Comparison of Methods for Immunocytochemical Detection of Rotavirus Infections

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Received for publication 10 August 1979

Rotavirus infections in intestinal tissues of animals or in tissue culture cells were detected by the immunocytochemical unlabeled soluble enzyme peroxidase antiperoxidase method. Comparison of the immunofluorescence and peroxidase antiperoxidase immunological staining techniques revealed that the two methods are equally sensitive for detection of rotavirus-infected cells. The peroxidase antiperoxidase technique offers the advantages of negligible nonspecific staining reactions, the use of a standard light microscope, the production of permanent slides, and the conservation of immunological reagents. The ability to detect antigens in paraffin-embedded tissues enhances the usefulness of the peroxidase antiperoxidase test for both prospective and retrospective studies.

Rotaviruses are a common cause of gastroenteritis in both animals and humans. A number of techniques with various sensitivities have been developed to detect rotaviruses in intestinal contents and feces, including electron microscopy (3, 10), immune electron microscopy (11, 15), immunodiffusion (22), counterimmunoelectrophoresis (20, 26), complement fixation (14, 24), immunofluorescence (IF) (4, 30), immune adherence hemagglutination (17), enzyme-linked immunosorbent assays (8, 29), radioimmunoassays (6, 13, 19), and most recently, a plaque assay to measure viral infectivity (16, 23). In contrast, only electron microscopy and the fluorescent-antibody assay have been used to directly demonstrate rotavirus particles or antigens in intestinal tissue biopsies or in infected cell cultures (1, 2, 5, 7, 18, 27, 28).

We report the application of an enzyme immunochemical technique, the peroxidase antiperoxidase (PAP) test, for the detection of rotavirus infections in tissue culture cells and in intestinal biopsy specimens. Similar in sensitivity, the PAP technique has several advantages over the IF technique. It can be employed without the use of an expensive fluorescence microscope and can be applied to paraffin-embedded tissues, thereby abrogating the need for fresh tissues.

MATERIALS AND METHODS

Materials examined by IF and PAP tests. Cover slip cultures of MA104 cells (Microbiological Associates, Bethesda, Md.) infected at various multiplicities (0.01 to 10 plaque-forming units per cell) with the simian rotavirus SA11 as previously described (9) were harvested 18 h postinfection by fixation in 100% ethanol for 10 min at −20°C. Frozen murine rotavirus (epizootic diarrhea of infant mice)-infected mouse intestines were kindly donated by John Shadduck and Lynn Little (University of Texas Medical School, Dallas). The frozen mouse intestines were embedded in optimal cutting temperature compound (Lab-Tek Products, Naperville, Ill.), and cryostat sections (8 to 10 μm) were mounted on cover slips and fixed in acetone at room temperature. Paraffin-embedded sections of a biopsy of an infant in whom rotavirus gastroenteritis had been proven by electron microscopy were kindly supplied by David Yawn (Texas Children’s Hospital, Houston). The intestinal biopsy was fixed in Zenker solution and embedded in paraffin, by routine histological procedures. Sections 4 μm thick were mounted on slides. The paraffin was removed by immersing the slides in the following solutions: xylene, 3 min twice; 100% ethanol, 2 min twice; 90% ethanol, 1 min; 70% ethanol, 1 min; distilled water, two changes. The deparaffinized tissues were finally rehydrated in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (TBS; 0.05 M Tris-hydrochloride, 0.15 M NaCl, pH 7.6) before staining.

Antisera. Hyperimmune guinea pig anti-SA11 serum was prepared as previously described (9). Fluorescein-labeled goat anti-guinea pig immunoglobulin G (IgG), unlabeled goat anti-guinea pig IgG, and guinea pig PAP-soluble complex were purchased from Cappell Laboratories (Cochranville, Pa.).

IF tests. The indirect IF technique was performed as previously described (9). Briefly, 15-mm round cover slip cultures fixed in ethanol were rehydrated in TBS, and 50-μl amounts of serial dilutions of hyperimmune guinea pig SA11 antisera were added. After incubation in a humidified chamber at 37°C for 30 min, the cover slips were washed in TBS, and 50 μl of fluorescein-conjugated goat anti-guinea pig IgG was added. After a second incubation at 37°C for 30 min,
the cover slips were drained, washed in TBS, air dried, and mounted in elvanol. Stained cover slips were observed in a Zeiss universal microscope equipped with an RSIII vertical illuminator by using a fluorescein filter pack.

