JOURNAL OF VIROLOGY, Oct. 1969, p. 521-527
Vol. 4, No. 4

Electron Microscopy of Equine Infectious Anemia Virus

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Received for publication 2 June 1969

Equine infectious anemia (EIA) virus was observed in thin sections of infected cultured horse leukocytes by electron microscopy. The virus particles had a spherical shape and were between 80 and 120 nm in diameter. Most of them contained an electron-dense nucleoid 40 to 60 nm in diameter. They were observed to form by a process of budding from the plasma membrane and appeared to have thin surface projections. The particles described were not detected in uninfected cultured cells, and their appearance could be prevented by adding EIA immune serum to the inoculum. The implications of these findings in the classification of EIA virus are discussed.

To the best of our knowledge, there has been no previous report on the morphology and intracellular development of equine infectious anemia (EIA) virus as revealed in thin sections by electron microscopy. With the recent development of a horse leukocyte culture system in which EIA virus causes a cytopathic effect (CPE), it has been possible to determine the infectivity of EIA virus in vitro and to study physicochemical and biological properties of the virus.

Nakajima et al. (*unpublished data*) succeeded in obtaining highly purified and highly infective materials from EIA virus-infected horse leukocyte cultures by the combined methods of ultracentrifugation, diethylaminoethyl cellulose column chromatography, and cesium chloride equilibrium density gradient centrifugation. They identified EIA virions in these materials in the electron microscope by negative staining and suggested that EIA virus is similar in size and shape to the myxoviruses. However, helical internal components characteristic of the myxoviruses were not seen in the EIA virions.

A study on the structure and development of EIA virus in cultured horse leukocytes, as observed in thin sections with the electron microscope, is reported here.

MATERIALS AND METHODS

Virus and infectivity assay. The Wyoming strain of EIA virus, passaged 43 times in horse leukocyte cultures and cloned by 4 repeated limiting-dilution passages, was used as the inoculum in the present work. At this passage level, the maximal infectivity titer of the culture medium was at least 10^6 $TCID_{50}$ per 0.5 ml, when tested in horse leukocyte culture.

Virus titrations were carried out by the technique described by Kobayashi and Kono (9).

Antiserum. Immune serum against EIA virus was obtained from a horse with chronic EIA. The serum had been mixed with an equal volume of undiluted bovine serum and had been heated at 56 C for 30 min to inactivate infective EIA virus before it was used. The titer of neutralizing antibodies, as determined by the technique of Kono (10), was 1:50.

Propagation of EIA virus in horse leukocyte culture. Horse leukocyte cultures were prepared and inoculated with EIA virus as described by Kobayashi and Kono (9). In brief, horse leukocytes were sedimented from plasma by low-speed centrifugation. They were washed with Hanks' balanced salt solution and suspended in inactivated (30 min at 56 C) undiluted bovine serum at a concentration of 2×10^7 to 4×10^7 cells per ml. The resultant suspension was distributed into rubber-stopped, rectangular bottles (50 by 50 by 120 mm) in 10-ml amounts and was incubated at 37 C for 20 hr. After removing the culture medium, 1 ml of virus in the medium containing about 10^6 $TCID_{50}$/ml was inoculated into each culture, and then 9 ml of fresh inactivated undiluted bovine serum was added. After incubation for 24 hr at 37 C, the medium was renewed, and the cultures were reincubated.

One group of horse leukocyte cultures was inoculated with the same quantity of EIA virus which had been incubated for 2 hr at 37 C with an equal volume of antiserum.

Preparation of specimens for electron microscopy. The cultures were harvested 3 and 4 days after inoculation, when a distinct CPE was observed in infected cultures. The culture medium was poured off. The cell layers were washed two times with phosphate-buffered saline, 2% glutaraldehyde (18) was added to them, and the cells were scraped from bottles and held for about 1 hr in the cold. The cells were then centrifuged at a low speed for 5 min to form a pellet. The cell pel-

lets were postfixed for 1 hr at 4 C in buffered 1% osmium tetroxide solution (15), dehydrated in a graded series of alcohols, and embedded in epoxy resin (12). Thin sections were cut with glass knives by using a Porter-Blum ultramicrotome, stained with uranyl acetate (19) and lead citrate (17), and examined with a JEM-6S electron microscope. Uninoculated cultures were similarly harvested and processed and served as controls.

