Intracellular Development and Mechanism of Hemadsorption of a Human Coronavirus, OC43 (36243)

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The coronaviruses are a group of medium-sized, RNA, enveloped viruses (1), which include avian infectious bronchitis virus (IBV), mouse hepatitis virus, and two groups of human respiratory viruses: the "obligate organ culture" group originally isolated only in organ cultures and consisting of the B814 strain of Tyrrell and Bynoe (2), six strains isolated at the National Institutes of Health (3–7), and strains LP and EVS (8–11), and the "229E-like" (12, 13) group originally isolated in monolayer tissue cultures. The coronaviruses are distinguished from the myxoviruses both by the structure of the virion and the way in which it is formed. The virion of a coronavirus carries on its surface a series of 200 A "drumstick" projections which are larger, and more widely spaced than the 70–100 A "spikes" of the myxoviruses. In negatively stained preparations, the surface projections of coronaviruses resemble a coronet, or crown, from which the name of the group is derived (*L. corona*, a crown). The intracellular development of three coronaviruses, IBV, 229E (14, 15), and MHV (16) has been studied, and all mature by budding into vesicles in the cytoplasm. This is in contrast to the myxoviruses, which bud from the external cytoplasmic membrane of the infected cell (17). During myxovirus replication, large areas of the external cytoplasmic membrane carry viral hemagglutinin, and red blood cells will adsorb to the surface of infected cells (18).

Until recently, the only coronavirus shown to carry a hemagglutinin in the virion was IBV, and this was only functional after the virions had been treated with trypsin or ether (19, 20). However, one of the NIH organ culture coronaviruses, OC43 (3), which has been adapted to grow in tissue culture (7), has been shown to hemagglutinate rat red blood cells directly without chemical activation (21). Furthermore, BS-C-1, rhesus monkey kidney, human embryonic kidney, and human diploid cell strain (HDCS) WI38 monolayer cultures infected with OC43 virus were found to adsorb rat red blood cells (22).

The findings that OC43-infected cells hemadsorb, is unexpected, since, if this virus develops in the same way as IBV and 229E by budding into cytoplasmic vesicles, one would not expect the surface of the infected cell to contain hemagglutinin, which would enable red cells to attach.

The present work was undertaken first, to study the intracellular development of the NIH organ culture coronavirus, OC43, for comparison with the development of IBV, 229E, and MHV, and second, to understand the mechanisms whereby cells infected with OC43 are able to adsorb rat red blood cells.

Materials and Methods. Viruses. The 229E coronavirus, kindly supplied by Dr. D. Hamre (12), and the HDCS WI38 tissue culture adapted coronavirus OC43 which was available from previous studies in the laboratory (22), were both maintained by serial passage in WI38 cells.

Virus growth curves. WI38 tubes were inoculated with approximately 10^6 TCD_{50} of OC43 or 229E, which was allowed to adsorb at room temperature for 2 hr. Tubes were washed 6 times with Hanks' saline and then

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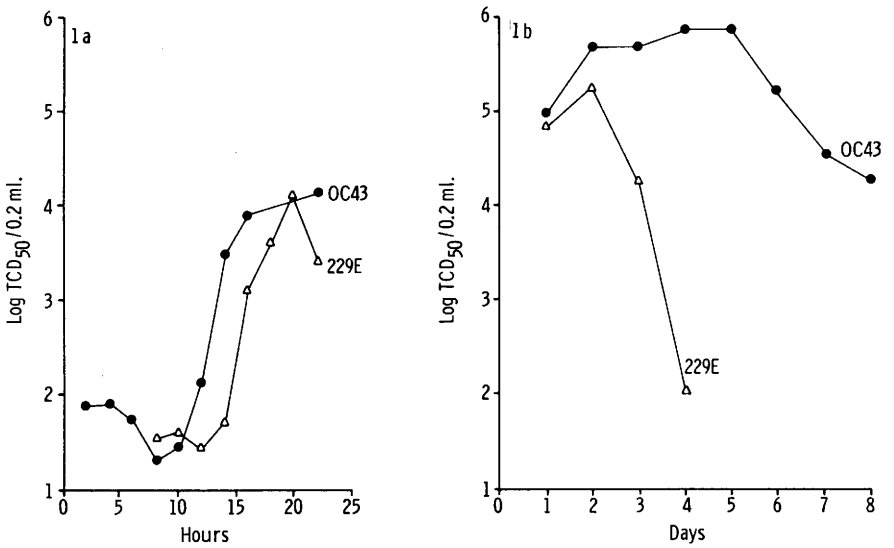


