TECHNICAL NOTE

Barbara H. Amaker,[1] *M.D.; Francis W. Chandler, Jr.*[2] *D.V.M., Ph,D., Lee O. Huey,*[2] *B.S.; and Rita M. Colwell,*[2] *B.S.*

Molecular Detection of JC Virus in Embalmed, Formalin-Fixed, Paraffin-Embedded Brain Tissue*

REFERENCE: Amaker BH, Chandler FW, Jr., Huey LO, Colwell RM. Molecular detection of JC virus in embalmed, formalin-fixed, paraffin-embedded brain tissue. J Forensic Sci 1997;42(6):1157–1159.

ABSTRACT: Embalmed tissues are adequate for the detection of JC virus in lesions of progressive multifocal leukoencephalopathy (PML) by immunohistologic and molecular methods. JC virus was readily detected in embalmed brain tissue using immunohistochemistry (IHC), *in situ* hybridization (ISH), and the polymerase chain reaction (PCR). Two brains were removed from bodies that had been embalmed at least 24 h prior to autopsy. They were subsequently post fixed in 10% buffered formalin for 10–14 days before dissection and molecular studies were performed. Though these techniques are not novel, their use in embalmed tissues is. Routine embalming should not eliminate these diagnostic procedures from consideration.

KEYWORDS: forensic science, forensic pathology, autopsy, embalmed tissues, JC virus, brain, immunohistochemistry, *in situ* hybridization, polymerase chain reaction

We are reporting the successful use of immunohistochemistry (IHC), *in situ* hybridization (ISH) and the polymerase chain reaction (PCR) in embalmed, post-fixed brain tissue. Though these techniques have been widely used as diagnostic aids in formalin-fixed, paraffin-embedded tissues, their use in embalmed tissues has not been previously reported. While searching for brain tissue known to be infected with JC virus for use as positive control tissue in another study, we serendipitously discovered that all three techniques can be used successfully in embalmed brain tissue. Deceased bodies at our institution which are known to be infectious (viral hepatitis, HIV) are routinely embalmed at least 24 h prior to autopsy. These included two cases of progressive multifocal leukoencephalopathy (PML) confirmed by electron microscopy (EM). One case was diagnosed during life and the other after death. Since both cases had been proven to be infected with papovirus ultrastructurally, we wished to see if immunohistologic and molecular methods for detecting JC virus would work in embalmed tissues. JC virus, a polyomavirus of the family Papovaviridae, is the most common causative agent of PML although rare cases caused by the SV 40 virus have been reported (1). JC virus, BK virus, and SV 40 virus are closely related and share some genomic sequences (2). JC virus is ubiquitous, and some authors estimate that approximately 80% of the adult population possess antibodies to JC virus (3).

Although sporadic cases of PML have been reported, it most commonly occurs when an individual becomes immunocompromised for any reason, such as: 1) AIDS (acquired immunodeficiency syndrome), 2) lymphoma, 3) leukemia, 4) non-neoplastic disorders treated with corticosteroids, and 5) organ recipients treated with immunosuppressive therapy (4). PML often presents as multifocal demyelinating lesions in the white matter which may coalesce. These lesions can be spotty in nature and give rise to a variety of symptoms, e.g., intellectual deterioration, hemiparesis, blindness, and signs of brainstem and cerebellar dysfunction (5). The external surface of the brain may appear normal. Once the brain is sectioned, the gross lesions tend to appear as discrete discolored lesions in the white matter which resemble those seen in multiple sclerosis. Histologically, the lesions of PML are characterized by rarefaction of myelin and, often, frank necrosis. Examination of the periphery of the lesions may show enlarged oligodendroglial cells with altered or hyperchromatic nuclei, sometimes containing viral inclusions. These are accompanied by astrocytes which are often bizarre and almost neoplastic in appearance (Fig. 1). The center of the lesion is apt to be necrotic and populated by macrophages.

Transmission electron microscopy (EM) was responsible for the discovery of the viral nature of PML (6,7) and is still considered to be the gold standard for diagnosis. Although JC virus has been previously identified in formalin-fixed, paraffin-embedded brain using immunohistochemistry (IHC) and *in situ* hybridization (ISH) (8,9), to our knowledge, these techniques have not been used before on embalmed tissue. Since prolonged formalin fixation may alter the results obtained with IHC or ISH, pathologists have been reluctant to apply these techniques to tissues fixed for an extended period of time.

Formalin fixation is achieved through a reaction with proteins. Methylene type linkages between polypeptide chains are at the basis of fixation. This type of fixation may structually alter some molecules, thus preventing the antigens from reacting with the applied antibody (10). Immunohistochemical results are optimal

[1]Medical College of Virginia, Department of Pathology, Division of Neuropathology, P.O. Box 980017, Richmond, VA.
[2]Department of Pathology, Medical College of Georgia, Augusta, GA.
*This work was supported by a grant from the Department of Pathology, Medical College of Georgia.
Received 21 June 1996; and in revised form 12 Feb. 1997, 12 March 1997; accepted 12 March 1997.

