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Hemadsorption of Mumps Virus Examined by Light and Electron Microscopy

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The hemadsorption (HAD) reaction of chick embryo cells infected with mumps virus was studied by means of light and electron microscopy, with special reference to the plasma membrane of the infected cell. The concomitant observation of membrane-free aggregates of viral nucleocapsid in the cytoplasm and attached red blood cells on the surface of the same cell indicated that only infected cells hemadsorbed and that hemagglutinin is confined within the infected cell. The attachment of red blood cells to morphologically intact cell membrane prior to its differentiation into viral envelope suggested that the HAD phenomenon, dependent on the presence of hemagglutinin, was independent of the viral maturation process. The gap of low electron density normally separating the morphologically intact membrane of the tissue culture cell and that of the red blood cell at the binding site was replaced by newly formed surface projections in HAD involving a segment of differentiated plasma membrane.

The hemadsorption (HAD) phenomenon was described in monkey tissue cultures infected with influenza A virus by Vogel and Shelokov in 1957 (17). The application of this reaction to tissue culture systems provided a useful tool for diagnostic studies of several other viruses (16) and for fundamental investigations of sequential events occurring on the surface of myxovirus-infected cells (13). Hotchin and co-workers observed, by means of electron microscopy, two types of red blood cell attachment and cytoplasmic changes in hemadsorbing monkey kidney cells infected with influenza A virus (10). In an ultrastructural study of the HAD of measles virus-infected cells, Baker and co-workers showed that the adsorbed erythrocytes were highly deformable (1). The purpose of the present study on the HAD induced by mumps virus was to examine ultrastructurally the infected cell membrane during the period of attachment of the red blood cell and after the induction of its elution. In an attempt to correlate the HAD phenomenon with different cellular changes following infection, the electron microscopic study was supplemented with a sequential light microscopic examination of the cultures.

MATERIALS AND METHODS

Cell and viruses. The Ricki strain of mumps virus at its ninth amniotic passage in chick embryos (CE) was used throughout the present study. To prepare inocula, samples of infectious amniotic fluid were clarified at 600 × *g* for 15 min. The resulting supernatant fluids were then pelletized by high-speed centrifugation (30,000 × *g*) for 60 min. A volume of balanced salt solution (BSS) equal to one-fifteenth of the original volume was added to each pellet after the removal of the clarified amniotic fluid. The container was then stored overnight for further softening of the pellet. On the next day, a homogeneous virus suspension was prepared by mixing the pellet and the added BSS. In a few experiments, infectious amniotic fluids were employed without being submitted to centrifugation.

CE fibroblast monolayers were prepared according to the method of Dulbecco (6). The embryos were derived from avian lymphomatosis virus-free eggs. The inoculation of the cultures and their maintenance throughout the study period were described elsewhere (5).

Cytology and electron microscopy. Monolayers grown on cover slips were washed in BSS, fixed in Formalin-Zenker solution, and then iron-hematoxylin stained.

For preparation of ultrathin sections, the monolayers grown in plastic petri dishes (Falcon Plastic, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) were washed with BSS and overlaid with 2 ml of a 0.6% suspension of chicken red blood cells in BSS. After 20 min at 4 C, the unadsorbed erythrocytes were removed by five washes with cold BSS. The cultures were fixed successively in chrome-osmium mixture and 0.5% aqueous uranyl acetate solution, scraped from the plastic surface, sedimented at 600 × *g* for 10 min, dehydrated with graded alcohol, and finally embedded in an Epon-araldite mixture (preparation type A).

The thin sections, double stained with uranyl acetate and lead citrate for 20 and 10 min, respectively, were examined in a Siemens Elmiskop I electron microscope.

To induce a partial elution of the red blood cells, the monolayer, with its overlaying fluid, was gently shaken for 5 sec at the end of the 20-min adsorption period. The eluted and unattached red blood cells were then removed by washing, and the monolayer was scraped from the plastic surface and sedimented, prior to its fixation in chrome-osmium mixture (preparation type B).

RESULTS

Light microscopic observations of the HAD pattern and associated cellular alterations. The earliest detectable changes observed in a CE fibroblast culture infected with mumps virus was the appearance of an HAD pattern. At an input multiplicity of approximately 9.5 plaque-forming units (PFU) per cell, numerous foci of hemadsorbing cells became apparent at 18 to 20 hr, and by 22 to 24 hr the entire monolayer hemadsorbed. The HAD reaction was markedly delayed when the input multiplicity was approximately 0.95 PFU per cell, and with an input multiplicity of 0.095 PFU per cell the HAD test became positive only at the end of the 3rd day. The HAD test remained positive through the 9th or 10th day when the monolayer became detached from the plastic surface. When the infected monolayer was gently washed after the 20-min adsorption period, clumps of red blood cells were generally observed on the hemadsorbed monolayer (Fig. 1). Complete spontaneous red blood cell elution occurred within the 12-hr period following the replenishment of the culture with maintenance medium and its reincubation at 37 C.

At an imput multiplicity of approximately 9.5 PFU per cell, cytological alterations revealed by iron-hematoxylin staining were observed at 38 hr. At this time, multinucleated cells of various sizes began to appear, and their number increased with time (Fig. 2). The nuclei of these cells were in general larger than those of the controls but, with rare exceptions, the number of nucleoli was limited to two per cell. In several of these syncytia, the nuclei were arranged in rows (Fig. 3). Eosinophilic inclusions, slightly smaller than most of the nuclei and surrounded by white halos, were observed in a small number of syncytia (Fig. 2, 4). At high magnification (Fig. 4), these ovoid inclusions showed areas having the size and the density of the nucleoli. At an input multiplicity of 0.95 PFU per cell or less, only rare multinucleated cells were observed. Interspersed with syncytia were elongated fibroblast-like cells with small and narrow nuclei (Fig. 2). By the 5th day, further cellular alterations became visible in unstained cultures inoculated with 0.95 PFU of virus per cell. These consisted of large masses of refractile amorphous materials interconnected by fine filamentous bridges. In the control cultures of the same age, these cytological changes were not observed.

Electron microscopic observations: association of HAD phenomenon and cytoplasmic viral nucleocapsid. In a previous communication, we described the accumulation, at the early stages of infection, of nucleocapsid (viral nucleoprotein) in the cytoplasm of mumps virus-infected cells prior to the differentiation of the plasma membrane into viral envelope (5). The differentiated membrane, consisting of an outermost layer of surface projections, a unit membrane with markedly increased electron density, and an inner layer of nucleocapsid strands aligned parallel to the cell surface were considered as the earliest detectable changes leading to formation of a viral bud (5). To determine whether a time relationship exists between the HAD phenomenon and the formation of cytoplasmic nucleocapsid, 200 CE cells of a culture having a moderately positive HAD reaction were examined at the 20th hr postinoculation. Inasmuch as the infected culture had been thoroughly washed to remove unadsorbed erythrocytes, a CE cell was considered to hemadsorb actively when a segment of its plasma membrane was in close contact with that of one or more red blood cells. It was found that 53 of the cells hemadsorbed; 48 of these (90.5%) contained nucleocapsid. Among the cells which failed to hemadsorb, only eight (5.44%) were found to carry nucleocapsid in their cytoplasm.

