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Immunoperoxidase Stain of Measles Antigen in Tissue Culture

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A specific electron microscopy staining technique for measles antigen has been developed by using Vero cells infected with a subacute sclerosing panencephalitis (SSPE) measles virus strain and fixed in glutaraldehyde or formaldehyde. Peroxidase-labeled antibody was prepared according to the method of Avrameas (4). Sera from SSPE patients with high measles antibody titer as well as normal human sera with and without measles antibody were used. With both fixatives, specific labeling was obtained on the surface of infected cells, on the budding site, and on complete viral particles. The cell membrane staining sometimes had a patchy distribution in that the reaction was most intense on the surface projections in front of each nucleocapsid. This suggests modification of the cell membrane in association with the nucleocapsids. In contrast, no label was detected on the membranes of the cells during the latent period from penetration through maturation of the virus. In formaldehyde-fixed cultures, cytoplasmic inclusions were stained, and this label was located on the "fuzzy" material around the nucleocapsids. The smooth type of nucleocapsids, mainly seen in the nucleus, were never labeled. These findings suggest that the antigenic nature of the "fuzzy" nucleocapsids in the cytoplasm may be different from that of the "smooth" nucleocapsids. The immunoperoxidase method gives good resolution of viral antigenic sites at high magnifications under electron microscopy and may be of value in studies on the immunopathogenesis of SSPE and other chronic viral infections.

The immunoperoxidase (IP) technique has been used recently for electron microscopy (EM) detection of viral antigens (1, 18-20, 35, 36). However, no study with peroxidase-labeled antibody (PLA) has yet been described for measles virus.

The enzyme peroxidase (mol wt 40,000) has been used as a tracer for extracellular space in the brain (7, 33) and other organs. It can be coupled to an antibody molecule by treatment with different agents including glutaraldehyde (4). The resulting conjugate is then applied to the fixed cells for the detection of intracellular as well as surface antigens.

In the present study the IP technique was used on Vero cells infected with a subacute sclerosing panencephalitis (SSPE) measles strain (14, 15, 29). The SSPE measles strains have been previously studied by the fluorescent antibody (FA) technique and by EM both in nervous tissue and in tissue culture (5, 10, 23, 27, 28, 30) (C.S. Raine et al., Lab. Invest., in press; R. Hamilton et al., J. Virol., in press). By using these techniques, viral components have been localized in the cytoplasm and in the nucleus of the infected cells.

The IP technique used in the present work permitted the identification of specific viral antigenic sites in a single cell at the ultrastructural level. The localization of the specific stain allowed a differentiation between the various viral components. The development of the surface antigen was studied along with morphological evidence of viral replication and cell fusion.

The relevance of these findings to the morphogenesis of measles virus will be discussed, and the potential value of the IP technique in studies of SSPE will be emphasized.

MATERIALS AND METHODS

Virus. The McClellan strain was used in this study. This virus was isolated from brain tissue of an adult case of SSPE (8). Primary brain cells were passed and cocultivated with HeLa cells. The isolate was used after the second passage in HeLa cells and had a titer of $10^{8.7}$ tissue culture infective dose ($TCID_{50}$)/ml.

Tissue culture and virus propagation. Vero cells

(an African green monkey kidney continuous cell line) were obtained commercially from Flow Laboratories, (Rockville, Md.). Plastic petri dishes (35 mm) were seeded with 2 ml of 3×10^5 cells/ml of suspension. The growth medium was Eagle minimal essential medium supplemented with 5% fetal calf serum (FCS), 100 U of penicillin G, and 100 μg of streptomycin sulfate per ml. Confluent monolayers were inoculated with the McClellan measles strain at a multiplicity of infection of 10:1 or more. The virus was allowed to adsorb for 1 h at 37 C. The cultures were subsequently refed with a 2% FCS maintenance medium and kept in a 37 C incubator with 5% CO_2 in air. They were repeatedly observed by phase microscopy to detect the appearance of syncytia.

Antibody and conjugate; direct method. The immunoglobulin fractions were purified from two human sera and coupled to peroxidase (Cappel Laboratories, Downingtown, Pa.) by Avrameas' method (4). One of the samples was from a patient with SSPE and had a measles antibody-hemagglutination inhibition (HI) titer of 128 and a complement fixation (CF) titer of 1,024. This conjugate was called "measles-positive PLA." The second sample was a normal human serum with no detectable measles antibody when tested by CF, HI, and neutralization tests. This conjugate was called "measles-negative PLA." Conjugates were lyophilized and stored at 4 C. Before use, they were suspended in saline and centrifuged at $20,000 \times g$. The supernatant fluid was used.

Indirect method. Three sera were used: (i) SSPE serum with measles antibody-HI titer (1,024) and CF titer (1,024); (ii) normal human serum with measles antibody-HI titer (512); and (iii) normal human serum with no detectable measles antibody when tested by HI and FA. Concentrated antihuman globulin of horse origin (Progressive Laboratories, Baltimore, Md.) was coupled to peroxidase type VI (Sigma) with purified glutaraldehyde by the method used for direct coupling (4). The final product was kept in solution at 4 C. This conjugate was called "antihuman PLA."

