

Use of Horseradish Peroxidase-Labeled Antibody for Light and Electron Microscope Localization of Reovirus Antigen

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Horseradish peroxidase-labeled antibody was used for light and electron microscopic localization of reovirus antigen in tissue culture. Reaction product in infected cells was easily detected in the cytoplasm, and the procedure was as sensitive as the fluorescent-antibody technique. At the electron microscopic level, infected and enzyme-labeled antibody-treated cells showed accumulations of reaction product at the sites of viral replication and around the viral particles. Reaction product was not detected in unstained infected cells, in stained uninfected cells, or in cells infected with an unrelated virus.

Recently, Nakane and Pierce (5, 6) described a method for labeling antibodies with enzymes by means of bifunctional reagents. The technique used horseradish peroxidase for enzyme and difluoro-*m,m'*-dinitrodiphenyl sulfone as bifunctional reagent. The reaction product is enzymatically and immunologically active and allows the localization of antibody by the demonstration of enzymatic activity (3). The reaction product is brown and very osmiophilic, and, when treated with osmium, it becomes an amorphous, distinct product easily demonstrable in light and electron microscopy. The localization of antibody is very clear, and the technique is sensitive because of the amplifying effect of enzymatic activity. The enzyme-labeled antibody technique (ELAT) has been successfully used for light and electron microscopic localization of antibody against epithelial basement membrane of parietal yolk sack carcinoma (4), leucine-binding protein from *Escherichia coli* (4), and lymphocytic choriomeningitis virus (1). This report describes the use of ELAT, both at light and electron microscopic levels, as a tool for the study of reoviruses.

MATERIALS AND METHODS

Virus. A reovirus isolated in this laboratory, serologically identified as mammalian type 1 (7), was cultivated in tissue cultures, purified, and concentrated in a suspension with a titer of 80 hemagglutinating units per ml.

Cell culture. A chimpanzee liver cell line (kindly provided by L. E. Carmichael, Cornell University) was employed. Monolayer cultures, grown in sealed

200-ml prescription bottles and on glass cover slips in Leighton tubes, were inoculated with 0.25 and 0.01 ml of viral suspension, respectively. After a virus adsorption period of 2 hr at 37 C, the viral suspension was replaced with Eagle's minimal essential medium supplemented with 3% calf serum and antibiotics (20 ml per bottle, 1.5 ml per tube). Infected and control cells were incubated at 37 C for the various examinations at 5 days postinoculation.

Enzyme and fluorescein-labeled antibody. Hyperimmune serum, produced in goats (2), was found to be specific for reovirus type 1 by agar-gel diffusion (no reaction to chimpanzee cells) and had a hemagglutination inhibition titer of 1:32,768.

Immune serum was processed as described by Nakane and Pierce (6) by using horseradish peroxidase (crude type VI, Sigma Chemical Corp., St. Louis, Mo.) and 4,4'-difluoro 3,3'-dinitro diphenyl sulfone (Pfaltz and Bauer Inc., Flushing, N.Y.) as the bifunctional reagent. Serum from the same batch was employed for conjugation with fluorescein isothiocyanate by the technique described by Campbell et al. (2).

Immunohistochemical preparation for light microscopy. Infected and control cells attached to cover slips were removed from the Leighton tubes and washed twice in phosphate-buffered saline (PBS; 0.1 M phosphate buffer, 0.15 M NaCl). (Washings were generally for 10-min periods.) After fixing in acetone for 15 min and washing three more times in PBS, the cover slips were covered with enzyme-labeled antibody. Staining was carried out in a moist chamber for 30 min. Cover slips were washed twice in PBS and then incubated in diaminobenzidine solution (3) for 20 min. Three washes in tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (0.05 M, pH 7.6) preceded a 15-min treatment with 2% osmium tetroxide in distilled water. After two more washings in

Tris buffer, the monolayers were dehydrated in graded alcohols, cleared in xylol, and mounted in balsam.

For specific stain control, the same procedure was applied to chicken kidney cell monolayers infected

with Marek's disease herpesvirus, JM strain (kindly provided by B. W. Calnek, Cornell University).