Morphological alterations of adsorbed erythrocytes. To avoid artificially induced red blood cell deformation which might be the result of processing procedures, the infected monolayer with adsorbed erythrocytes was fixed with a chrome-osmium solution prior to removal of the erythrocytes from the plastic surface. Under these experimental conditions, two types of red blood cell configuration were observed. The erythrocytes, in several instances, showed tentacle-like processes which were in close contact with cellular microvilli (Fig. 5) or occupied spaces existing between the latter (Fig. 6). However, approximately 90 to 95% of the red blood cells examined remained fairly undistorted and were attached to the tissue culture cell either over a considerable segment (Fig. 7, 8) or at several small sites (Fig. 10) of its plasma membrane. There were no unusual intracellular alterations in the adsorbed erythrocytes. The configuration of the nucleus remained grossly undeformed even in instances in which marked distortion of the red cell contour occurred.

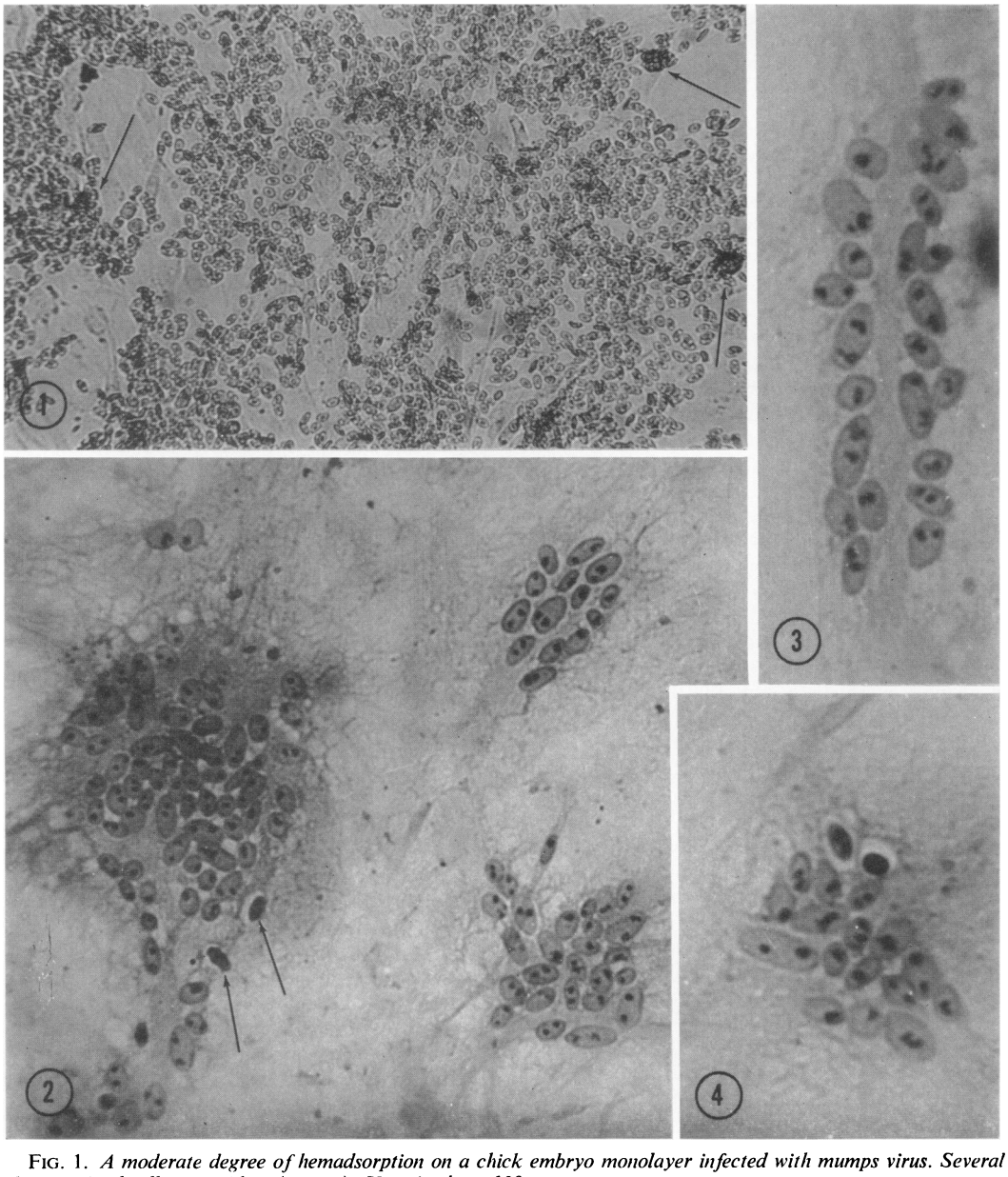


FIG. 1. *A moderate degree of hemadsorption on a chick embryo monolayer infected with mumps virus. Several clumps of red cells are evident (arrows). Unstained. × 100.*

FIG. 2. *Multinucleated giant cells in a chick embryo monolayer infected with mumps virus. Three small pycnotic nuclei, surrounded each by a white halo, are shown in the left lower quadrant of the micrograph (arrows). Note the presence of an elongated cell with a small and narrow nucleus in the right lower quadrant. Iron-hematoxylin stain × 400.*

FIG. 3. *Same culture as in Fig. 2. Note the linear arrangement of nuclei. Iron-hematoxylin stain. × 500.*

FIG. 4. *Same culture as in Fig. 2. A dark and small inclusion, surrounded by a white halo, is lying next to a small pycnotic nucleus. Iron-hematoxylin stain. × 500.*

In type B preparations, fine filamentous materials of various lengths (Fig. 11, 12) were often observed on the red blood cell surface lateral to the HAD site. As will be described in the next paragraph, the same filamentous materials were also encountered on the opposite side of the hemadsorbing CE cell. In contrast, this was not observed on the surface of red blood cells added to uninfected controls (Fig. 13).