FIG. 1. The growth of OC43 and 229E in W138 cells at 33°: (a) during the first 24 hr; (b) between 1 and 8 days after infection.

changed onto a mixture of half Eagle's minimal essential medium in Earle's BSS and half medium 199 in Hanks' BSS containing 2% fetal calf serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin. Fluids were harvested at suitable intervals, and virus infectivity was titrated in WI38 tube cultures.

Preparation of specimens for electron microscopy. WI38 tube cultures were infected with approximately 10^6 TCD_{50} of either 229E or OC43, which was allowed to adsorb at room temperature for 2 hr. Tubes were then incubated on a roller drum at 33°, and at suitable times later, groups of five tubes were washed twice with Tyrode's solution, fixed for 30 min with 1% glutaraldehyde at pH 7.2, and washed 5 times with isotonic phosphate buffer at pH 7.2. Cells were gently scraped from the glass, pooled and pelleted at 2000 rpm. The pellet was fixed in 1% osmium tetroxide for 1 hr, dehydrated through graded alcohols, and transferred to propylene oxide. The pellet was embedded in Epon resin according to the method of Mollenhauer (23). After sectioning, ribbons were stained first with 1% uranyl acetate, and then Reynolds' lead citrate (24). Sections were examined in a Siemens Elmiskop 1A.

Rat red cells agglutinated with OC43 were prepared for sectioning by the same general method. Negatively stained grids of OC43 and 229E were prepared by placing drops of virus suspension on Formvar-coated copper grids, blotting off the surplus fluid, and staining with 1% phosphotungstic acid at pH 5.2.

Results. The growth of OC43 and 229E in WI38 cells. The infectivity of the virus seeds which were available was not high enough to permit simultaneous infection of all cells in a tube culture. However, the first cycle of virus growth was discernible from the first part of the growth curves. Virus progeny appeared in the culture fluids after 13 hr with OC43 and 15 hr with 229E (Fig. 1a). These progeny infected the remaining cells, which continued to produce virus for over 6 days with OC43, and 3 days with 229E. No cytopathic effects were seen until 5–6 days with OC43, and 3–4 days with 229E. The peak virus titers in the culture fluids were about the same for each virus (Fig. 1b).

The intracellular development of OC43 in WI38 cells. During the first 18 hr after infection, it was diffcult to find intracellular structures in electron micrographs which could be unequivocally associated with virus growth. However, between 18 and 24 hr the production of virus by infected cultures increased greatly, and most cells showed structures which appeared to be related to virus

