

Immunoperoxidase Localization of Herpes Zoster Virus and Simian Virus 40 in Cell Culture

ALAN L. SHABO, JOHN C. PETRICCIANI, AND RUTH L. KIRSCHSTEIN

Laboratory of Pathology, Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 11 January 1972

The immunoperoxidase technique was used in an electron microscopy study to localize the virions of herpes zoster virus and simian virus 40 in cell cultures. Intranuclear and intracytoplasmic virions of herpes zoster virus were easily and specifically identified due to intense staining by the finely granular, black reaction product. With simian virus 40, intranuclear virions were not stained, whereas intracytoplasmic particles appeared densely black. There was essentially no background staining. Advantages of this technique over the ferritin-labeled antibody method include simpler preparative procedures for reagents, greater penetrability of the antibody conjugate, and internal amplification which substantially improves the ability to localize sites of antigen-antibody reaction. We believe that the immunoperoxidase method can be successfully applied to a wide variety of problems involving viral antigens.

The use of techniques for labeling antibody against viral antigen has provided information essential to the understanding of microbiological systems. By coupling antibody to fluorescein, as originally described by Coons (2), fluorescence microscopy has been used universally as a method for identifying and localizing viral antigen in infected host cells. For electron microscopy, the conjugation of heavy metals such as mercury (15) and uranium (23) to antibody has been attempted with little success. At present, the method used most widely and effectively involves the coupling of ferritin (molecular weight, 850,000; diameter, 11 nm) to antibody with reagents such as diisocyanates (5, 8, 9, 13, 14, 19, 22) or *p,p'*-difluoro-*m-m'*-dinitrodiphenyl sulfone (FNPS) (18). Recently, Nakane and Pierce (10, 11), employing FNPS, conjugated antibody against tissue antigens to horseradish peroxidase and demonstrated that these peroxidase-antibody conjugates retained their enzymatic and immunologic activity. Horseradish peroxidase (molecular weight, 43,000; diameter, 2.5 to 3 nm) is a protein which, after incubation with the appropriate substrate (3-3' diaminobenzidine), can be localized specifically with light and electron microscopes. The accumulation of reaction product amplifies the ability to localize sites of

peroxidase activity. Previous ultrastructural studies on viral antigens (1, 25) have encountered problems in fixation that may alter microanatomy and diminish immunohistochemical staining.

This report describes the ultrastructural identification of herpes zoster virus (HZV) and simian virus 40 (SV40) in cell cultures with peroxidase-labeled antibodies and improved fixation. Observations are presented that relate to the specificity and technical simplicity of the immunoperoxidase technique in the localization of viral antigen.

MATERIALS AND METHODS

Viruses. Plaque-purified SV40 small plaque virus (24) was a gift of S. Aaronson, National Cancer Institute. HZV strain HM was obtained from H. Hopps, Division of Biologics Standards, National Institutes of Health.

Infection of cell cultures. All cell lines used in this study were maintained in 250-ml plastic Falcon flasks with medium MAB 87/3 (3) supplemented with 5% fetal bovine serum and penicillin (100 units/ml) at 37 C in a humidified incubator with 5% CO₂ and air. Confluent monolayers of African green monkey (*Cercopithecus aethiops*) diploid fibroblast cell line, DBS-FCL-1 (16), were inoculated with SV40 at a multiplicity of infection (MOI) of 5, and monolayers of the human diploid cell line, CCL-72

(American Type Culture Collection, Rockville, Md.), were inoculated with HZV (MOI of 5). After inoculation with SV40 or HZV, the cultures were refed without rinsing and maintained in medium MAB 87/3 without serum and reacted with peroxidase-labeled antibody at 4 days and 18 hr, respectively.

Preparation of peroxidase-antibody conjugates. Rabbit SV40 antiserum (Grand Island Biological Co.) contained 84 mg of protein/ml and had a neutralization titer of 1:1,280. Human immune globulin (Lederle Laboratories) was used as the source of antibody to HZV, contained 290 mg of protein/ml, and had a neutralization titer of 1:288. The respective antibodies were conjugated with horseradish peroxidase by a method modified after Nakane and Pierce (10, 11) without column fractionation or absorption with liver powder. Equal amounts by weight of horseradish peroxidase (Sigma, type II) and antibody were dissolved in 0.5 M cold Na_2CO_3 , adjusted to pH 10 with HCl (2 ml per 50 mg of protein of the antibody). To this solution, 0.5% FNPS in acetone was added (0.1 ml of FNPS per 1.0 ml of peroxidase-globulin solution). The mixture was stirred for 4 to 6 hr at 4 C and then was dialyzed against phosphate-buffered saline (PBS) overnight at 4 C. The dialyzed material was centrifuged at $3,000 \times g$ for 10 min, and the supernatant extract was combined with an equal volume of an aqueous solution of saturated ammonium sulfate. The precipitate was collected by centrifugation at $3,000 \times g$ for 20 min, washed twice with a solution of 50% saturated ammonium sulfate in PBS, dissolved in PBS, and dialyzed against PBS overnight at 4 C. The dialyzed material contained the peroxidase-labeled antibody and was stored in portions at -20 C.

