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Combined Fluorescent-Antibody and Electron Microscopy Study of Marek's Disease Virus-Infected Cell Culture[1]

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Duck embryo fibroblast (DEF) and chicken embryo fibroblast (CEF) cultures infected with Marek's disease virus were studied by combined fluorescent antibody and electron microscopy techniques. In both DEF and CEF cultures, cells containing immunofluorescent (IF) antigen also contained herpesvirus particles; conversely, cells lacking this antigen lacked herpesvirus particles. Two morphologically distinct IF antigens were detected in the cytoplasm. (i) A granular antigen in the perinuclear region was brightly stained with the conjugated antibody. This antigen was composed of a granular mass of osmiophilic material and did not contain virions. (ii) A diffuse antigen, present throughout the cytoplasm of infected cells, was less brightly stained. The area of the cell with the highest concentration of this antigen contained small vesicles, folded membranes, and fine electron-dense granules. Naked virions were occasionally seen in these areas. A diffuse nuclear IF antigen was occasionally seen in infected cells. This antigen was often separated from the nuclear membrane and the nucleolus by a clear margin. The intranuclear IF antigen was composed of a fine granular aggregate and naked herpesvirus particles which were randomly distributed throughout the nucleus. Viral capsids in antibody-treated cells were coated with fine filamentous material.

On the basis of circumstantial evidence relating virus isolation to the presence of the disease (2, 5) and serological reactions (1, 6), a herpesvirus is now considered to be the etiological agent of Marek's disease. Sera from infected and hyperimmunized chickens reacted with antigens in cells infected with this herpesvirus in the agar gel precipitin (1) and fluorescent antibody [FA (6)] tests. The purpose of the present study was to relate immunofluorescent (IF) antigens to herpesvirus infection of individual cells by combined FA and electron microscopy, and to determine the ultrastructure of the IF antigen.

A similar study on EB virus-infected lymphoblasts demonstrated a positive relationship between IF antigens and the presence of the virus (3).

MATERIALS AND METHODS

Virus. High cell culture-passaged JM strain of Marek's disease virus (MDV) was used in these studies. The virus was isolated in duck embryo fibroblast (DEF) cultures and propagated in DEF and chicken embryo fibroblast (CEF) cultures for over 18 months. It had lost its pathogenicity for chickens (K. Nazerian, *in preparation*).

Propagation of the virus in cell culture. Monolayer cultures of DEF or CEF grown in 35-mm Falcon plastic petri dishes were inoculated with several dilutions of growth fluid from highly infected CEF cultures. After 2 hr of virus adsorption, the inoculum was washed and replaced with liquid medium consisting of a mixture of medium F10 and medium 199 (8) with 2% bovine fetal serum. Typical plaques formed in 48 and 72 hr in CEF and DEF cultures, respectively. Cultures with well-isolated plaques in the center of the petri dish were then prepared for FA staining and electron microscopy as described below.

Preparation and purification of specific antibody. Serum was obtained from chickens hyperimmunized against blood from MDV-infected chickens (6). Globulin was precipiated by sodium sulfate fractionation (9) and dissolved in 0.85% NaCl.

Indirect FA staining. Monolayer cultures in petri dishes were thoroughly washed in phosphate-buffered saline (PBS), *p*H 7.4, fixed in cold absolute ethyl alcohol for 30 sec, and washed again five times in PBS. They were treated with 450 μg of specific globulin in 0.5 ml of PBS for 30 min at room temperature

[1] Preliminary results of this study were presented at the 27th Annual Meeting of the Electron Microscopy Society of America at St. Paul, Minn., August 1969.

and then washed thoroughly in five changes of PBS for 15 min. Cultures were then incubated at room temperature with 0.5 ml of a 10% solution of fluorescein-conjugated anti-chicken globulin (Roboz Surgical Instruments Co., Inc., Washington, D.C.) for 30 min. Excess conjugate was removed by washing in PBS for 15 min, and cultures were temporarily mounted in PBS and immediately examined under a fluorescence microscope. Suitable plaques were photographed and marked for further examination with the electron microscope.

Electron microscopy. Immediately after examination with the fluorescence microscope, cultures were fixed in situ in 1% osmium tetroxide for 90 min. They were dehydrated in increasing concentrations of ethyl alcohol and then covered with 5 mm of Epon 812 and incubated at 60 C for 24 hr. Areas of the monolayer containing the microplaques previously examined and photographed with the fluorescence microscope were cut out and mounted with the cell layer facing up, on top of gelatin capsules containing fresh Epon. Capsules were incubated for another 72 hr at 60 C. Sections of the plaques were made with a MT-2 Porter Blum ultramicrotome. They were stained with uranyl acetate and lead citrate and were examined with an Elmiskop 1A electron microscope.

RESULTS

Localization of the virus and IF antigens. In DEF cultures, well-developed, isolated microplaques were seen 72 hr postinoculation. These microplaques were composed of one or more syncytia in the center and many rounded and fusiform refractile cells in the periphery. IF antigens were observed only in the central syncytium and surrounding cells and not in the remaining cells in the monolayer (Fig. 1). Many of the morphologically unaltered cells adjacent to the microplaque contained IF antigens, so that the stained area observed under the fluorescence microscope was much larger in size than the microplaque seen with the conventional light microscope, indicating that the IF antigens appeared before any major morphological alteration. IF antigens occurred more frequently in the cytoplasm than in the nucleus of infected cells. On electron microscopic examination, numerous herpesvirus particles were found in sections of cells containing IF antigens, whereas none was found in sections of cells free of these antigens.