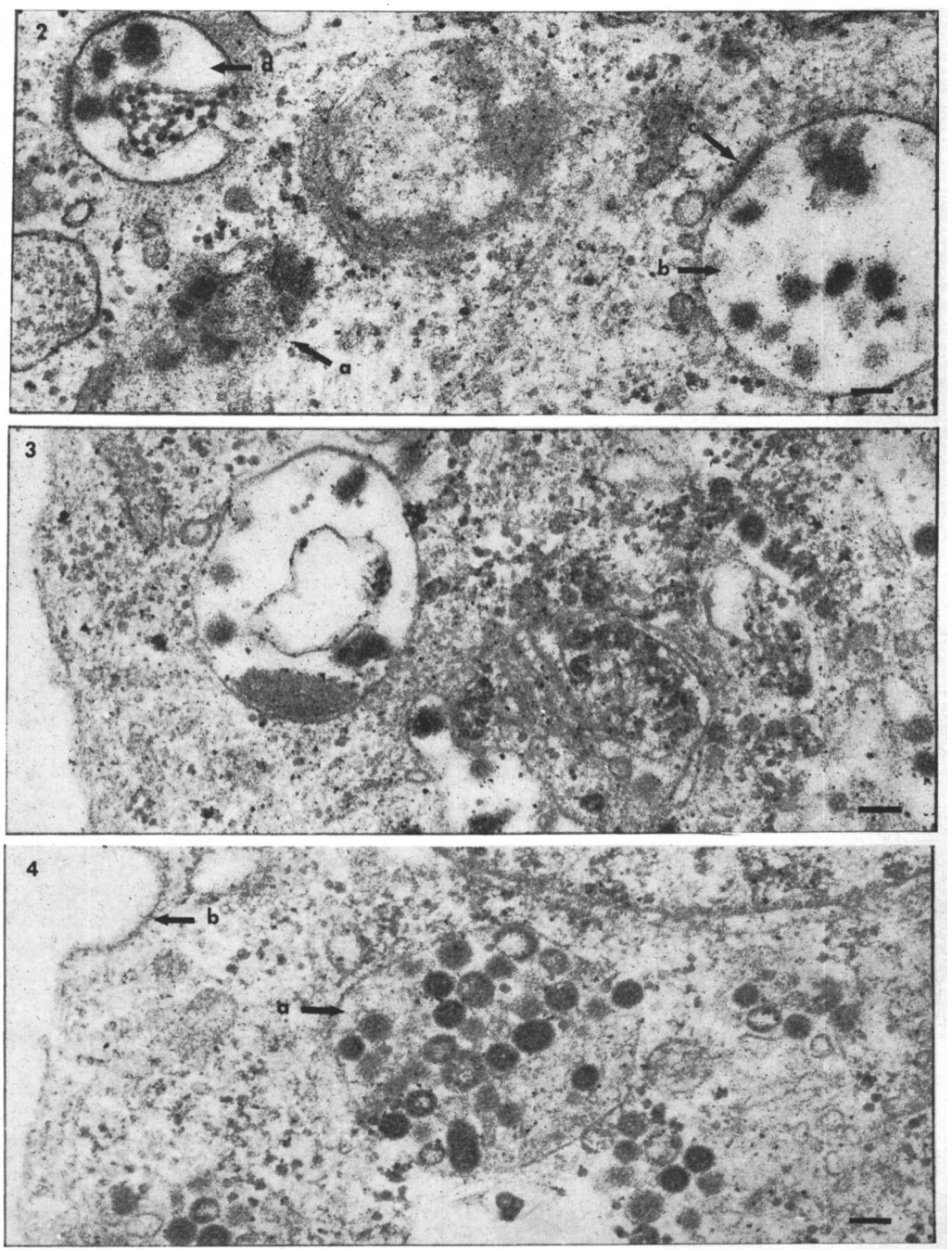


FIG. 2. OC43-infected WI38 cells 48 hr after infection: (a) early vesicles with poorly defined (virus?) particles; (b) later vesicle containing virus particles; (c) thickening of vesicle wall—possibly a site of virus budding; (d) vesicle in formation with virus particles; bar = 100 nm.

FIG. 3. OC43-infected WI38 cells 60 hr after infection: "doughnut" vesicle containing viruses between concentric membranes; bar = 100 nm.

FIG. 4. OC43-infected WI38 cells, 72 hr after infection: The cytoplasm contains vesicles (a), which enclose a number of virus particles. An empty ruptured vesicle (b) is shown at the cell surface; bar = 100 nm.

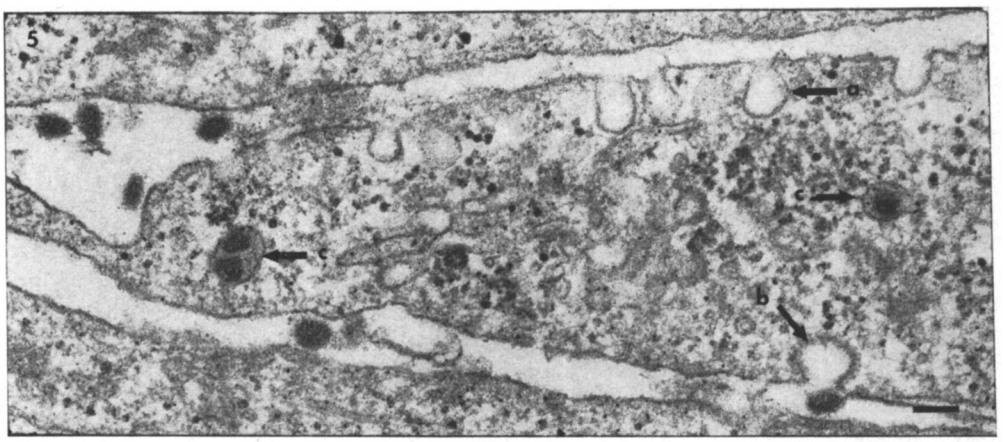


FIG. 5. OC43-infected WI38 cells 36 hr after infection: A cell showing many empty ruptured vesicles at the cell surface (a); a vesicle from which a virion is just escaping (b); also virions enclosed in vesicles within the cytoplasm (c); bar = 100 nm.

morphogenesis.