Application of peroxidase-labeled antibodies. Infected cells were fixed in situ for 1 hr in cold 4% paraformaldehyde containing 0.1 M cacodylate buffer and 0.01% CaCl_2 at pH 7.3. The cells were rinsed three times and allowed to wash overnight at 4 C in 0.1 M cacodylate buffer at pH 7.3. The peroxidase-antibody conjugate (20 ml of a 1:2 dilution) was then added for 24 to 48 hr at 4 C. After exposure to the peroxidase-antibody conjugate, the cells were rinsed in three changes of cold 0.1 M cacodylate buffer (pH 7.3) and fixed for an additional 45 min in cold 4% glutaraldehyde in the same buffer at pH 7.3. Then, the cells were rinsed three additional times with 0.1 M cacodylate buffer (pH 7.3) prior to 15 min of incubation in 25 ml of substrate solution at room temperature. The substrate consisted of 3-3' diaminobenzidine tetrahydrochloride (0.5 mg/ml) in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer at pH 7.6 containing 0.01% hydrogen peroxidase (24). After the substrate reaction, the cells were rinsed in three changes of distilled water and post-fixed for 1 hr in 1% osmium tetroxide containing 0.1 M cacodylate buffer at pH 7.3. The cells were scraped gently from the flask surface, suspended in 50% ethyl alcohol, and centrifuged ($2,000 \times g$ for 5 min) into pellets. Then, the pellets were rapidly dehydrated in ethyl alcohol and embedded in Araldite. Thin sections were cut, stained with lead cit-

rate and uranyl acetate, and examined in a Hitachi HU11E electron microscope.

Studies of control cell cultures. Uninfected cells and cells infected with HZV (CCL-72) or SV40 (DBS-FCL-1) were examined after application of the peroxidase-antibody conjugate or substrate alone. Additionally, DBS-FCL-1 cells infected with SV40 were examined after exposure to peroxidase-labeled human immune globulin plus substrate. All of these cells were fixed and prepared for electron microscopy as described above.

RESULTS

Figures 1-3 illustrate CCL-72 cells infected with HZV, and Fig. 4-6 illustrate DBS-FCL-1 cells infected with SV40.

Figure 1 is a low-power electron micrograph of a cell infected with HZV after application of the peroxidase-antibody conjugate and substrate. The nucleus contains a number of round, black virions which are stained by dense, black peroxidase reaction product. This figure demonstrates how readily the virions may be localized at low magnification. There is no background staining.

In Fig. 2, the nucleus of a cell contains several well defined virions of HZV (arrows). The cell has been incubated in substrate (3-3' diaminobenzidine) which results in a slight general increase in cellular density and a diminution of membrane definition which was noted in all cells exposed to the substrate. As can be seen, the virions are unaffected by the substrate; capsid membranes enclose cores of low to moderate density that appear irregular in shape. Between the capsid and the core, characteristically one can identify a zone of diminished density which appears clear.

Figure 3 illustrates a cell infected with HZV which has been exposed to peroxidase-labeled antibody and incubated in substrate. In contrast to Fig. 2, the virions appear as round profiles which are stained densely by black, granular peroxidase reaction product. The peroxidase activity is specific for the virion and there is minimal background staining. The normal zone of diminished density between the capsid and the core has been obliterated by the dense reaction product. Adjacent to one of the virions, a circumscribed, granular clump of reaction product is present (arrow) and may localize another HZV virion. Intracytoplasmic HZV virions were identified in several of the cells and demonstrated comparable specific peroxidase labeling.

Figure 4 shows a cell heavily infected with SV40 that has been incubated in substrate alone. The SV40 virions fill the nucleus, and

