JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1990, p. 2775–2778
0095-1137/90/122775-04$02.00/0
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Vol. 28, No. 12

Diagnosis of Epstein-Barr Virus Infection in Hairy Leukoplakia by Using Nucleic Acid Hybridization and Noninvasive Techniques

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Received 4 May 1990/Accepted 24 September 1990

The presence of Epstein-Barr virus (EBV) DNA in the epithelial cells of oral hairy leukoplakia is the confirming criterion in the diagnosis of this lesion, which occurs mainly in persons infected by the human immunodeficiency virus. Because hairy leukoplakia often presages the development of the acquired immune deficiency syndrome, it is important that suspicious lesions be accurately diagnosed. Commonly, biopsy tissue is removed for detection of EBV DNA by in situ hybridization, but biopsy is contraindicated in some patients. This study evaluated filter and cytospin in situ hybridization, two noninvasive techniques that examine epithelial cells swabbed from the surfaces of the lesions, for their sensitivity in detecting EBV DNA. As compared with tissue in situ hybridization, the filter and cytospin techniques had sensitivities of 100 and 92%, respectively. We conclude that these two noninvasive techniques can provide the clinician with an accurate alternative to biopsy whenever this human immunodeficiency virus-associated lesion is suspected.

Oral hairy leukoplakia, a white lesion of the oral mucosa (4), is a primary site of epithelial Epstein-Barr virus (EBV) infection and replication in its natural host (2, 7, 8, 11, 14; A. E. Friedman-Kein, Letter, Lancet **ii**:694, 1986; Y. G. De Souza et al., Letter, N. Engl. J. Med. **320**:1559, 1989). This lesion is found predominantly on the oral mucosa of persons who exhibit evidence of human immunodeficiency virus (HIV) infection or who are in a high-risk category for the acquired immune deficiency syndrome (AIDS) (4, 6, 12, 13, 18). It is an early clinical indicator of infection with HIV (6). The Centers for Disease Control (1) have classified hairy leukoplakia as a disease associated with HIV infection (group IV-C secondary infectious diseases). In our study (D. Greenspan et al., Abstr. IVth Int. Conf. AIDS 1988, book 2, p. 373) of 198 men with hairy leukoplakia, 17 had AIDS at the time of diagnosis and 197 of 198 were seropositive for HIV infection. The probability that AIDS would develop in these patients was 57%, with a median time of 2.2 years. Thus, it is important that hairy leukoplakia be identified accurately. Differential diagnosis to discriminate this condition from other oral lesions with similar clinical appearances requires a biopsy for microscopic examination as well as the demonstration of EBV DNA by in situ hybridization. However, biopsy requires patient compliance and may be contraindicated for young children and persons with hemophilia. Rarely, cases of hairy leukoplakia are seen in HIV-seronegative patients (5, 9, 15).

The purpose of this study was to evaluate two noninvasive diagnostic techniques for their usefulness in detecting the presence of EBV DNA in the epithelial cells of hairy leukoplakia lesions. These diagnostic procedures were filter in situ hybridization (FISH) and cytospin in situ hybridization (CISH). To assess the sensitivity of EBV detection by these methods, we collected biopsy specimens and performed tissue in situ hybridization (TISH) as previously described (De Souza et al., Letter) and used the results as the standard against which to compare CISH and FISH. The methodologies of Wagner et al. (19) for the FISH and CISH protocols were modified for this study.

MATERIALS AND METHODS

Subjects. The study group, members of a population described previously (4, 6, 8), consisted of 25 homosexual male patients being seen at the Oral AIDS Clinic of the University of California, San Francisco. The 25 patients were diagnosed as having hairy leukoplakia; they volunteered after being informed of the possible risks and benefits of participating and were entered into the study sequentially. The diagnostic criteria for hairy leukoplakia were as follows: a white lesion that (i) was located on the lateral margin of the tongue, (ii) did not rub off, and (iii) had the characteristic histological features of the hairy leukoplakia lesion. All participants gave informed consent to have a biopsy specimen taken as well as material collected for the noninvasive diagnostic procedures.

Collection and preparation of specimens. (i) Cells. Cells were collected for FISH and CISH by firm swabbing of the hairy leukoplakia lesion (right and left sides of the tongue) and nonlesional areas (buccal mucosa) with a sterile Dacron swab, which was then submerged in sterile phosphate-buffered saline. The cells were centrifuged and resuspended in 1 ml of RPMI 1640 and 10% fetal calf serum.

For CISH, a Shandon-Southern (Pittsburgh, Pa.) cytocentrifuge was used to spin the cells onto 0.01% poly-D-lysine-coated slides. One hundred microliters of the cell suspension was cytocentrifuged at 7,000 × *g* for 10 min. Slides were removed from the cytocentrifuge, air dried, fixed in 10% buffered Formalin, rinsed in distilled water, air dried again, and stored at −80°C in sealed slide boxes until needed.

