Immunohistochemical Method for Detection of Vaccinia Virus* (34014)

NANCY J. SIVERD AND NEHEMA SHARON
(Introduced by Morris Pollard)
Lobund Laboratory, University of Notre Dame, Notre Dame, Indiana 46556

Viral inclusion bodies have been observed in cells by staining methods which required ordinary light- and ultraviolet microscopy. For groups of viruses which do not produce inclusion bodies or which destroy cells, differential cytological methods have been more difficult to apply. The specific immunofluorescent staining method developed by Coons (1) and modified by others (2, 3) provided an important practical and experimental procedure for locating and identifying viruses in cells. Although the fluroescein-labeled antibody technique has many advantages, there are some limitations in its use: It requires dark field ultraviolet microscopy; stained cells have a tendency to fade after a short time; naturally occurring autofluorescence can obscure the specific reaction, and paraffin-embedded tissues cannot be used because of nonspecific fluorescence (4).

The enzyme-conjugated antibody staining technique has the advantages of the immunofluorescent technique without some of its limitations (5). The method appears to be as specific and as sensitive as the immunoflourescent technique, but the preparations are permanent. They can be prepared from paraffin-embedded tissues, and they can be examined with the ordinary light microscope.

Two enzymes have been conjugated to antibodies in the search for epithelial basement membrane antigens in yolk sac carcinoma: they are acid phosphatase (6), and horseradish peroxidase (7, 8). Since horseradish peroxidase is available commercially and in relatively pure form, this enzyme was employed in our study, which describes the adaptation of an immunohistochemical method for detection of vaccinia virus in infected cell cultures.

Material and Methods. Antiserum. Three rabbits were inoculated repeatedly with vaccinia virus. The first two injections were given intradermally, with a 1-week interval between infections, to a shaved area on the abdomen. The rabbits were then inoculated intravenously six times each at biweekly intervals. At 1 week after the last injection the rabbits were bled and serum was separated. Vaccinia antibody content of the serums was determined by the complement-fixation test. Gamma globulins were precipitated from the serum specimens with 50% solution of saturated ammonium sulfate, and their purity was determined by immunoelectrophoretic analysis with goat anti-rabbit serum.

Conjugation of horseradish peroxidase and gamma globulins. Twenty-five mg of horseradish peroxidase (Sigma type II) was added to 25 mg of γ globulins in 1 ml of 0.5 *M* carbonate buffer, pH 10.0. The enzyme was conjugated to the antibody by adding 0.13 ml of 0.5% bifunctional reagent *p,p'*-difluoro-*m-m'*dinitrophenyl sulfone (FNPS-General Biochemicals) dissolved in acetone. The mixture was agitated gently at 4° for 4 hr. Unreacted peroxidase was separated from the γ globulins by precipitation of the latter with equal amounts of saturated ammonium sulfate in distilled water. After centrifugation the tagged globulin precipitate was washed twice with 50% solution of saturated ammonium sulfate–buffered saline; and this was dialyzed against 0.5 *M* phosphate buffer pH 7.2 containing 45% sucrose until the ammonium sulfate was removed. The conjugation technique was basically that described by Nakane (personal communication).

Virus. Vaccinia virus (strain WR204-mouse neurotropic) was propagated in "L" cells on coverslips with Eagle's minimum essential medium supplemented with 100 U/ml of penicillin, 100 U/ml of streptomycin sulfate, and 5% heat-inactivated calf serum. Confluent sheets of "L" cells were infected with 2×10^5 inclusion-forming

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particles or 3×10^8 pox-forming units of virus and were incubated at room temperature for 1 hr to allow virus adsorption. The infected cells were then washed twice, nutrient medium was added, and they were incubated at 37° for various periods of time.

Staining of infected cultures. Infected cultures at time 0, 6, 18, and 48 hr after onset of infection were washed and fixed in cold acetone for 10 min. The fixed cultures were washed in three changes of phosphate-buffered saline (PBS), 3–4 min for each change. The peroxidase-labeled γ globulin was added and incubated for 30 min in a moist chamber at room temperature. The treated coverslips were washed in three changes of phosphate-buffered saline for a total of 30 min, and then stained by the method of Graham and Karnovsky (9): 25 mg of 3, 3′-diaminobenzidine (Sigma) was dissolved in 100 ml of 0.05 *M* Tris buffer pH 7.6 + 0.001% peroxide. This solution was added to the reacted cultures and they were incubated at room temperature for 30 min. The coverslips were thoroughly washed in distilled water and then mounted on slides in glycerol–saline and examined by conventional light microscopy. Infected cultures at 18 hr after onset of infection were washed and fixed as above. After fixation the infected cell cultures were divided into four groups: Group 1 was treated with the peroxidase-labeled γ globulin as described above. Group II was treated with normal rabbit γ globulin for 30 min at room temperature, washed three times in phosphate-buffered saline pH 7.2, and treated with the peroxidase-labeled γ globulin. Group III was treated with unlabeled immune rabbit γ globulin for 30 min at room temperature, washed, and treated with peroxidase-labeled γ globulin. Group IV was treated for 30 min at room temperature with peroxidase-labeled γ globulin from rats which had been immunized against LCM virus. They were then washed and mounted for examination. Group V was uninfected cells treated with peroxidase-labeled γ globulin and they served as controls.