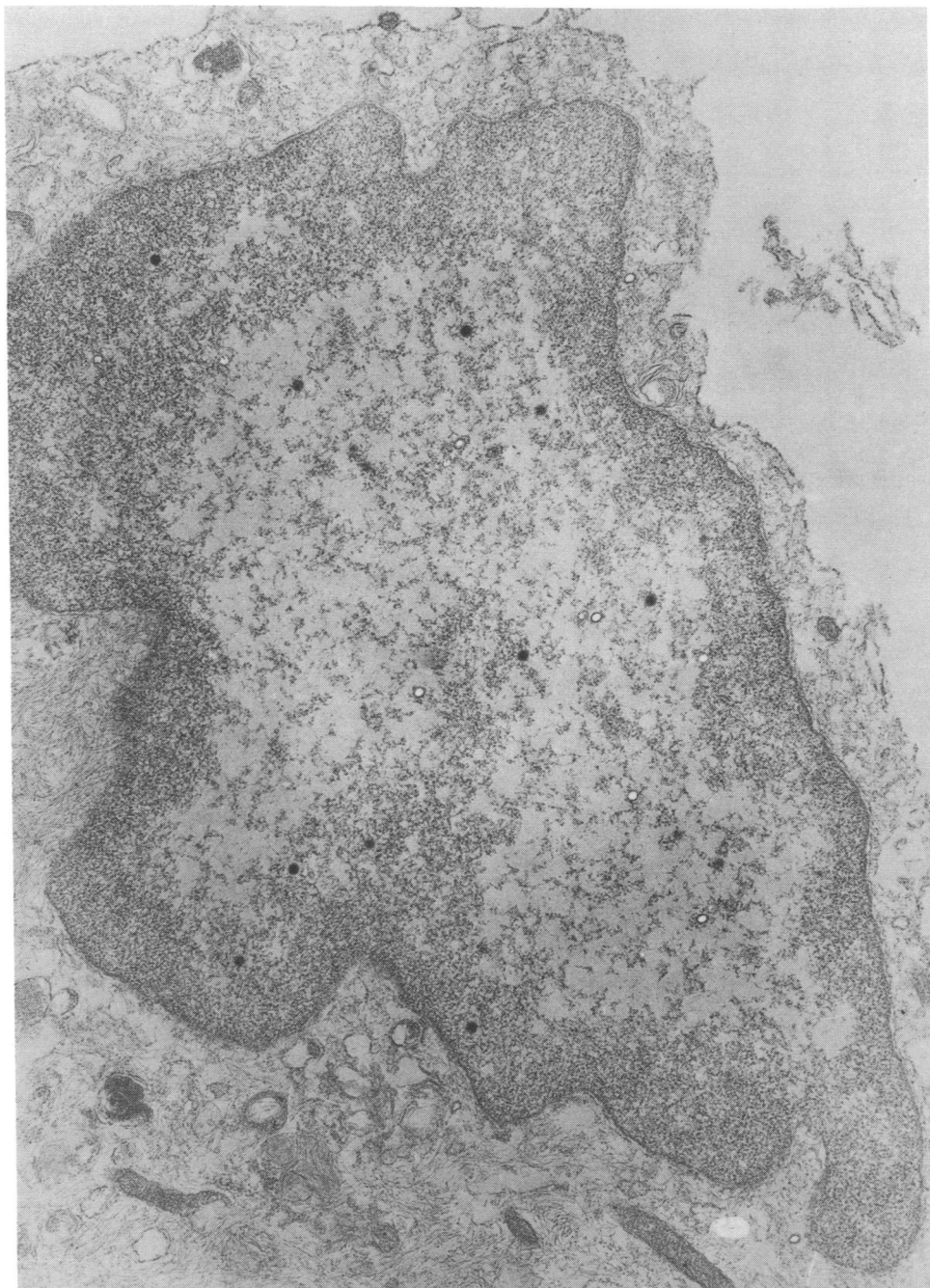


FIG. 1. *Low-power electron micrograph of a fibroblast infected with herpes zoster virus; peroxidase-labeled antibody and substrate. Herpes zoster virions, which are scattered throughout the nucleus, are stained densely and are easily identified as round, black profiles at low magnification. $\times 10,200$.*

one virion (arrow) is present in the cytoplasm. The intracytoplasmic virions are usually slightly larger and denser than intranuclear ones, but, in both locations, they have a stip-

pled granularity and are outlined distinctly.

In Fig. 5, the cytoplasm of a substrate-incubated cell contains several SV40 virions (arrows) adjacent to tubular profiles. The virions