Preparations for electron microscopy. For immunohistochemical preparations, infected and control

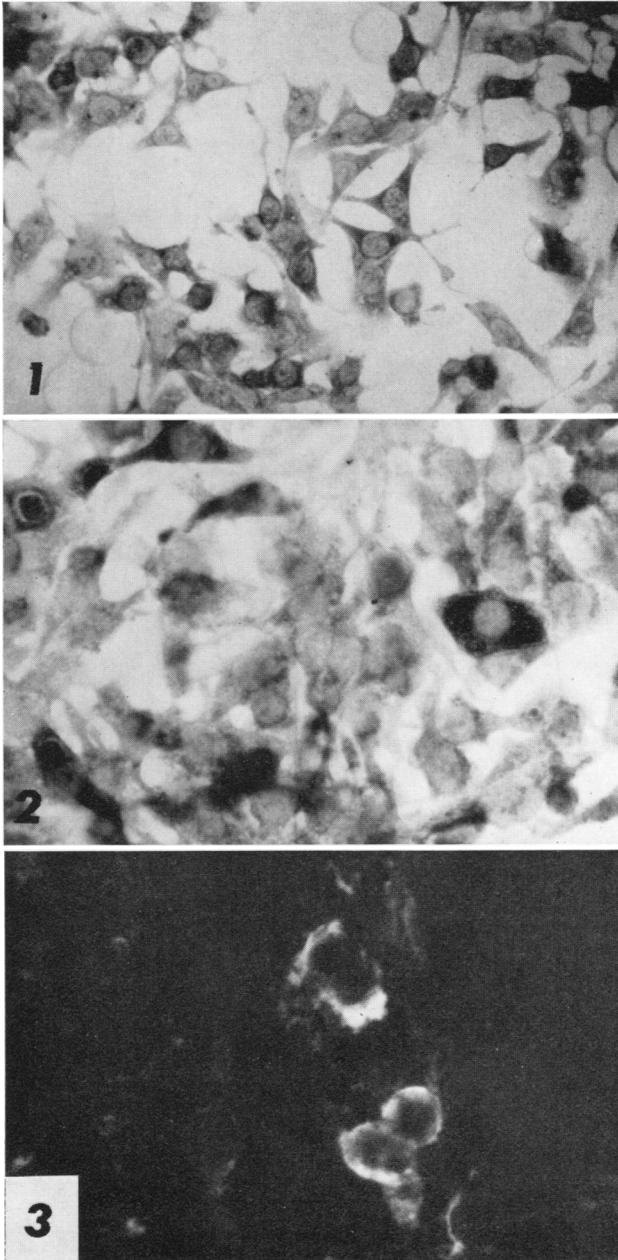


FIG. 1. Infected monolayer treated for peroxidase activity without prior exposure to enzyme-labeled antibody. No reaction product is detected. $\times 500$.

FIG. 2. Infected cells first treated with enzyme-labeled antibody and then treated for peroxidase activity. Irregular intracytoplasmic masses of reaction product. $\times 900$.

FIG. 3. Similar infected cell culture treated with fluorescent antibody. Intracytoplasmic areas of fluorescence. $\times 900$.