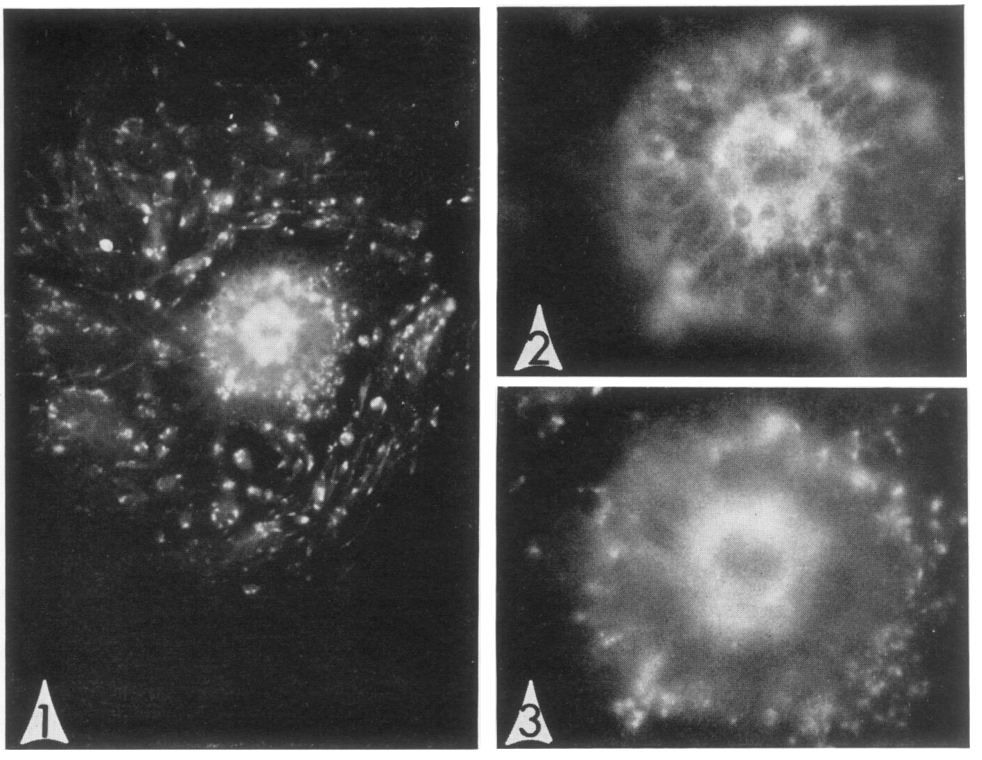


FIG. 1–3. *Fluorescence micrographs of a microplaque in DEF culture. The entire microplaque is composed of a large syncytium in the center and many smaller cells in the periphery (Fig. 1; × 300). The remainder of the monolayer is not stained. The central syncytium in Fig. 1 is seen at a higher magnification in Fig. 2 and 3 (× 750). Two kinds of cytoplasmic antigens can be seen in this cell: a diffuse antigen particularly concentrated in the central portion (Fig. 2) and a discrete granular antigen in the perinuclear region and periphery of the cell (Fig. 3).*

Two morphologically distinct antigens were found in the cytoplasm (Fig. 2 and 3): (i) well-separated, discrete granules which were common in the perinuclear region of the cell, and (ii) a diffuse powdery mass distributed throughout the cytoplasm and concentrated particularly in the center of the syncytium. This central mass of diffuse antigen was surrounded by a large number of nuclei which in most cells did not contain an appreciable amount of IF antigen. In sections of these cells examined with the electron microscope, the granular antigen was more commonly found in the perinuclear region (Fig. 4–6) of the cell than in other areas of the cytoplasm. It consisted of a mass of closely packed osmiophilic particles 10 to 20 nm in size and was easily detected in cells prepared for electron microscopy in the usual manner (Fig. 7). Herpesvirions were not seen within the granular antigen or in areas of the cell containing this antigen. The diffuse cytoplasmic antigen present in the center of the syncytium was composed of small vesicles, fragmentary membrane-like structures, and irregularly shaped, small, electron-dense granules (Fig. 8). These granules were much smaller than the perinuclear antigen. Herpesvirions were found only occasionally in areas of the cell containing this diffuse antigen (Fig. 8, 9).

The nuclear IF antigen was diffuse and homogeneous and was usually separated from the nuclear membrane and the nucleolus by a clear margin (Fig. 10, 11). No granular antigens were detected in the nucleus. On examination with the electron microscope, the nuclear antigen was found to be composed of naked herpesvirions embedded in a diffuse mass of fine granular material (Fig. 12, 13). This material was different in morphology and electron density from granular antigen in the cytoplasm and was less closely packed. Herpesvirions were randomly distributed throughout the nucleus and were not seen in high concentrations or aggregations.

In CEF cultures, the virus produced a microplaque composed mainly of rounded and fusiform refractile cells and a few multinucleated cells which contained only a few nuclei. The IF antigen more commonly was seen in the cytoplasm than in the nucleus (Fig. 14). Bright, granular, diffuse staining was observed in the cytoplasm of rounded cells and fibroblastic cells adjacent to rounded cells. The diffuse cytoplasmic staining, however, was brighter in rounded cells than in fibroblastic cells. Herpesvirions were found in the nuclei of most of the round cells in the microplaque and in the fibroblastic cells adjacent to the microplaque which contained IF antigens, but they were not found in cells lacking the IF antigens. The perinuclear granular antigen was composed of aggregates of closely packed, small, electron-dense particles 10 to 20 nm in size (Fig. 14, 15). Herpesvirions were not associated with the cytoplasmic antigens.