RESULTS

The virus titer in the supernatant fluid of cultures inoculated with EIA virus was 10^6 to $10^{6.5}$ $TCID_{50}$ per 0.5 ml, 3 and 4 days after inoculation, when the cultures were harvested for electron microscopy. Thin sections of cells in these cultures contained numerous particles presumed to be those of EIA virus. On the contrary, neither infectivity nor particles could be detected in uninoculated cultures or those inoculated with a mixture of EIA virus and EIA antiserum.

In infected cultures, virus particles were observed in membrane-bound cytoplasmic vesicles or vacuoles (Fig. 1, 2) or in extracellular spaces (Fig. 3, 4), but not free within the cytoplasm or nucleoplasm. Some of the extracellular particles were observed to lie free, but others were intimately associated with the external surface of the cell membrane (Fig. 3). Large numbers of the extracellular particles sometimes appeared to be caught in cellular debris (Fig. 4). The particles were circular or ellipsoidal in profile and varied in size, ranging from 80 to 120 nm. They were composed of a dense central nucleoid, viroplasm, and outer coat. The nucleoids showed considerable pleomorphism, although most of them were roughly circular in outline with sizes ranging from 40 to 60 nm. Elongated bar-shaped nucleoids, with a diameter of about 35 by 90 nm, were frequently seen (Fig. 4). Some particles were devoid of the nucleoid, but they were presumed to be produced by the plane of section which might exclude the part of the particles containing the nucleoid. The viroplasm was a moderately electron-dense zone separating the nucleoid and outer coat. In the majority of the particles, the nucleoid was separated from viroplasm by a narrow electron-transparent zone. At higher magnification, some particles exhibited a structure suggestive of inner particle shell around the nucleoid (Fig. 5, 6). The outer coat appeared to be a single limiting membrane and, in some instances, to be covered with thin surface projections (Fig. 5, 6).

Particles seen in the cytoplasmic vesicles or vacuoles were morphologically indistinguishable from those observed extracellularly. The vesicles or vacuoles in which the particles were lodged varied in shape and size and were set off from the surrounding cytoplasm by a membrane. Most of the vacuoles contained not only virus particles but also various amounts of cellular debris and amorphous material (Fig. 1, 2), suggesting that they were the result of phagocytosis by cells.

In addition to the virus particles described above, many particles in the process of budding were observed in infected cells (Fig. 7). Most of the buds were seen at the cell surface, and, on rare occasions, the particles budded into the cytoplasmic vacuoles from the bounding membrane. The viral buds possessed an outer coat continuous with the plasma membrane of the cell. Various stages of virus development were observed, and the earliest event appeared to be the development of a crescent. At this stage, the plasma membrane at the site of crescents increased in density and thickness and was associated with an outer layer of surface projections (Fig. 8). At higher magnification, an electron-dense membranous structure was recognizable beneath and along the bulging portion of the plasma membrane (Fig. 9). Some buds which seemed to be at advanced stages of maturation contained an electron-dense nucleoid and were connected with the cell surface by a narrow stalk (Fig. 7). Intracytoplasmic or intranuclear accumulations of what might be interpreted as viral internal components were not resolved in any of cells examined.

DISCUSSION

There are three reasons for believing that the particles which we have observed and described in the present study are EIA virus. First, the particles were found only in the cultures infected with EIA virus, but not in the uninfected controls. Second, in the cultures inoculated with neutralized virus, the production of infective virus was completely suppressed, and no particles could be detected by electron microscopy. Third, the particles were similar, in size and shape, to EIA virions observed in negatively stained preparations of purified samples (Nakajima et al., *unpublished data*).

The diameter of EIA virus measured in the present work is 80 to 120 nm. This value is slightly smaller than that of negatively stained particles of EIA virus (90 to 140 nm; Nakajima et al., *unpublished data*) and larger than that determined by ultrafiltration (60 to 95 nm; 11). This degree of disparity may be explained by the differences in the methods used.

