JOURNAL OF VIROLOGY, Oct. 1968, p. 1172-1184

Vol. 2, No. 10
Printed in U.S.A.

Electron Microscopy of Herpes Simplex Virus

IV. Studies with Ferritin-conjugated Antibodies

S. NII,[1] C. MORGAN, H. M. ROSE, AND K. C. HSU

Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Received for publication 26 July 1968

Small aggregates of viral antigen were encountered in the nuclear matrix. The capsids did not tag with antibodies specific for the virus or for the host cell. This observation remains unexplained. Nuclear and cytoplasmic membranes, as well as the envelope of the virus, reacted with both types of antibodies and appear, therefore, to contain host cell and viral protein. Large amounts of viral antigen are synthesized within the cytoplasm. This antigen was either diffusely spread or localized at the surface of membranes. The surface of infected cells contains viral antigen, which accumulates as infection progresses. At circumscribed sites, the cell wall becomes altered antigenically and structurally so as to resemble the envelope of the virus. Hypotheses are presented regarding the manner in which cell fusion occurs.

Studies of infected cells by the use of fluorescein-labeled antibodies have resulted in conflicting reports. Lebrun (7), Ross and Orlans (22), Kaufman (6), and Vozza and Balducci (28) observed fluorescence in both nucleus and cytoplasm, whereas Roizman (19) and Nii and Kamahora (14) noted fluorescence only in the cytoplasm. Recently, Roizman et al. (21) reported that "the antigenic products specified by HSV in HEp-2 cells form at least five immunofluorescent elements, differing with respect to immunological specificity, physical properties and with respect to the cellular compartment in which they localize." Such results are hardly surprising when one considers that 10 proteins specific for the virus have been demonstrated by acrylamide electrophoresis and immunoelectrophoresis (29). As a further complication, it would appear that there are differences in antigenic specificity between various lots of sera. For example, human immune gamma globulin coupled with fluorescein stained only the cytoplasm in two instances (19, 21), and both cytoplasm and nucleus in four (6, 7, 22, 28). Antibodies prepared in rabbits stained only the cytoplasm in one study (14), and both cytoplasm and nucleus in two (6, 21). It is evident that the results of staining infected tissues with labeled antibodies must be interpreted with caution, particularly at the ultrastructural level where individual components of the virus and cell are under consideration. This report, therefore, is presented as a preliminary investigation of the complex antigenic alterations induced in cells by infection with herpes simplex virus.

[1] Postdoctoral Fellow supported by grants from the Rockefeller Foundation, a special Lederle International Fellowship, and The Jane Coffin Childs Memorial Fund for Medical Research. Present address: The Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

MATERIALS AND METHODS

Virus. The Miyama strain (11) of herpes simplex virus was used.

Cells. HeLa cells, as well as a stable line of human amnion (FL) cells, were grown in Leighton tubes or in 30-ml plastic flasks as described by Morgan et al. (10). There seemed to be little difference between the two cell lines in the results obtained by employing antiherpes antibody. Only FL cells were used to study the localization of anti-FL cell antibody.

Antibodies. Two antisera specific for the virus were employed. One was obtained from the serum of Shiro Nii (neutralization titer, 1:256). The other was provided by R. A. Malmgren of the National Cancer Institute. This serum was obtained from rabbits that had received, over a period of 4 months, 15 intracutaneous and intraperitoneal injections of virus grown in human WI-38 fibroblasts. To provide antibodies specific for FL cells, rabbits were injected intramuscularly three times, at weekly intervals, with 10^7 washed cells. Since preliminary studies of these sera conjugated with ferritin showed only weak tagging of control cells, the

same rabbits received another course of five weekly injections with 5×10^7 sonically treated cells. After centrifugation, the supernatant fluid was injected intravenously; the sediment, intramuscularly.

Globulins were prepared by the method of Strauss et al. (26). The virus-specific globulins were absorbed with HeLa or FL cells before conjugation with ferritin. Conjugation was carried out by the method of Rifkind et al. (17).

Application of ferritin-conjugated antibodies. Cells were infected with a high multiplicity of virus and treated with conjugated antibody 24 to 48 hr later. Surface tagging was accomplished by washing infected cells in Sorenson's buffer and by flooding the cells in situ with the conjugate. After 1 hr, the cells were washed, scraped from the surface of the container, centrifuged, fixed, and embedded as previously described (10).

To tag the interior, infected cells were washed, scraped, pelleted, fixed in 4% Formalin for 5 min, washed again, immersed in 10% dimethyl sulfoxide for 30 min, frozen in a CO_2-alcohol bath, thawed, suspended in the conjugate for 1 hr, washed by pelleting and resuspending three times in Sorenson's buffer, fixed, and embedded as previously described.

RESULTS

Figures 1–10 illustrate cells which were frozen and thawed before treatment with ferritin-conjugated rabbit antibody specific for the virus. Figure 1 shows part of the nuclear matrix. Ferritin has tagged a large aggregate of antigen at the upper left, as well as small aggregates of finely granular antigen scattered between the capsids. Figure 2 shows dense, amorphous collections of antigen. In neither micrograph are the viral capsids specifically tagged. Figure 3 illustrates aberrant capsid formation (also 15, Fig. 10). The location of ferritin granules suggests that there may be slight tagging of the internal aspect of the capsids, but there is insufficient ferritin to be sure. An enveloped particle is tagged just beneath the nuclear membrane at the upper right. In Fig. 4 ferritin is localized on the nuclear and cytoplasmic membranes. The sparse filaments and granules of the nuclear matrix below are largely free of ferritin, and the mitochondrial membrane at the top is untagged. Although no virus is present within this field, the cell was heavily infected. Figure 5 illustrates at higher magnification a striking tag of the two membranes bordering a nucleus. The cytoplasm is above; the nuclear matrix, below. Figure 6 shows part of the nucleus and cytoplasm of an infected cell. All membranes except those enclosing vacuolated mitochondria are labeled. In this instance, the fused membranes appear to derive from the nuclear membrane, the thin membranes from cytoplasmic membranes. As noted in Fig. 1 and 2, the capsids are not tagged. Figure 7 illustrates capsids at differing stages of envelopment at the nuclear surface. The internal aspect of membranes enclosing the capsids are tagged. Ferritin is scattered throughout the cytoplasmic matrix on the right, suggesting the presence of soluble antigen. There is considerably less ferritin within the nuclear matrix on the left. Figure 8 shows numerous ferritin granules in the cytoplasm. The nucleus of this cell has ruptured, and capsids with cores of differing density are present in the cytoplasm. Ferritin is not specifically localized on the surface of the capsids. Figure 9 illustrates fused membranes of the type previously illustrated (15, Fig. 20). These membranes, as well as nearby thin single membranes, are heavily tagged. Figure 10 shows part of the cytoplasm of a cell at a late stage of infection. There has been extensive proliferation of membranes, all of which are tagged. In most cases, ferritin lines the internal aspect of the viral envelopes. There is no specific tag of the capsids or of the mitochondrion at the lower left.