Morphological alterations of hemadsorbing CE

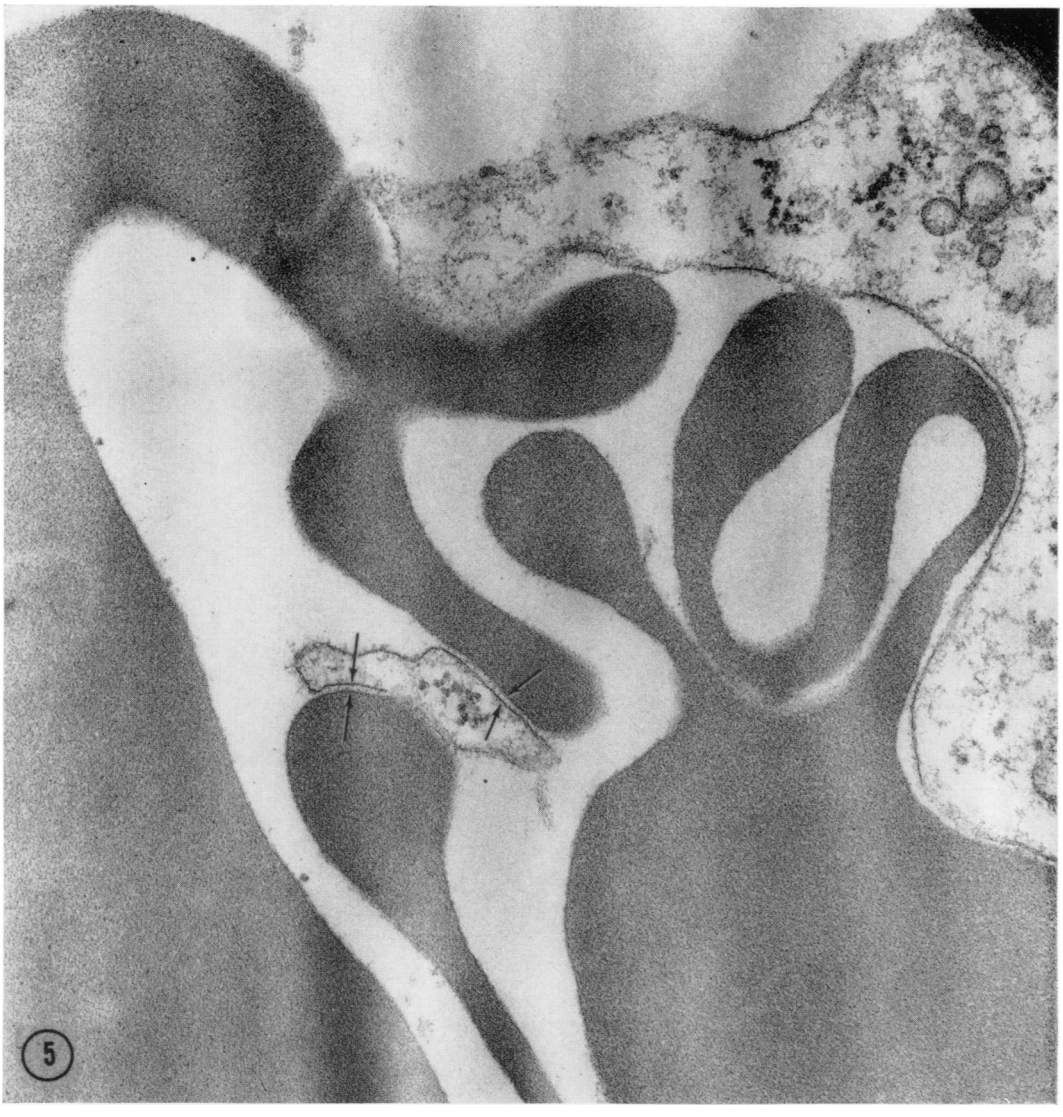


FIG. 5. *Portion of an adsorbed chick red blood cell showing tentacle-like processes. The presence of a gap of low density separating the unit membrane of the chick embryo cell and that of the red blood cell is indicated by arrows. Approximately × 52,500.*

cells. At an early stage of infection, most of the red blood cells were found attached to morphologically unaltered segments of CE cell membrane (Fig. 6, 7, 10). The surface of the red blood cell was separated from that of the CE cell by a gap of low electron density. Later, when most of the infected cells revealed large aggregates of cytoplasmic nucleocapsid, a type of HAD involving a red blood cell and a morphologically differentiated segment of a CE cell membrane was frequently encountered (Fig. 8, 11, 14). When the plane of section passed perpendicular to the surface of both red and infected CE cells at the site of HAD, thus revealing the double layer of the two apposed unit membranes, the surface projections of the differentiated plasma membrane appeared to be in direct contact with the red blood cell surface (Fig. 9). At this stage of infection, it was not uncommon to find the same red blood cell attached to viral particles and to CE cells (Fig. 15). The virion appeared either simply in contact with the red blood cell surface over a small segment of its outercoat (Fig. 16) or lodged in an invagination of the red blood cell mem-