In the labeled preparations, the molar ratio of peroxidase to antibody was approximately 0.5. The enzymatic activity, as tested by the gaiacol assay (13), was one-third of that of the uncoupled enzyme; this loss of activity was more pronounced (1:10) in lyophilized material than in the one kept in soluble form. Before use, the globulin concentration of PLA was adjusted to 0.1 to 0.6 mg/ml.

Application of conjugate to the cultured cells. The entire procedure was performed on monolayers in situ (1). Tissue culture dishes were fixed at different intervals postinoculation (PI). These intervals varied from 1 h to 4 days. For each preparation, after several rinses in Hanks solution, cells were covered for 30 min at 4 C with 1% purified glutaraldehyde solution or with freshly prepared 2% formaldehyde solution in 0.1 M phosphate or cacodylate neutral buffer. The cells were then rinsed in buffer for several hours. Nonspecific factors which can be present in serum or conjugates were eliminated by absorption on fixed monolayers of uninfected Vero cells. PLA preparations were then applied onto the fixed cultures for 2 to 3 h on a rocking machine at room temperature. For the indirect method, the unlabeled serum was first applied for 1 h, plates were then rinsed, and the antihuman PLA was added. The antibody was then decanted and the cells were washed several times for up to 16 h. In most cases, the plates were then fixed a second time in 2% glutaraldehyde before incubating for 30 min at 20 C in diaminobenzidine (DAB) substrate at pH 7.6 for cytochemical stain of peroxidatic activity (33).

The intensity of deposit of the brown reaction product which occurs with fixation of PLA was then determined under a light microscope, and paired control and infected plates were selected for EM. For this purpose, the cells were rinsed again in neutral buffer before being postfixed for 1 h in 1% osmium tetroxyde and stained with uranyl at pH 5.0. They were then quickly dehydrated in graded alcohol and embedded in Epon. Thin sections were cut tangential to the monolayer surface in order to observe the in situ cell-to-cell and cell-to-virus relationship. Electron micrographs were taken either with an Itashi HU12 or a Philips 201 microscope.

Controls for specificity. Uninfected cells were treated with sera or conjugate positive for measles antibody in each experiment.

By the direct method, the following control preparations were included by using virus-infected cells: (i) incubation medium alone or exposure to unconjugated peroxidase at a concentration identical to that of the PLA; (ii) measles-negative PLA, at identical dilutions in globulin and peroxidase to those used for the measles-positive PLA; and (iii) pretreatment with SSPE serum with high measles antibody titer in order to block antigenic sites for the subsequent treatment by measles-positive PLA (blocking experiment) and pretreatment with measles-negative serum, which should not block antigenic sites during the subsequent treatment by measles-positive PLA.

By the indirect method we included the following controls: (i) antihuman PLA only; and (ii) measles-negative serum, then antihuman PLA.

RESULTS

Specificity. There was a good correlation between the presence or absence of stain by both light and electron microscopy. Uninfected cells were never labeled with measles-positive sera and conjugates, except for a patchy nonspecific nuclear stain.

By the direct method the specificity of the IP label was shown by: (i) the absence of any label when the infected cells were treated with the substrate or the enzyme alone. However, in formaldehyde-fixed preparations, incubation medium stained nonspecifically an area located under the cellular and viral membrane. This stain was readily distinguished from the IP-induced stain by its lower electron density and by the fact that it was present only when no attachment of PLA occurred, so that DAB was not consumed during the enzymatic reaction. This artifact was absent from glutaraldehyde-fixed preparations. (ii) IP label specificity also

was shown by the inability of the measles-negative PLA to label the infected cells, and (iii) the complete block of the viral antigenic sites by a measles-positive serum, whereas a measles-negative serum failed to produce the same effect.

By the indirect method, the specificity of the IP labeling was demonstrated by the inability of the antihuman PLA alone to label the viral-infected cells and by a dramatic difference in the intensity of the reaction between measles-positive and measles-negative sera. Thus, sera with no detectable measles HI or FA antibodies caused some label of viral sites by the indirect IP technique. This observation might be related to the higher sensitivity of this method to detect human antibodies than the HI, FA, or even direct IP tests.

Surface antigen. A specific surface reaction was observed in both glutaraldehyde- and formaldehyde-fixed cultures. However, the former fixative allowed better preservation and localization of the label, whereas the latter induced better preservation of immunological activity as indicated by the more intense staining.