The evidence obtained in this study indicates that EIA virus replicates primarily by a process of budding from the plasma membrane of infected cells. The virus particles within membrane-bound cytoplasmic vesicles or vacuoles are interpreted as those phagocytized by membrane invagination of cells after being liberated from some cells into the medium. This assertion is supported by the ob-

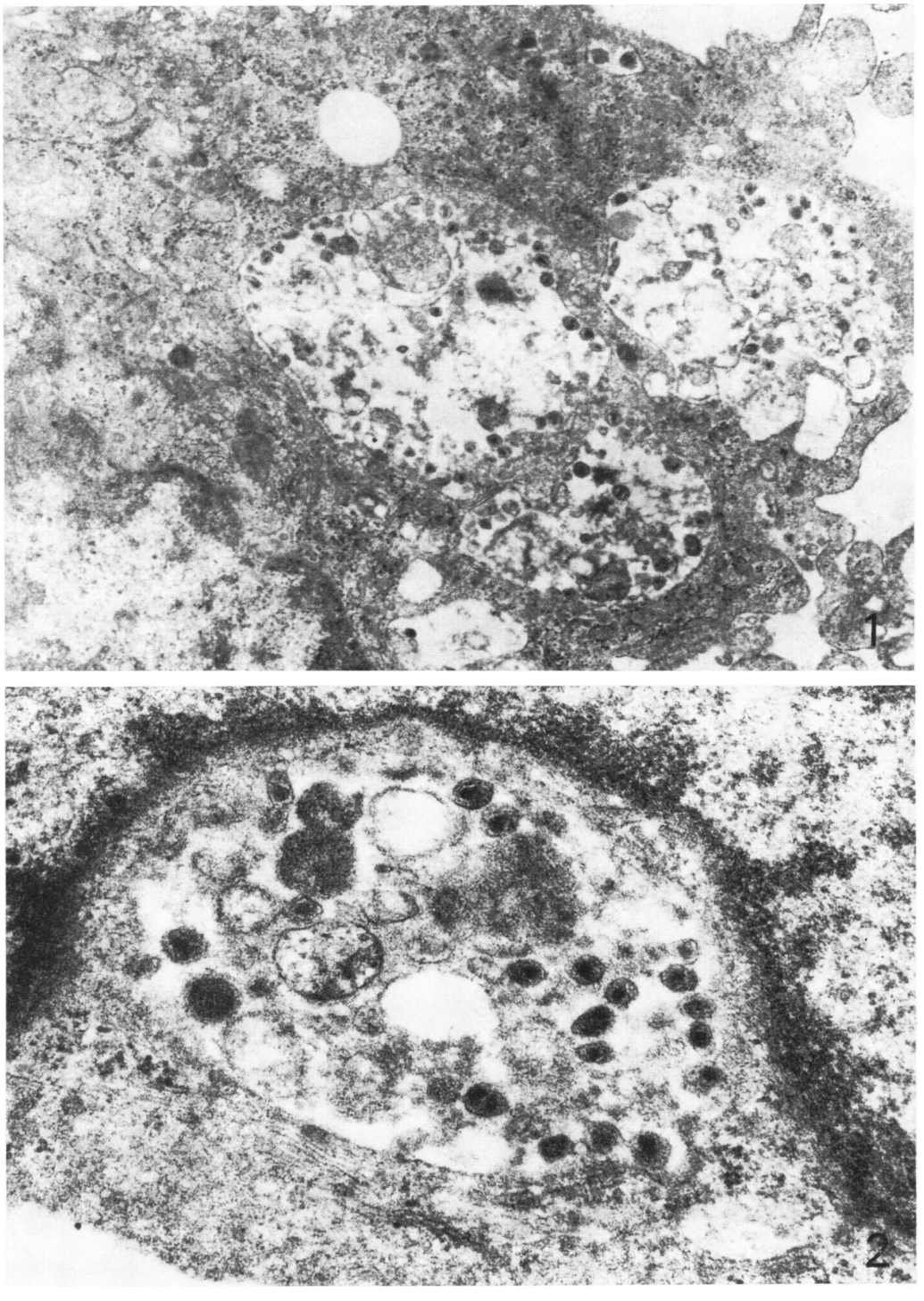

FIG. 1 and 2. *Cultured horse leukocytes 4 days after inoculation with EIA virus. Numerous virus particles and a variety of cell organelles are seen within membrane-bound cytoplasmic vacuoles which are presumably the result of phagocytosis by the cell. In Fig. 1, most particles are distributed around the periphery of the vacuoles. Fig. 1, × 20,000; Fig. 2, × 56,000.*

servation that, in spite of the presence of numerous virus particles in the vacuoles, virus production by budding at the vacuolar membrane was seen only in a limited number, and the vacuoles were filled with virus particles along with a variety of cell organelles and amorphous material. Since the cultured horse leukocytes studied in the present work had many irregular cytoplasmic extensions and deep indentations of the cell membrane, it is also possible that some virus-filled vacuoles represent cross sections of cytoplasmic invagination of the plasma membrane which communicate directly with the extracellular space.