PAP immunological staining. The Sternberger technique of PAP immunological staining (25) was used with minor modifications. All antisera were diluted in 10% fetal bovine serum (FBS) previously shown to be free from rotavirus antibody by complement fixation and IF tests. Cover slips were washed in TBS which contained 2% FBS (TFBS) until the wash before the addition of enzyme substrate. Before staining, the cells on cover slips were rehydrated with TBS and immersed in 10% FBS in TBS or in 10% egg albumin (Sigma Chemical Co., St. Louis, Mo.) in TBS. After washing, 50-μl amounts of various dilutions of guinea pig anti-SA11 antiserum were added, and the cover slips were incubated for 30 min in a moist chamber at 37°C. After draining off the serum and dipping them in TBS, we washed the cover slips extensively with TFBS with constant stirring. A 50-μl amount of goat anti-guinea pig heavy- and light-chain IgG (Cappell Laboratories) diluted 1:10 in TFBS was then added. After incubation at 37°C for 30 min, the cover slips were washed in TBS. A 50-μl amount of guinea pig PAP diluted 1:50 in TFBS was added, followed by a 30-min incubation in a moist chamber at 37°C. The cover slips were washed in 0.05 M Tris-hydrochloride (pH 7.6) without FBS and treated for 5 min in the dark with a solution of 0.05% 3,3'-diaminobenzidine tetrachloride (Sigma) freshly prepared in 0.05 M Tris-hydrochloride (pH 7.6). Hydrogen peroxide was added to 0.01%, and the cells were allowed to react in the dark for 3 min with constant stirring. The cover slips were then rinsed in TBS without serum, counterstained for 1 min with Gill hematoxylin no. 3 (Polysciences, Inc., Warrington, Pa.), rinsed in tap water, immersed for 1 min in saturated lithium carbonate, and rinsed again in tap water. After a final dipping in distilled water, the cover slips were dehydrated through a graded series (15, 50, 75, 95, and 100%) of ethanol, cleared with xylene, and mounted in Permount (Fisher Scientific Co., Fair Lawn, N.J.).

PAP staining in intestinal tissues. The highly differentiated enterocytes of intestinal villi contain endogenous peroxidases which interfere with the interpretation of the PAP test. A modification of the method of Isobe et al. using periodic acid and sodium borohydride (12) was used to destroy this activity in cryostat sections of mouse intestinal tissues. Tissue sections fixed in acetone were washed in TBS for 15 min and treated with 0.005 M periodic acid for 10 min at room temperature. They were washed again in TBS for 15 min and with 0.003 M sodium borohydride for 30 min at room temperature. The sections were finally washed in TBS, immersed in TBS containing 10% egg albumin for 30 min, and stained by the PAP procedure described above.

RESULTS

Detection of rotavirus infections in tissue culture cells by using IF and PAP tests. Duplicate rotavirus (SA11)-infected cover slip cultures were stained by the IF or PAP test. The general pattern of reaction by the specific SA11 antiserum was similar with either technique. Cytoplasmic staining and perinuclear inclusions were observed by both the IF and PAP tests. In some cells, nuclear stained structures could be discerned by the PAP test which were not distinguishable in the IF slides. These nuclear reactions were not observed in uninfected cells or in infected cells treated with preimmune guinea pig serum.

Comparative sensitivities of the IF and PAP tests for detection of rotavirus infections. The comparative sensitivities of the IF and PAP tests for detection of rotavirus infections were investigated in two ways. First, the primary antiserum used to detect the infection was titrated. The antiserum could be diluted 1: 40 to 1:80 in the IF test, whereas the endpoint of the antiserum in the PAP test ranged from 1:200 to 1:400. The ability to detect positive reactions at high dilutions of the antiserum shows the PAP test to be more sensitive and suggests a potential saving of valuable immunological reagents.