The first reactive sites were detected approximately 20 h PI. Under light microscopy, the stain was very weak at this time, but increased considerably during the following 2 days. At 2 to 3 days PI, with glutaraldehyde-fixed cultures, the measles-positive PLA was labeling the virus, and the modified membrane of cells was showing active signs of virus replication (Fig. 1). The same components were devoid of any specific stain when measles-negative PLA was used (Fig. 2) or when a blocking experiment was performed. The surface labeling was either discontinuous or continuous in mononuclear and giant cells containing cytoplasmic nucleocapsids (Fig. 1, 3, 4, and 5). The discontinuous label was more often seen at 20 h than at a later time, but even at advanced stages of infection, cells could be either unlabeled, periodically labeled, or continuously stained, depending on the stage of maturation of the virus (Fig. 1, 3 and 5). When the staining had a periodic distribution, the reaction was most intense on the surface projections in front of each nucleocapsid (Fig. 4). However, the latter viral component was not identified under all positive patches on the cell membrane (Fig. 3). A similar periodic surface labeling was also observed in some mature virus particles (Fig. 5). The surface projections disposed in front of each underlying tubule are shown unstained in Fig. 6, where the cells were exposed to the enzyme only.

The reactivity of cell surface immediately after virus adsorption was explored. In rare instances, one viral particle was seen free in the extracellular space or fused with the cell membrane, allowing nucleocapsids to spread into the cytoplasm. The surface labeling on these viral sites, if any, was very weak (Fig. 7). Through the latent period, when no viral structural components were detected, the surface of the cells remained entirely unreactive as in uninfected cells treated with measles-positive PLA.

Cellular antigen. Labeling of the cytoplasmic nucleocapsids was observed most frequently in formaldehyde-fixed preparations. The intensity of the label varied from one cell to another and from one inclusion to another depending qpon membrane preservation and penetration. Alternatively, the PLA might have been consumed entirely on the first antigenic sites encountered on the periphery of the cell. In Fig. 8, a perinuclear tubular inclusion is shown unlabeled in a culture treated with a fluorescent conjugate. An intense labeling of a similar perinuclear inclusion was obtained with the measles-positive PLA (Fig. 9). The staining has a patchy appearance, but in some places a tubular shape can be identified (Fig. 9, arrow). Figures 10 and 11 illustrate at higher magnification the differences between an unlabeled and labeled group of cytoplasmic nucleocapsids by the indirect method. In Fig. 10, the nucleocapsids have a diameter of about 16 nm and are surrounded by a less-electron-dense granular material so that the total width of these fuzzy tubules is 30 to 45 nm. In Fig. 11, dark labeled tubular formations 35 to 50 nm large can be recognized. This indicates that the label is located on the fuzzy material ensheathing the cytoplasmic nucleocapsids. In a glutaraldehyde-fixed preparation (Fig. 12) the same type of labeling is seen on nucleocapsids at the surface of the cell, cut tangentially. Inside the labeled surface, the tubule can still be detected (Fig. 12, arrow).

The smooth type of nucleocapsids observed in many nuclei of heavily stained preparations were never labeled. Lack of penetration alone could not account for this absence of reactivity; indeed, on rare occasions smooth and fuzzy tubules were seen together in the cytoplasm, and only the latter were stained. In addition, unstained smooth tubules were seen in complete particles having a heavy label on their interrupted membrane (Fig. 13).

DISCUSSION

Until now all studies dealing with specific staining of viral antigens of paramyxoviruses by using EM have been done with the immunoferritin technique (9, 11, 12, 16, and 21). Compared with these observations, the present use of the