For FISH, the 1-ml cell suspensions were collected onto 0.45-μm Nytran (Schleicher & Schuell, Keene, N.H.) filter disks placed on a Hoefer (San Francisco, Calif.) 10-place filter manifold. The disks were then placed onto 3MM Whatman paper strips saturated with 1.5 M NaCl and 0.5 M NaOH for 30 min to denature cellular DNA. The disks were transferred twice to paper strips saturated with 1 M Tris, pH 7.5, and 1.5 M NaCl for 30 min. Finally, the disks were baked at 60°C for 1 h and stored at room temperature in an airtight container for later use.

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(ii) Biopsy specimens. Incisional biopsy specimens of hairy leukoplakia lesions were obtained under local anesthesia. Samples to be used for routine histopathology and TISH were fixed in Formol-saline (0.9% NaCl) and embedded in paraffin. For TISH, sections were cut at 4 μm and placed on 0.01% poly-D-lysine-coated slides.

Probe preparation. For CISH and TISH, the recombinant probe p*Bgl*2-U (a generous gift of G. W. Bornkamm), which is specific for the large internal repeat sequence of the EBV genome, was transfected into *Escherichia coli* HB101, amplified, and purified according to the protocols of Maniatis et al. (10). The purified probe was labeled with biotin by nick translation with the dTTP analog Bio-11-dUTP (Bethesda Research Laboratories, Gaithersburg, Md.). This probe was selected because the large internal repeat sequence is present at more than 10 copies per EBV genome and is thus more readily detected than unique sequences, making the probe sensitive for very small amounts of EBV DNA. A negative-control probe of the plasmid vector pBR322 alone was also labeled with Bio-11-dUTP by nick translation to ensure that any positive reaction detected within the tissue sections was not due to nonspecific binding of the probe.

To separate the labeled DNA from unincorporated nucleotides, the reaction mixture was chromatographed through a 4-ml column of Bio-Rad Bio-Gel P-60 (100/200 mesh) in Tris EDTA, pH 7.5, and 250-μl fractions were collected. A 1-μl aliquot from each fraction was collected onto nitrocellulose paper and baked for 10 min at 80°C. Biotinylated probe was detected by using the Bethesda Research Laboratories DNA detection system. The two fractions showing the strongest reactions were pooled for use as a probe.

For FISH, 1 mg of probe was radiolabeled with ^{32}P by using the Du Pont NEN nick translation system. The reaction mixture was incubated for 2 h at 14°C. Labeled DNA was separated from unincorporated nucleotides on a column prepared from the same materials as were used in the biotin-labeling protocol. Fractions were collected and emissions were counted on a Beckman (Fullerton, Calif.) scintillation counter.

CISH and TISH. (i) Prehybridization. Before undergoing the prehybridization steps, sections for TISH were deparaffinized and rehydrated, whereas cytospin preparations (for CISH) were incubated in a 1:1 solution of methanol and acetone for 5 min at −20°C. Unless otherwise stated, all prehybridization steps were performed at room temperature. The prehybridization and hybridization techniques were modified from the method of Unger et al. (18).

First, sections were incubated in 0.2 M HCl for 10 min, rinsed in 50 mM Tris (pH 7.5)–5 mM EDTA, and digested in 0.4-mg/ml protease (from *Streptomyces griseus*)–50 mM Tris (pH 7.5)–5 mM EDTA for 15 min. Next, to denature the DNA within the tissue, the sections were incubated in 0.07 M NaOH for 2 min and then refixed in 4% formaldehyde–0.1 M Tris (pH 7.5)–0.1 M NaCl for 5 min. Finally, sections were washed twice for 5 min in 2-mg/ml glycine–0.1 M Tris (pH 7.5)–0.1 M NaCl (this prevents further protease digestion) and incubated for 30 min in a prehybridization solution (50% formamide, 0.6 M NaCl, 0.01 M Tris [pH 7.4], 0.001 M EDTA, 1 ml of bovine serum albumin per ml, 0.02% Ficoll [M_r 400,000], 0.02% polyvinylpyrrolidone-360, 0.2 mg of sheared salmon sperm DNA per ml, and 0.5 mM dithiothreitol).

(ii) Hybridization. The hybridization solution was composed of 50% formamide, 6× standard saline citrate (SSC) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02 M sodium phosphate buffer (pH 6.5), 1× Denhardt solution, 0.3 mg of salmon sperm DNA per ml, 10% dextran sulfate, and 0.5 μg of probe per ml. The hybridization solution was heated to above 90°C for 5 min to denature the DNA and then placed on ice immediately.

