Identification of herpes simplex and vaccinia viruses in corneal cell cultures with immunoperoxidase: a light and electron microscopic study*

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Peroxidase-labeled antibody (pooled human immune globulin) was employed to localize herpes simplex and vaccinia viruses in corneal cells maintained in culture. With the light microscope, intranuclear and paranuclear staining were noted in cells infected with herpes simplex while intracytoplasmic staining was found in cells infected with vaccinia. Electron microscopy confirmed the differential, specific viral antigen labeling by peroxidase marker. The immunoperoxidase method, utilizing more specific antibodies in conjunction with electron microscopy, could presumably localize viral precursor antigens during different phases of infection. The relative advantages of this method in comparison to immunofluorescence and immunoferritin are discussed. Due to the specific staining achieved and its dual microscopic potential, we believe that the immunoperoxidase method may be useful as a diagnostic tool and could assist in understanding the immunopathology of corneal viral infections.

Key words: immunoperoxidase, substrate, reaction product, virion, herpes simplex, vaccinia.

The immunopathology of cells infected with herpes simplex or vaccinia viruses has been examined by various methods designed to localize intracellular viral antigens. For light microscopy, immunofluorescence has proved to be a valuable research and diagnostic tool.1-8 With the electron microscope, the immunoferritin technique has been employed widely, particularly in studies of herpes simplex virus.9-11 However, until recently12-14 no single method was available for both light and electron microscopic localization of intracellular antigens. Horseradish peroxidase is an enzyme which, following exposure to the appropriate substrate, forms an electron-dense reaction product. The enzymatic activity of peroxidase is retained after conjugation to antibody and the nature of the substrate reaction allows for the specific localization of reaction product with the light and electron microscopes.

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This report describes the application of the immunoperoxidase method to the identification of herpes simplex and vaccinia viruses in corneal cell cultures. Data are presented which suggest that this method could benefit immunopathologic studies of corneal viral infections.

Materials and methods

Viruses. Herpes simplex virus (HSV), strain HP-490, was obtained from Dr. G. Silver, Microbiological Associates, and a pool was prepared from this material in rabbit kidney cells. Vaccinia virus, strain CVI-78, was obtained from Dr. R. Dunlap, Division of Biologies Standards (DBS), National Institutes of Health.

Infection of cell cultures. An epithelial-like cell line derived from rabbit cornea, SIRC, was obtained from Mrs. H. Hopp, Division of Biologies Standards, National Institutes of Health. Cultures were maintained in 250 ml. plastic Falcon flasks with medium MAB 87/3 supplemented with 5 per cent fetal bovine serum without antibiotics at 37°C in a humidified incubator with 5 per cent CO₂ and air. Nearly confluent monolayers of SIRC cells were inoculated with HSV or vaccinia at a multiplicity of infection (MOI) of 25 to 100. Following inoculation, the virus was allowed to adsorb for 1 hour at 37°C and the cultures were then re-fed without rinsing with medium MAB 87/3 without serum. Cultures for electron microscopy were reacted with peroxidase-labeled antibody 24 to 48 hours after viral inoculation, while cultures for light microscopy were studied at 24 hours for vaccinia, and 18 to 72 hours for HSV.

Preparation of peroxidase-antibody conjugate. Immune globulin (human, Lederle Laboratories) was used as the source of antibody to both vaccinia and HSV. It had a protein content of 280 mg per milliliter and neutralization titers of 1:1024 and 1:512, respectively, against HSV and vaccinia. Antibody was conjugated with horseradish peroxidase by a method modified after Nakane and Pierce. Details of this procedure have been described previously. In summary, horseradish peroxidase (Sigma Type II) was dissolved with an equal amount (by weight) of antibody protein in 0.5 M. cold Na₂CO₃, pH 10. This was followed by the addition of 0.5 per cent p,p'-difluoro-m,m'-dinitro-diphenyl sulfone (FNPS), stirring for 6 hours at 4°C, and overnight dialysis against phosphate-buffered saline (PBS). The dialysate was then centrifuged and the supernate combined with an equal volume of saturated ammonium sulfate. The precipitate was washed twice with 50 per cent saturated ammonium sulfate in PBS, then dissolved in PBS, and dialyzed against PBS overnight at 4°C.