Morphology of the virus. Virus particles seen in cells treated with antibody for FA examination had the same general morphology of a herpesvirus. They had capsids 90 to 100 nm in diameter, and some contained nucleoids 40 to 70 nm in size. In addition, a fine filamentous substance was seen on the surface of viral capsid (Fig. 9) in cells treated with antibody. Enveloped particles were rarely seen.

DISCUSSION

Techniques used in the present study allow a combined study of infected cell cultures by both FA staining and electron microscopy. These techniques permit (i) identification of IF antigens, (ii) demonstration of fine structure of these antigens, and (iii) demonstration of viral specificity of the antigens, their relationship to the virus, and its site of replication.

Several steps in the routine FA and electron microscopy techniques were modified to allow a combined study of infected cells. These modifications consisted of the use of plastic petri dishes instead of coverslips for growth of cultures, the substitution of cold ethyl alcohol for acetone for fixation of cells for FA, and the elimination of dehydration in propylene oxide for electron microscopy preparations. These modifications slightly influenced the brightness of staining of the FA preparations because the plastic dish absorbed some of the incident light and the autofluorescence of the plastic reduced the contrast. The resolution of the electron micrographs was slightly reduced by the suboptimal fixation. These alterations, however, did not obscure the presence of the antigen or cause a drastic change in the ultrastructure of cellular organelles, viral antigens, or viral particles.

The results reported here demonstrate that IF antigens detected in DEF and CEF infected with MDV are related to the infection and are viral specific.

The presence of herpesvirus particles in cells containing IF antigens and their absence in cells free of IF antigens demonstrate the viral specificity of these antigens. They are, therefore, structurally or functionally related to the virus. However, structurally intact virus particles were not found in the granular cytoplasmic antigen, and the small number of particles found or associated with the diffuse cytoplasmic antigen could have been incidental and released into the cytoplasm as a result of rupture of the nuclear membrane. The presence of structurally

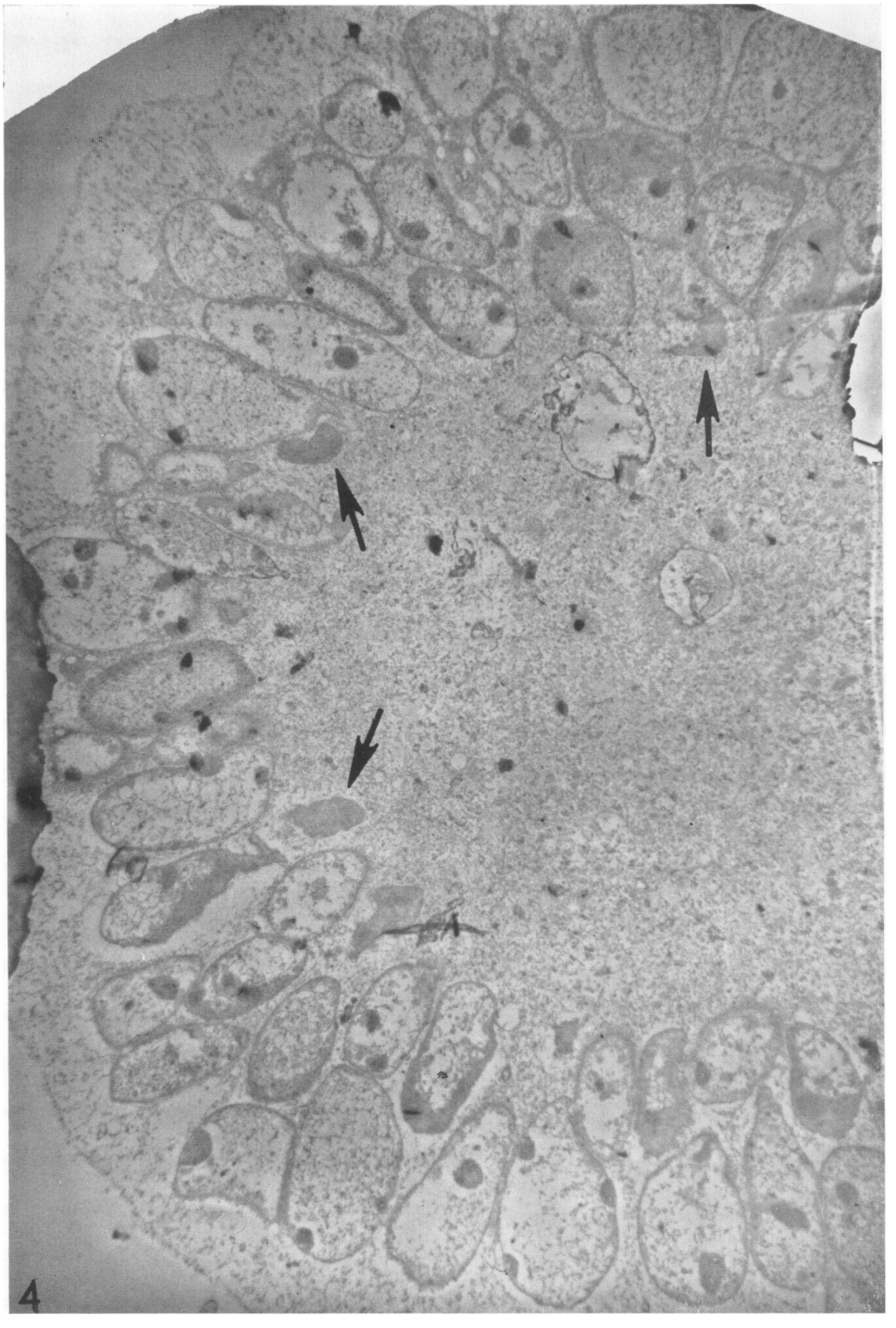


FIG. 4. *Electron micrograph of a portion of the large syncytium seen in Fig. 1–3. A large number of nuclei can be seen in this section of the cell and they correspond well with the nuclei in Fig. 2. The granular antigen is in the vicinity of some of the nuclei (arrows). The central region of the cell contains the diffuse antigen as in Fig. 2.* × *3,000.*

