Rapid Detection of Herpes Simplex Virus DNA in Human Brain Tissue by In Situ Hybridization

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A commercial system for detection of herpes simplex virus (HSV) DNA by in situ hybridization gave positive results on 16 of 17 stored human brain specimens that were positive for HSV on initial testing by virus isolation and immunofluorescence staining, and the hybridization system gave negative results on 13 brain specimens that showed no evidence of HSV by isolation or immunofluorescence staining.

There has been increasing interest in the application of in situ nucleic acid hybridization to the detection and identification of viral genomes in clinical specimens because of the sharp specificity and potentially high sensitivity of this technology and also because of the ability to produce large quantities of probes of the desired specificity by molecular cloning procedures (1, 4, 5). The use of radiolabeled probes combined with autoradiography is feasible only in large research institutions or medical centers. However, new advances in the development and application of biotinylated probes together with immunofluorescence (IF) or immunoenzymatic methods (1-3) offer considerable promise for wider adaptation of in situ hybridization in clinical virology laboratories.

In the present study we evaluated a commercially available biotinylated herpes simplex virus (HSV) DNA probe for detection of HSV genomic material in human brain tissue and compared results with those obtained by virus isolation and direct IF staining.

The HSV DNA hybridization kit was purchased from Enzo Biochem, Inc., New York, N.Y. Literature provided by the manufacturer indicated that the probe consisted of a mixture of three fragments, each of which was cloned into the *Bam*HI site of phage vector pBR322. These included two HSV type 1 (HSV-1) fragments of 3.0 and 8.0 kilobase pairs and one HSV-2 fragment of 16.0 kilobase pairs. The probe was biotinylated by nick translation with the thymidine analog, 5-[*N*-(*N*-biotinyl- ϵ -aminocaproyl)-3-aminoallyl]-deoxyuridine triphosphate, and streptavidin-biotinylated horseradish peroxidase was used as the detector system (2).

Human brain tissues examined were 30 autopsy or biopsy specimens received in this laboratory for diagnosis of suspected HSV or other viral encephalitides. They included 17 specimens which were positive on original testing for HSV by viral isolation (6) and IF staining (6) and 13 that were negative for HSV or other viral agents. The specimens had been stored for 10 to 20 years at -70°C . All hybridization testing was done without knowledge of original test results or results of repeat IF testing done at the time of the hybridization studies. Virus isolation attempts were not repeated on the specimens.

To prepare smears of brain tissue for hybridization, slides were first treated with Gelman adhesive preparation (Gelman Sciences, Inc., Ann Arbor, Mich.) to prevent loss of tissue during the process. A drop of the solution was

added to each slide and spread with a piece of filter paper. Slides were then air dried at room temperature and stored at room temperature in a closed, dust-free container.

Slip smears of brain tissue were prepared by pressing a 2- to 3-mm piece of the tissue between two slides and pulling the slides apart. After air drying, one set of slides was fixed in cold acetone for IF staining, and the other set was fixed in Carnoy fixative (60% ethanol, 30% chloroform, 10% acetic acid) for hybridization. Fixed slides were air dried and stored at -70°C .

In situ hybridization for detection of HSV genomic material was done by the kit protocol, with minor modifications. Briefly, a circle approximately 15 mm in diameter was outlined around the fixed brain tissue with a liquid embroidery pen, after which the tissue was treated with 3% hydrogen peroxide at 37°C for 5 to 10 min to inactivate endogenous peroxidase which might be present in some types of cells. After a brief rinse in 0.01 M phosphate-buffered saline (pH 7.3, PBS) containing 5 mM EDTA, 10 to 15 μl of the probe was added to the tissue and covered with a 15-mm round cover slip. The slides were placed on a moistened filter paper in a plastic petri dish, and the dish was covered and floated in an 80°C water bath for 10 min, followed by incubation in a 37°C water bath for 60 min. Slides were washed once for 5 min in $2\times$ SSC (0.3 M sodium chloride, 0.03 M sodium citrate) buffer, once in $2\times$ SSC containing 50% formamide, and once again in $2\times$ SSC. The slides were then immersed in PBS containing 0.1% Triton X-100 and then rinsed briefly in PBS.

To detect the probe hybridized to viral DNA in the tissue, 2 drops of the streptavidin-biotinylated horseradish peroxidase complex was added to the tissue and incubated for 30 min in a 37°C water bath. The slides were then washed once with $2\times$ SSC buffer for 5 min, rinsed briefly in PBS containing 0.1% Triton X-100, and rinsed in PBS for 3 min. The slides were placed in substrate solution (2.5 mg of diaminobenzidine tetrahydrochloride in 5 ml of PBS with 0.1 ml of 1% hydrogen peroxide), and a brown color developed within 4 to 5 min. The enzymatic action was stopped by placing the slides into distilled water. Incubation times for the hybridization procedure totaled approximately 120 min. The slides were air dried, mounted in polyvinyl alcohol (Elvanol; Du Pont Co., Wilmington, Del.) and examined with a light microscope for evidence of hybridization (brown reaction product at sites of viral infection).