Figures 11–18 illustrate surface tagging of unfrozen cells. In Fig. 11, the surface of the cell is vertical with the cytoplasm on the left. One particle of extracellular virus near the bottom is central to the plane of section and shows ferritin granules at its surface. At three circumscribed sites, the wall of the host cell is thickened and heavily tagged with ferritin. Just beneath the thickened wall there are dense, finely granular material and a thin irregular membrane. Figure 12 shows the surface of two contiguous cells. There is no ferritin at the surface of the uninfected cell on the right. The infected cell on the left exhibits numerous regions that closely resemble those seen in the preceding micrograph. Extracellular virus, as well as a thin walled vesicular component, is tagged. Three empty capsids are evident within the nucleus at the left margin. One may ask why digestion of virus adjacent to the normal cell has not occurred. The reason is that the cells were removed from the surface of the container and centrifuged after antibody had been applied. Figures 13 and 14 illustrate the surface of four cells at relatively advanced stages of infection. Ferritin granules are evident not only at sites where the cell wall has undergone characteristic alteration but also along most of the remaining portions, which otherwise look entirely normal. Ovoid cytoplasmic fragments, which are tagged with ferritin, lie scattered

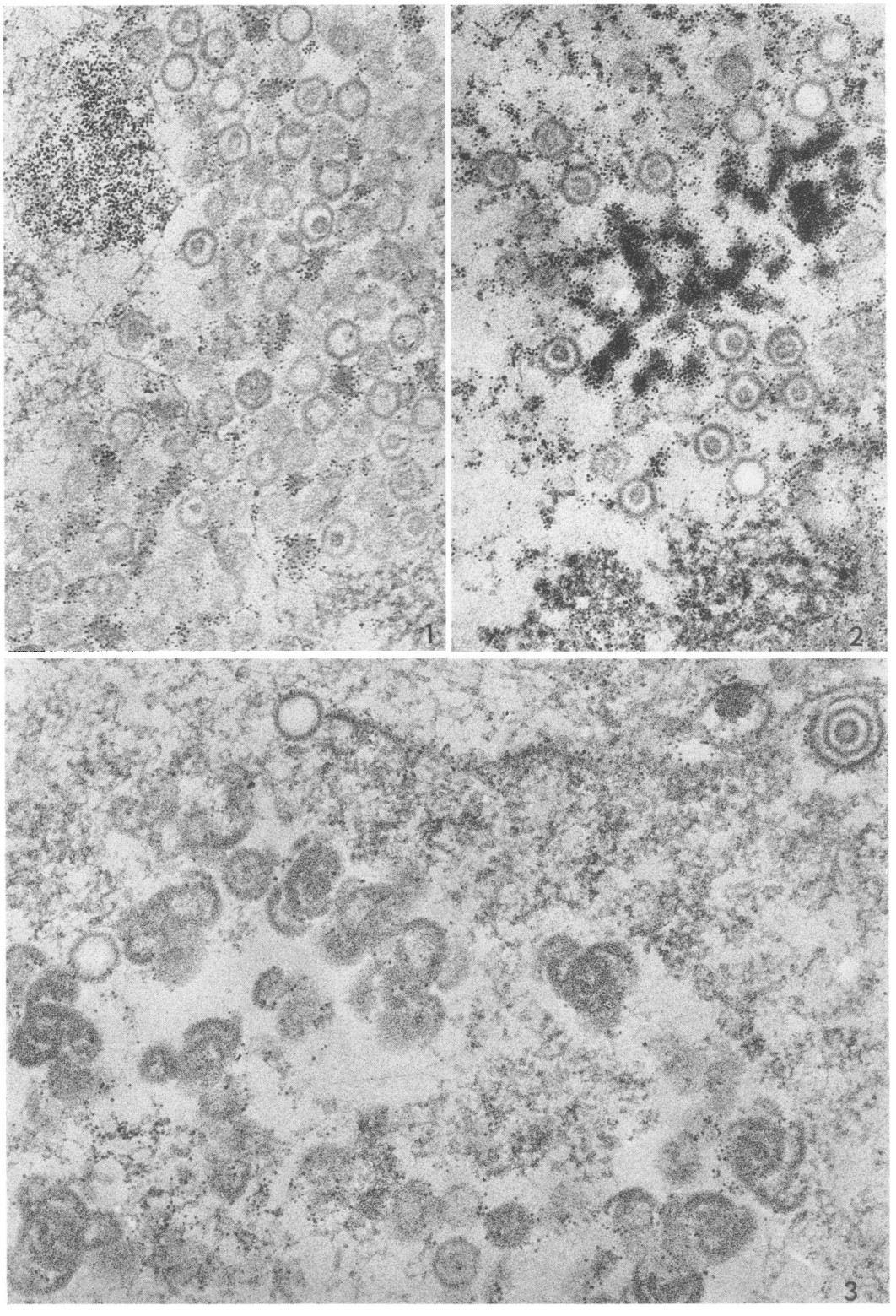


FIG. 1 and 2. *Intranuclear viral antigen tagged with ferritin-conjugated antibody. The capsids are not labeled.* × *58,000.*

FIG. 3. *Aberrant forms of the capsid with questionable tagging. The envelope of the virus at the upper right is tagged.* × *70,000.*