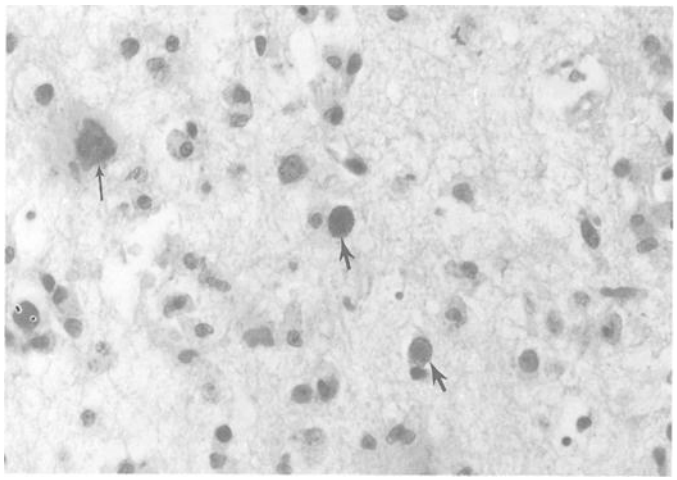

FIG. 1—*Region of demyelination with enlarged virus-infected oligodendrocytes (large arrows). Reactive astrocytes are seen with abundant cytoplasm (small arrow). (H&E, 400×)*

if the tissue to be studied has been fixed in 10% formalin for 8–24 h prior to processing (10), though recently described microwave-citrate antigen retrieval may make this a less critical issue (11). The effects of prolonged fixation prior to ISH may be remedied by increased treatment with Proteinase K (12,13). Prolonged fixation may also alter the results obtained with PCR depending on the type of fixative and the length of the DNA sequence to be amplified (14). The purpose of embalming is to retard decomposition of the body; in essence, embalming fluid acts as a tissue fixative.

Methods

Brains were removed at autopsy from the embalmed bodies of two patients with PML. The brains were then post-fixed in 10% neutral buffered formalin for 10–14 days prior to dissection. Hematoxylin and eosin-stained sections of the fixed, paraffin-embedded brains were systematically examined for the presence of demyelinating and necrotizing lesions of PML. Replicate sections of lesions characteristic for PML were then studied utilizing IHC, ISH, and PCR.

Immunohistochemical procedures were performed on deparaffinized tissue sections, using a polyclonal antibody (anti-SV40, Lee BioMolecular Research, Inc., San Diego, CA) and the peroxidase-labeled streptavidin-biotin detection system (15). Antigen availability was enhanced by microwave pretreatment of mounted tissue sections in a citrate buffer (pH 6.0) for 5 min (11).

In situ hybridization was carried out on deparaffinized tissue sections using a commercially prepared biotinylated cDNA probe for JC virus, and an enhanced detection system utilizing an antibody sandwiching technique (ENZO Diagnostics, Farmingdale, NY). A biotinylated DNA probe for human genomic DNA was used as an endogenous positive control to ensure adequacy of digestion, denaturation, and hybridization with each run of slides.

PCR was carried out using 20-mer primers P5 (5′ AGT CTT TAG GGT CTT CTA CC 3′) and P6 (5′ GGT GCC AAC CTA TGG AAC AG 3′) that amplify a 172 base pair segment of the JC virus genome (2,16). A fragment of brain tissue taken by stereotactic biopsy and proven by transmission electron microcopy to be PML was used as a positive control and sterile water as a negative control. DNA was extracted from paraffin-embedded tissues as follows. Following inspection of the appropriate blocks, 15 sections each were cut at 10 μm thickness. The blocks were cut with a clean microtome blade, and technologists changed gloves between each specimen. As an added precaution, surfaces on the microtome coming into contact with each block were cleansed with a 0.525% sodium hypochlorite solution (Clorox® diluted 1:10) between each specimen. Each rolled brain section was picked up from the microtome blade with forceps and then placed into a sterile 1.5 mL microcentrifuge tube. A xylene/ethanol paraffin extraction was then performed. This was followed by digestion in 10 mM Tris (pH 8.3) that contained proteinase K (1 mg/mL). The digestion mixture was incubated in a 50°C water bath for 24 h. The proteinase K was heat inactivated by boiling at 100°C for 10 min. Specimens were then centrifuged at 14,000 rpm for 5 min. The supernatant was collected and used for amplification by PCR. Each 50 μL reaction mixture contained 5.0 μL of sample DNA, 200 μM each of the deoxynuclotide triphosphates (Promega, Madison, WI), 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 pmol of each appropriate oligonucleotide primer, and 1.25 units of *Taq* DNA polymerase (Promega). All specimens were subjected to 40 cycles of two temperature PCR amplification (94°C for 1.5 min, 60°C for 3 min using a Perkin Elmer/Cetus 9600 thermal cycler, Norwalk, CT). The PCR-amplified products were electrophoresed through an 8% acrylamide gel and stained with silver nitrate (17). The gels were air dried between ultra clear cellophane (Tut's Tomb, Idea Scientific, Minneapolis, MN) and then photographed for a permanent record.