Results and Discussion. Uninfected "L" cells, treated with the peroxidase-labeled γ globulin and stained with diaminobenzidine failed to show any staining reaction: nuclei and cytoplasms remained pale although they were clearly detected (Fig. 1). At time 0, the cytoplasms of the infected cells showed a brownish coloring, with no distinct localization of viral antigens, and the nuclei did not show any changes. At 6 hr after infection, the reaction products of the enzyme were localized in the cytoplasm of many cells. The inclusions were stained distinctly dark brown (Fig. 2). The localization was still distinct at 18 hr after onset of the infection; but after 48 hr all of the cytoplasm was darker, and localization of the enzyme–antigen complex was not well defined. The infected cells were damaged and a virus-related cytopathic effect could be recognized. Duplicate sets of infected cultures were stained for comparison by Giemsa and by acridine orange fluorochrome stains. The number of cells with lo-

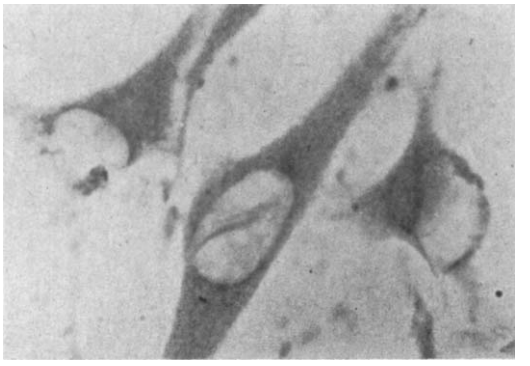

FIG. 1. Uninfected "L" cells treated with peroxidase-labeled rabbit antivaccinia γ globulin and stained with diaminobenzidine. (× 1000)

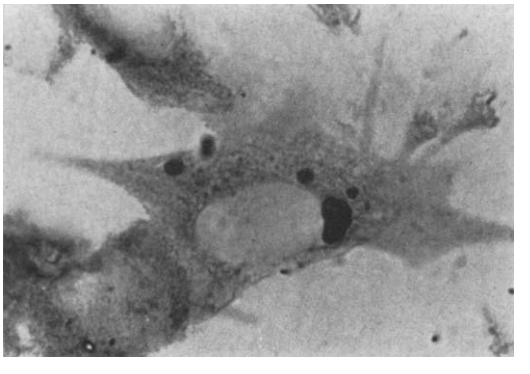

FIG. 2. "L" cells infected with vaccinia virus, treated as described in Figure 1. (× 1000)

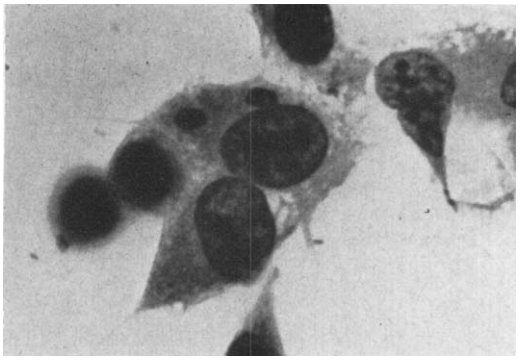

FIG. 3. "L" cells infected with vaccinia virus and stained by Giemsa. (× 1000)

calized inclusions at 6 hr, as demonstrated by the enzyme–antibody method, was as clearly defined as those which were stained by either of the other two methods (Fig. 3) which were not antigen-related stains. In the peroxidase staining procedure the nuclei and the cytoplasms were pale, only the viral antigen–enzyme complex appeared as dark-brown inclusions; in contrast to the cells which were stained by the acridine orange or with Giemsa stains, in both of which the vaccinia virus had the same color as the nucleus of the cell. In many uninfected cells stained by acridine orange or Giemsa, some nuclear "blebs" could possibly be mistaken for viral inclusion bodies. Therefore a more specific differentiation was achieved by peroxidase staining procedure.

Additional controls included the treatment of the infected cultures with unlabeled vaccinia immune rabbit γ globulin prior to the treatment with the labeled antiserum, for comparison with the treatment procedure with unlabeled normal rabbit γ globulin. The unlabeled immune γ globulin blocked the staining of the viral inclusions by the labeled γ globulin, whereas treatment with normal rabbit serum did not interfere with the specific staining. Treatment of the infected cultures with LCM immune γ globulin did not stain the vaccinia inclusions.

The clear picture observed by light microscopy with enzyme labeled antibody technique should be applied to examinations by electron microscopy. Peroxidase is an enzyme with a small molecular weight and the conjugate should easily penetrate through tissue or cell membranes. The electron opacity of the reaction products should make this method suitable for examination by electron microscopy. The use of light microscopy for examination of the stained slides, the stability of the preparations, and the sensitivity of the immunohistochemical method make it an additional important technique to be employed.

Summary. An adaptation of the enzyme-conjugated antibody technique for detection of vaccinia virus in tissue cultures was described. Gamma globulin, precipitated from rabbit antiserum, was conjugated to horseradish peroxidase with the aid of the bifunctional reagent FNPS. Vaccinia virus–infected cells on coverslips which were reacted with the enzyme-antibody complex and stained with diaminobenzidine, demonstrated the dark-brown cytoplasmic locatization of the antigen–antibody enzyme complex. Pretreatment with unlabeled antibody blocked the appearance of the cytoplasmic inclusions. The cell preparations were examined by ordinary light microscopy.

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