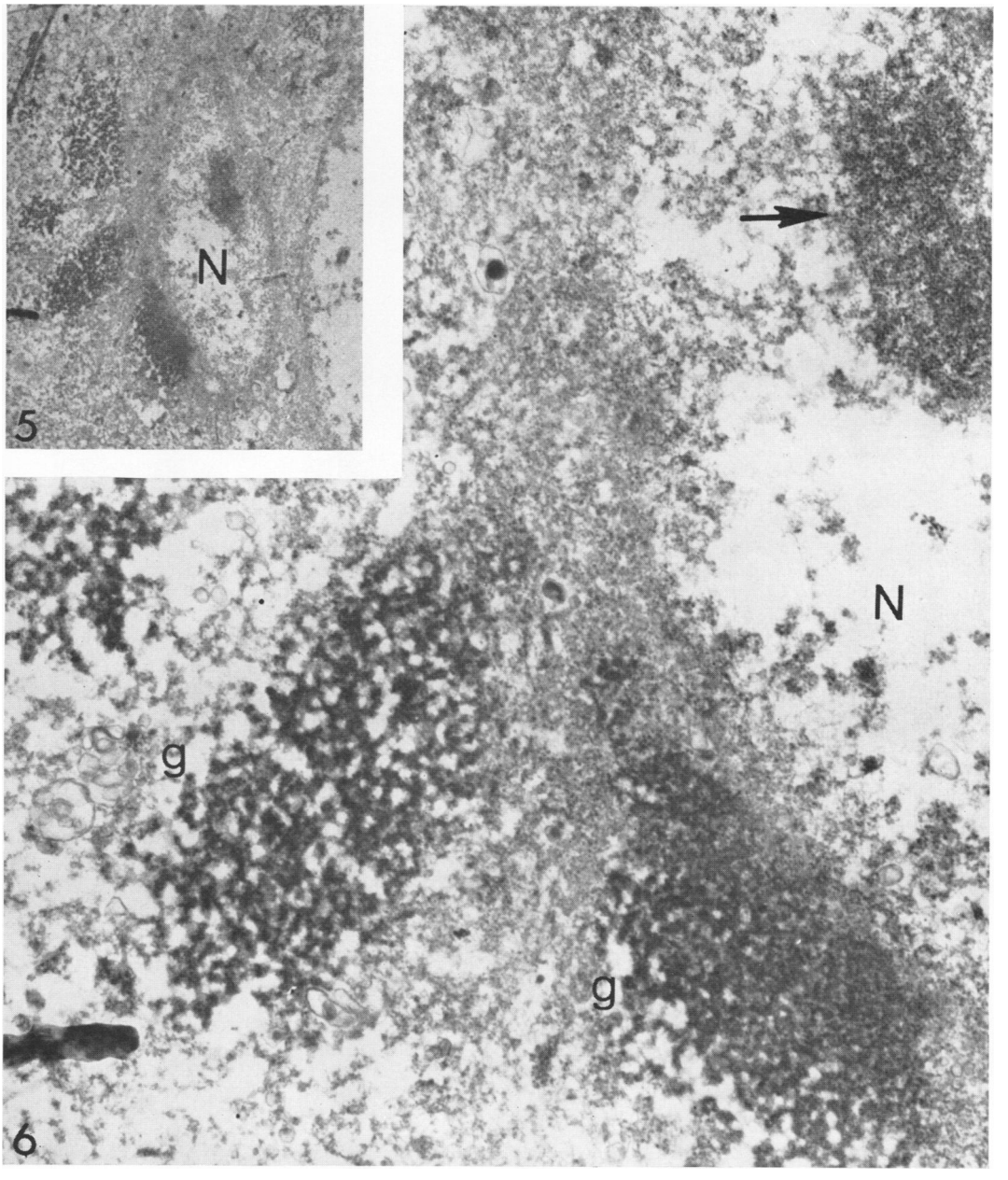


FIG. 5. *Portion of the cell examined in Fig. 4. Three granules of antigen are seen adjacent to one of the nuclei (N).* × *3,000.*

FIG. 6. *Higher magnification of Fig. 5. The granular antigen (G) is composed of electron-dense material different in texture from the nucleolus (arrow).* × *30,000.*

intact virus particles in nuclei containing IF antigen could not be considered the only factor responsible for staining since other nuclei which also contained virus particles did not stain.

On the other hand, the fine filamentous structures seen on the surface of viral capsid in antibody-treated cells are indicative of a reaction between antibody molecules and capsid protein. Similar reaction has been noted by other investigators (G. Schidlovsky, *personal communication*). Therefore, viral capsid, cytoplasmic, and nuclear antigens all react with the specific antibody.

FIG. 7. *Section of an infected DEF prepared for electron microscopy in normal fashion. Granular antigen (G) is near the nuclear membrane. The fine granularity of this antigen is quite clearly demonstrated in this micrograph. Naked virus particles are seen in the nucleus and not in the granular antigen.* × *30,000.*

IF antigens in the cytoplasm may represent structural components of the virus synthesized in excess and later transferred into the nucleus for assembly of the virus. Such a phenomenon has been demonstrated (4) in cells infected with pseudorabies virus.