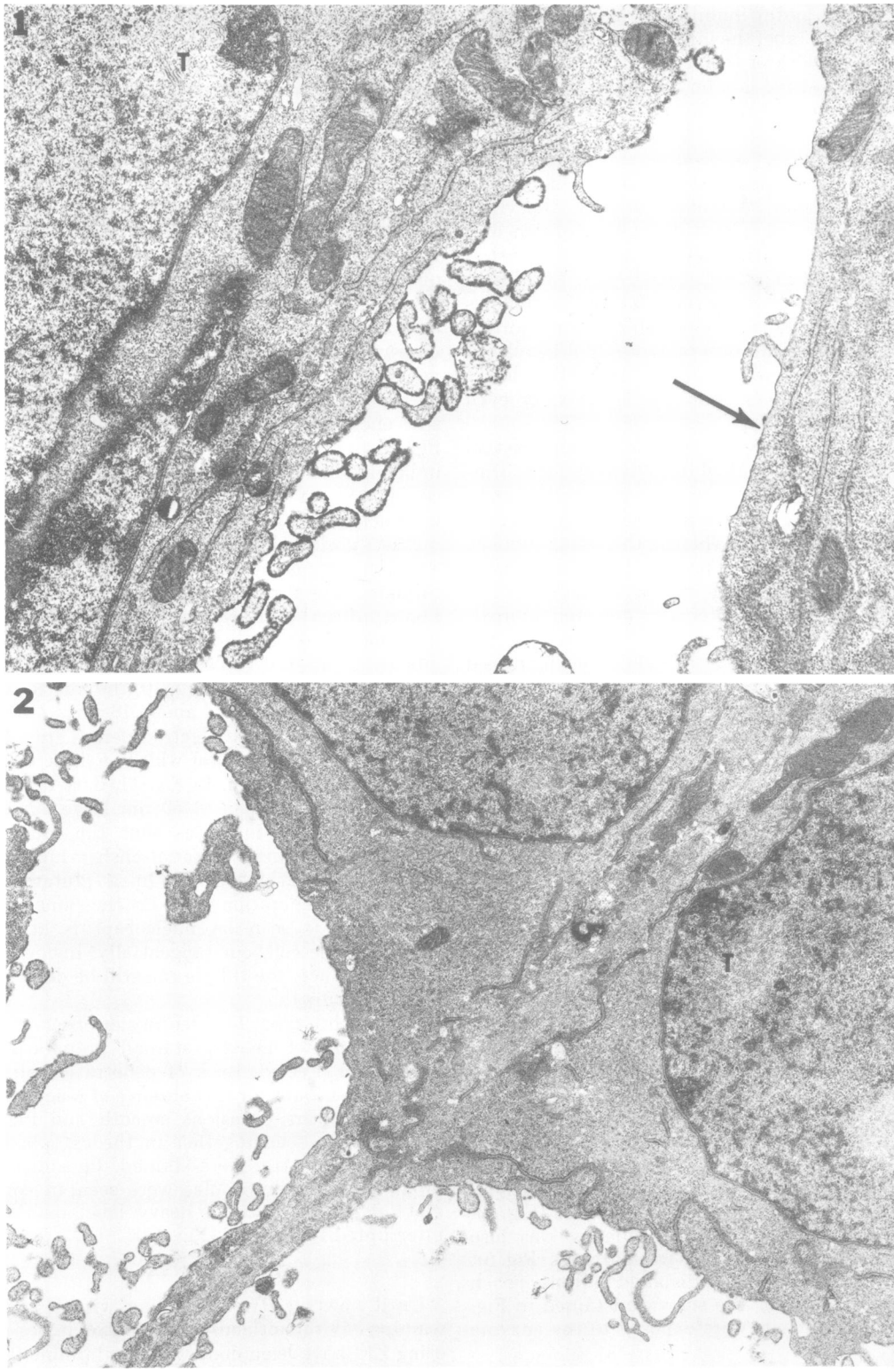
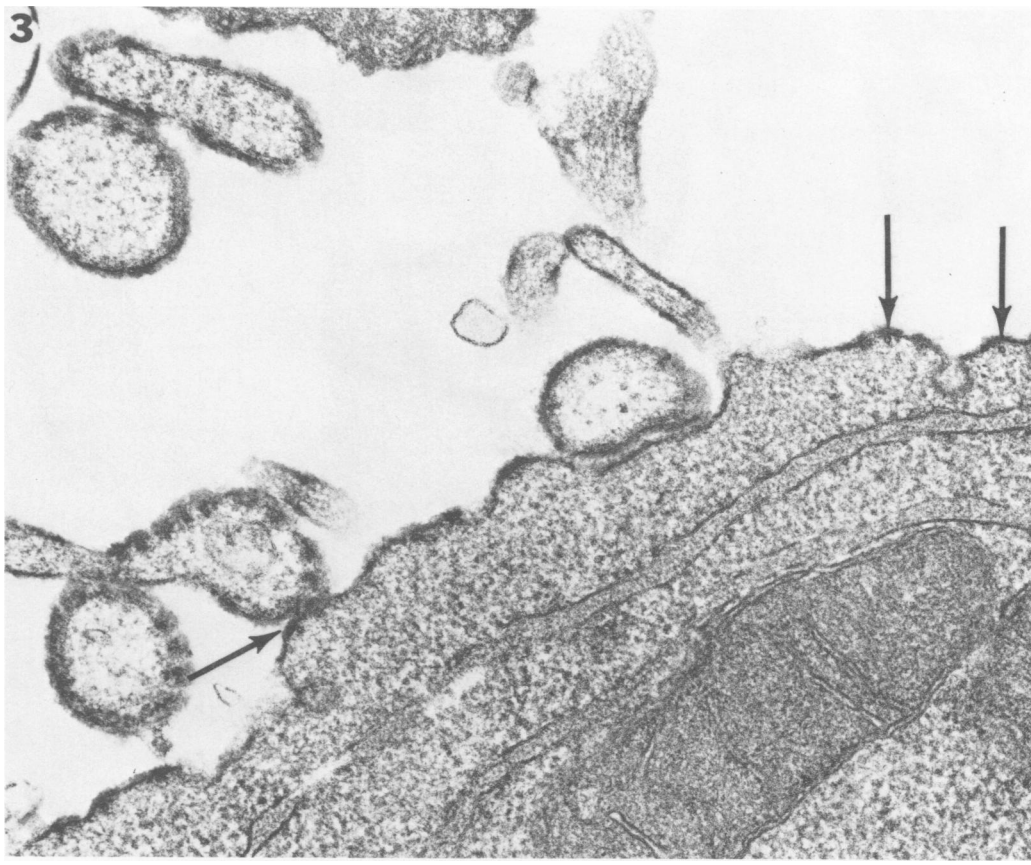