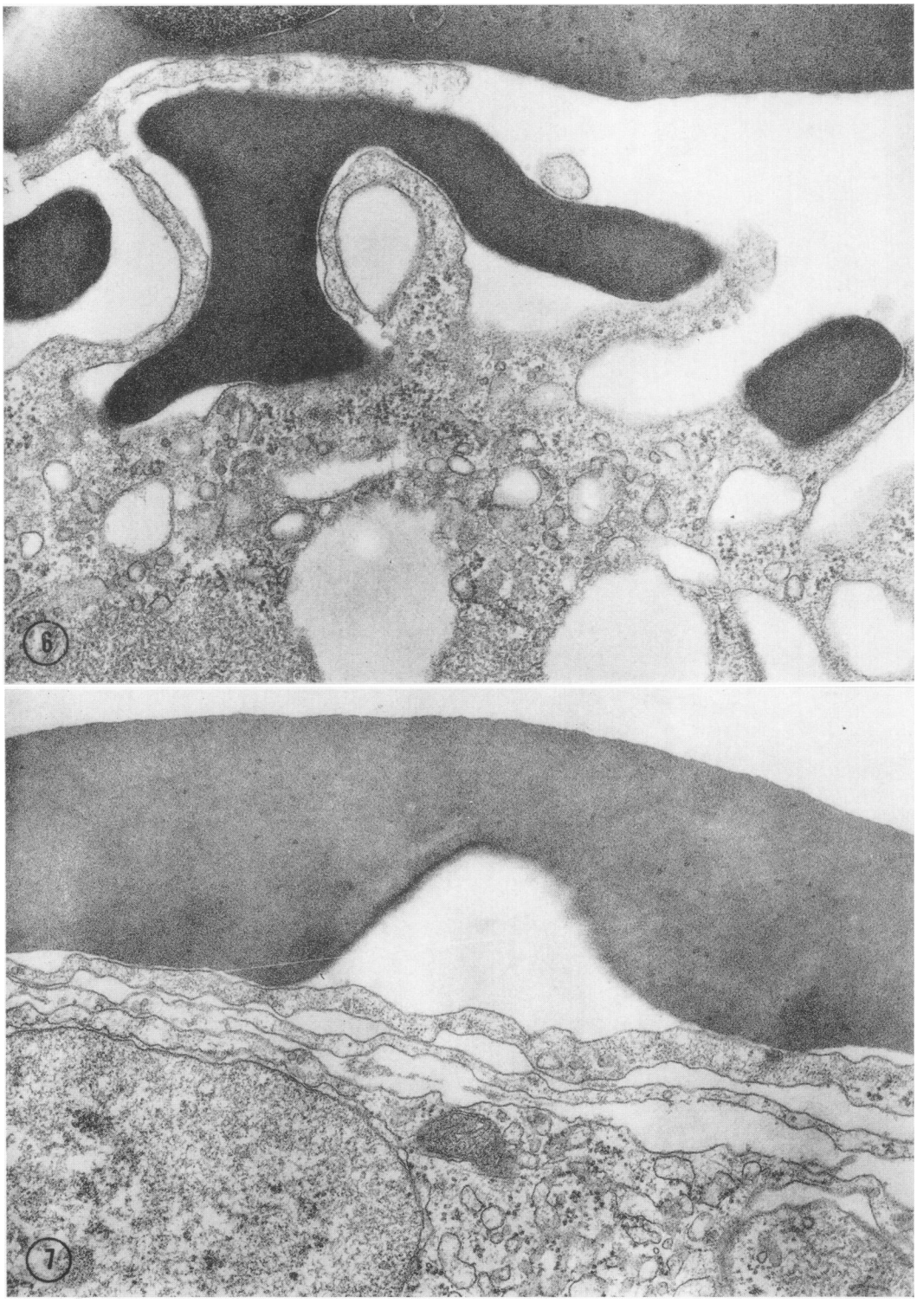
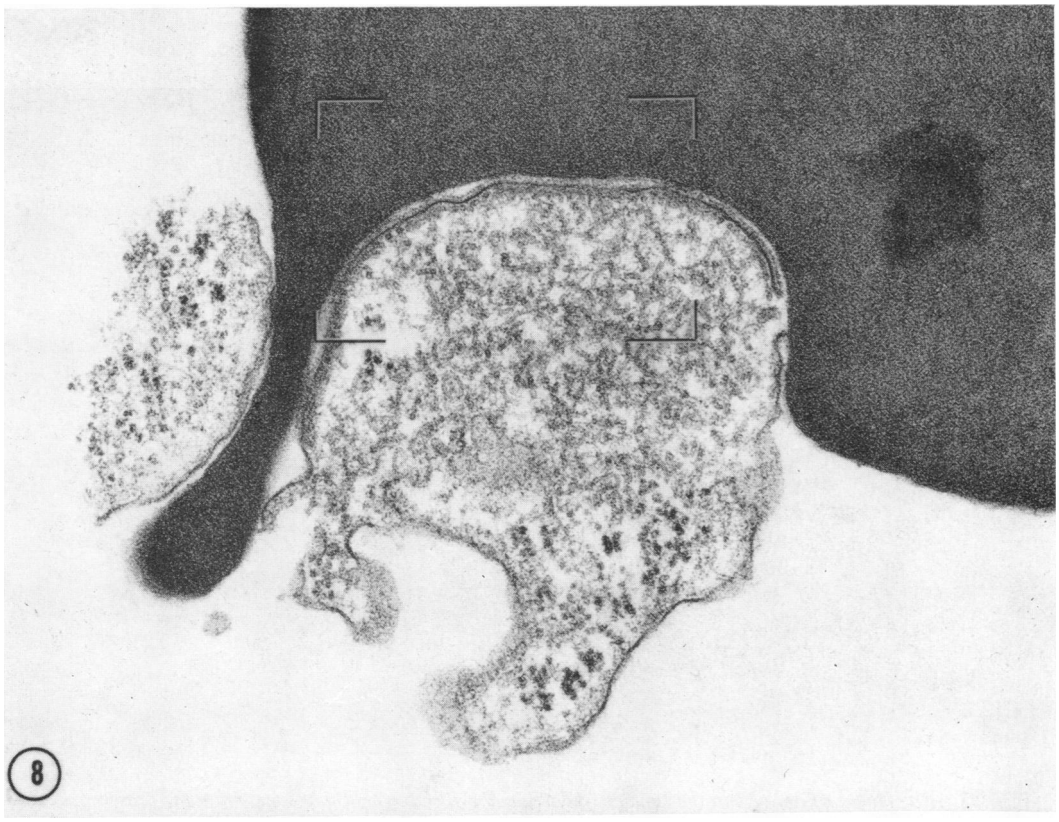


FIG. 6. *Attachment of red cell to microvilli of an infected CE cell. A nucleated red cell is partially shown in the uppermost part of the micrograph. The red blood cell cytoplasm occupies spaces existing between microvilli. The bottom half of the micrograph shows cytoplasmic vesicles, vacuoles, and nucleocapsid strands. Approximately* × *35,000.*

FIG. 7. *Attachment of red blood cell to chick embryo cell over a considerable segment of their plasma membranes. Note the intact appearance of the chick embryo cell membrane at site of hemadsorption. Approximately* × *35,000.*



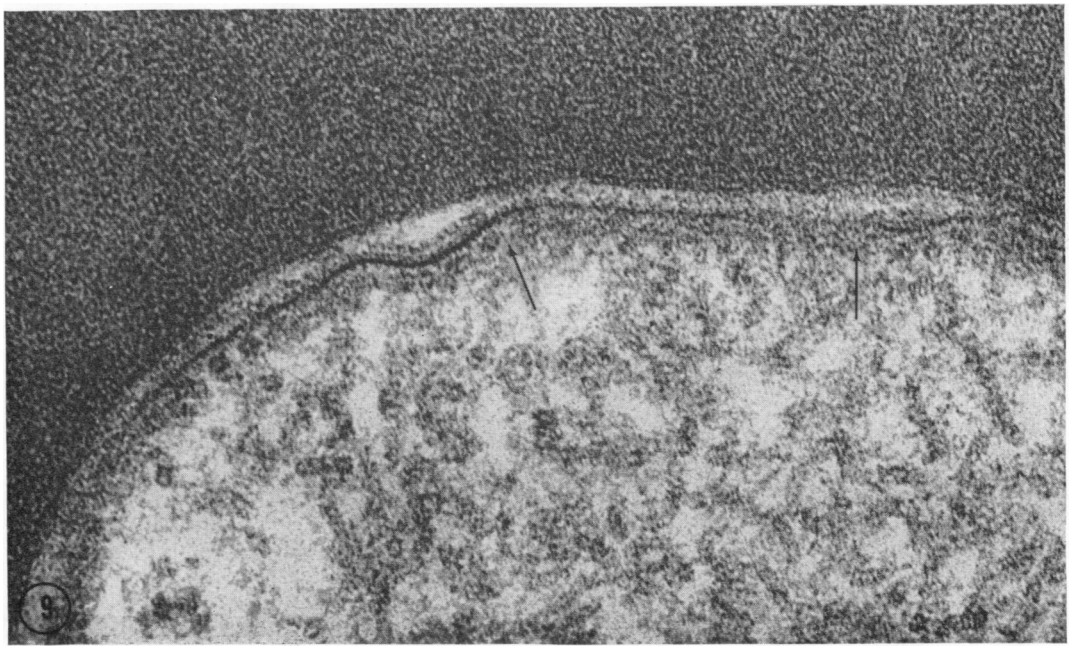


FIG. 8. *Attachment of a partially shown red blood cell to two fragments of chick embryo cell cytoplasm. On the left fragment, the plasma membrane appears morphologically intact. On the right fragment, the cellular membrane shows morphological differentiation. Approximately* × *52,500.*