Virus-associated structures were first seen in cells 12–18 hr after infection. These were poorly defined vesicles containing a fairly dense amorphous material, in which recognizable virus particles were occasionally seen. With time, these vesicles increased in number and size, and contained more virus particles and less amorphous material. Vesicles of both types could be seen together in cells between 24 hr and 72 hr after infection (Fig. 2).

Virus particles were approximately 100 nm in diameter, and were often seen to be surrounded by a halo of fine radiating threads which seemed to be more like a coating of the amorphous material which originally filled the vesicle than part of the virion structure. Many of the vesicles looked like "doughnut rings," with virus particles in the space between the concentric membranes (Fig. 3). Figure 2 shows one of these "doughnuts" in formation. A spherical vesicle formed from a point on the endoplasmic membrane system and grew back enclosing a projection of cytoplasm. When such a structure was cut transversely, it produced the characteristic "doughnut" vesicle seen in many preparations. These cytoplasmic vesicles contained a variety of virus, and virus-like particles. These particles ranged from 60 to 130 nm in diameter and were either completely, or partly filled with an electron-dense material, or else they were empty (Fig. 4). Occasionally, a vesicle could be found which contained mostly empty particles. Despite an intensive search at all stages of virus growth, there was no clear evidence to show how the virus particles were formed in the vesicles, though occasionally thickenings in vesicle walls were seen which could be an early stage in virus budding into the vesicle (Fig. 2). Virus was released from the cell by the migration of virus-filled vesicles to the cell membrane where they ruptured and released their contents (Fig. 5). From about 30 hr after infection, virus was seen to accumulate on the outside of the cell membrane. The virus was not seen to bud from the cell membrane, and the cell membrane did not show any morphological changes to suggest that virus components were forming there (Fig. 6).

Seventy-two hours after infection the cytoplasm was filled with vesicles of all sizes which mainly appeared circular or oval in sections. This suggests that the vesicles were spherical or ovoid structures and not elongated or tubular. The mitochondria were seen to be degenerating, and the cell was clearly being damaged by the effects of the virus infection, although no cytopathic effect was seen in tube cultures until 5–6 days after infection.

The mechanism of hemadsorption by OC43-infected cells. The virions of OC43 readily agglutinate rat red blood cells, and,