FIG. 1 and 2. *Glutaraldehyde fixation; 2 days PI.*



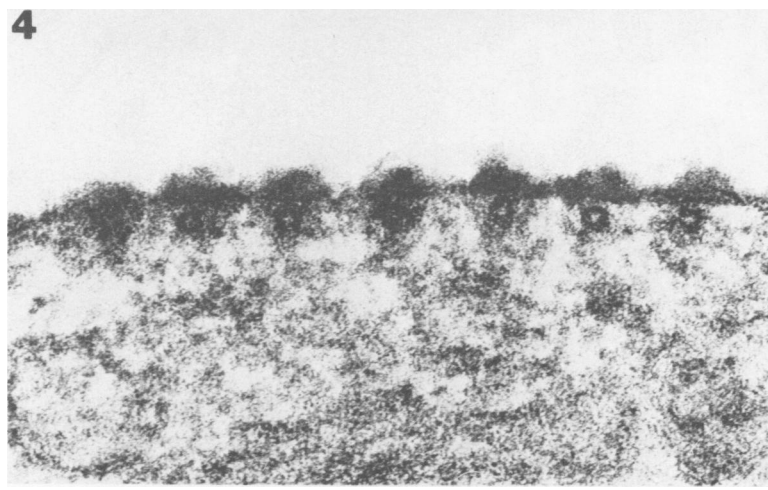


FIG. 3 and 4. *Glutaraldehyde fixation; 2 days PI. Measles-positive PLA.*

FIG. 3. *Discontinuous label is seen on the surface of an infected cell. Nucleocapsids underlying the patchy surface stain can be identified in three places (arrows). ×60,000.*

FIG. 4. *The periodic surface labeling is the thickest in front of each underlying nucleocapsid. ×217,000.*

FIG. 1. *Measles-positive PLA. The label is seen on the surface of a giant cell on the left and on the viral particles that have been produced by this cell. Nuclear tubules are present (T). On the right is another cell surface which is reactive only at two points (arrow). ×12,600.*

FIG. 2. *Measles-negative PLA. The modified surface and the viral particles produced by this giant cell are devoided of any label. Nuclear tubules in T. ×9,000.*