Three different antigens shown in these studies were similar in distribution and morphology

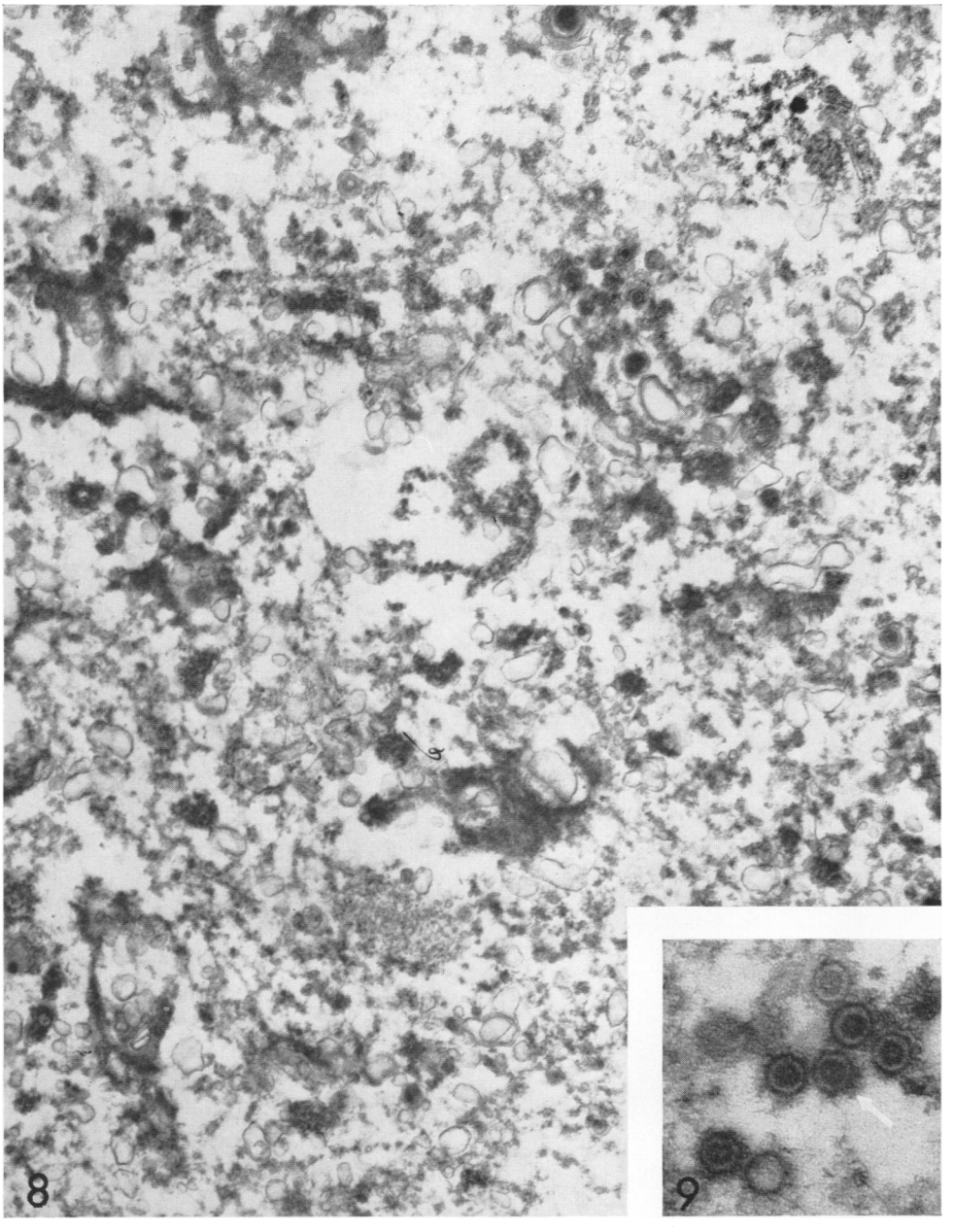
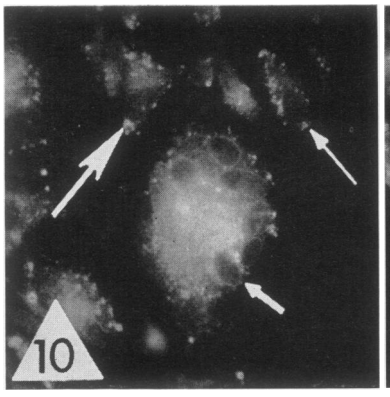


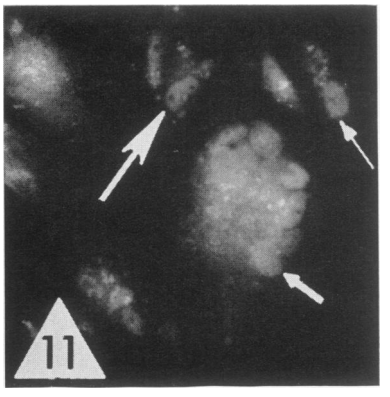
FIG. 8. *Higher magnification of the central portion of Fig. 4. This area contains the diffuse antigen in Fig. 2. It is composed of small vesicles, membrane-like structures, irregularly shaped electron-dense granules, and occasional virus particles (arrows).* × *30,000.*

FIG. 9. *High magnification of several naked virus particles in the nuclei of syncytium in Fig. 2. Notice the presence of a fine filamentous substance around the viral capsid (arrow).* × *69,000.*

to those of other herpesviruses (7). However, in the experiments performed here, nuclear antigen was not as frequent as reported in other herpesviruses and was composed of a uniform mass rather than small and large granules seen in the nucleus of other herpesvirus-infected cells (7).