FIG. 9. *Higher magnification of the area indicated in Fig. 8. The plane of section passes perpendicular to the surface of both red and chick embryo cells. The surface projections of the morphologically differentiated membrane are in direct contact with the red cell membrane (arrow). Approximately* × *150,000.*

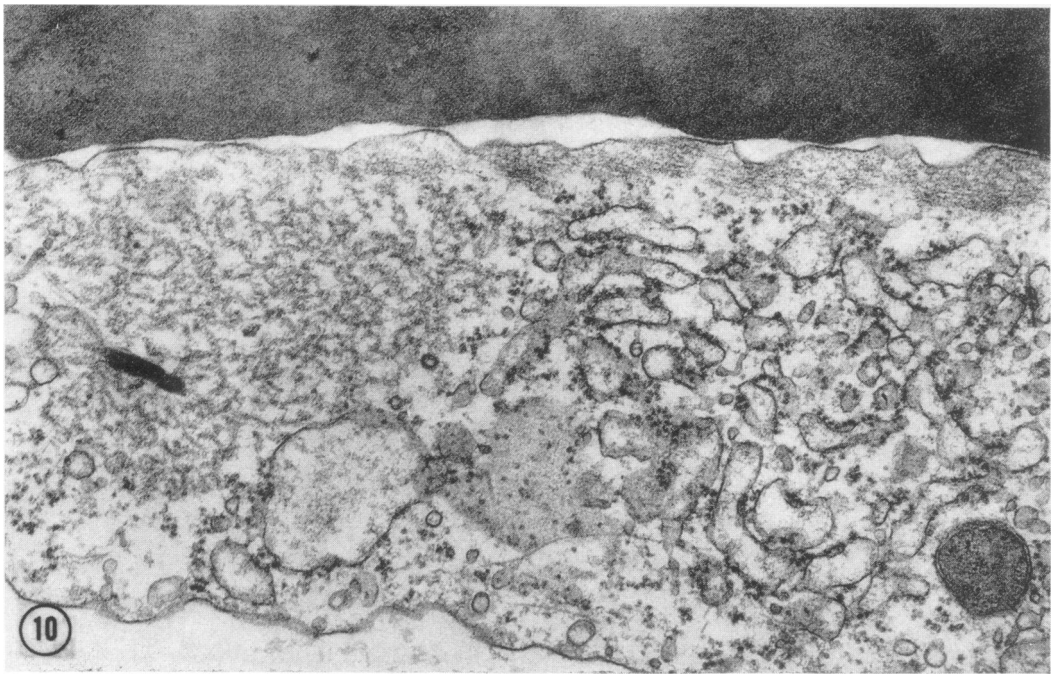


FIG. 10. *Attachment of red cell to the surface of chick embryo cell at multiple sites. Note the presence of a gap between the unit membranes at these sites. An aggregate of cytoplasmic nucleocapsid strands are evident on the left half of the micrograph. Approximately* × *40,000.*

brane. Filamentous materials described for partially eluted red blood cells were also encountered on the CE cell surface lateral to the HAD site (Fig. 11, 12). In contrast, these materials were not observed on the surface of either type of cells after spontaneous red blood cell elution. Tearings of the plasma membrane of CE cells were frequently observed at sites of red cell attachment (Fig. 17, 18). The segments of membrane lying between two tearings, together with subjacent cytoplasmic materials, were in most instances pulled toward the red blood cell (Fig. 17, 18) and remained attached to the latter when erythrocytic elution occurred (Fig. 19). These tearings, frequently encountered in type B preparations, but rarely in type A preparations, involved morphologically intact, as well as differentiated, plasma membranes (Fig. 17, 18).

DISCUSSION

The cytopathic effect induced by the Ricki strain of mumps virus was very similar to that described for the Enders strain (7). The only difference was the absence of extensive cellular destruction in CE cultures inoculated with large doses of the former. This was probably due to the small number of passages of the virus in embryonated eggs. The size, the shape, the density, and the localization of the eosinophilic inclusions described here and previously reported by Gresser and Enders (7) suggested that the inclusions represented nuclei undergoing degeneration. These isolated inclusions did not seem to represent the aggregates of cytoplasmic nucleocapsid observed by electron microscopy (5), for the aggregates were not ovoid in shape and were found in almost all infected cells. Although the formation of syncytium has been observed with the larger paramyxovirus group, including parainfluenza virus (14, 15), Newcastle disease virus (11), and measles virus (3), the linear arrangement of nuclei has been described previously only for parainfluenza virus SV5 (9).

The ultrastructural survey revealed that 90.5% of the hemadsorbing cells contained cytoplasmic nucleocapsid, and only a small fraction (5.44%) of the nonhemadsorbing cells carried this nucleoprotein. Since HAD areas or nucleocapsid aggregates were not expected to be always on the plane of section, the result strongly indicated that only infected cells with identifiable viral nucleoprotein hemadsorbed.

In the present mumps virus-CE system, only a small percentage of adsorbed chick red blood