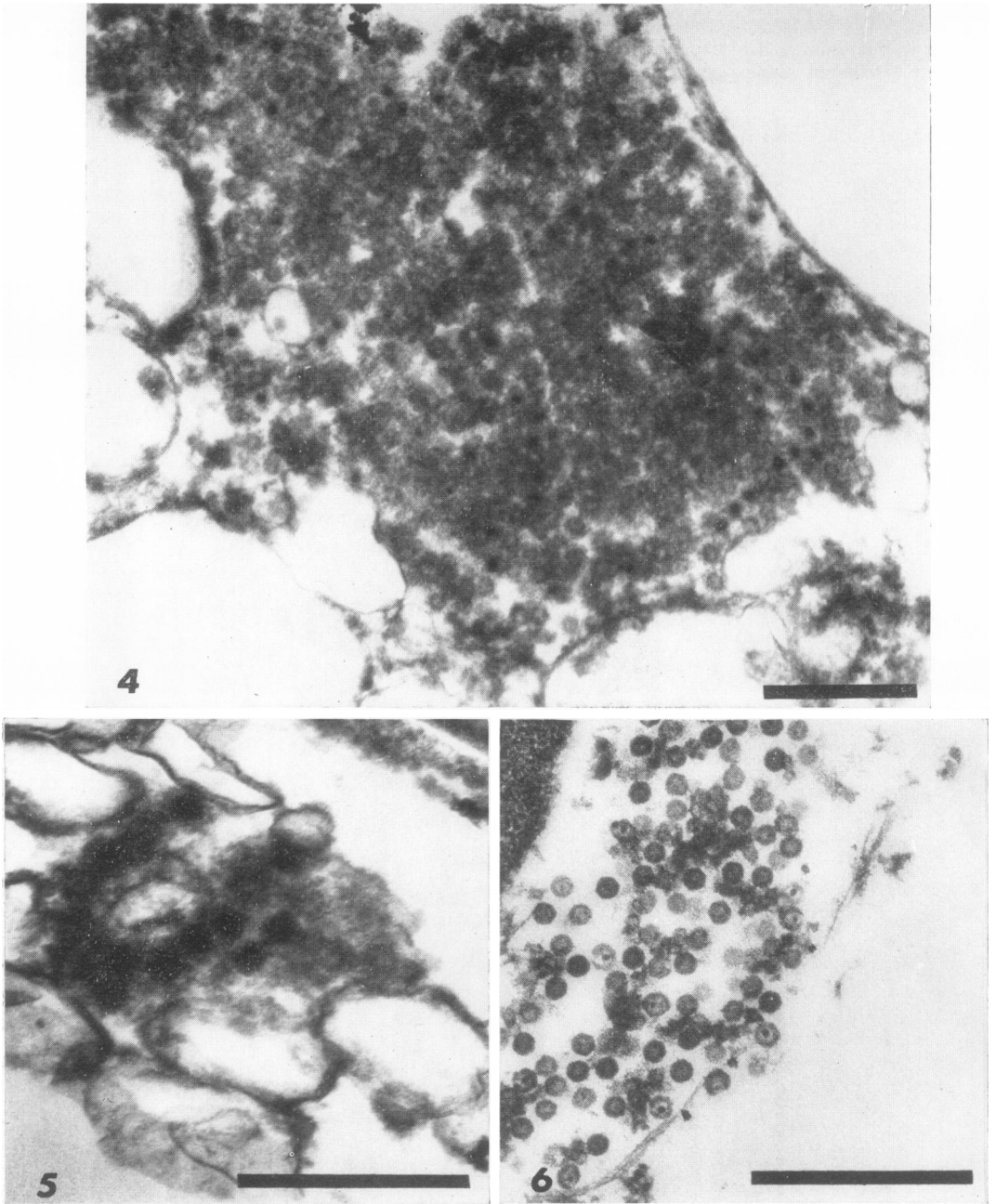


FIG. 4. Mature particles and empty capsids surrounded by reaction product in the cytoplasm of an infected and immunoenzymatically treated cell. Bar = 1 μ m.

FIG. 5. Extra cytoplasmic viral particles of immunoenzymatically treated cells. The virions are clearly surrounded by the reaction product. Bar = 0.5 μ m.

FIG. 6. Portion of an infected cell treated for peroxidase activity without prior exposure to enzyme-labeled antibody. Viral particles in the cytoplasm, none surrounded by reaction product. Bar = 1 μ m.

monolayers were scraped from the prescription bottles into the medium, centrifuged, and washed with PBS. (This and other washes were all by low-speed centrifugation after the cells had been suspended in the wash fluid for 5 or 10 min.)

Cells were fixed for 10 min in 10% acetate-buffered Formalin and washed three times with PBS. After staining with enzyme-labeled antibody for 30 min and washing twice in PBS, the cells were incubated for 20 min in diaminobenzidine. Three 10-min changes of Tris buffer preceded a 20-min treatment in 2% osmium tetroxide in distilled water. After three more changes (5 min) in Tris buffer, the cells were stained with 5% uranyl acetate in 25% alcohol, dehydrated in graded alcohols and propylene oxide, and then embedded in an Epon-Araldite mixture. For conventional preparation for electron microscopy, infected cells were scraped from the bottles, pelleted by low-speed centrifugation, and fixed overnight in 3% buffered glutaraldehyde at 4 C. Pelleted material was stained with 1% buffered osmium and dehydrated in graded alcohols before staining with uranyl acetate. Propylene oxide dehydration was followed by Epon-Araldite embedding.

Conventional and immunohistochemically treated, embedded cells were thin-sectioned at 60 to 90 nm with a Porter & Blum MT-1 ultramicrotome and collected on 200-mesh Formvar and carbon-coated grids. Post-staining was carried out with lead citrate. Prepared sections were observed with an RCA-EMU-3G electron microscope.

Fluorescent-antibody techniques. Staining of infected and control cells on cover slips was carried out by using the direct technique. The monolayers (5 days postinoculation) were fixed in acetone and processed by the method described by Campbell et al. (2). Buffered glycerol-mounted, stained cover slips were observed by dark-field illumination with an HB 200 W Osram mercury vapor lamp, a BG 12 excitor filter, and a 510-nm barrier filter.