The presence of antibody to Marek's disease herpesvirus particles in sera of hyperimmunized chickens indicates that the virus or its antigens





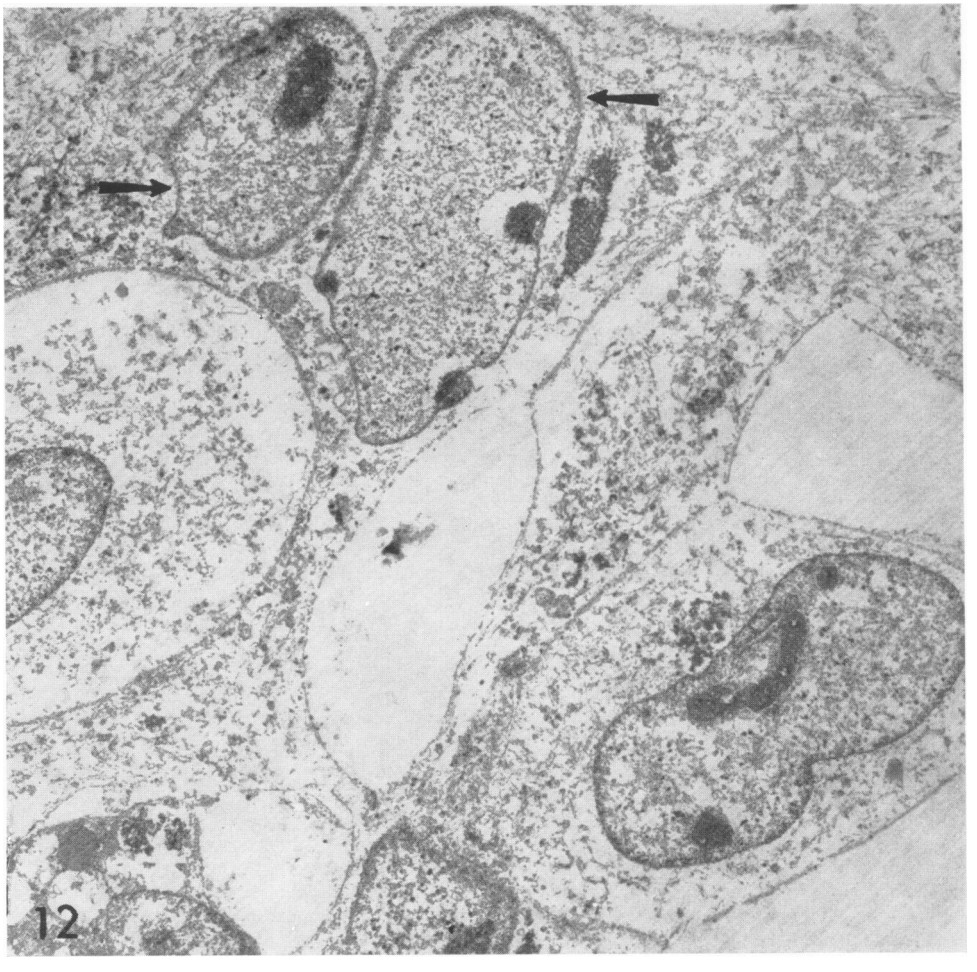


FIG. 10 and 11. *Light micrographs of an area of an infected DEF culture. Fig. 10 is a dark-field-visible light micrograph, and it clearly shows the location of the nuclei in the syncytium and some other smaller cells. Figure 11 fluorescent micrograph of the same area and shows specific staining of many of the nuclei. Similar arrows are used in both micrographs to show identical nuclei. Nuclear staining is diffuse and is not present in the nucleolar region (large arrow in Fig. 11).* × *750.*

FIG. 12. *Electron micrograph of an infected DEF culture. Nuclei indicated by arrows showed diffuse staining when examined with the fluorescence microscope.* × *6,000.*