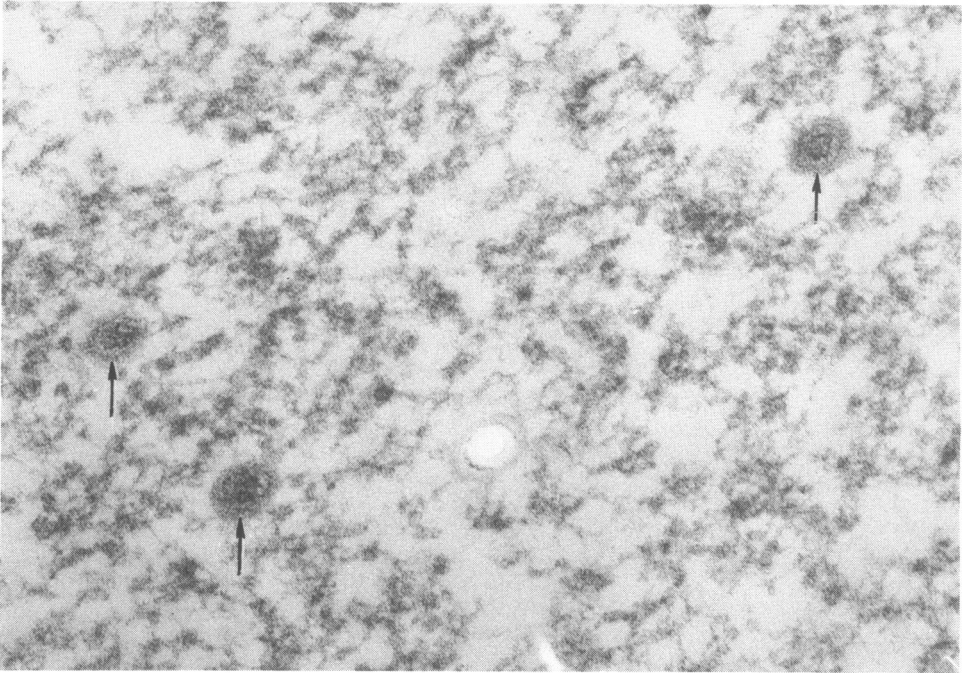


FIG. 2. Nucleus of a fibroblast; substrate alone. Three herpes zoster virions (arrows) are seen to possess irregularly shaped cores of low to moderate density and distinct capsid membranes. $\times 59,000$.

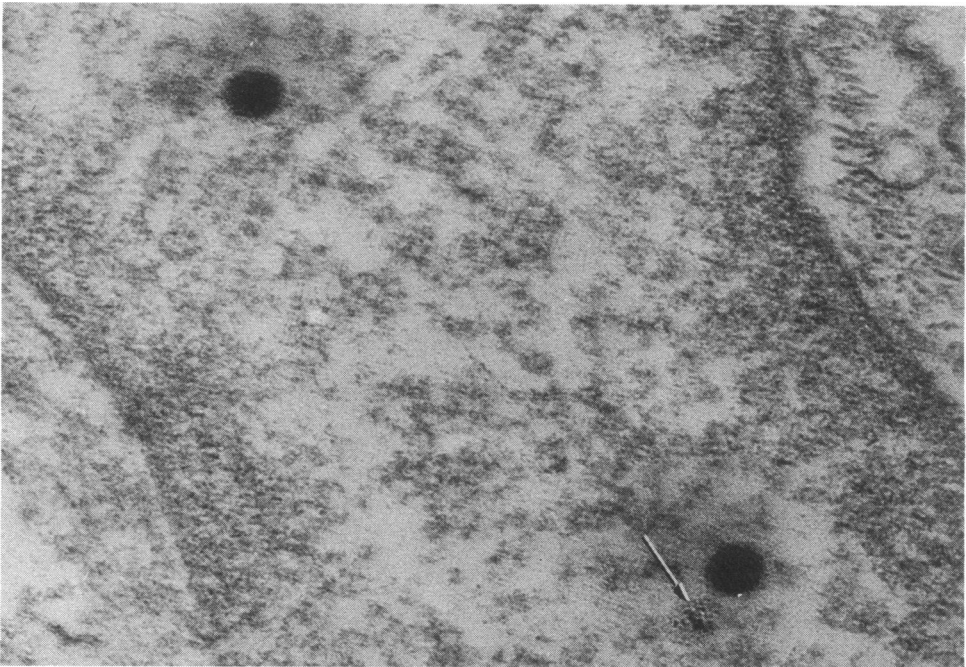


FIG. 3. Fibroblast infected with herpes zoster virus; peroxidase-labeled antibody and substrate. Two intranuclear virions are stained intensely with dense, black peroxidase reaction product. A circumscribed, granular clump of reaction product (arrow) is adjacent to one of the stained virions and probably represents another virion. $\times 52,000$.