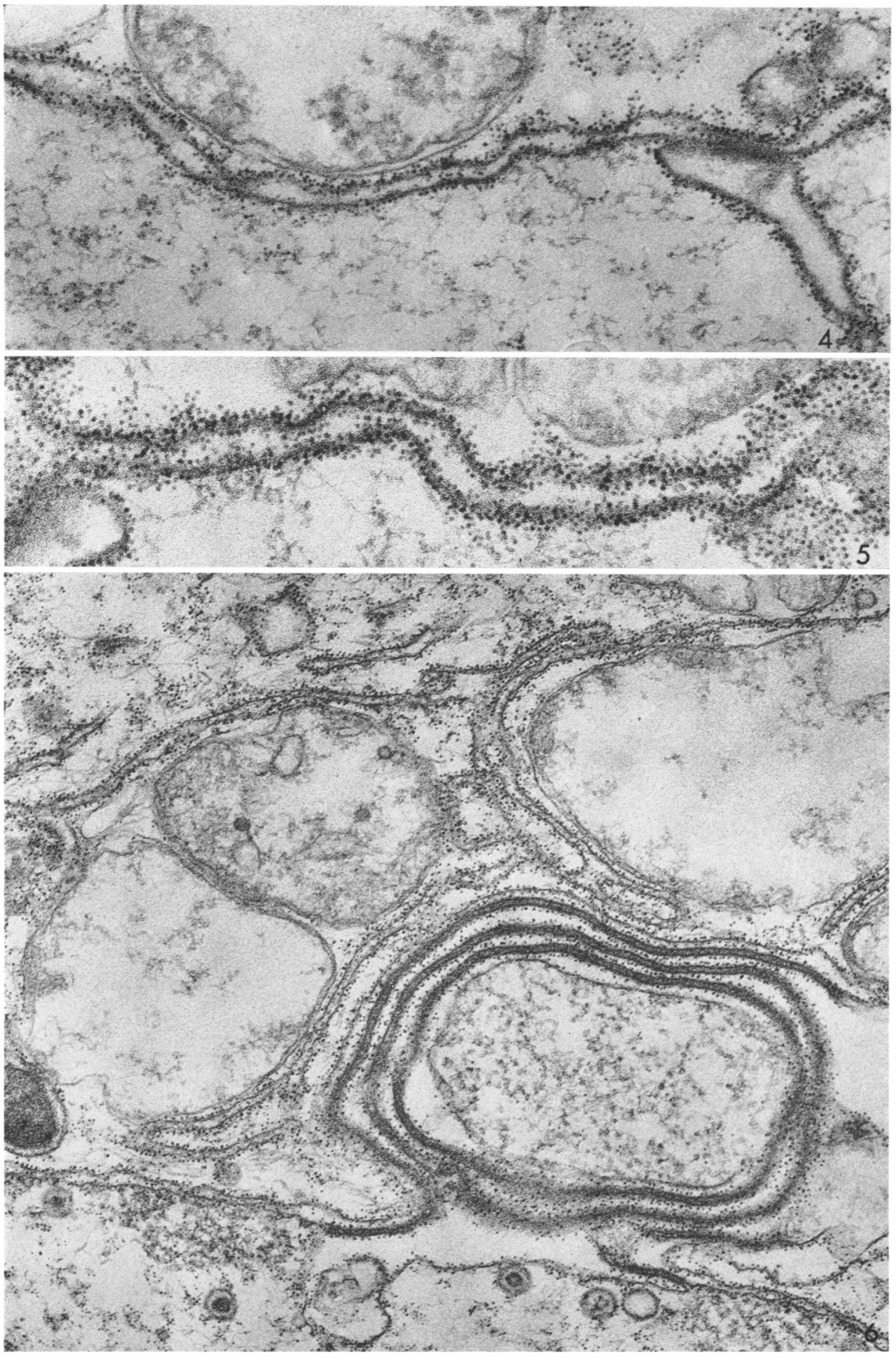


FIG. 4. *Tagging of the nuclear and cytoplasmic membranes. The mitochondrial membrane above is not tagged.* × *78,000.*

FIG. 5. *Nuclear and cytoplasmic membranes at higher magnification showing heavy labeling.* × *110,000.*

FIG. 6. *Fused nuclear membranes extending upward into the cytoplasm. These as well as the cytoplasmic membranes are labeled with ferritin-conjugated antibodies. The vacuolated mitochondria are not tagged.* × *57,000.*

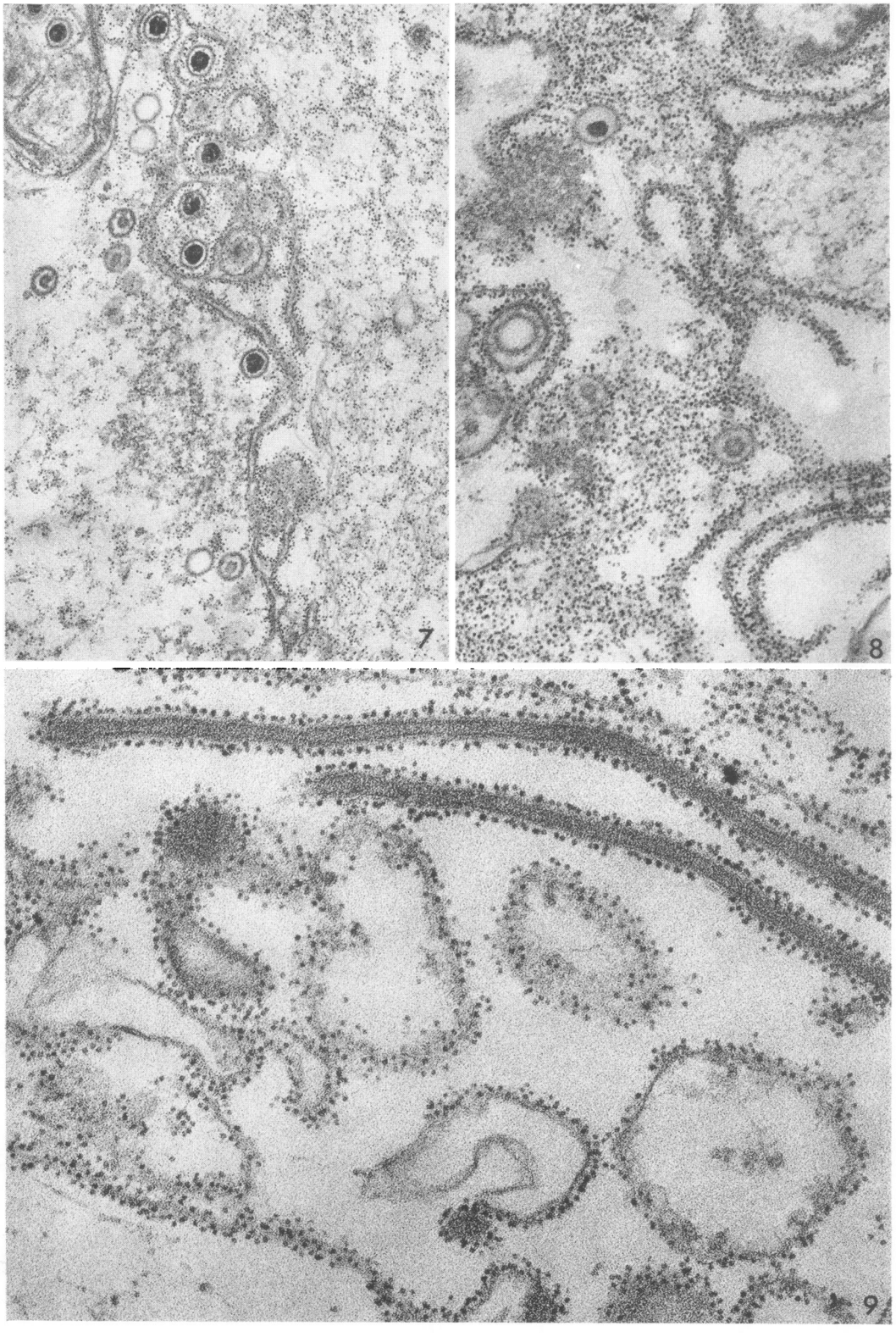


FIG. 7. *Margin of the nucleus with the nuclear matrix to the left, cytoplasm to the right. There is diffuse labeling of the cytoplasm.* × *47,000.*