Although the morphogenesis of EIA virus resembles that of the myxoviruses (1, 2, 6, 8, 13, 14, 16), viral internal fibrillar components characteristic of the latter were not seen in the cytoplasm subjacent to budding areas. Moreover, negatively stained particles of EIA virus did not contain helical internal components, and surface projections of EIA virions were not as prominent as those seen in the myxoviruses (Nakajima et al., *unpublished data*). The size, fine structure, and morphogenesis of EIA virus seem to bear a closer resemblance to the corresponding characteristics of viruses associated with avian leukosis (5, 7, 20) and murine leukemia (3, 4, 22) and of RE virus (21), although the inner particle shell was not prominent in EIA virus. Thus, EIA virus may be considered as belonging to a presently unclassified group of viruses. However, further information is

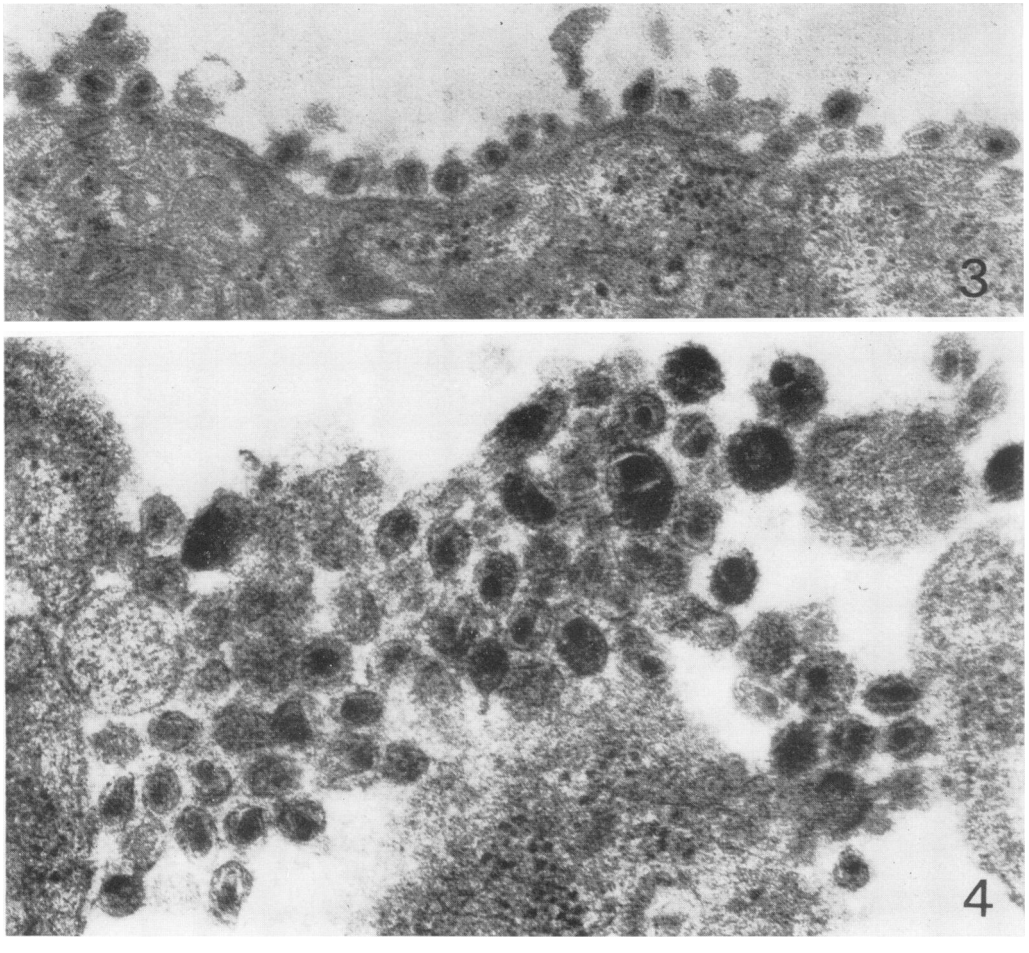

FIG. 3 and 4. *Cultured horse leukocytes 3 days after infection with EIA virus, showing extracellular virus particles. In Fig. 3, particles are seen in a row along the cell surface, and some of them are in intimate contact with the plasma membrane. Fig. 4 shows a group of particles apparently trapped in cellular debris. Nucleoids are variable in shape and size. Fig. 3, × 45,000; Fig. 4, × 72,000.*