The sensitivities of the two methods were also compared by staining cultures infected with SA11 at various multiplicities of infection (multiplicity of infection = 0.01, 0.1, 1.0, and 10 plaque-forming units per cell). The percentage of cells positive for rotavirus antigen at a given multiplicity of infection was the same when measured by either the IF or PAP test. It should be noted, however, that the minimal background seen with the PAP test facilitated interpretation of the cover slips, particularly when the percentage of infected cells was low.

Detection of rotavirus infection in tissue sections. The high nonspecific background staining encountered with IF tests often limits their usefulness in monitoring tissue sections. For this reason, the applicability of the PAP test to tissue sections was examined. High levels of endogenous peroxidase activity were observed in the differentiated columnar epithelial cells on the sides and tips of the villus, but not in the crypts (Fig. 1A). This endogenous reaction was successfully eliminated by pretreatment with periodic acid and sodium borohydride (see Materials and Methods), thereby permitting ready identification of the rotavirus-infected cells (Fig. 1B). This technique not only localized the viral antigen within the cell, but could also establish the cell type infected by the virus. Paraffin-embedded intestinal biopsy specimens obtained from a child with a rotavirus infection and from piglets with experimentally induced rotavirus
infection were also stained by the PAP test. Rotavirus antigen was easily demonstrated, emphasizing that this technique can be applied in retrospective studies.

DISCUSSION

Rotavirus antigens have previously been detected in tissue culture cells or in intestinal biopsies by electron microscopy or by IF methods (2, 3, 6, 8, 20, 28, 30). This study showed that the IF and the PAP tests are equally sensitive, in that the percentage of positive cells detected in tissue culture samples was identical by both techniques. However, the PAP test has the advantage that the primary antiserum can be diluted five- to tenfold higher than with the IF technique, thus allowing a saving of valuable reagents. The minimal background reactions obtained with the PAP test as compared with the IF technique also permits easy evaluation of slides and the potential detection of minor viral antigens (or antigens in intestinal biopsy or extraintestinal tissues) which may remain undetectable by the IF technique.

Rotavirus antigens appear to be relatively stable. We were able to demonstrate rotavirus-infected cells in tissues that had been embedded in paraffin for several years. In general, formalin-fixed tissues can be processed into thinner sections, exhibit better preservation of cellular architecture, and are preferred for evaluation of changes in intestinal morphology. Utilization of the PAP technique with paraffin sections not only can localize the viral antigen within a cell, but it can also establish the cell type infected by the virus. The ability to utilize the PAP test on paraffin-fixed tissue sections eliminates the need to divide specimens for cryostat samples and for Formalin fixation; the amount of biopsy material available for fixation in Formalin and histological interpretation is thus considerably increased. The applicability of the PAP test on paraffin-embedded sections would allow a retrospective examination of such tissues for rotavirus antigens. The capability to easily examine multiple sections of Formalin-fixed tissue will also allow adequate sampling of tissue before an area of intestine is labeled rotavirus negative.

Additional advantages of the PAP test over the IF technique are that permanent slides are produced, expensive fluorescence microscopes are not required, and identification of infection of only a small percentage of cells is not equivocal. Although the PAP procedure is slightly more time consuming to perform than the IF test, multiple samples can easily be processed in

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**Fig. 1. Immunocytochemical (PAP) localization of rotavirus infected cells in frozen mouse intestinal sections. (A) Intestinal sections showing strong endogenous peroxidase reaction in epithelial cells along the villus. (B) Intestinal section after treatment to destroy endogenous peroxidase. Arrows denote epithelial cells containing rotavirus antigens. ×180.**
one day. The PAP test can be modified to include a second antibody directed against another antigen if a second substrate yielding a different-colored reaction product is used (21). This methodology could be valuable in studying the role of dual infections in gastroenteritis. Finally, the application of the PAP procedure to electron microscopic studies permits detailed ultrastructural studies of rotavirus replication and morphogenesis in infected cells (B. Altenberg, D. Y. Graham, and M. K. Estes, J. Gen. Virol., in press).

ACKNOWLEDGMENTS

The excellent technical assistance of Candace Roberts is gratefully acknowledged. We thank Janet Butel for her helpful advice regarding the PAP methodology.

This work was supported in part by grant no. AM 25,198 from the National Institutes of Health and by a grant from Vick Division of Research and Development, Mount Vernon, N.Y.

LITERATURE CITED