FIG. 8. *Soluble intracytoplasmic antigen labeled with ferritin.* × *70,000.*

FIG. 9. *Two fused membranes showing the presence of viral antigen at their surface.* × *120,000.*

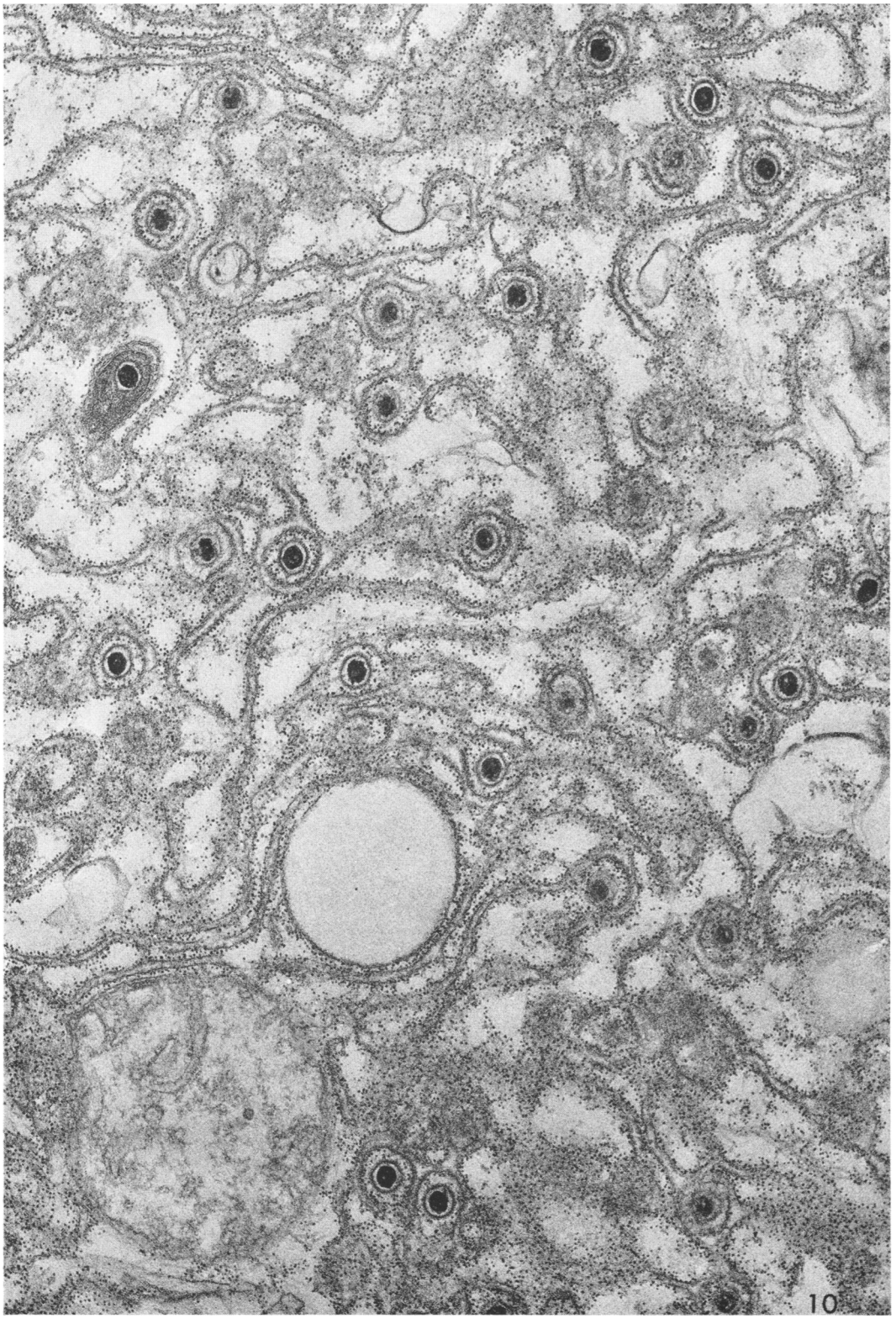


FIG. 10. *Virus within the cytoplasm. Note that in the case of the clearly budding form at the upper left the ferritin has tagged the inner aspect of the envelope. The presence of ferritin at this same site within most of the other particles would suggest that these, too, were in process of budding at the time of treatment with ferritin. Only rarely does the stalk of the bud appear within the plane of section.* × *50,000.*