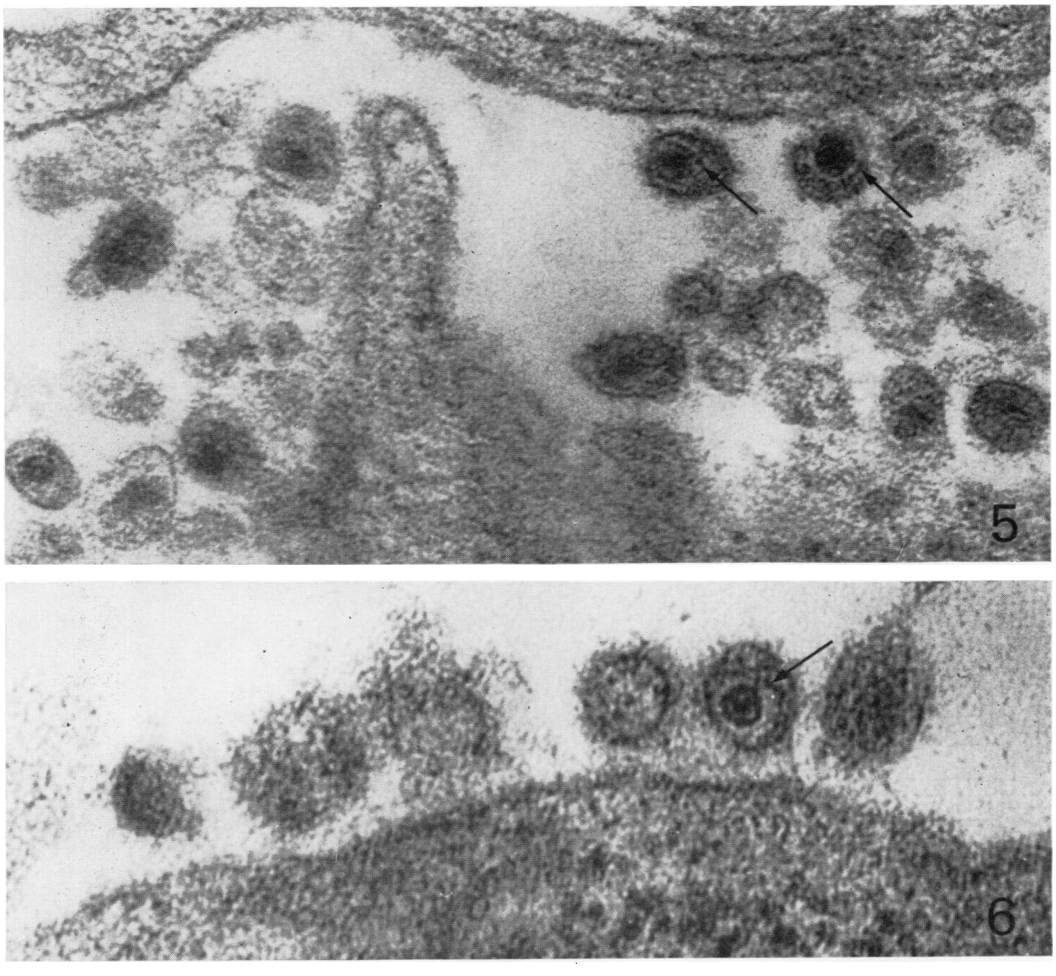

FIG. 5 and 6. *Cultured horse leukocytes 4 days after infection with EIA virus, showing extracellular virus particles at higher magnification. The outer coat of some particles appears to be covered with thin surface projections. In some particles, inner particle shell can be seen around the nucleoid (arrows). Fig. 5, × 120,000; Fig. 6, × 150,000.*