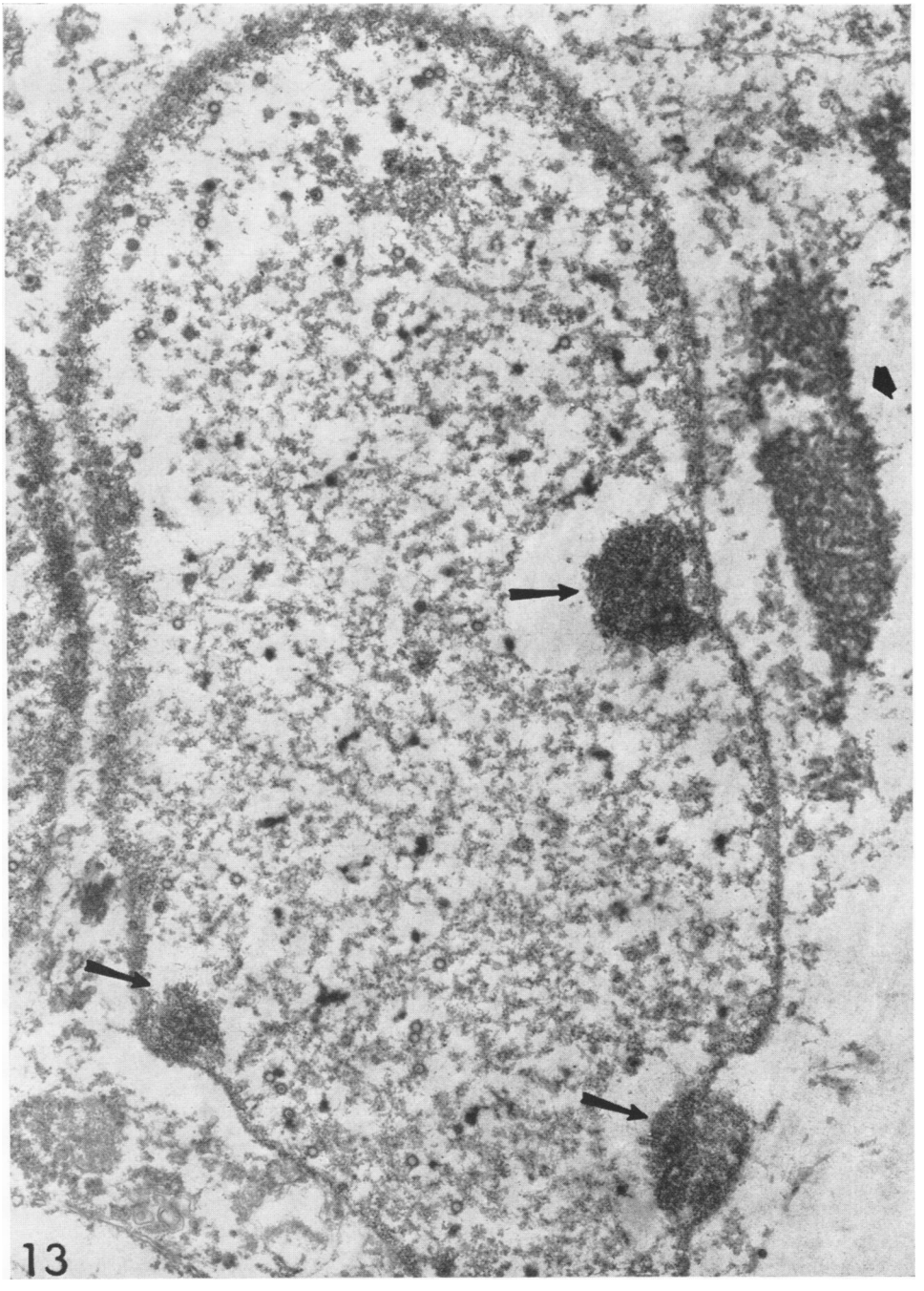


FIG. 13. *Higher magnification of one of the nuclei in Fig. 12. This nucleus contained IF antigen. Nucleoli are indicated by arrows. No staining was observed in the nucleolar region or in the area immediately adjacent to the nuclear membrane. The entire nucleus, except the nucleolar region, is composed of a homogeneous granular substance with naked herpesvirus particles randomly distributed throughout.* × *15,000.*