Twenty microliters of the hybridization solution was placed on each section, which was then covered with a polypropylene autoclave bag cover slip (VWR Scientific, Brisbane, Calif.) to distribute the probe evenly and prevent the sections from drying. The sections were placed in a humidified chamber and incubated for 2 h at 37°C to allow hybridization to occur.

(iii) Washing. All washing steps were performed at room temperature. Cover slips were removed from sections, and excess probe was washed off in 2× SSC–0.1% sodium dodecyl sulfate (SDS). The slides were then washed at room temperature for 3 min each step, twice in 2× SSC–0.1% SDS, twice in 0.2× SSC–0.1% SDS, twice in 0.16× SSC–0.1% SDS, and once in 2× SSC–0.1% SDS.

(iv) Detection of EBV DNA. The target hybrid was detected with streptavidin and a biotin-conjugated alkaline phosphatase system (DNA Detection Kit; Bethesda Research Laboratory). This system is sensitive enough to detect 2 to 5 pg of biotinylated probe that has been hybridized to target DNA. We followed the manufacturer's protocol with the exception that color was developed by incubating slides vertically in a dark humid chamber for 2 h at 37°C in a solution of McGadey reagent. This solution was made by adding 44 μl of 75-mg/ml Nitro Blue Tetrazolium in 70% *N*,*N*-dimethyl formamide, 33 μl of 50-mg/ml 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt in *N*,*N*-dimethyl formamide, and 10 ml of buffer (0.1 M Tris [pH 9.5], 0.1 M NaCl, 50 mM $MgCl_2$). Sections were removed from the chromogenic substrate solution, rinsed in distilled water, counterstained in nuclear fast red for 5 min, rinsed again in distilled water, dehydrated through a graded series of ethanol, cleared in xylene, and mounted in Permount (Fisher Scientific).

FISH: prehybridization and hybridization. In the FISH, filters were cut in half and labeled A and B. Both halves were incubated overnight at 45°C in prehybridization solution (5 ml of 10-mg/ml tRNA, 50 ml of formamide, 8 ml of water, 5 ml of 1 M sodium phosphate [pH 6.6], 2 ml of 250× Denhardt solution, 5 ml of 20% SDS, 25 ml of 20× SSC). The hybridization solution was identical to the prehybridization solution except for the addition of the labeled probe and a reduction of tRNA to a concentration of 1:100. Filters A were hybridized with ^{32}P-labeled p*Bgl*2-U, and filters B were hybridized with ^{32}P-labeled pBR322 for 3 days at 45°C. After hybridization, the filters were washed five times for 30 min each in 2× SSC–1% SDS in a hot (70°C) shaking water bath. Filters were blotted dry, placed onto stamp holders, and autoradiographed. Films were developed with an automatic X-ray developer (Kodak RP X-Omat).

RESULTS

Of the 25 cases examined, all were positive for EBV DNA by TISH (Fig. 1) and FISH (Fig. 2) and 23 were positive by CISH (Fig. 3). In both the TISH and CISH specimens, the hybridization signal was seen focally in the nuclei as a dark purplish-blue stain. All nonlesional swabs (buccal mucosa) were negative for EBV DNA by both the CISH and FISH techniques. All sections, filters, and cytospin slides probed with pBR322 uniformly showed no hybridization.

The sensitivities of FISH and CISH for the presence of EBV were 100 and 92%, respectively. That some CISH

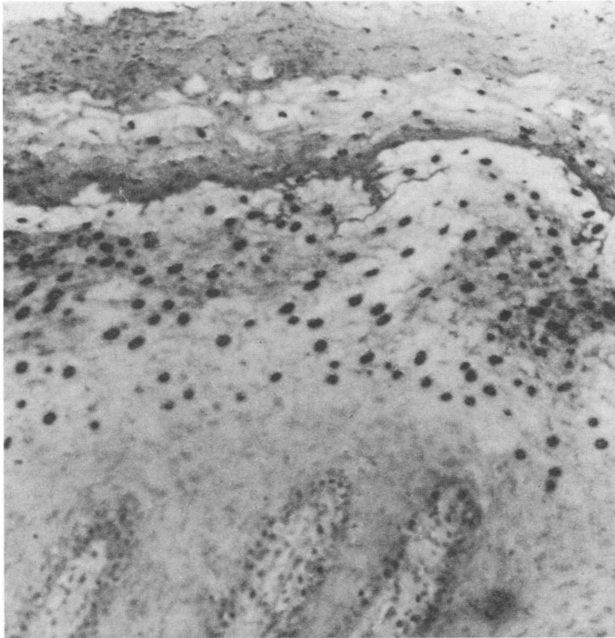

FIG. 1. Demonstration of EBV DNA in hairy leukoplakia by TISH. Hybridization is confined to the upper portions of the epithelium and is seen focally in the nuclei as a dark purplish-blue stain. Magnification, ×40.

cases were negative for EBV DNA was due to an error in specimen preparation. Because of the failure of the epithelial cells to adhere to the slides, two cases were negative for EBV by CISH but positive for EBV DNA by TISH and FISH.