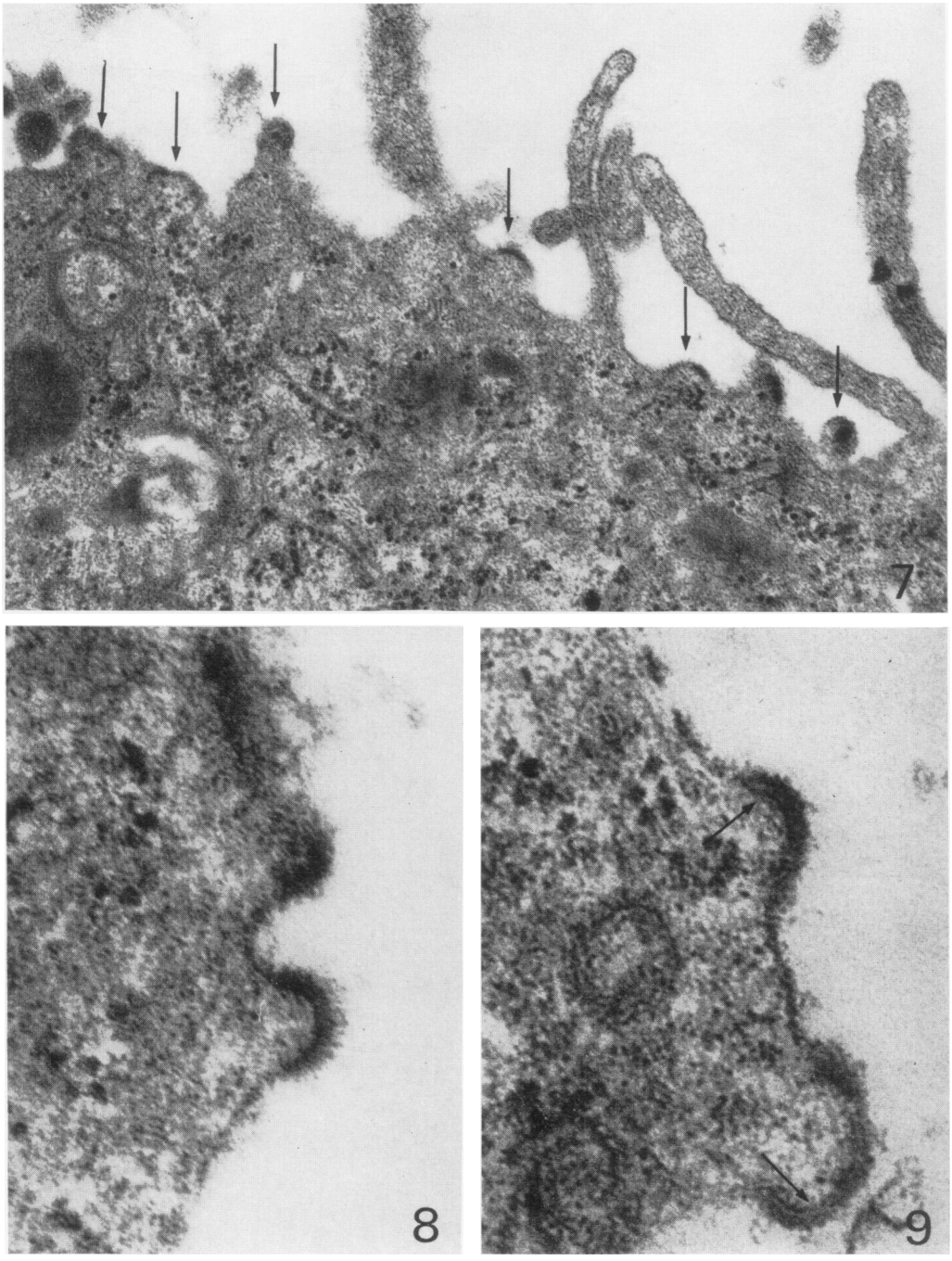

FIG. 7–9. *Cultured horse leukocytes 3 days (Fig. 7) and 4 days (Fig. 8 and 9) after infection with EIA virus, showing particles in the process of budding from the plasma membrane. In Fig. 7, the plasma membrane bulges outward at several points to form crescents, and bulging portions of it show increased electron density and appear to have been thickened (arrows). Two buds contain an electron-dense nucleoid. One bud in Fig. 8 evidently shows newly formed layer of surface projections on its outer edge. In Fig. 9, an electron-dense membranous structure is visible in parallel with the bulging portion of the plasma membrane (arrows). Fig. 7, × 50,000; Fig. 8, × 140,000; Fig. 9, × 150,000.*

necessary for recognition of its true position in virus classification.

ACKNOWLEDGMENTS

The authors express their appreciation to Y. Kono, National Institute of Animal Health, for providing the antiserum used in the present study.

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