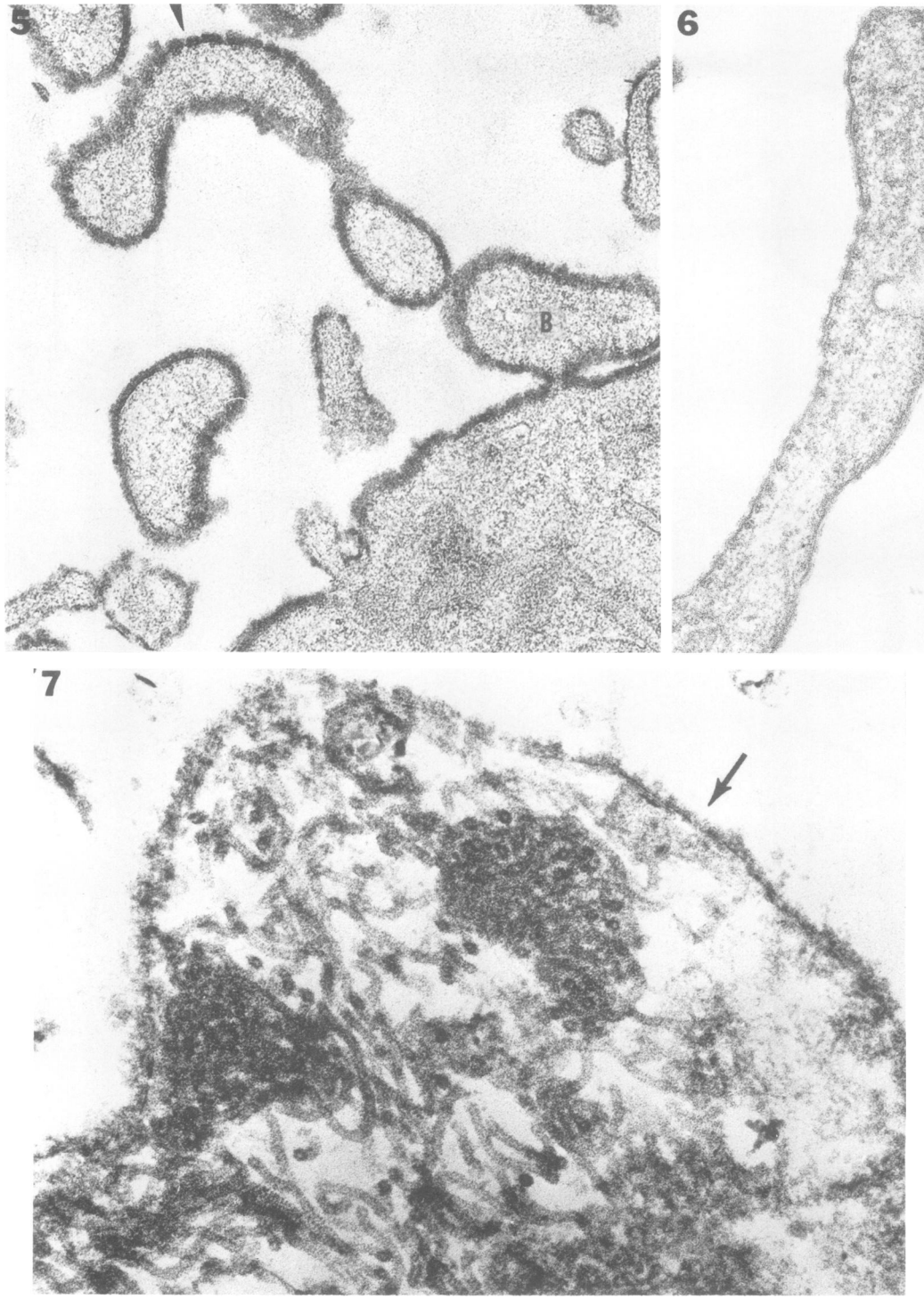


FIG. 5 and 6. *Glutaraldehyde fixation; 2 days PI.*

FIG. 5. *Measles-positive PLA. A continuous labeling is present on the membrane and the budding site of an infected cell (B). Periodic surface stain is seen on one side of a complete viral particle (arrow).* ×*45,000.*

FIG. 6. *Peroxidase alone. Unlabeled surface projections are periodically present in front of each underlying nucleocapsid.* ×*54,000.*

FIG. 7. *Glutaraldehyde fixation. Measles-positive PLA; 1 h PI. The membrane of a viral particle has fused with a cell membrane allowing nucleocapsids to spread into the cell. Note a very weak label on the viral membrane (arrow).* ×*124,000.*