Application of peroxidase-labeled antibodies. Light microscopy. Infected cells with 2 to 3 + CPE were fixed in situ for 15 minutes in cold acetone and then rinsed without air drying in three changes of PBS. The peroxidase-antibody conjugate (1 ml. of a 1:2 dilution) was applied to the cells for 30 minutes in a moist chamber at 37°C. After exposure to this conjugate, the cells were rinsed again in three changes of PBS and incubated for 15 minutes in 25 ml. of substrate solution at room temperature. The substrate consisted of 3-3' diaminobenzidine tetrahydrochloride (0.5 mg. per milliliter) in 0.05 M. Tris-HCl buffer at pH 7.6 containing 0.01 per cent hydrogen peroxide. Following the substrate incubation, the cells were rinsed in three more changes of PBS, stained with Giemsa, and examined with the light microscope.

Electron microscopy. Infected cells with 1 to 2 + CPE were fixed in situ for 1 hour in cold 4 per cent paraformaldehyde containing 0.1 M. cacodylate buffer and 0.01 per cent CaCl₂ at pH 7.3. The cells were rinsed three times in the same buffer and allowed to wash overnight at 4°C. The next morning, the peroxidase-antibody conjugate (20 ml. of a 1:2 dilution) was added for 24 to 48 hours at 4°C. Following exposure to the conjugate, the cells were rinsed in three changes of 0.1 M. cacodylate buffer (pH 7.3) and fixed for an additional hour in cold 4 per cent glutaraldehyde in the same buffer. Then the cells were rinsed three times with 0.1 M. cacodylate buffer prior to a 15 minute incubation in substrate as described for light microscopy. After the substrate reaction, the cells were rinsed three additional times in buffer and postfixed for 1 hour in 1 per cent osmium tetroxide containing 0.1 M. cacodylate buffer at pH 7.3. The cells were scraped gently from the flask surface, suspended in 75 per cent ethyl alcohol, and centrifuged (1,800 x g for 5 minutes) into pellets. Finally, the pellets were rapidly dehydrated in ethyl alcohol and embedded in Araldite. Thin sections were cut, stained with lead citrate and uranyl acetate, and examined in the Hitachi HU11E electron microscope.

Studies of control cell cultures. Uninfected cells and cells infected with HSV or vaccinia were examined following application of the peroxidase-antibody conjugate or substrate alone. An additional control to demonstrate the specificity of the virus-antibody reaction consisted of exposing African green monkey cells, DBS-FCL-1, infected with SV-40 virus to the peroxidase-labeled immune globulin plus substrate.

Results

Light microscopy. Fig. 1A illustrates SIRC cells that have been infected with vaccinia virus and incubated in substrate...
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Fig. 1A. Corneal cells infected with vaccinia and incubated in substrate alone as a control. Multiple, small, intracytoplasmic inclusions or Guarnieri bodies (arrows) are present. Note the absence of large, dense intranuclear or intracytoplasmic inclusions. Giemsa, ×700, oil immersion.

Fig. 1B. Following exposure to the peroxidase-antibody conjugate and substrate, cells infected with vaccinia reveal diffuse, dense-black intracytoplasmic inclusions of peroxidase reaction product that localize viral antigen. No intranuclear reaction product is present. Giemsa, ×800, oil immersion.