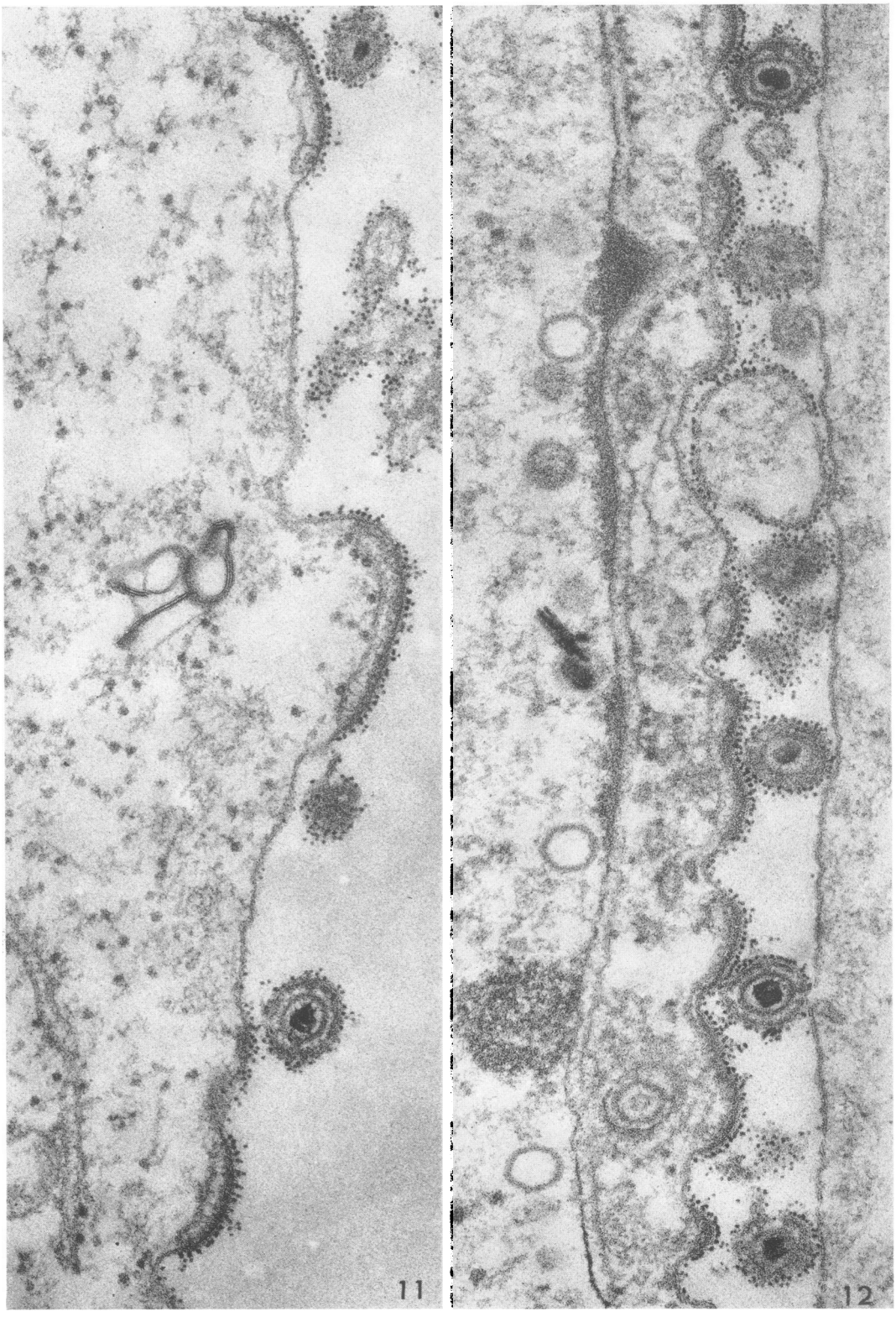
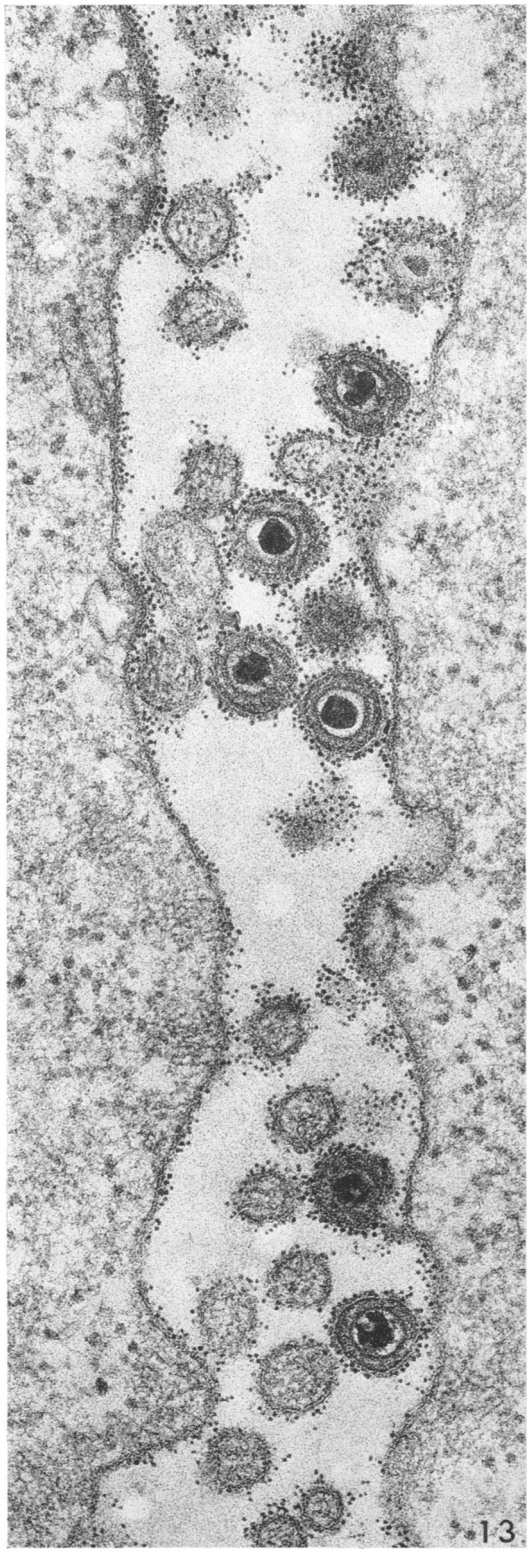


FIG. 11. *Surface tag of an unfrozen cell. At several sites, the cell surface is altered structurally and contains viral antigen.* × *78,000.*

FIG. 12. *An infected cell on the left showing extensive surface alteration. The uninfected cell at the right is not tagged.* × *78,000.*



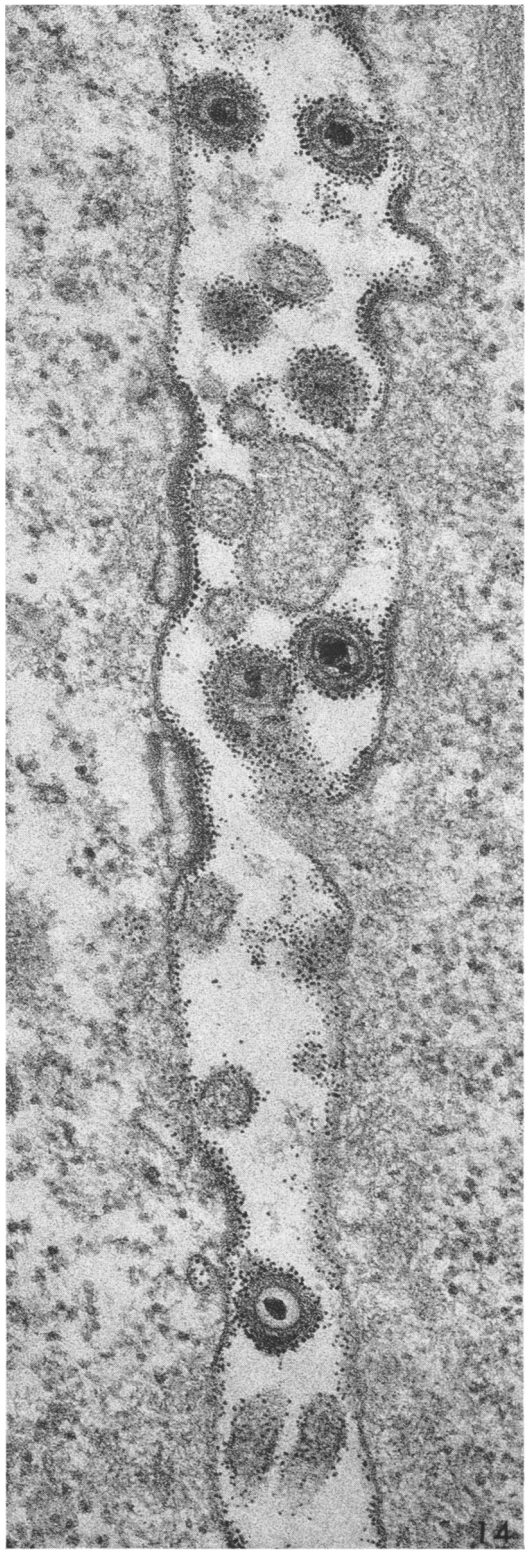


FIG. 13. *Two cells that exhibit viral antigen at their surface. Detached cytoplasmic fragments are also labeled.* × *75,000.*