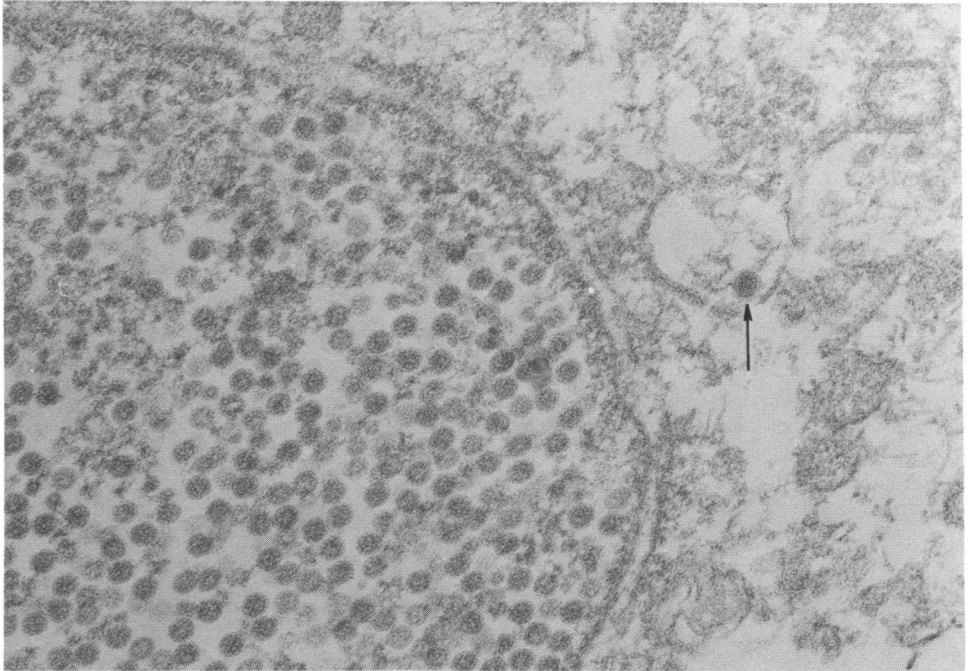


FIG. 4. *Fibroblast infected with SV40; substrate alone. The nucleus is packed with SV40 virions, and one virion (arrow) is found in the cytoplasm. The virions appear slightly granular and are outlined distinctly. $\times 52,000$.*

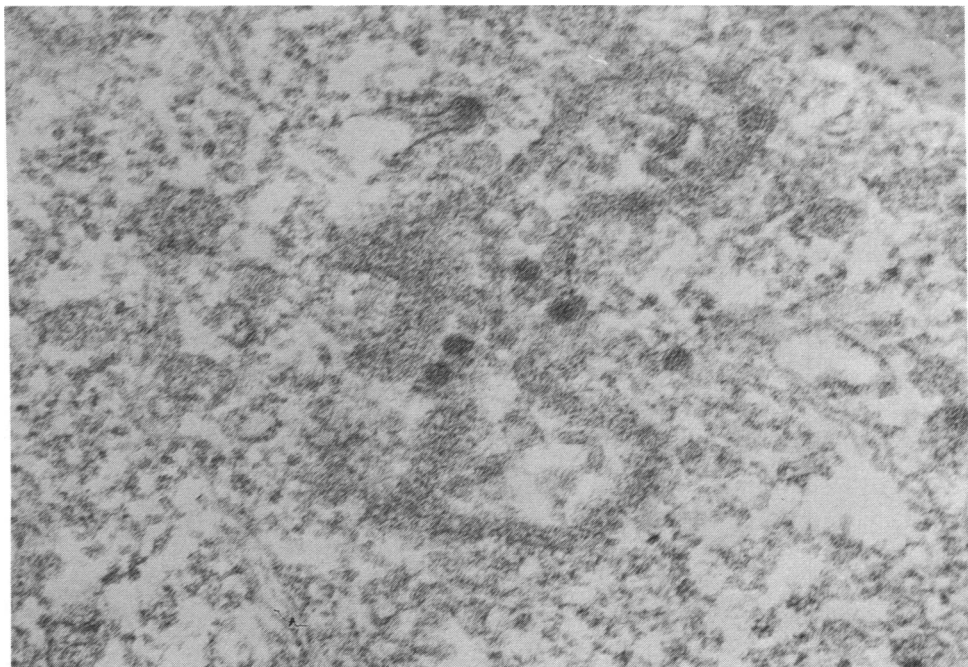


FIG. 5. *Cytoplasm of a fibroblast; substrate alone. Several intracytoplasmic SV40 virions appear near a tubular profile. $\times 67,000$.*

appear slightly granular and their outlines are evident.

Figure 6 illustrates the nucleus and adjacent cytoplasm of a disintegrating, heavily infected

cell that has been exposed to peroxidase-labeled antibody and substrate. The nucleus is filled with SV40 virions that are not stained with peroxidase reaction product. Distinct