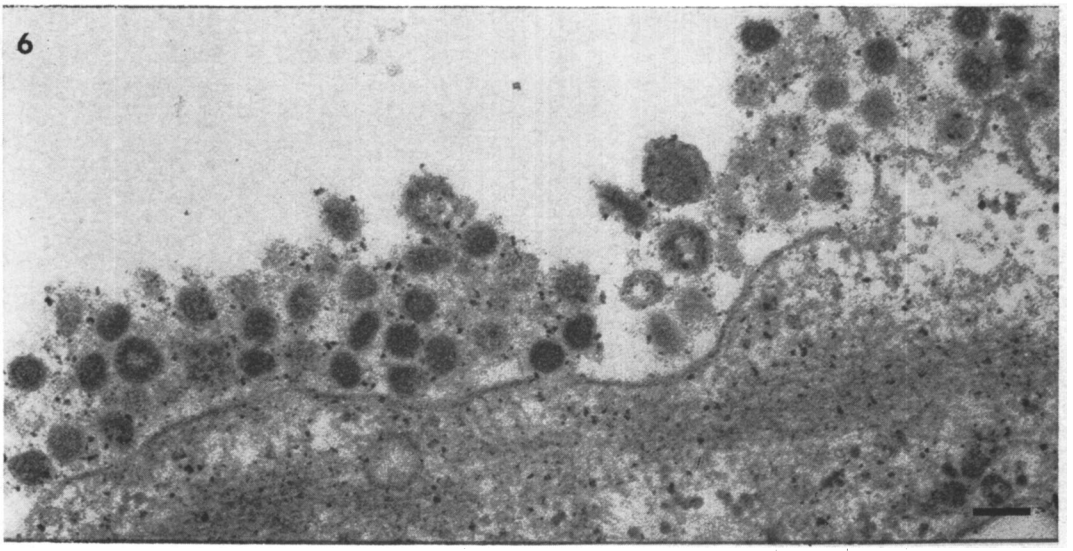
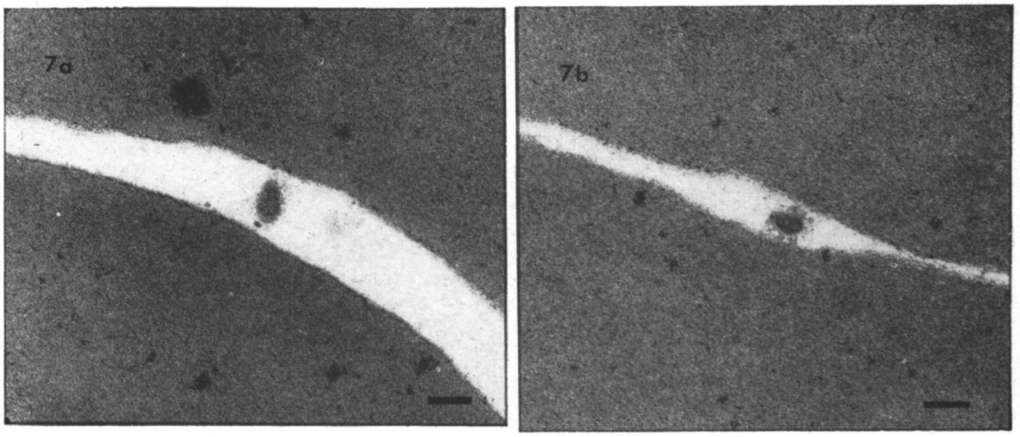


FIG. 6. OC43-infected WI38 cells 60 hr after infection: Masses of virus progeny are attached to the external cell surface, but the cell membrane is virtually intact and there is no evidence of virus budding; bar = 100 nm.

sections of pellets of agglutinated cells show clearly how virus particles bridge between red cells, attaching by the outermost edge of the "halos", which are usually seen in isolated sectioned viruses (Figs. 7A and 7B). This "halo", may be the "corona", which presents so characteristic an appearance in negatively stained preparations. Rat red cells agglutinated with OC43 do not dissociate even after 24 hr at 37°, and Kaye and Dowdle (21) have shown that neuraminic acid residues are not involved in the agglutination reaction. However, although strong, irreversible bonds are formed between the OC43 virion and the rat red blood cell, red cells are not strongly bound to the surface of WI38 cells infected with OC43. The OC43 hemadsorption technique as described by Kapikian *et al.* was modified in this study (22). OC43 hemadsorption reactions were carried out by leaving a dilute suspension (0.1%) of rat red blood cells in contact with infected WI38 cell sheets for 1 hr. The tubes were then gently rocked to suspend unadsorbed red cells and the fluids were decanted. The OC43-infected cells in the sheet could be clearly seen by their dense hemadsorption reaction. If the tubes were rinsed gently with saline, or if the tubes were shaken moderately, most of the specifically adsorbed red cells were detached, and if fresh red cells were added to the tube, the hemadsorption reaction was very much weaker. These observations, together with the evidence from electron microscope sections that OC43 accumulates on the outside surface of infected cells, provide a mechanism for the hemadsorption reaction of OC43-infected cells.

Discussion. The foregoing study has shown that OC43, like the coronaviruses IBV, 229E, and MHV, is formed in intracellular vesicles, and not by budding at the external cell membrane in the manner of the myxoviruses. Although many cells in all phases of virus production were examined, we saw no clear evidence of virus budding into the cytoplasmic vesicles such as has been reported by Becker *et al.* (15) for 229E and IBV, and by David-Ferreira and Manaker for MHV (16). However, the general similarity of the cytoplasmic development of OC43 and 229E suggests that OC43 should go through a phase of budding into the cytoplasmic vesicles, although we were not able to see it. Since we were able to find many examples of 229E budding we conclude that during the development of OC43, budding is much less frequent, or is confined to fewer centers, pos-



FIGS. 7A and B. OC43 virions forming bridges between rat red blood cells; bar = 100 nm.

sibly only one, in each vesicle.