Results

All three methods consistently detected JC virus in multiple brain sections from each case. IHC and ISH showed comparable sensitivities in areas of brain that demonstrated typical demyelinating and necrotizing lesions of PML (Fig.1). Viral antigen (Fig. 2) and nucleic acid (Fig. 3) were localized to nuclei of oligodendrocytes. Repeated PCR assays of multiple brain sections for known JC virus DNA sequences were positive in both autopsy cases of PML (Fig. 4).

Discussion

Reluctance to use molecular and immunohistochemical techniques on embalmed tissues may be due to the concerns that the

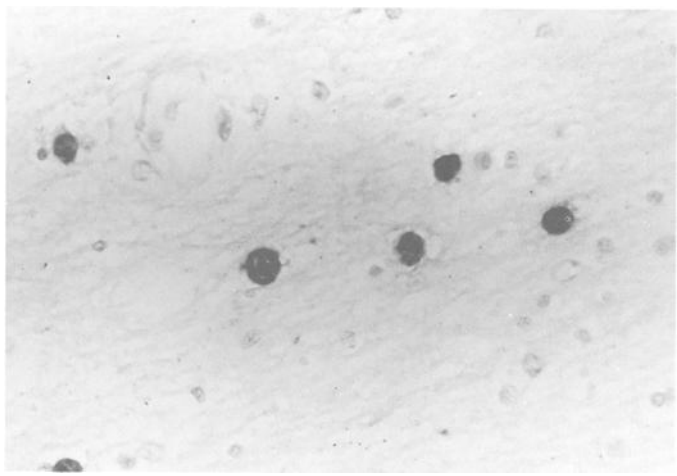

FIG. 2—*Positive immunohistochemical staining for JC virus in oligodendoglial cells. Immunostained with anti-SV 40 antibody. (400×)*

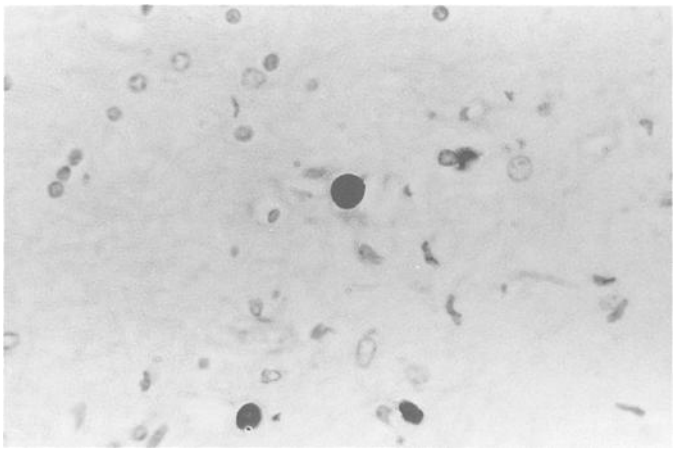

FIG. 3—*JC virus detected with DNA* in situ *hybridization using a commercially prepared biotinylated cDNA probe (ENZO Diagnostics, Farmingdale, NY). (400×)*

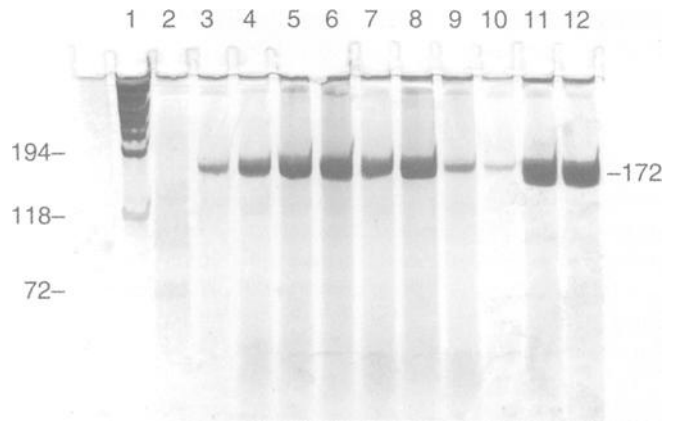

FIG. 4—*Polymerase chain reaction analysis of embalmed brain tissue with PML. Lane 1 contains the ϕX 174 marker, lanes 2 and 3 contain the negative and positive controls, respectively. Lanes 4–8 and 9–12 contain samples from 2 cases of PML (see methods). The amplified product is 172 base pairs.*

many different brands of embalming fluids contain various amounts and combinations of tissue fixatives, e.g., formalin, glutaraldehyde, and methanol, as well as anticoagulants, lanolin, and various types of perfumes and natural and synthetic dyes (18). The exact contents, other than the hazardous chemicals, may not be known as some brands are patented and the ingredients are confidential. Another potential variable is that a cadaver may be perfused with a concoction of many fluids from the mortician's shelf, depending on the post mortem condition of the body.