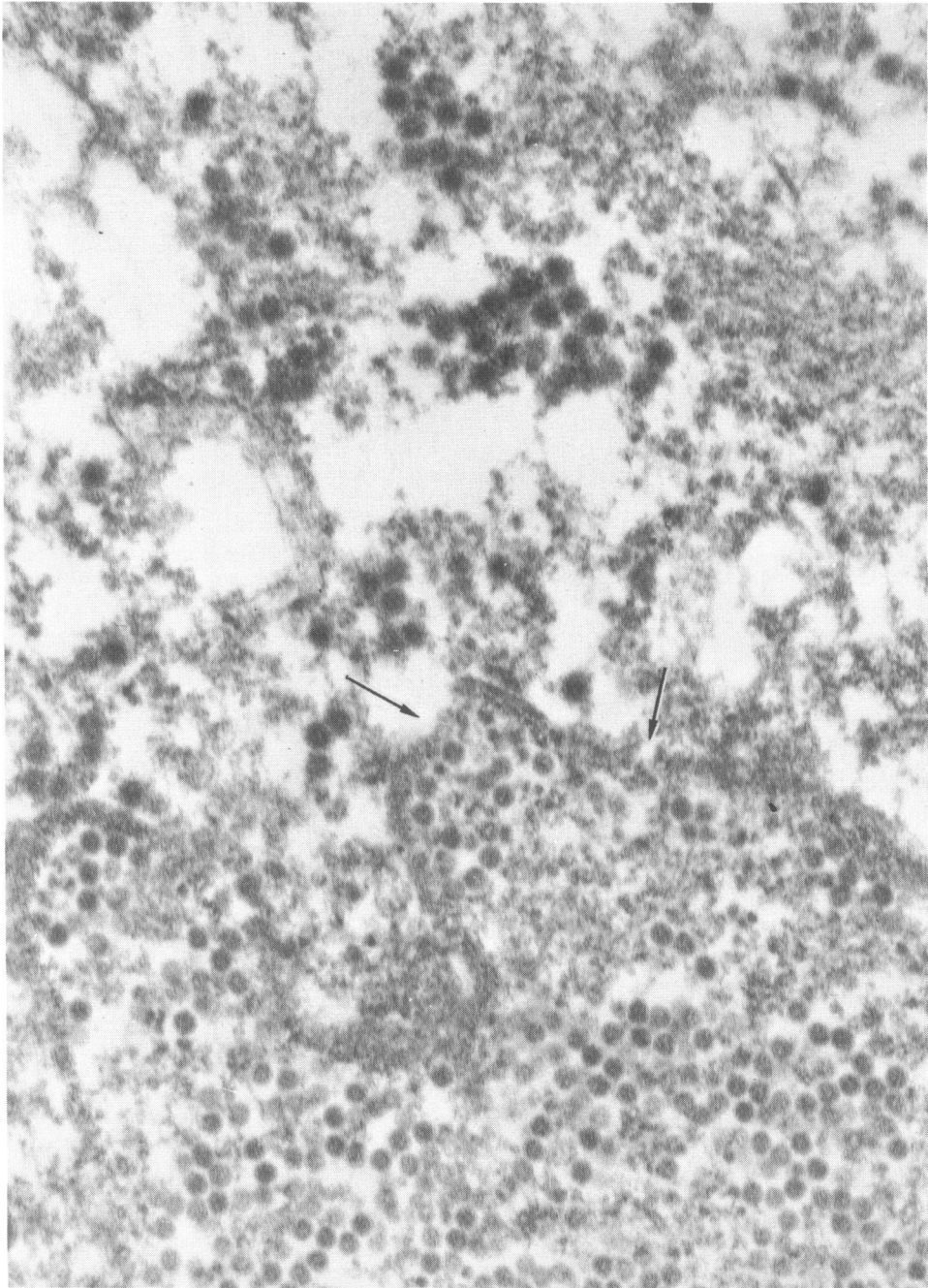


FIG. 6. Fibroblast heavily infected with SV40; peroxidase-labeled antibody and substrate. The nucleus is filled by SV40 virions that are not stained, and several breaks in the nuclear membrane (arrows) are evident. The intracytoplasmic virions are stained by dense, black peroxidase marker. $\times 58,000$.

breaks in the nuclear membrane are evident (arrows) through which intranuclear SV40 virions are presumably able to enter the cytoplasm. In the cytoplasm, several clumps of virions that are stained densely with peroxidase reaction product are identified. The virions appear somewhat irregular in shape and size and are not outlined clearly due to adherent reaction product. The restriction of peroxidase labeling to intracytoplasmic virions is a consistent finding in SV40-infected cells.

In addition to the cells infected with HZV or SV40 and exposed to peroxidase-labeled antibody or substrate, or both (as described above), control cells (uninfected and infected) were examined after exposure to labeled antibody alone, substrate alone, and SV40-infected cells to labeled human immune globulin plus substrate. In all of these control cells there was no nonspecific peroxidase staining of uninfected cells or labeling of SV40 virions with peroxidase-labeled immune globulin in SV40-infected cells.

DISCUSSION

The results of this study demonstrate that the immunoperoxidase technique is useful in the specific localization of viral antigen.