The hemadsorption reaction of OC43-infected WI38 cells is an interesting anomaly and is not caused by the insertion of viral hemagglutinins into the external cell membrane. Instead, newly formed virus accumulates at the external cell surface, as though it were "sticky" or held in a glutinous matrix, possibly the early dense material seen filling the vesicles in which viruses later appear (Fig. 2). When rat red cells are allowed to settle on these layers of loosely attached virus particles, they are bound to the virus mass by a strong irreversible reaction through receptors on the red cells. Provided the excess, unbound red cells are gently removed, the bound red cells remain attached to the cell surface and give a clear indication of which cells in a sheet have adsorbed virus attached. However, quite mild forces, such as washing the hemadsorbing WI38 cell sheet, or shaking the tube, can detach the virus mass with its attached red cells from the cell surface. If fresh red cells are then added and allowed to settle, the hemadsorption reaction is very much less because most of the virus has been removed. It is noteworthy that in OC43 virus-infected BS-C-1 cell cultures, Kapikian *et al.* (22) found that the hemadsorption reaction was quite stable.

Because of the fragile nature of the WI38 cell–OC43 virus–rat red cell complex, it is difficult to obtain convincing electron microscope evidence that it exists. However, in Fig. 8 a rat red cell is shown in association with a mass of OC43 virus particles at the surface of an infected WI38 cell. In contrast to the fragile nature of red cells adsorbed to WI38 cells infected with OC43 virus, red cells adsorbed to cells infected with myxoviruses may be repeatedly washed with saline and may be shaken quite vigorously without being detached. This is because large areas of the red cell surface are held directly on the infected cell membrane (18) by bonds between virus hemagglutinin and mucoprotein receptors on the red cells (25). The mechanism whereby cells infected with coronavirus OC43 adsorb red cells is unique, as far as we know, and we propose that this be called "pseudohemadsorption" to distinguish it from the true hemadsorption of the myxo- and paramyxoviruses. The accumulation of newly formed virus particles at the external cell surface has been noted in two other electron microscope studies with coronaviruses. Hamre *et al.* (14), working with coronavirus 229E, and Oshiro *et al.* (26), working with a 229E-like strain, found aggregates of virus at the cell surface. Perhaps this will emerge as a characteristic feature of certain coronaviruses.

Summary. The human respiratory coronavirus OC43, grown in WI38 cells matures in cytoplasmic vesicles, and its development resembles that of coronavirus 229E. However, we did not observe budding of OC43 virus into the vesicles, although this is a characteristic feature of the morphogenesis of 229E virus. In OC43-infected cells, progeny virus

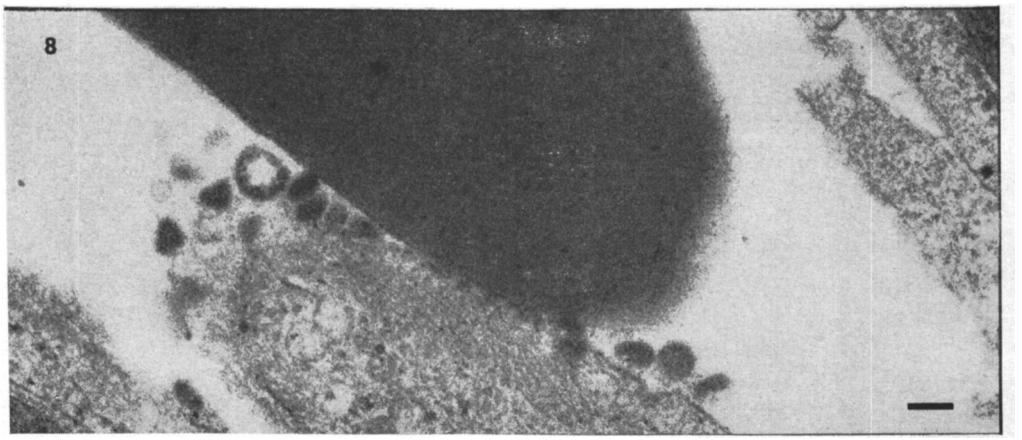


FIG. 8. Rat red blood cell in association with an OC43-infected WI38 cell. bar = 100 nm.

accumulate on the outside surface of the cell in sufficient quantity to bind rat red blood cells and give rise to a "pseudohemadsorption" reaction.

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