In preliminary studies, the probe was evaluated by applying it to human fetal diploid lung cell cultures infected with

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FIG. 1. Detection of HSV infection in cell culture by in situ hybridization. (A) Uninfected human fetal diploid lung cell culture; (B) cell culture infected with HSV-1.

the McIntyre strain of HSV-1, the MS strain of HSV-2, the AD169 strain of human cytomegalovirus, the CaQu strain of varicella-zoster virus, and the BS-C-1 line of grivet monkey kidney cells infected with the DHV and LBI strains of simian varicella virus. Uninfected cell cultures were also included as controls. The monolayer cell cultures in eight-chamber Lab-Tek slides (Miles Laboratories, Inc., Elkhart, Ind.) were prepared for hybridization by rinsing in distilled water for a few seconds, and then, without drying, the monolayers

were fixed in Carnoy fixative for 5 to 10 min and then air dried. The probe was also tested on smears of brains of 6- to 8-week-old mice infected intracerebrally with HSV-1 or HSV-2 and on brains of uninfected mice. Mouse brain smears were prepared as described above for human brain tissue. The probe reacted only with cell cultures or mouse brains infected with HSV. Figure 1 shows the typical nuclear staining of HSV-infected cell cultures. HSV-infected cells in mouse brains showed a similar nuclear staining pattern (Fig.

FIG. 2. Detection of HSV infection in mouse brain by in situ hybridization. (A) Uninfected mouse brain smear; (B) brain smear from mouse infected with HSV-2.

FIG. 3. Detection of HSV infection in human brain by in situ hybridization. (A) Brain negative for HSV; (B) brain positive for HSV by IF staining and virus isolation.

2). Occasionally, certain cells in brain tissue, such as squamous cells or blood cells, showed some staining in both infected and uninfected preparations. However, these cells easily could be differentiated by their size and shape from infected nerve cells in brain tissue, and further, they did not show typical nuclear staining but rather a diffuse, faint staining in the nucleus and cytoplasm. Intensity of reactions with the probe was the same in HSV-1- and HSV-2-infected cell cultures and mouse brains.

Figure 3 shows typical staining observed with HSV-positive human brain specimens, again with strong staining in the nucleus and very light staining in the cytoplasm of infected cells. Strong staining was seen in the axons of some nerve cells.

In comparative testing of 30 human brain specimens by IF staining and in situ nucleic acid hybridization, 11 of 17 specimens that were positive for HSV by virus isolation and IF staining years earlier were positive by IF in the current study, whereas 16 of these specimens were positive by hybridization. None of the 13 brain specimens which were negative for HSV on original testing gave positive results by either IF staining or hybridization in the present study.

Results obtained with this small series of specimens illustrated the feasibility of applying commercially available, nonisotopic probes for HSV DNA to the routine detection of HSV infection in human brain tissue. Sensitivity of nucleic acid hybridization for virus detection was comparable to that of the original direct IF staining in that only a single specimen that was positive originally by IF staining failed to show hybridization. On the other hand, six specimens failed to show positive reactions by IF staining on repeated testing. Viral antigen may have been more labile than viral DNA upon years of storage of the specimens in the frozen state. Specificity of the DNA probe was good, as evidenced by the fact that all of the brain specimens which were negative for HSV gave negative hybridization results.

Additional testing on a variety of different types of clinical

specimens containing HSV will be required to establish whether DNA hybridization with commercial kits of the sort employed for this study will offer any significant advantage in terms of sensitivity or specificity over immunoassays, such as IF staining, or enzyme immunoassays for viral diagnosis. One possible advantage might prove to be the ability to produce DNA probes more economically than immune reagents by molecular cloning procedures.

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LITERATURE CITED

1. Brigati, D. J., D. Myerson, J. J. Leary, B. Spalholz, S. Z. Travis, C. K. Y. Fong, G. D. Hsiung, and D. C. Ward. 1983. Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. *Virology* 126:32-50.
2. Langer, P. R., A. A. Waldrop, and D. C. Ward. 1981. Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. U.S.A.* 78:6633-6637.
3. Leary, J. J., D. J. Brigati, and D. C. Ward. 1983. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. *Proc. Natl. Acad. Sci. U.S.A.* 80:4045-4049.
4. Richman, D. D., P. H. Cleveland, D. C. Redfield, M. N. Oxman, and G. M. Wahl. 1984. Rapid viral diagnosis. *J. Infect. Dis.* 149:298-310.
5. Richman, D. D., N. Schmidt, S. Plotkin, R. Yolken, M. Chernesky, K. McIntosh, and M. Mattheis. 1984. Summary of a workshop on new and useful methods in rapid viral diagnosis. *J. Infect. Dis.* 150:941-951.
6. Schmidt, N. J., D. Gallo, V. Devlin, J. D. Woodie, and R. W. Emmons. 1980. Direct immunofluorescence staining for detection of herpes simplex and varicella-zoster virus antigens in vesicular lesions and certain tissue specimens. *J. Clin. Microbiol.* 12:651-655.