Because many institutions may embalm biohazardous bodies prior to autopsy and pathologists are sometimes called upon to autopsy recently embalmed or exhumed bodies, we are reporting that JC virus can be detected in embalmed, post-fixed tissue utilizing immunohistologic and molecular techniques. Routine embalming should not eliminate these diagnostic procedures from consideration. The techniques described can be used to detect JC virus and perhaps other microorganisms and antigens even after elaborate and prolonged fixation.

Acknowledgments

We thank Janet Stewart for performing immunohistochemical procedures, Kathy Mangold, Ph.D., for her support, patience, and technical advice, and Dr. M. Gary Hadfield for help in revising the manuscript.

References

1. Weiner LP, Herndon RM, Narayan O, et al. Isolation of virus related to SV40 from patients with progressive multifocal leukoencephalopathy. N Engl J Med 1972;286:385–90.
2. Frisque RJ, Bream GL, Cannella MT. Human polyomavirus JC virus genome. J Virol 1984;51:458–69.
3. Padgett BL, Walker DL. Prevalence of antibodies in human sera against JC virus: an isolate from a case of multifocal from a case of multifocal leukoencephalopathy. J Inf Dis 1973;127:467–70.
4. Esiri MM, Kennedy PGE. In: Greenfield's Neuropathology, Adams JH, Duchen LW, editors, Oxford University Press, New York, New York. 1992:367–70.
5. Burger PC, Scheithauer BW, Vogel FS. Surgical pathology of the nervous system and its coverings. Churchill Livingstone, New York, Edinburgh, London, Melbourne, Tokyo. 1991:155–7.
6. Silverman L, Rubinstein LJ. Electron microscopic observations on a case of progressive multifocal leukoencephalopathy. Acta Neuropathol 1965;5:215–24.
7. ZuRhein GM, Chou SM. Particles resembling papova viruses in human cerebral demyelinating disease. Science 1965;148:1477–9.
8. Hair LS, Nuovo G, Powers JM. Progressive multifocal leukoencephalopathy in patients with human immunodeficiency virus. Hum Pathol 1992;23:663–7.
9. Aksamit AJ, Sever JL, Major EO. Progressive multifocal leukoencephalopathy: JC virus detection by *in situ* hybridization compared with immunohistochemistry. Neurology 1986;36:499–504.
10. Taylor CR, Shi SR. Fixation, processing, special applications in immunohistochemistry. In: Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist. Saunders, Philadelphia, PA. 1994; 42–5.
11. Brown RW, Chirala R. Utility of microwave-citrate antigen retrieval in diagnostic immunohistochemistry. Modern Pathol 1995; 8:515–20.
12. Mc Allister HA, Rock DL. Comparative usefulness of tissue fixatives for in situ viral nucleic acid hybridization. J Histochem Cytochem 1985;33:1026.
13. Elias, JM. Immunohistopathology a practical approach to diagnosis, ASCP Press, Chicago, Ilinois. 1990:418–9.
14. Greer CE, Peterson HT, Kiviat NB, Manos MM. PCR amplification from paraffin-embedded tissues: effects of fixative and fixation time. Am J Clin Pathol 1991;95:117–24.
15. Elias JM, Margiotta M, Gaborc O. Sensitivity and detection efficiency of the peroxidase antiperoxidase (PAP), avidin-biotin peroxidase complex (ABC) and peroxidase-labeled avidin-biotin (LAB) methods. Am J Clin Pathol 1989;92:62–7.
16. Aksamit AJ, Jr. PCR detection of JC virus. In: Persing DH, Smith TF, Tenover FC, White TJ. Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, D.C. 1993;361–6.
17. Bassam BJ, Caetano-Anolles G, Giesshoff PM. Fast and sensitive silver staining of DNA in polyacrylamide gels. Analytical Biochemistry 1991;196:80–3.
18. Hanzlick R. Embalming, body preparation, burial, and disinterment. Am J Forensic Med Pathol 1994;15(2):122–31.

Additional information and reprint requests:
Barbara H. Amaker, M.D.
Medical College of Virginia
Department of Pathology
Division of Neuropathology
P.O. Box 980017
Richmond, VA 23298-0017