Fig. 1C. Cells infected with herpes simplex and incubated in the peroxidase-antibody conjugate plus substrate demonstrate intranuclear and paranuclear staining by black peroxidase marker. Giemsa, ×625, oil immersion.
alone as a control. The corneal cells possess round or oval nuclei and pale cytoplasm. In the cytoplasm of many cells, small, round, eosinophilic inclusions (Guarnieri bodies)\textsuperscript{21} are identified that are characteristic of vaccinial infections of epithelial cells. No large, dense intranuclear or intracytoplasmic inclusions are present, however. The exposure to substrate does not affect the appearance of these cells other than to increase slightly the cellular density. In Fig. 1B, corneal cells have been infected with vaccinia virus and incubated in both the peroxidase-antibody conjugate and substrate. Several large, dense-black inclusions of peroxidase reaction product are identified in the cytoplasm. These localize the sites of vaccinia antigen. The nuclei are not labeled and often appear displaced by the diffuse, irregularly shaped cytoplasmic inclusions. Fig. 1C shows corneal cells that have been infected with herpes simplex virus and exposed to the peroxidase-antibody conjugate plus substrate. The nuclei of many of the cells contain dense-black inclusions of the peroxidase marker. There is a spectrum of nuclear labeling evident with some nuclei containing larger accumulations of reaction product than others. In several of the cells, paranuclear labeling was observed but no large inclusions were found in the cytoplasm as had been found in cells infected with vaccinia virus. Counterstains assist in identifying cellular detail but do not influence the appearance of peroxidase reaction product.

**Electron microscopy.** Figs. 2A and 2B illustrate infected corneal cells that have been exposed to the substrate alone as controls. At the ultrastructural level, substrate incubation results in decreased membrane definition but the virions are otherwise unaffected. In Fig. 2A, two round intranuclear virions of herpes simplex possess capsid membranes that enclose cores of moderate density. A clear zone is characteristically seen between the core and the capsid. The background chromatin appears relatively homogeneous and is of approximately the same density as the virions. Fig. 2B illustrates the cytoplasm of a corneal cell which contains immature vaccinia virions with incomplete membranes that surround a focus of granularity (the "virus factory\textsuperscript{22}."

A mature virion (arrow) is also shown and is composed of a central nucleoid enveloped by distinct membranes.

Fig. 3A demonstrates the nucleus and adjacent cytoplasm of a cell infected with herpes simplex that has been exposed to the peroxidase-antibody conjugate and substrate. Several well-defined virions of herpes simplex are present and labeled by dense, black, granular peroxidase reaction product. Despite the relative low magnification, the virions are easily identified in contrast to the background which is not labeled except for some focal accumulations of apparent reaction product that may localize sites of precursor antigen. As can be seen in Fig. 3B with higher magnification, one intracytoplasmic and several intranuclear virions are stained densely by black peroxidase reaction product. The normal clear zone between the core and the capsid has been obliterated by accumulated reaction product.

Figs. 4A and 4B illustrate the cytoplasm of cells infected with vaccinia and incubated in the antibody-conjugate plus substrate. In Fig. 4A, several virions surround a cytoplasmic "virus factory." These virions appear to be coated by dense, peroxidase-reaction product and the "virus factory" is specifically more dense than the surrounding cytoplasm due to labeling by the peroxidase marker. Fig. 4B shows several vaccinia virions under higher magnification that are coated completely by black reaction product that selectively adheres to the enveloping surface membranes and obliterates their normal definition. This membrane labeling of vaccinia virions corresponds to the apparent site of the antigens that elicit the production of serum neutralizing antibodies.\textsuperscript{23}

Examination of uninfected cells exposed to the antibody conjugate or substrate alone failed to reveal peroxidase staining. Similarly, African green monkey cells infected
Fig. 2A. Electron micrograph of the nucleus and adjacent cytoplasm of a cell infected with herpes simplex and incubated in substrate alone. Two intranuclear virions (arrows) possess cores of irregular shape that are moderately dense. Capsid membranes surround these cores and are separated from them by clear zones. Substrate incubation diminishes membrane definition slightly but otherwise does not affect the virions. ×52,500.

Fig. 2B. Cytoplasm of a cell infected with vaccinia and exposed only to the substrate. A "virus factory" is surrounded by immature vaccinia virions, some of which have incomplete membranes. A mature virion (arrow) is adjacent to this "factory." ×26,500.
Fig. 3A. Nucleus and adjacent cytoplasm of a cell infected with herpes simplex and exposed to the peroxidase-antibody and substrate. Despite low magnification, multiple round virions are easily identified due to specific staining by black, peroxidase reaction product. Focal accumulations of reaction product (arrows) may localize precursor intranuclear antigen. x22,200.