RESULTS

ELAT-treated uninfected monolayers had a characteristic light background staining involving all of the cells. No reaction product could be seen in cells in these monolayers or in those of infected monolayers reacted cytochemically for peroxidase activity without prior exposure to enzyme-labeled antibody (Fig. 1). Marek's disease virus-infected cells were likewise negative. In contrast, reovirus-infected, ELAT-treated monolayers had cells with areas containing reaction product (Fig. 2). These intracytoplasmic areas were dark brown, polymorphic, and corresponded to the sites of antigen localization that were detected in cells stained by the fluorescent-antibody technique (Fig. 3).

Infected cells, conventionally treated for electron microscopic observation, contained characteristic reoviruses, either aggregated as intracytoplasmic inclusion bodies or free outside of the cells. The particles were often surrounded by viral

matrix; however, single virions, most often outlined by a halo, could be easily distinguished.

In infected, immunohistochemically treated cells, the areas of viral synthesis were surrounded by an amorphous osmiophilic material which accumulated most heavily around the virions (Fig. 4). Amorphous osmiophilic material was also clearly associated with extracellular viral particles (Fig. 5).

On infected cells not exposed to enzyme-labeled antibody, the particles present in aggregates within the cytoplasm were poorly osmiophilic, distinctly separated from one another, and not surrounded by osmiophilic material (Fig. 6). Nucleoids were only faintly stained.

DISCUSSION

Our findings indicated that the enzyme-labeled antibody technique is suitable for studies on reovirus infection. In light microscopy, ELAT, applied to cells in monolayers, appeared to be as satisfactory as the fluorescent-antibody technique. With ELAT, an ordinary light microscope could be used and thus there was better visualization of microstructural details than could be distinguished with a dark-field condenser used for fluorescent microscopy.

The ELAT also seemed useful for the ultrastructural localization of virus. The localization of the antigen antibody reaction was very clear, possibly due to the high specificity and titer of the serum we used. On the other hand, in cells not reacted with enzyme-labeled antibody, the morphology of the virions, and especially nucleoids, was poorly defined. This may have been because of the short (20 min) reaction period with osmium. In cells treated with enzyme-labeled antibody, there was probably enhanced osmiophilia of the viral particles.

Attempts are being made in our laboratory to improve the fixation procedure applied to this system and to adapt the ELAT to other viral antigen-antibody systems.

In recent experiments (Ubertini et al., *in preparation*), we applied the ELAT for localization of the feline leukemia-sarcoma group-specific antigens in infected tissue cultures at the light and electron microscopic levels. In these experiments, the ELAT was useful for locating and visualizing nonparticulate viral materials.

The ELAT seems to be, as pointed out by Nakane and Pierce (5, 6), very useful for localization of antigens because, at the light microscopy level, its sensitivity is comparable to that of the fluorescent-antibody procedure, and at the electron microscopic level it is easier to use than the ferritin-conjugated antibody technique.

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LITERATURE CITED

1. Abelson, H. T., G. H. Smith, H. A. Hoffman, and W. P. Rowe. 1969. Use of enzyme labeled antibody for electron microscope localization of lymphocytic choriomeningitis virus antigen in infected cell culture. *J. Nat. Cancer Inst.* **42**:497-515.
2. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1963. Detection of antigens by fluorescein-labeled antibody, p. 209-215. *In Methods in immunology*. W. A. Benjamin, Inc., New York.
3. Graham, R. C., and M. J. Karnovsky. 1966. The early stages of adsorption of injected horseradish peroxidase in the proximal tubules of mouse kidney, ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291-302.
4. Nakane, P. K., G. E. Nicoalds, and D. L. Oxander. 1968. Cellular localization of leucine binding protein from *Escherichia coli*. *Science* **161**:182-183.
5. Nakane, P. K., and G. B. Pierce, Jr. 1966. Enzyme labeled antibodies. Preparation and application for localization of antigens. *J. Histochem. Cytochem.* **14**:929-931.
6. Nakane, P. K., and G. B. Pierce, Jr. 1967. Enzyme labeled antibodies for the light and electron microscopic localization of tissue antigens. *J. Cell. Biol.* **33**:307-318.
7. Rosen, L. 1968. Reoviruses, p. 73-107. *In* S. Gard, C. Hallauer, and K. F. Mayer (ed.), *Virology monograph no. 1*. Springer Verlag, New York.