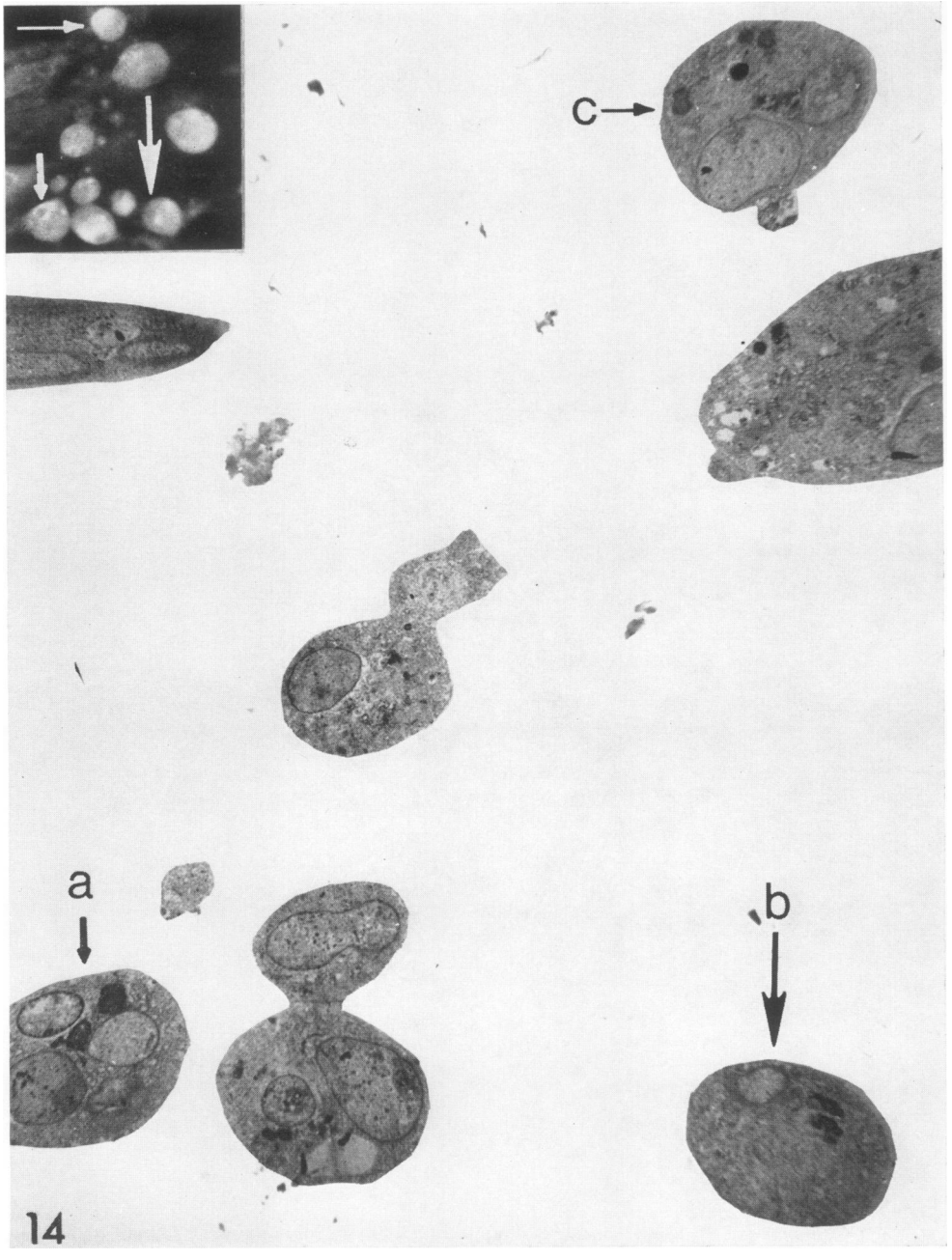


FIG. 14. *Fluorescence (inset) and electron micrographs of portion of a microplaque in an infected CEF culture. Diffuse and granular cytoplasmic antigens are seen in round cells and fusiform cells (inset). The nuclear staining is not as bright as the cytoplasmic staining. Similar arrows are used to show identical cells in both micrographs. In cells a, b, and c, electron-dense granular bodies in the perinuclear region correspond to brightly stained areas in identical cells (inset).* × *3,000. Inset,* × *750.*

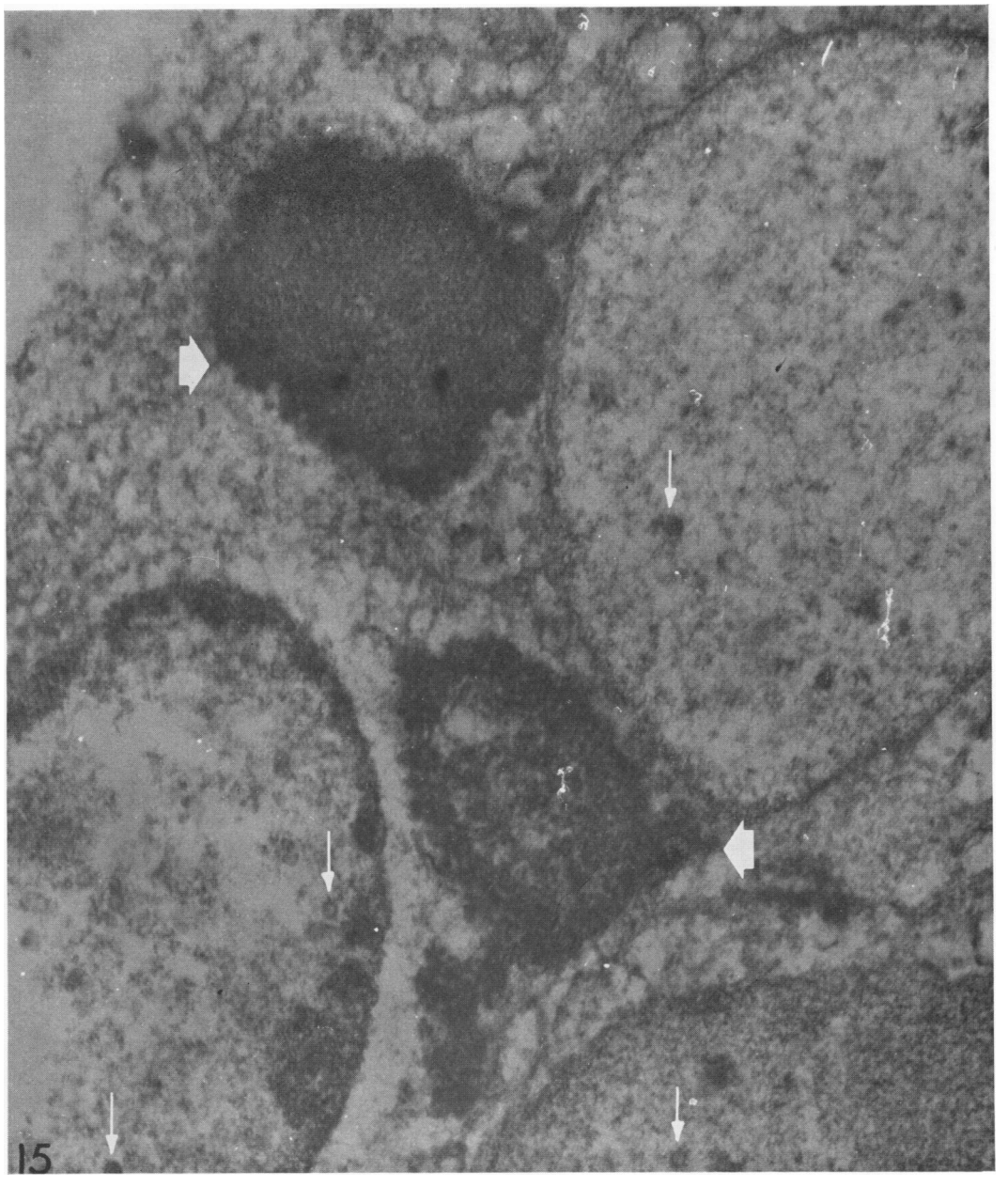


FIG. 15. *Higher magnification of one of the cells in Fig. 14 (cell a). The large arrows point to two granular antigens in this cell. This antigen is very similar to the antigen seen in infected DEF. It is highly granular and does not contain any virus particles. The small arrows point to naked herpesvirus particles in the nucleus.* × *30,000.*

were present in the inoculum (whole blood from MDV-infected chicken) used for preparation of the antibody. Direct electron microscopic examination of cells and plasma from infectious blood has failed to reveal the presence of the virus (K. Nazerian, *unpublished data*). It is possible that the virus is present in these cells in a very low concentration or in an inapparent form and, therefore, is not detectable by electron microscopy. The specificity of antibody from im-

munized chickens for Marek's disease herpesvirions further substantiates the hypothesis that this virus is the cause of the disease.

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