphocyte transformation assay but positive by the hybridization assay, showing the presence of EBV DNA in the mouthwash in excess of 10^6 genome equivalents per ml. Cyclosporine was discontinued on day 134. Samples obtained from day 157 onward were consistently positive by lymphocyte transformation assay. These samples were also positive by the hybridization assay but showed a decrease in the concentration of EBV genome equivalents to 10^5 /ml.

DISCUSSION

We have demonstrated that the pSL76 EBV DNA-containing probe is sensitive and specific for EBV DNA. It does not have interfering homology with the other viral DNAs tested, and unlike some herpesvirus DNA sequences, it does not have homology with human cell DNA (15). The feasibility of applying the assay to clinical mouthwash specimens has been shown, and in contrast to the lymphocyte transformation assay, the hybridization assay is semiquantitative, facilitates batch processing of samples, and can yield a result in 72 h rather than the 3 to 8 weeks required for lymphocyte transformation.

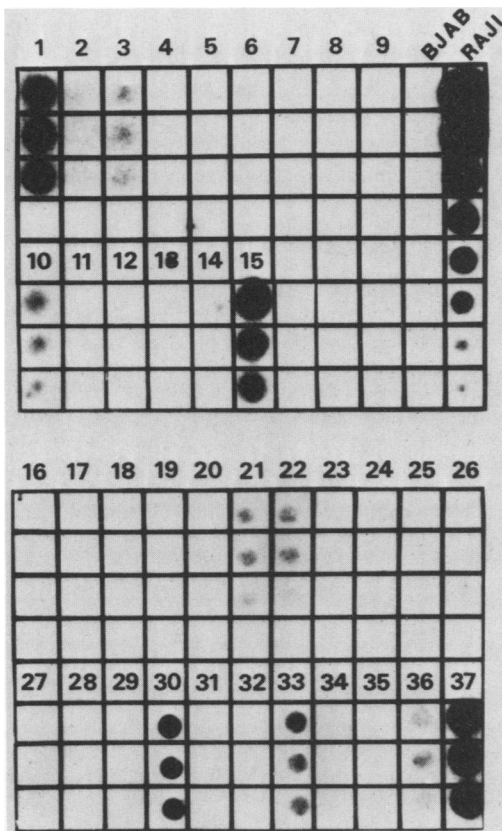


FIG. 2. Mouthwash dot blot. Thirty-six mouthwashes were applied to the nylon filter as described and probed with pSL76. Positive results were recognized by comparing the signal to a positive control (Raji cells applied in serial twofold dilutions with the first spot corresponding to 100,000 Raji cells) and a negative control (BJAB cells applied in serial twofold dilutions with the first spot corresponding to 100,000 BJAB cells). Specimens 1, 3, 10, 21, and 36 were positive by both hybridization and lymphocyte transformation assays. Specimens 15, 30, and 33 were positive by hybridization only, and specimen 7 was positive by transformation assay only. Sample 37 was supernatant fluid from B95-8 (EBV producer cell line) cell culture.

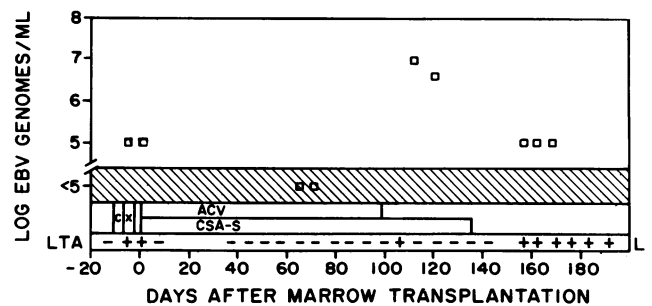


FIG. 4. EBV shedding in a patient with acute myelogenous leukemia treated with cyclophosphamide (C), total body irradiation (X), high dose steroids (S), cyclosporine (CSA), and acyclovir (ACV). The \log_{10} of the EBV genomic concentration, as calculated on the basis of hybridizations, is shown, as well as the results of lymphocyte transformation assays (LTA), indicated simply as positive (+) or negative (-).

The three samples which were positive by hybridization but not by lymphocyte transformation cannot be easily accounted for in terms of nonspecific hybridization. Other viral DNAs tested against the EBV probe did not substantially cross-hybridize, and repeat hybridization of the same filter with a non-EBV-containing plasmid probe did not yield a signal. This leads us to believe that there were no false-positive results in the sense of a hybridization signal when EBV DNA was absent. The findings that these three samples were from patients in the intermittent excretor group and that there were no hybridization-positive signals from the nonexcretor group are consistent with the possibility that hybridization-positive lymphocyte transformation-negative samples may represent viral shedding missed by the transformation assay. Such a result might occur if there was material in saliva which inhibited the growth of the lymphocytes or inhibited their viral transformation or if the virus in the mouthwashes lost viability in handling. Another intriguing possibility is that patients may at certain times within the course of their infection shed defective or nontransforming virus such as is produced by the P3HR1 cell line. Such viruses would not be detected by the lymphocyte transformation assay but would be detected by hybridization. Mouthwashes which transformed cord blood lymphocytes but in which EBV was not detected by hybridization may have contained a quantity of EBV genomes below our lower limits of detection. The use of probes with higher specific activity or the use of a DNA extraction procedure for the clinical specimens might increase the sensitivity. The ease with which the hybridization assay can be performed on multiple samples and its semiquantitative nature make it especially useful for the quantitative assessment of the impact of various interventions on EBV secretion in immunocompromised populations. Thus serial sampling of mouthwash specimens (Fig. 4) should facilitate assessment of the effects of radiation, cytotoxic chemotherapy, antiviral chemotherapy, and immunosuppressive chemotherapy on EBV in vivo and perhaps shed light on the possible role of the virus in the occurrence of monoclonal and polyclonal lymphoproliferative disease in immunocompromised patients.

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