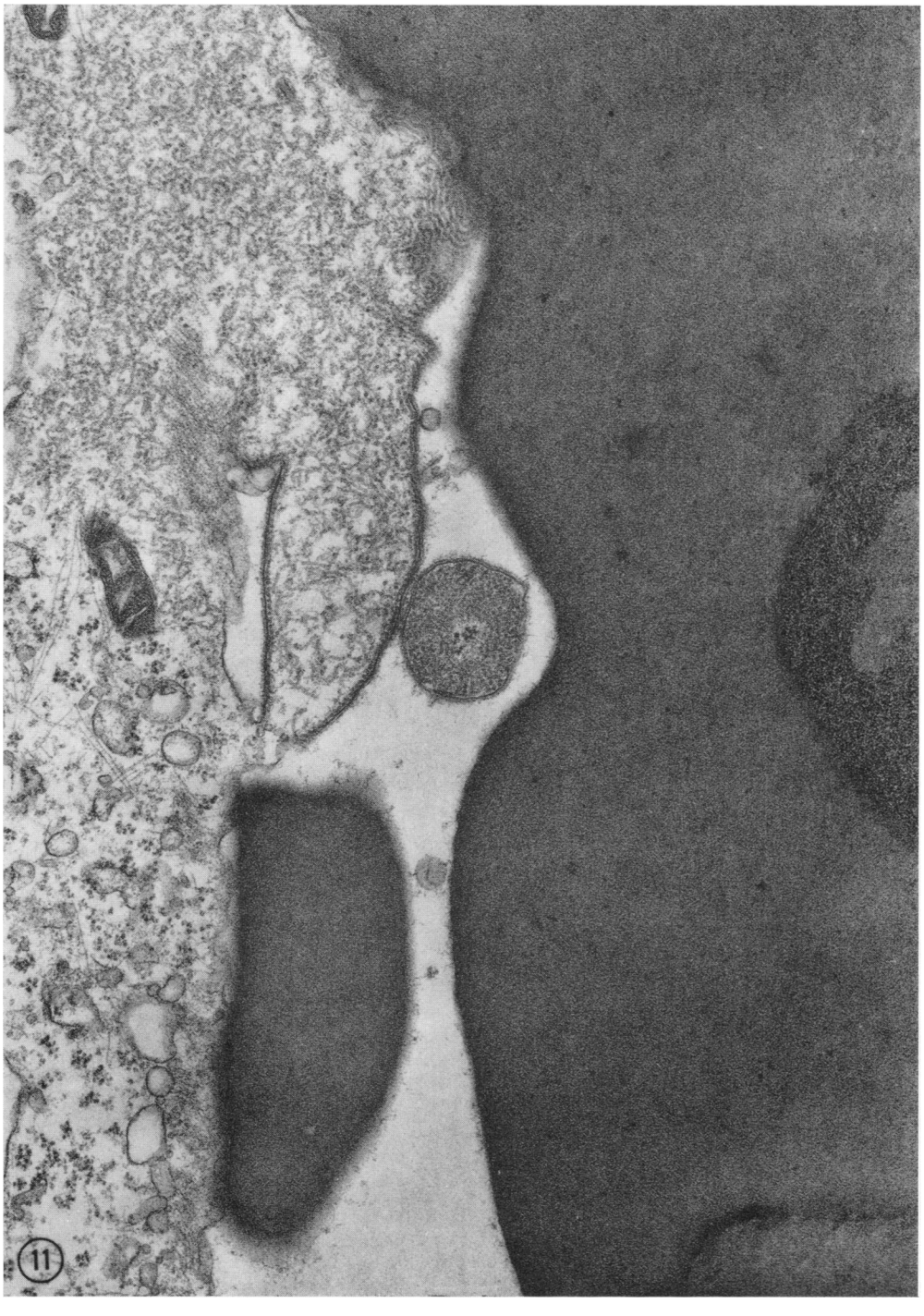


FIG. 11. *Attachment of nucleated red blood cell to chick embryo cell surface at two sites. The cell membranes cross the micrograph from the top to the bottom. The upper attachment site involves a differentiated chick embryo plasma membrane; the lower one, an intact segment of the same membrane. A viral particle (in the center) which is in contact with the differentiated membrane appears separated from the red blood cell surface on the plane of section. Note the presence of filamentous materials on the surface of red blood cells. Approximately* × *35,000.*

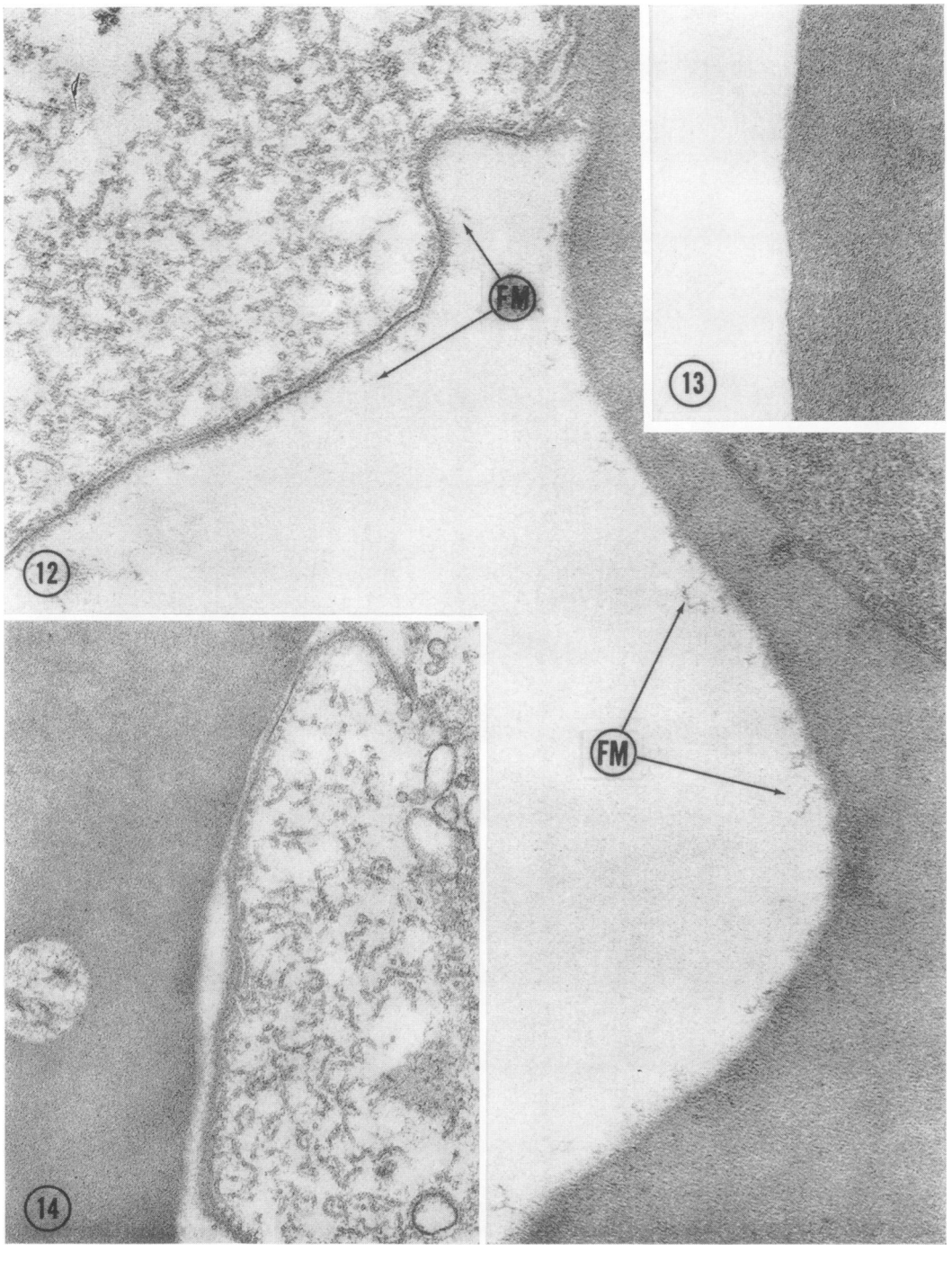


FIG. 12. *Red cell partially eluted from chick embryo cell. On the upper left part of the micrograph, a portion of chick embryo cell cytoplasm with nucleocapsid strands and differentiated membrane is still bound to a red blood cell partially shown on the right. Note the presence of filamentous materials on opposite surface of both cells. Approximately* × *75,000.*

FIG. 13. *Absence of filamentous materials on the surface of red cells added to uninoculated control chick embryo cultures. Approximately* × *45,000.*

FIG. 14. *Attachment of a red blood cell to a segment of differentiated CE plasma membrane at two sites. The plane of section passes slightly outside the lower binding site. Approximately* × *52,500.*