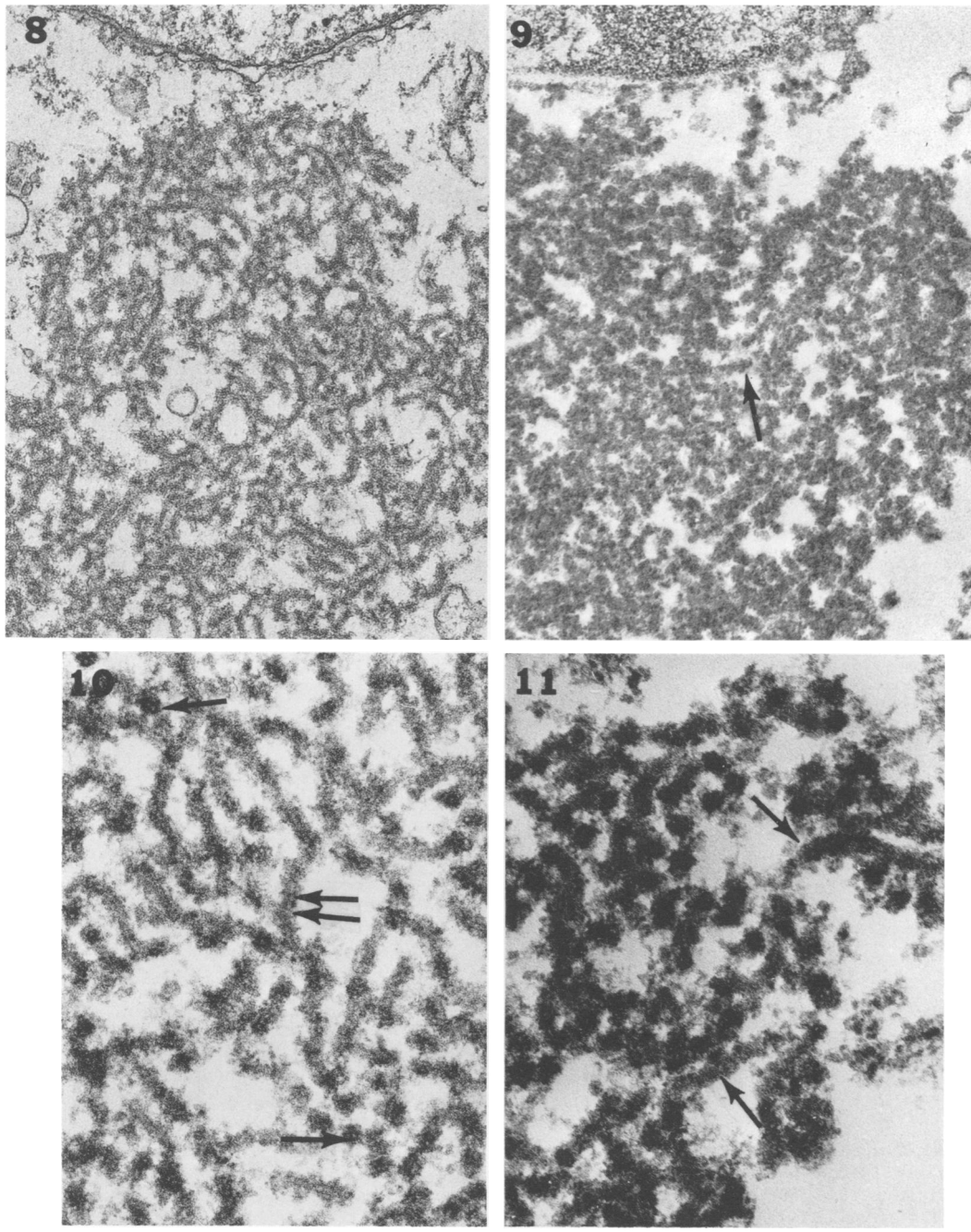


FIG. 8 and 9. *Formaldehyde fixation; 2 days PI.*

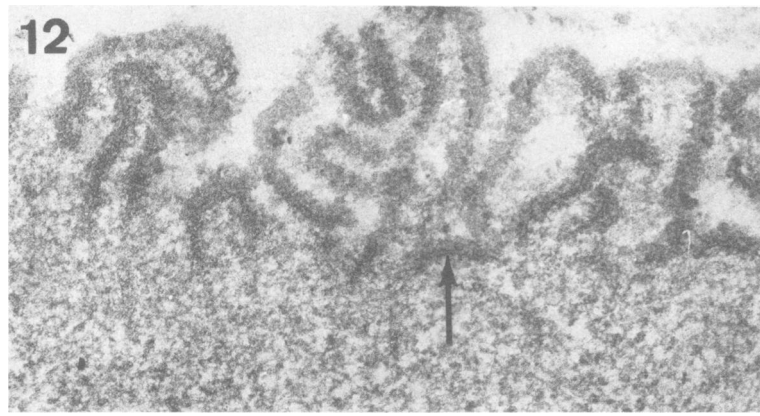
FIG. 8. *FA preparation. A perinuclear inclusion is composed of tubules and granular material. ×32,000.*

FIG. 9. *Measles-positive PLA. A patchy labeling is seen on a similar perinuclear inclusion. A tubular shape can be detected in some places (arrow). ×33,000.*

FIG. 10 and 11. *Formaldehyde fixation; 4 days PI. SSPE serum indirect method. Cytoplasmic inclusion. ×75,000.*

FIG. 10. *Unlabeled nucleocapsids approximately 16 nm in diameter are seen cut transversally (arrow) or longitudinally (double arrow). They are surrounded by a less-electron-dense granular material.*

FIG. 11. *The granular material ensheathing the nucleocapsids is labeled so that black tubular shapes of 35 to 50 nm large are recognized.*



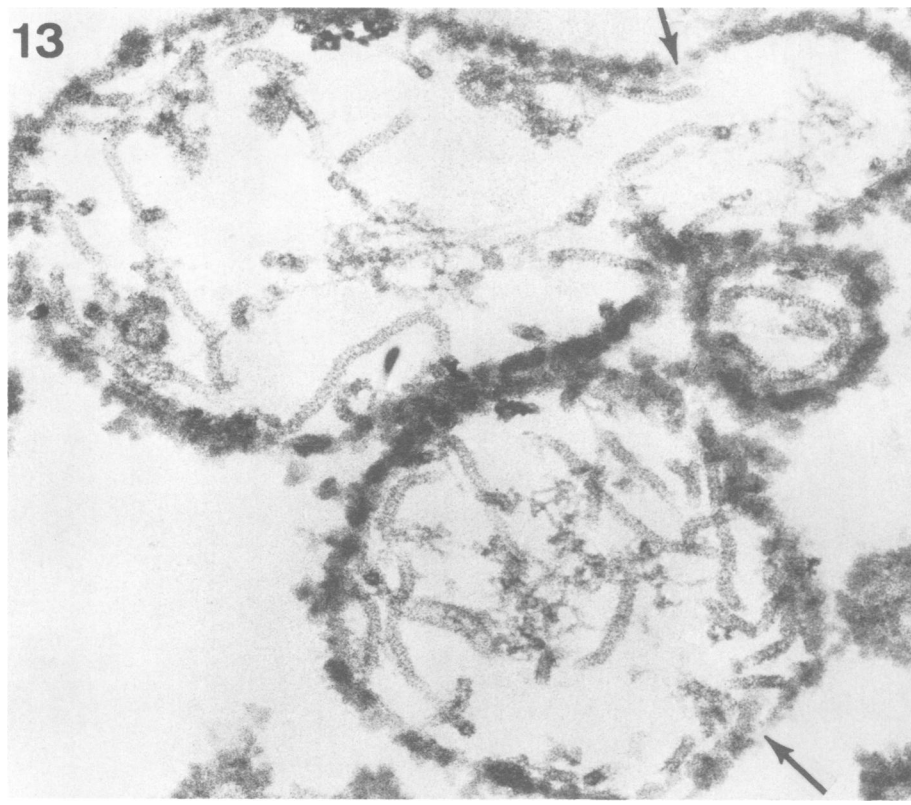


FIG. 12 and 13. *Measles-positive PLA; 2 days PI.*

FIG. 12. *Glutaraldehyde fixation. "Fuzzy" nucleocapsids located at the surface of a cell are seen in a tangential section. The label is located on the fuzzy material around the tubule. The latter can be detected within the label in some places (arrow). ×64,000.*

FIG. 13. *Formaldehyde fixation. Smooth nucleocapsids located in two complete virions are devoid of any stain, although the surface is heavily stained. Note interrupted membranes in some places (arrows). ×124,000.*

IP technique for the localization of measles antigen by using EM has allowed a more precise resolution of the reactive sites on the cell membrane and in the cytoplasm.