Fig. 3B. Higher magnification of nucleus and adjacent cytoplasm of another cell containing one paranuclear (arrow) and several intranuclear virions of herpes simplex. These virions are specifically stained by dense peroxidase marker which obliterates the clear zone between the core and capsid membrane. x53,500.
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Fig. 4A. Cytoplasm of a cell infected with vaccinia and incubated in the peroxidase-antibody conjugate plus substrate. A “virus factory” is stained by black peroxidase reaction product. Incomplete membranes of two immature virions (arrows) are partially obscured by the peroxidase marker. Several mature virions surround the “factory” and are coated by reaction product, ×22,000.

Fig. 4B. Higher magnification of mature vaccinia virions illustrating the coating of their surface membranes by the dense peroxidase label, ×46,000.
with SV-40 and incubated in the immune globulin conjugate and substrate did not demonstrate virion labeling as had been seen previously with specific anti-SV-40 antibody conjugate.18

Comment

Based on the results of this study, the immunoperoxidase method appears to have great potential as an investigative tool in studying the immunopathologic aspects of corneal viral infections. Differential peroxidase labeling occurred with herpes simplex and vaccinia viruses using human immune serum globulin as the single source of antibody. For herpes simplex, intranuclear and paranuclear labeling was identified with the light microscope and specific virion labeling in the nucleus and cytoplasm was confirmed with the electron microscope. With vaccinia, dense inclusions were restricted to the cytoplasm by light microscopy and labeling of virions and "virus factories" was verified by electron microscopy. Although labeling with this pooled antibody-peroxidase conjugate was primarily confined to intact virions, the use of more specific antibodies with electron microscopy could presumably allow precursor viral antigen localization during different phases of infection. The nature, reproducibility, and specificity of labeling achieved in this study confirm previous observations18 of immunoperoxidase localization of herpes zoster virus and Simian virus-40 in human and monkey fibroblasts, respectively.

The ability to study infected cells at both the light and electron microscopic levels with the same antibody-conjugate offers important technical advantages. With immunofluorescence, in herpes simplex infections, some investigators have noted only cytoplasmic staining6,5 while others have reported both cytoplasmic and nuclear staining.4,6,8 In an interesting study of cells infected with herpes simplex and EB viruses, Hampar and co-workers10 noted that rabbit antibodies to these viruses bound ferritin to intranuclear virus capsids, but identical virus particles could not be detected by immunofluorescence using the same antibodies. It appears that in cells infected with herpes simplex or EB viruses, the nature of the intranuclear antigens that stain with fluorescein-labeled antibody is not resolved and might benefit from evaluation with immunoperoxidase.

Additional advantages of the immunoperoxidase method as opposed to immunofluorescence include minimal background staining, permanency of slides, stability of storage of the conjugate, and evaluation by ordinary light microscopy. However, whereas positive immunofluorescence is often readily evident, the immunoperoxidase method requires a histopathologic approach to the interpretation of cellular involvement. Moreover, although background staining is minimal, it is essential to distinguish the sites of peroxidase localization from normal morphologic structures or endogenous peroxidatic activity by using substrate controls.

In comparison to immunoferritin, the peroxidase-antibody conjugate appears to penetrate cells more readily as the peroxidase molecule (MW = 43,000) is much smaller than ferritin (MW = 800,000). Furthermore, the immunoperoxidase conjugation is simpler and the nature of the peroxidase-substrate reaction amplifies the ability to localize viral antigen due to the accumulation of reaction product. However, as the ultrastructural identification of viral antigen is based on the differing densities of accumulated reaction product, a significant amount of antigen must be present in order to be certain of its localization.

The role of the immune system in viral corneal infections has not been fully delineated. Due to the specificity of peroxidase staining achieved in this study and the potential for conjugation to more specific antibodies, we believe that the immunoperoxidase method can provide valuable information in immunopathologic studies of corneal viral infections, and could be used as a diagnostic tool.
REFERENCES