Peroxidase staining of the virions of HZV was observed by using human immune globulin as the source of antibody. It is interesting that our results with HZV differ from those of Nii et al. (13), who studied herpes simplex virus with ferritin-labeled antibody to both virus and host cell. These investigators failed to observe capsid labeling with antibodies specific for either the virus or the host cell. Although the variance from our results may reflect the differing nature of the viral antigens or antibodies employed, an additional possibility is that the large size of the ferritin molecule (molecular weight, 850,000; diameter, 11 nm) precludes the consistent penetration of cellular and viral membranes. On the other hand, peroxidase (molecular weight, 43,000; diameter, 2.5 to 3 nm) apparently can penetrate cellular and viral membranes more consistently. Recently, Miyamoto et al. (9), using four different antibody fractions conjugated to ferritin, noted a varying reactivity of herpes simplex virion-associated antigens. They postulated that capsids with cores of low density can be labeled only with the early 7S antibody fraction and might be those in the process of assembly, whereas capsids with dense cores might be completely closed, preventing penetration of ferritin-conjugated antibody. It is possible that the lack of labeling of viral an-

tigen may represent a technical defect in the immunoferritin method that complicates the interpretation of viral antigen localization and immunologic activity.

In addition to the easier penetrability of the peroxidase-antibody conjugate, there are several other important advantages of the immunoperoxidase technique over immunoferritin. First, the method for conjugation of peroxidase to antibody is simple. Second, the smaller size and easier penetrability of peroxidase-labeled antibody obviates the need for agents (for example, dimethyl sulfoxide) or methods (for example, freezing) which are likely to damage cells and distort microanatomy as are used in immunoferritin techniques (5, 9, 13). Third, the accumulation of reaction product amplifies the ability to localize antibody at the sites of peroxidase activity, which in turn results in easy identification of viral antigen with minimal background staining.

Our observations concerning SV40 with specific peroxidase-labeled anti-SV40 antibody indicate that intranuclear virions are not stained by peroxidase, whereas intracytoplasmic virions are stained. These results agree with previous immunoferritin studies. It appears likely, as postulated by Oshiro et al. (14) and Levinthal et al. (8), that SV40, upon release from the nucleus, acquires a new "antigenic reactivity" in the cytoplasm. The finding that rabbit antibody to SV40 does not stain normal monkey cells even though the SV40 was propagated in monkey cells might be explained by the fact that the rabbits were immunized with SV40 without adjuvant. In such a system, the antigenicity of any contaminant monkey cell components apparently is very slight compared to that of the SV40 virions, and anticell antibodies are not produced in sufficient titer to result in staining of the monkey cells.

At the light microscopy level, the immunoperoxidase technique appears equal in specificity to immunofluorescence (11, 25). In studies of herpes simplex-infected cells with fluorescein-labeled antibodies, conflicting results have been described. For example, some authors (6, 7, 13, 17, 20, 21, 26) noted intranuclear and/or intracytoplasmic fluorescence. In a comparative examination, Hampar et al. (5) reported that rabbit antibodies to herpes simplex and EB viruses bound ferritin to intranuclear virus capsids, but that these same virus particles could not be demonstrated by the immunofluorescence method using the same antibodies. As these investigators emphasized, the nature of the intranuclear antigens which

have been detected by immunofluorescence in cells infected with herpes simplex or EB viruses remains open to question. As the same tissue blocks can be used for light and electron microscopy with the immunoperoxidase technique, this method may be useful in resolving the disparity in results between immunofluorescence and immunoferritin. Furthermore, in comparison to immunofluorescence, the sections are permanent and may be studied with an ordinary light microscope without special illumination; there is minimal background staining; and the peroxidase-antibody conjugates can be stored indefinitely at -20°C or below.

The principal problem associated with the peroxidase-antibody technique, as was experienced in previous studies on viral antigen (1, 25), is the preservation of maximal enzymatic and immunologic activity as well as microanatomical integrity. It has been shown previously (4) that a combined paraformaldehyde-glutaraldehyde fixative provides optimal structural preservation for electron microscopy with horseradish peroxidase. However, paraformaldehyde diminishes the enzymatic reaction, and glutaraldehyde tends to inhibit immunologic activity. We have found that the best sequence for retaining the enzymatic, immunologic, and structural parameters is to fix the cells in paraformaldehyde, wash overnight, apply the peroxidase-antibody conjugate, and then fix in glutaraldehyde. Postfixation in osmium tetroxide after the substrate reaction, as noted by Graham and Karnovsky (4), intensifies peroxidase staining. Although the substrate incubation tends to diminish membrane definition, pending the development of improved fixatives and substrates, we recommend this method for ultrastructural studies with peroxidase-labeled antibody in cell culture.

The results of this study suggest that the immunoperoxidase technique has important practical potential in electron microscopy studies of virus-cell interactions and may be useful for light microscopy as well. This technique may aid in the resolution of a wide variety of problems involving cellular and viral antigens.