FIG. 14. *Cells containing viral antigen on their surface at sites which appear normal in structure and at sites which are thickened and resemble the envelope of the virus.* × *65,000.*

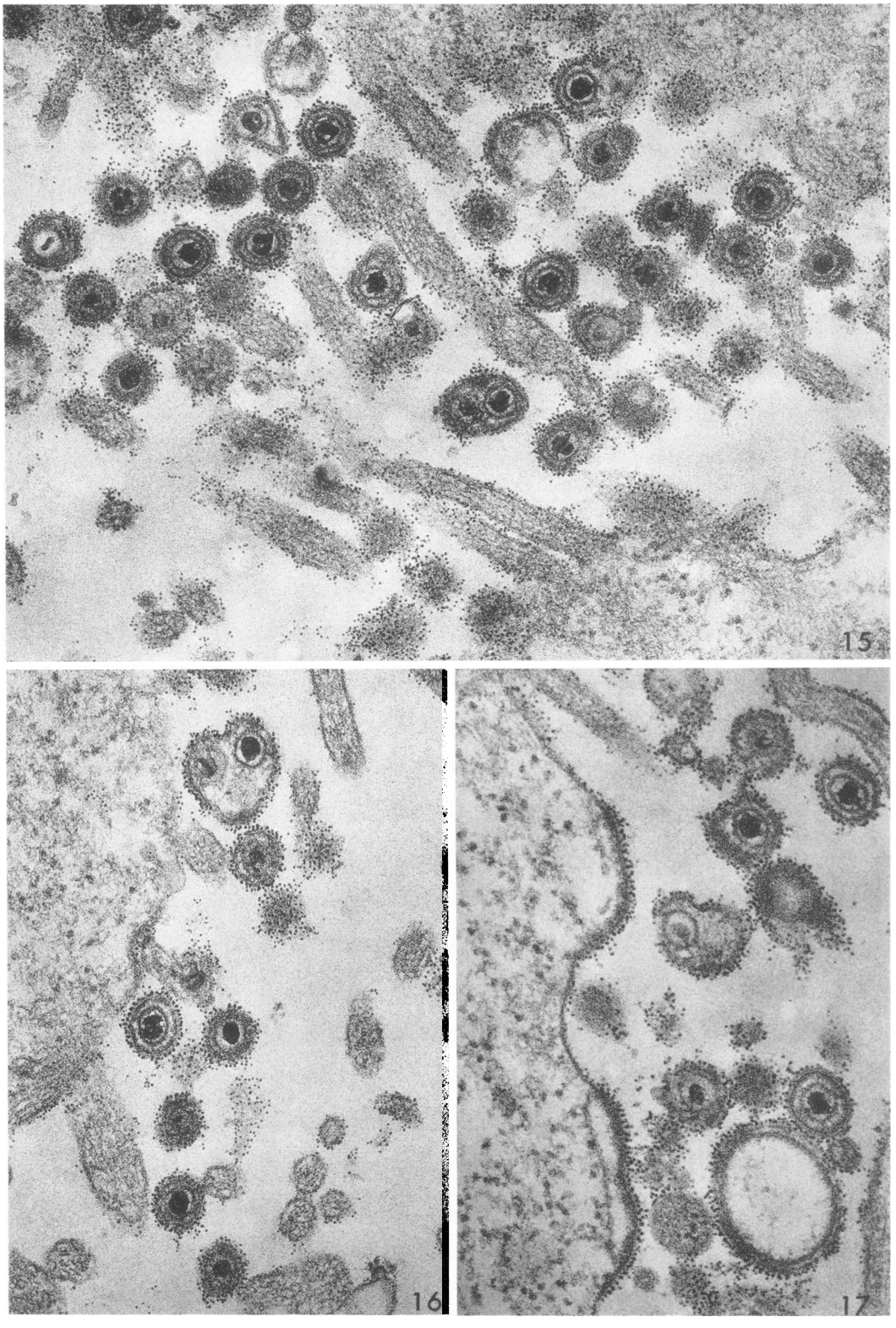


FIG. 15. *Extracellular virus tagged with ferritin-conjugated antibody specific for FL cells.* × *57,000.*

FIG. 16. *A similar preparation treated with ferritin-conjugated antibody specific for the virus. The unusual twin capsid form at the top resembles an intracytoplasmic twin capsid particle previously illustrated by Nii et al. (15, Fig. 24).* × *50,000.*

FIG. 17. *Two altered regions at the surface of a cell. Note that in the lower example the thickened wall is separated from the cytoplasm, which is lined by a thin membrane. Presumably such regions detach and thus form the oval extracellular structure seen to the right.* × *50,000.*