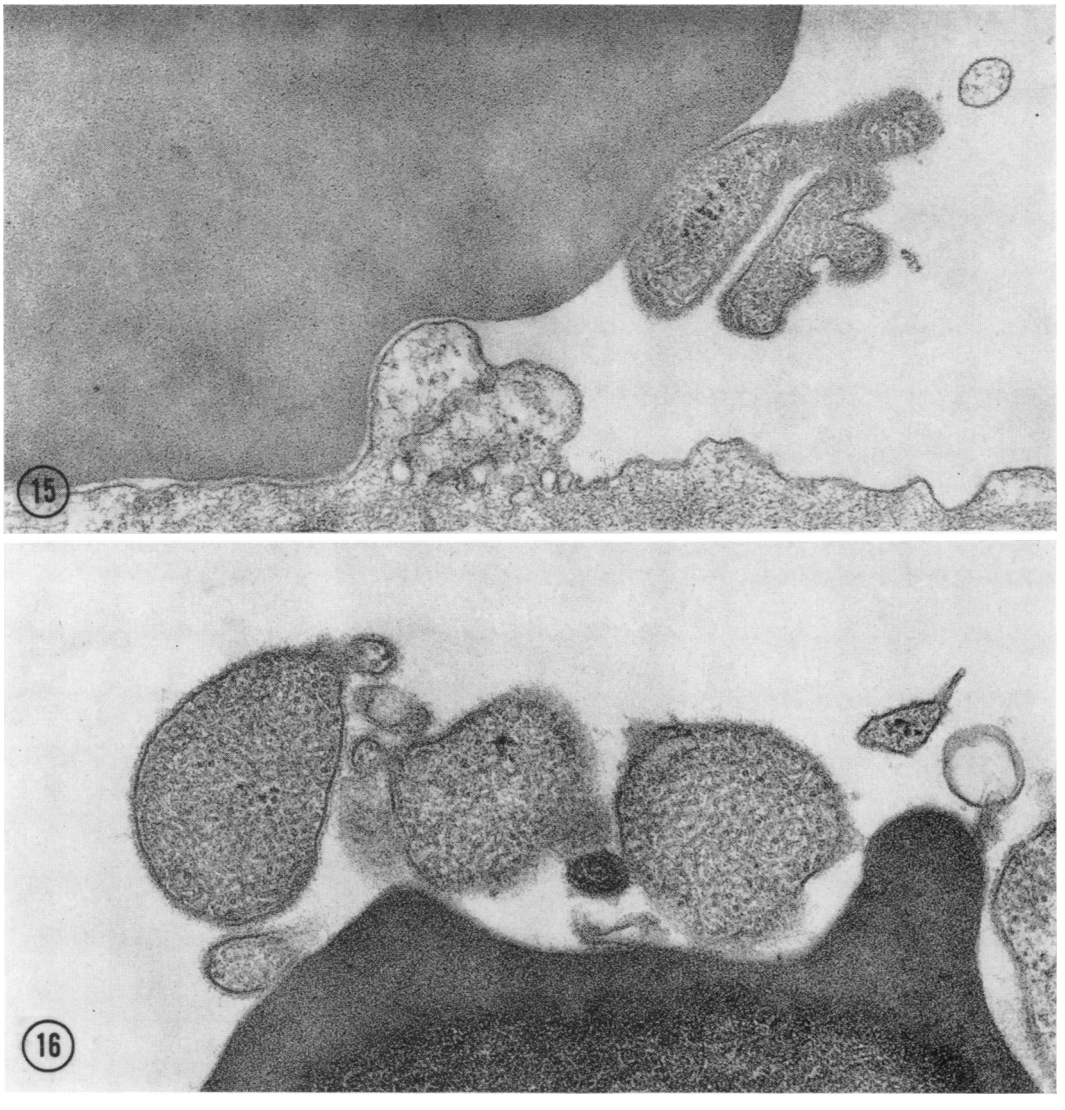


FIG. 15. *Attachment of the same red blood cell to the membrane of a chick embryo cell and to a viral particle. Approximately* × *52,500.*

FIG. 16. *Adsorption of viral particles to a nucleated red blood cell. After five washings, the virions still remain attached to the surface of the red blood cell over a small segment of their outercoat. Approximately* × *52,500.*

cells exhibited contour distortion. This is in contrast to the observation of Baker et al. (1) on the HAD of measles virus-infected FL amnion cells. These investigators reported that the monkey red blood cells were usually found "wrapped around" microvilli of infected cells but remained undistorted in control cultures following centrifugation. Probably the absence of nuclei in the monkey red blood cell was responsible, at least in part, for its extreme deformation, which could be readily demonstrated in the presence of host cells with numerous microvilli.

Heretofore, all electron microscopic studies of the HAD reaction had been carried out during the binding phase (1, 2, 10). The present examination of the HAD site after a mechanically induced, partial elution of the red blood cells has yielded interesting data. The tearing involving the plasma membrane and the cytoplasm of infected cells at the site of HAD, frequently encountered in type