LITERATURE CITED

- 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.
- Coons, A. H., and M. H. Kaplan. 1950. Localization of antigen in tissue cells. II. Improvement in method for detection of antigen by means of fluorescent antibody. *J. Exp. Med.* **91**:1-13.
- Gorham, L. W., and C. Waymouth. 1965. Differentiation *in vitro* of embryonic cartilage and bone in chemically defined medium. *Proc. Soc. Exp. Biol. Med.* **119**:287-290.
- Graham, R. C., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291-302.
- Hampar, B., P. Gerber, K. C. Hsu, L. M. Martos, J. L. Walker, R. F. Sigüenza, and G. Wells. 1970. Immunoferritin and immunofluorescent studies with Epstein-Barr virus and herpes simplex virus by use of human sera and hyperimmune rabbit sera. *J. Nat. Cancer Inst.* **45**:75-85.
- Kaufman, H. E. 1960. The diagnosis of corneal herpes simplex infection by fluorescent antibody staining. *Arch. Ophthalmol.* **64**:382-384.
- Lebrun, J. 1956. Cellular localization of herpes simplex virus by means of fluorescent antibody. *Virology* **2**:496-510.
- Levinthal, J., R. Wicker, and J. C. Cerottini. 1967. Study of intracellular SV-40 antigens by indirect immunoferritin technique. *Virology* **31**:555-558.
- Miyamoto, K., C. Morgan, K. C. Hsu, and B. Hampar. 1971. Differentiation by immunoferritin of herpes simplex virion antigens with the use of rabbit 7S and 19S antibodies from early (7-day) and late (7-week) immune sera. *J. Nat. Cancer Inst.* **46**:629-646.
- 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.
- 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.
- Nii, S., and J. Kamahora. 1963. Localization of herpetic viral antigen in interphase cells. *Biken J.* **6**:145-154.
- Nii, S., C. Morgan, H. M. Rose, and K. C. Hsu. 1968. Electron microscopy of herpes simplex virus. IV. Studies with ferritin-conjugated antibodies. *J. Virol.* **2**:1172-1184.
- Oshiro, L. S., H. M. Rose, C. Morgan, and K. C. Hsu. 1967. Electron microscopic study of the development of simian virus 40 by use of ferritin-labeled antibodies. *J. Virol.* **1**:384-399.
- Pepe, F. A., and H. Finck. 1961. The use of specific antibody in electron microscopy. II. The visualization of mercury-labeled antibody in the electron microscope. *J. Biophys. Biochem. Cytol.* **11**:521-531.
- Petricciani, J. C., H. H. Hopps, and D. E. Lorenz. 1971. Subhuman primate diploid cells: possible substrates in viral vaccine production. *Science* **174**:1025-1027.
- Pettit, T. H., S. J. Kimura, and H. Peters. 1964. The fluorescent antibody technique in diagnosis of herpes simplex keratitis. *Arch. Ophthalmol.* **72**:86-98.
- Ram, J. S., S. S. Tawde, G. B. Pierce, Jr., and A. R. Midgley, Jr. 1963. Preparation of antibody-ferritin conjugates for immuno-electron microscopy. *J. Cell Biol.* **17**:673-675.
- Rifkind, R. A., K. C. Hsu, and C. Morgan. 1964. Immunological staining for electron microscopy. *J. Histochem. Cytochem.* **12**:131-136.
- Roizman, B. 1961. Virus infection of cells in mitosis. I. Observations on the recruitment of cells in karyokinesis into giant cells induced by herpes simplex virus and bearing on the site of virus antigen formation. *Virology* **13**:387-401.
- Ross, R. W., and E. Orlans. 1958. The redistribution of nucleic acid and the appearance of specific antigen in HeLa cells infected with herpes virus. *J. Pathol. Bacteriol.* **76**:393-402.

22. Singer, S. J. 1959. Preparation of an electron-dense antibody conjugate. *Nature (London)* **183**:1523.
23. Sternberger, L. A., E. J. Donati, and C. E. Wilson. 1963. Electron microscopic study on specific protection of isolated *Bordetella bronchiseptica* antibody during exhaustive labeling with uranium. *J. Histochem. Cytochem.* **11**:48.
24. Takemoto, K. K., G. J. Todaro, and K. Habel. 1968. Recovery of SV40 virus with genetic markers of original inducing virus from SV40-transformed mouse cells. *Virology* **35**:1-8.
25. Ubertini, T., B. N. Wilkie, and F. Noronha. 1971. Use of horseradish peroxidase-labeled antibody for light and electron microscopic localization of reovirus antigen. *Appl. Microbiol.* **21**:534-538.
26. Vozza, R., and D. Balducci. 1961. The technique of fluorescent antibodies in ophthalmology: a study of herpes simplex and vaccinia keratoconjunctivitis and human trachomatous infection. *Amer. J. Ophthalmol.* **52**:72-77.