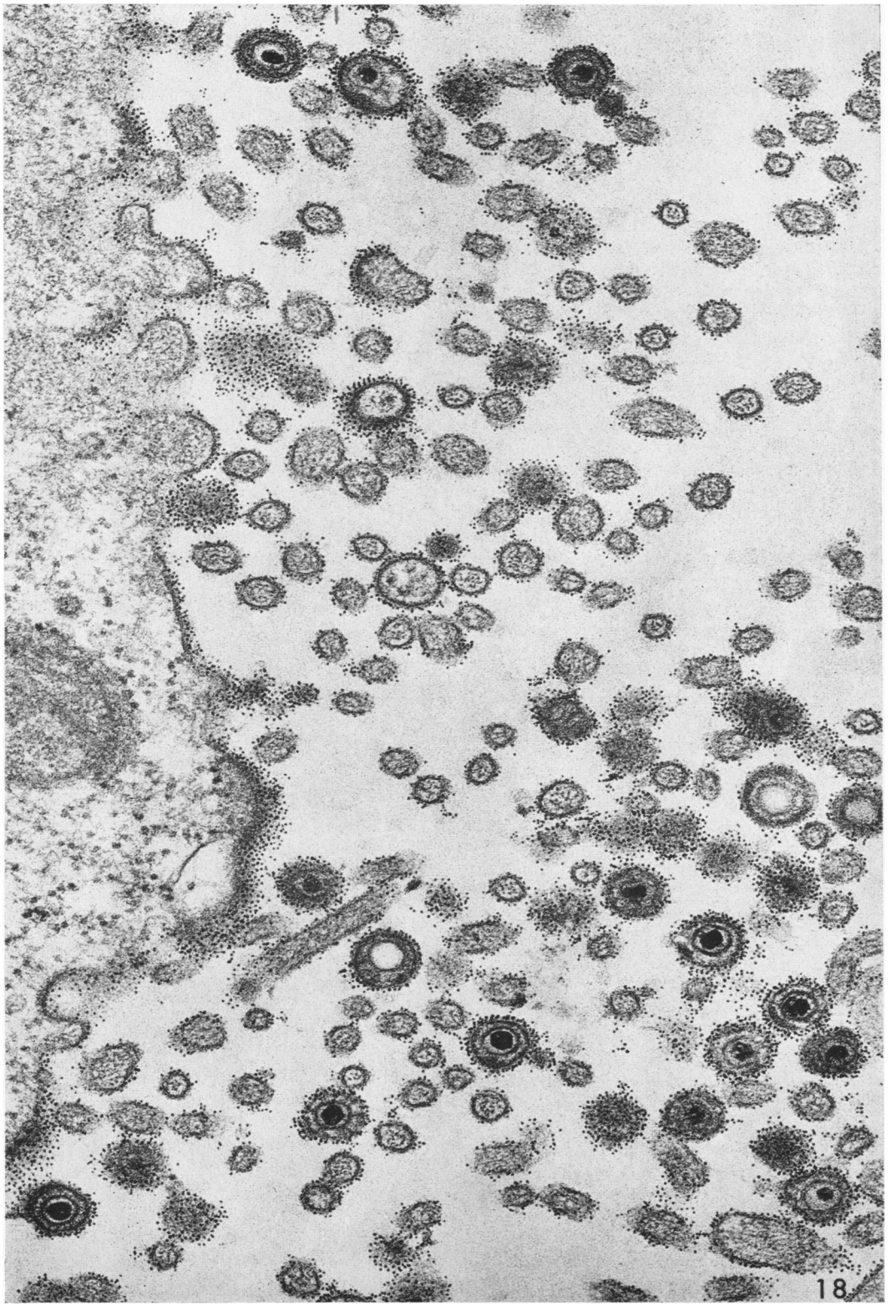


FIG. 18. *Host cell surface, detached cytoplasmic fragments, and virus labeled with ferritin-conjugated antibody.* × *57,000.*

between the viral particles. This alteration in antigenicity of the cell wall, together with the detachment of cytoplasmic fragments carrying viral antigen at their surface, is reminiscent of the changes that occur at the surface of cells infected with influenza virus (3, 9). Moreover, the thickened regions, which resemble the viral envelopes, are remarkably similar to the changes associated with parainfluenza virus (1, 2, 5), although no nucleoprotein filaments were observed. Figure 15 illustrates extracellular virus tagged with ferritin-conjugated antibody specific for FL cells. Cytoplasmic protrusions of adjacent FL host cells are also tagged. A capsid containing a dense bar (also in 15, Fig. 7) is visible at the left; a double capsid form is evident near the center. In Fig. 16, which shows a cell treated with ferritin-conjugated antibody specific for the virus, the virus is tagged but there is little ferritin at the surface of the cell. Figure 17 illustrates characteristic changes at the cell surface. In addition to extracellular virus there is a large, oval, vesicular structure lined by a thickened membrane similar to the altered wall of the host cell. Figure 18 shows the surface of a cell at an advanced stage of infection. Antibody specific for the virus has tagged much of the surface of the cell to the left, as well as numerous cytoplasmic fragments in process of detachment.

Although not illustrated in this report, antibodies specific for the host cell tagged the nuclear and cytoplasmic membranes of infected cells, thus indicating that these structures contained cellular antigen in addition to the viral antigen.

DISCUSSION

One of the most perplexing aspects of this study has been the failure of capsids to tag. Experiments using cells that were unfixed before freezing and omitting dimethyl sulfoxide were no more successful, nor did the addition of guinea pig complement in association with the ferritin-conjugated antibody result in labeling of the capsids. The possibility that the capsids became altered antigenically on passage to the cytoplasm in a manner analogous to SV40 virus (16) could not be substantiated. The possibility that the capsids were composed of host cell protein was excluded by their failure to tag with the anti-FL cell antibody. Conceivably, the capsid protein is so weak an antigen that the sera we employed were not sufficiently strong, at least after ferritin conjugation, to cause a recognizable tag.

The relatively small, localized aggregates of intranuclear antigen (Fig. 1 and 2) compared with the massive accumulation of intracytoplasmic antigen, both diffusely spread (Fig. 7 and 8) and localized on membranes (Fig. 6, 9, and 10), are in accord with the findings by Sydiskis and Roizman (27) and Fujiwara and Kaplan (4) that the bulk of viral antigen is synthesized in the cytoplasm. Evidence bearing on the hypothesis that the antigen is transported from the cytoplasm to the nucleus (4, 21) must await time sequence studies.

Examination of the data reported here suggests the following conclusions. Small aggregates of viral antigen accumulate in the nucleus near collections of capsids. The capsids, although they did not tag, are probably composed of viral protein, which is weakly antigenic. The nuclear membrane, as well as most of the cytoplasmic membranes, acquires viral antigens while maintaining specificity for host cell antibodies. Such membranes envelope the virus, which react both within and without the cell with antibodies specific for the host cell and antibodies specific for the virus. The characteristics of the membranes of different host cells may explain the differing densities of herpes simplex virus in cesium chloride gradients reported by Spear and Roizman (25). The differences in specificity of antisera previously alluded to may account for the finding of Watson and Wildy (30) that enveloped virus was agglutinated by antihost cell antibody but not by antivirus antibody. There is an enormous excess of viral antigen synthesized within the cytoplasm. These antigens are either diffusely spread (probably soluble) or localized to membranes, which proliferate extensively in the course of infection. The fusion of membranes (Fig. 9) presumably results from the accumulation of viral antigen at their surface. The viral antigen does not appear to be synthesized within cisternae of the endoplasmic reticulum, but rather to aggregate on the external surface of the lamellae (15, Fig. 20).