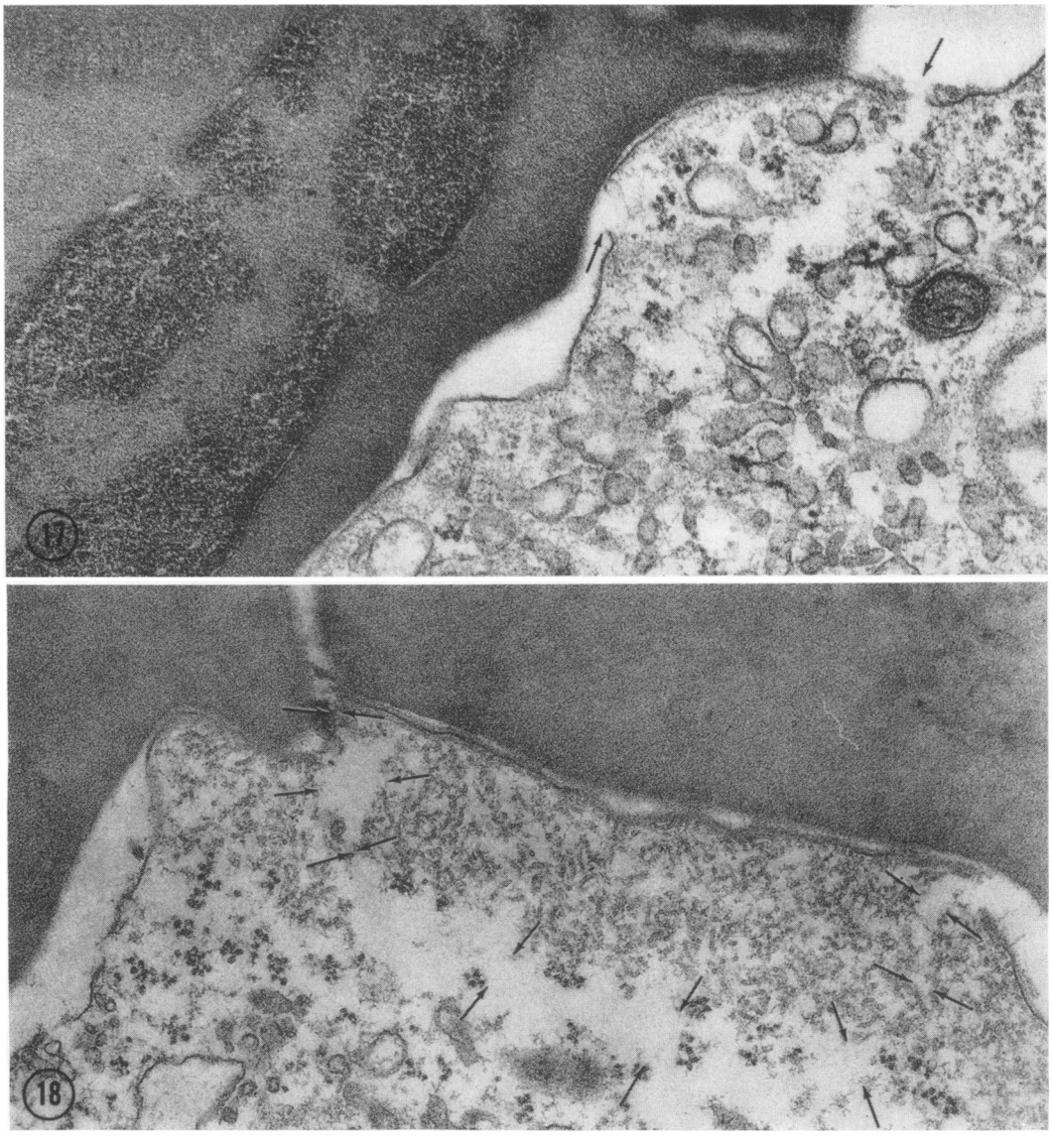


FIG. 17. *Rupture (arrows) of morphologically intact chick embryo membrane and separation of resulting fragment of cytoplasm firmly attached to displaced red blood cells. Approximately × 50,000.*

FIG. 18. *Rupture of cytoplasmic membrane occurring at site of hemadsorption, involving two red blood cells. The firm attachment of the red blood cells to the differentiated segment of chick embryo membrane is evidenced by the two ruptures in the latter and the displacement (arrows) of a fragment of cytoplasm containing nucleocapsid strands. Approximately × 45,000.*

B preparations, indicated that a substantial binding force existed between the red blood cell and the host cell. This force was not overcome by 5 sec of gentle shaking of the flask followed by five additional washings. The filamentous materials observed on the surface of partially dissociated red cells and on that of infected cells could result from the attachment of cell debris to the cell surface. However, this was very unlikely, for these materials were limited to certain areas on the cell surface. Since these filamentous materials were consistently observed on opposing surfaces and

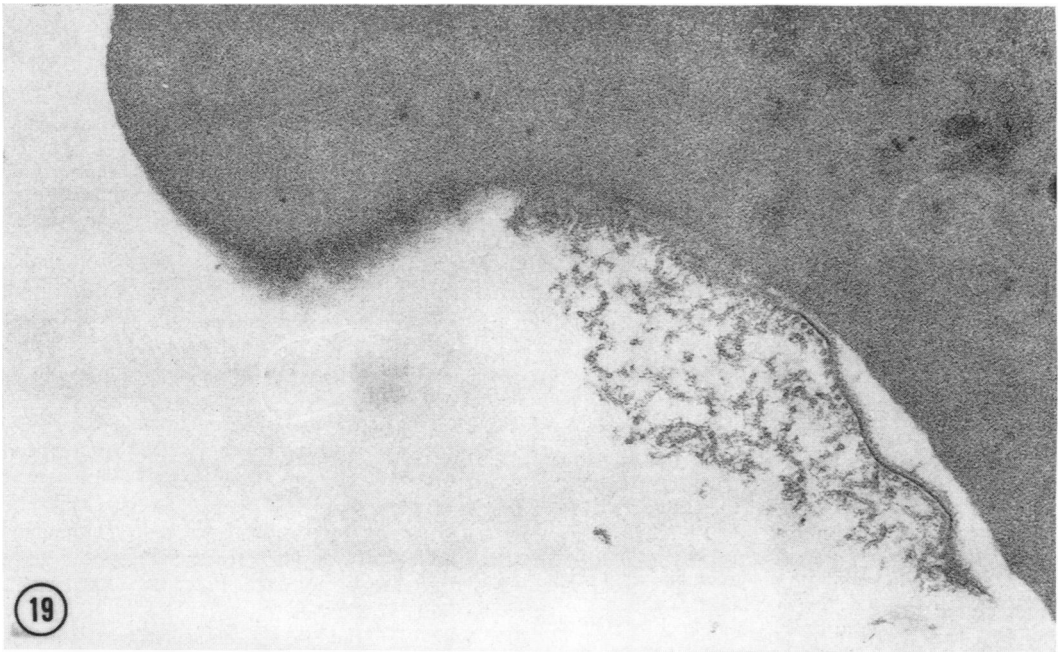


FIG. 19. *Attachment of a fragment of cytoplasm with differentiated membrane to red blood cell surface. The red blood cell was recovered from the wash fluid of a hemadsorbed culture. Approximately* × *52,500.*

were almost always adjacent to the binding region, they probably represented some sort of disrupted link existing between the two cells. The absence of these filamentous materials on the surface of cells after spontaneous red cell elution suggested that their destruction leads to the liberation of the latter. The question of whether the filamentous materials resulted from stretched and ruptured surface projections or whether they represented undigested red cell receptors which were normally destroyed by an enzyme associated with the virus (8) remained unanswered.

The adsorption of red blood cells not only to morphologically differentiated plasma membranes but also to intact ones indicated that the HAD reaction is solely dependent on the presence of hemagglutinin which migrates from the Golgi region to the cell surface (12, 13). This observation, which suggests that HAD is a separate phenomenon independent of the viral maturation process, is not in agreement with that of Berkaloff and Colobert (2) on the HAD of calf kidney cells infected with *Myxovirus influenzae* B (Lee strain). According to these investigators, the HAD phenomenon occurs exclusively in the presence of morphologically differentiated plasma membrane, and the peripheral accumulation of hemagglutinin is directly related to this morphological alteration.

The gap separating the morphologically intact CE cell surface and that of the erythrocyte was previously described for the measles virus-FL amnion cell (1) and mouse pneumonia virus-BHK21 cell (4) systems. Similar observation was made in the present study. However, when the HAD involved a segment of morphologically differentiated plasma membrane, as frequently encountered in the present study, the inherent surface projections were in direct contact with the red blood cell unit membrane and a gap was not observable. Such an intimate contact was also observed between the red blood cell and the spikes of the mumps virion. These data suggest that the surface projections of the differentiated membrane and viral spikes are "bathed" in a layer of proteinlike material, possibly mucoprotein (1), which is normally present on the surface of the infected cell.

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