Recently it has been shown that PLA always labels more antigenic sites at the cell surface than the ferritin antibody conjugate does at an equal globulin concentration (6). This might be explained by a deeper penetration of the PLA into the membrane and by an amplification factor, for the enzyme is not consumed in the reaction. Consequently, higher dilutions can be

utilized with peroxidase than with ferritin conjugates. Provided the molar coupling ratio is high, 1:1 in Avrameas' work (4) or one molecule of peroxidase for two molecules of globulin in our hands, and the enzyme activity is well preserved, the intensity of the reaction can be controlled accurately on identical cellular systems. The direct method, in which two conjugates have to be made and compared in identical conditions is more delicate to use than the indirect techniques, but the localization of the specific stain is the same in both cases.

A striking event with SSPE measles virus-infected cells was that, after a silent period of 20 h, we observed simultaneous increase in (i) intensity of the IP stain at the light microscopy level, (ii) intensity of fusion, (iii) number of cytoplasmic nucleocapsids, and (iv) number of antigenic sites on the cell membrane. Labeled areas of the membrane apparently contain viral proteins and they may be precursors to the modified membrane which will become the envelope of the virion (9). All particles and viral buds were specifically tagged by the measles-positive PLA. This observation suggests that unmodified host membrane is not used as part of the viral envelope in measles infection as previously shown for influenza virus (11). The periodic label of the surface in front of each underlying nucleocapsid suggests an uneven distribution of the membrane surface antigen and a close relationship between the reactive sites and the position of the nucleocapsid under the membrane. These surface antigenic sites are probably related to the spikes or surface projections which have been found to be glycoprotein in vesicular stomatitis virus (VSV) infection (22). This glycoprotein is the antigen that gives rise to and reacts with neutralizing antibody (17). A specific interaction between regions of the cell membrane which contain viral envelope proteins and the measles nucleocapsids was shown by our observations. Bio-chemical studies of measles virus proteins like the one performed on VSV (22) should be conducted to further confirm this concept.

After the virus adsorption period, fusion of the virion and the host cell membrane with release of the nucleoprotein into the cytoplasm occurred as described with Sendai virus (3). The very weak surface label on this site is probably related to the disappearance of the viral surface projections (3), the innermost layer of the virus being replaced with cell membranes.

After formaldehyde fixation, the measles-positive PLA labeled cytoplasmic inclusions more completely and regularly than the immunoferritin conjugate used in mumps virus infection (12). The reaction product had a distinct form so that it was possible to identify the label as located on the granular sheath surrounding the cytoplasmic nucleocapsids. In contrast the smooth type of nucleocapsid was never labeled. These results suggest that the two types of nucleocapsids as described in recent EM studies on measles and SSPE (27, 28, 31, and 32) (R. Hamilton et al, J. Virol., in press) have a different antigenic nature. Earlier studies on measles internal component using the negative staining technique have shown the presence of an extratubular structure surrounding some of the internal components (2, 27). This external structure was thought to be related to the cytoplasmic synthesis and assembly of the nucleocapsid. However, ensheathed tubules have not been seen by others (24, 25). Physical separation of the two types of nucleocapsid of measles virus should be performed to elucidate their possible differences in chemical composition and antigenicity. Interestingly, measles nucleocapsids are the antigens that react with a major fraction of antibodies detected by CF and immunodiffusion tests (34).

There is a good correlation between the IP stain after formaldehyde fixation and immunofluorescent stain after acetone fixation as far as surface and cytoplasmic viral components are concerned. The absence of specific nuclear stain with the PLA even at advanced stages of infection is in accord with FA studies performed by one of us (14, 15), but is in contrast to the specific fluorescent stain of nuclear inclusions described by others in SSPE (10) (23, 27, and 28).

It appears that the IP method might be of value in the study of the immunopathogenesis of SSPE. No qualitative differences were detected in our system between the stain induced by SSPE and normal human sera with high measles antibody titer in the indirect method. The suppressed state of virus infection in this disease does not seem to be related to a different type of antigen-antibody reaction as detected by the IP technique. Also, the antigenic sites should be investigated in a system where measles virus is in a "suppressed" state. An antiserum against purified SSPE nucleocapsids (37) should be used, for the most frequent viral components in this disease are the smooth nucleocapsids; these elements were never labeled by measles-positive human sera.

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