Regarding polykaryocyte formation, Roane and Roizman (18) noted that the surface of infected cells acquired a new specificity because such cells were killed by the addition of complement and antiinfected cell serum absorbed with uninfected cells. Roizman (20) reported further that infected cells generally fused with uninfected neighbors. These observations, when considered in the light of the changes at the surface of the cells illustrated by Fig. 11–14, suggest that the surface of infected cells becomes altered so that it contains viral antigen and resembles structurally the envelope of the virus. Assuming, as was previously discussed (10), that the envelope of the virus is digested by an enzyme at the surface of uninfected cells and that the wall of the cell is digested by a second enzyme activated at the site

of attachment, it becomes apparent that an infected cell whose surface closely resembles the envelope of the virus would attach, fuse, and merge upon contact with any normal cell. Under these circumstances, the synthesis of infective virus is not necessary for polykaryocytes to form (11, 24) provided only that the surface of the cell becomes altered. Finally, those segments of the cellular surface which are contiguous in confluent monolayers would probably be removed from antibody in the culture media, thus explaining why fusion with the direct passage of capsids from the cytoplasm of infected to uninfected cells can occur in the presence of antibody, a phenomenon mentioned previously (15).

The report by Nii and Kamahora (14) that rapid fusion (by the 2nd hr) is dependent on a high multiplicity of inoculum and that fusion occurs in the absence of other cytopathic changes when virus partially inactivated by ultraviolet light is used would suggest a slightly different mechanism. In this case, it seems not unlikely that virus which happens to lodge between two adjacent cells may become digested and fuse to both. Under such circumstances, a bridge would form, and with the digestion of the cell walls fusion could occur. A similar mechanism has been suggested in the case of Sendai virus (23) and Newcastle disease virus (8).

The foregoing hypotheses are presented as being consistent with the observations reported to date, but it should be emphasized that an important problem remains unexplained, namely, the differing propensity of viral strains to induce polykaryocytosis. One can only suppose that the surface of infected cells is altered to a lesser extent by strains that fail to cause cell fusion. Whether modifications in the degree or nature of the change occurring at the cell surface can be visualized must await further studies. However, preliminary experiments with a strain of virus that does not result in the formation of giant cells revealed no structural alterations of the surface of the host cell.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service research grant AI-06814 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Berkaloff, A. 1963. Étude au microscope électronique de la morphogènese de la particule du virus Sendai. J. Microscopie **2**:633–638.
2. Compans, R. W., K. V. Holmes, S. Dales, and P. W. Choppin. 1964. An electron microscopic study of moderate and virulent virus-cell interactions of the parainfluenza virus SV5. Virology **30**:411–426.
3. Duc-Nguyen, H., H. M. Rose, and C. Morgan. 1966. An electron microscopic study of changes at the surface of influenza-infected cells as revealed by ferritin-conjugated antibodies. Virology **28**:404–412.
4. Fujiwara, S., and A. S. Kaplan. 1967. Site of protein synthesis in cells infected with pseudorabies virus. Virology **32**:60–68.
5. Howe, C., C. Morgan, C. deVaux St. Cyr, K. C. Hsu, and H. M. Rose. 1967. Morphogenesis of type 2 parainfluenza virus examined by light and electron microscopy. J. Virol. **1**:215–237.
6. Kaufman, H. E. 1960. The diagnosis of corneal herpes simplex infection by fluorescent antibody staining. Arch. Ophthalmol. **64**:382–384.
7. Lebrun, J. 1956. Cellular localization of herpes simplex virus by means of fluorescent antibody. Virology **2**:496–510.
8. Meiselman, N., A. Kohn, and D. Danon. 1967. Electron microscopic study of penetration of Newcastle disease virus into cells leading to formation of polykaryocytes. J. Cell Sci. **2**: 71–76.
9. Morgan, C., K. C. Hsu, and H. M. Rose. 1962. Structure and development of viruses as observed in the electron microscope. VII. Incomplete influenza virus. J. Exptl. Med. **116**:553–564.
10. Morgan, C., H. M. Rose, and B. Mednis. 1968. Electron microscopy of herpes simplex virus. I. Entry. J. Virol. **2**:507–516.
11. Munk, K., and G. Sauer. 1964. Relationship between cell DNA metabolism and nucleocytoplasmic alterations in herpes virus-infected cells. Virology **22**:153–154.
12. Nii, S., and J. Kamahora. 1961. Cytopathic changes induced by herpes simplex virus. Biken's J. **4**:51–58.
13. Nii, S., and J. Kamahora. 1961. Cytopathic changes induced by herpes simplex virus. Biken's J. **4**:255–270.
14. Nii, S., and J. Kamahora. 1963. Location of herpetic viral antigen in interphase cells. Biken's J. **6**:145–154.
15. Nii, S., C. Morgan, and H. M. Rose. 1968. Electron Microscopy of herpes simplex virus. II. Sequence of development. J. Virol. **2**: 517–536.
16. 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.
17. Rifkind, R. A., K. C. Hsu, and C. Morgan. 1964. Immunochemical staining for electron microscopy. J. Histochem. Cytochem. **12**:131–136.
18. Roane, P. R., Jr., and B. Roizman. 1964. Studies of the determinant antigens of viable cells. II. Demonstration of altered antigenic reactivity of HEp-2 cells infected with herpes simplex virus. Virology **22**:1–8.
19. 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.
20. Roizman, B. 1962. Polykaryocytosis. Cold Spring Harbor Symp. Quant. Biol. **27**:327–342.
21. Roizman, B., S. B. Spring, and P. R. Roane, Jr. 1967. Cellular compartmentalization of herpes virus antigens during viral replication. J. Virol. **1**:181–192.
22. 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.
23. Schneeberger, E. E., and H. Harris. 1966. An ultrastructural study of interspecific cell fusion induced by inactivated Sendai virus. J. Cell Sci. **1**:401–406.
24. Siminoff, P. 1964. The effect of 5-bromodeoxyuridine on herpes simplex infection of HeLa cells. Virology **24**:1–12.
25. Spear, P. G., and B. Roizman. 1967. Buoyant density of herpes simplex virus in solutions of cesium chloride. Nature **214**:713–714.
26. Strauss, A. J. L., B. C. Seegal, K. C. Hsu, P. M. Burkholder, W. L. Nastuk, and K. F. Osserman. 1960. Immunofluorescence demonstration of a muscle-binding, complement-fixing serum globulin fraction in myasthenia gravis. Proc. Soc. Exptl. Biol. Med. **105**:184–191.
27. Sydiskis, R. J., and B. Roizman. 1966. Polysomes and protein synthesis in cells infected with a DNA virus. Science **153**:76–78.
28. Vozza, R., and D. Balducci. 1961. The technique of fluorescent antibodies in ophthalmology: a study of herpes simplex and vaccine kerato conjunctivitis and human trachomatous infection. Am. J. Ophthalmol. **52**:72–77.
29. Watson, D. H., W. I. H. Shedden, A. Elliot, T. Tetsuka, P. Wildy, D. Bourgauxramoisy, and E. Gold. 1966. Virus specific antigens in mammalian cells infected with herpes simplex virus. Immunology **11**:399–408.
30. Watson, D. H., and P. Wildy. 1963. Some serological properties of herpes virus particles studied with the electron microscope. Virology **21**:100–111.