DISCUSSION

Hairy leukoplakia is often an early clinical indicator of infection with HIV; thus, it is important that suspicious lesions be accurately diagnosed. Lesions that mimic hairy leukoplakia in clinical and histological appearance are sometimes found (3). Consequently, it is critical to test for the presence of EBV DNA and to recommend HIV antibody testing. We and others (7, 9, 16) have suggested that for patients whose HIV antibody status is unknown, the presence of EBV in lesions should be demonstrated immunohistochemically or by molecular biology techniques. The disadvantage of immunohistochemistry is that because of the spotty distribution of EBV expression in oral mucosa, one may not be able to detect viral capsid antigen (10), especially in the case of a very small biopsy specimen. Thus, DNA hybridization techniques performed on samples collected by biopsy or on swabs are more reliable and sensitive in the detection of EBV.

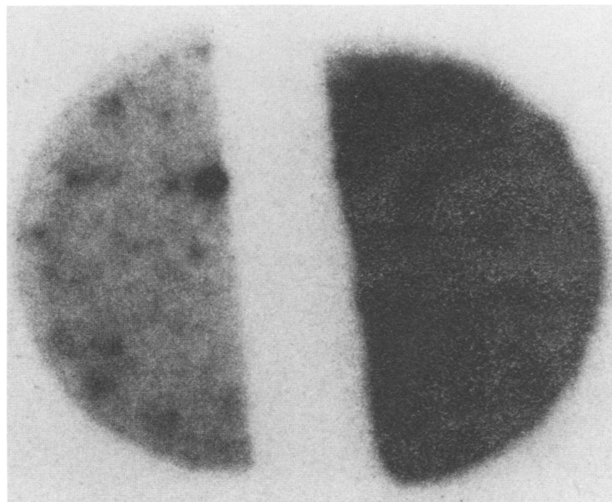

FIG. 2. Demonstration of EBV DNA in hairy leukoplakia by the FISH technique. The right filter half (dark half) was probed with p*Bgl*2-U, and the left half was probed with the control plasmid, pBR322.

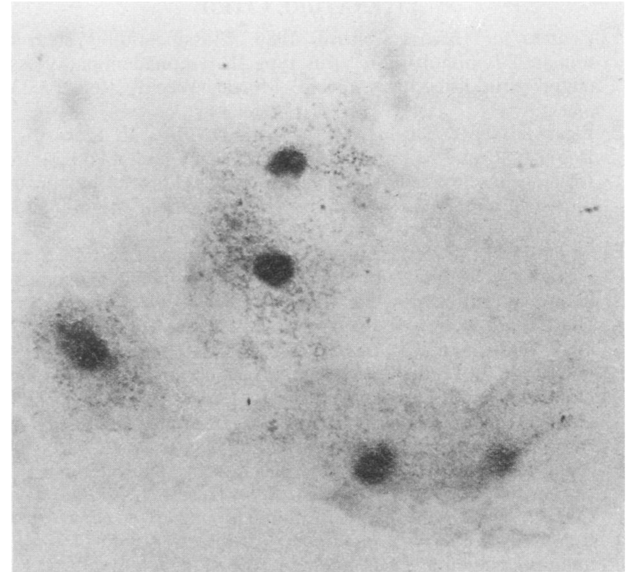

FIG. 3. Demonstration of EBV DNA in epithelial cells collected from a hairy leukoplakia lesion. The cells were probed for EBV DNA by CISH. Magnification, ×1,000.

Either of the noninvasive procedures CISH and FISH can provide the clinician with valuable diagnostic information for a questionable lesion as well as an alternative to biopsy. The advantage of CISH over FISH is that CISH uses a nonisotopically labeled probe; thus, no radioactive waste is generated. Biotinylated probes have a shelf life of up to 1 year, whereas a probe labeled with ^{32}P has a half-life of 14 days. When an immediate diagnosis is required, the CISH method is more convenient to use than the FISH method.

In our laboratory and clinic, we routinely perform either TISH or CISH on questionable oral lesions that we suspect may be hairy leukoplakia, especially when the patient's HIV status or AIDS risk status is unknown. This approach was used, for example, in diagnosing the first case of hairy leukoplakia seen in a child (J. S. Greenspan et al., Letter, AIDS 2:143, 1988). Thus, the use of a noninvasive procedure provides the clinician with a flexible alternative to biopsy whenever this HIV-associated lesion is suspected.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant PO1-DEO7946 from the National Institutes of Health.

We thank Evangeline Leash for editing the manuscript, Eileen Wong and Tammy Fell for technical support, and Marjorie Standow and Debra Stephenson for clinical assistance.

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