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Morphogenesis of Avian Infectious Bronchitis Virus and a Related Human Virus (Strain 229E)

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Avian infectious bronchitis virus (IBV) and strain 229E, a virus recently recovered from patients with colds, have been shown to possess a similar distinctive morphology in negatively stained preparations. An electron microscopic study of the morphogenesis of IBV in the chorioallantoic membrane and of strain 229E in WI-38 cells was performed. In infected cells, round electron-dense particles 82 mμ in diameter were observed to form by a process of budding from membranes of the endoplasmic reticulum and cytoplasmic vesicles. The particles in IBV-infected cells were similar in size and shape to those in strain 229E-infected cells but showed certain differences in internal structure. The evidence that the particles represent virions and the implications of these findings in the classification of this virus group are discussed.

Recent studies in this and other laboratories (1, 2, 11) have indicated the emergence of a morphologically distinct group of ether-labile, medium-sized, ribonucleic acid (RNA)-containing viruses for which avian infectious bronchitis virus (IBV) is the prototype. One member of the group, strain 229E, was recovered in tissue culture by Hamre and Procknow from students with upper respiratory disease (10). Tyrrell and Bynoe (18), using human embryonic tracheal organ cultures, isolated an ether-labile virus from patients with colds; this virus, strain B814, was subsequently examined by Almeida and Tyrrell (1) in negatively stained preparations and found to resemble IBV and strain 229E. Six strains of "IBV-like" viruses with a similar morphology were recovered in this laboratory from adults with upper respiratory disease through use of tracheal organ cultures (11). In negatively stained preparations, the members of this group appear roughly spherical with widely spaced, club-shaped surface projections, moderate pleomorphism, and an overall diameter, including projections, of 160 ± 40 mμ (11). This characteristic morphology differs distinctly from that of the myxo- and paramyxoviruses.

In an early electron microscopic study of IBV in the chorioallantoic membrane (CAM) of embryonated hens' eggs, spherical particles 180 mμ in diameter were observed in the cytoplasm of infected cells (7). In view of advances in the techniques of electron microscopy and as a further step in characterizing members of this virus group, a comparative study of the morphogenesis of strain 229E and IBV in ultrathin tissue sections was undertaken and forms the basis of this report.

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MATERIALS AND METHODS

Viruses. Dorothy Hamre kindly supplied strain 229E which had been purified in human diploid cell strain WI-38 by the terminal dilution technique. Virus pools used for the inoculation of tissue cultures contained $10^{5.5}$ to $10^{6.5}$ plaque-forming units (PFU)/ml.

IBV, Beaudette strain 66579, was kindly supplied by Harold DeVolt, and Beaudette strain 42 by Charles H. Cunningham. Seed pools were initially passaged four or five times at a dilution of 10^{-3} in leukosis virus free (LVF) embryonated eggs (National Institutes of Health), and once more after incubation for 1 hr with antiserum against avian leukosis virus group 1 (supplied by M. Katherine Cook). Seed pools prepared in this way were free from detectable avian leukosis virus when examined by the complement fixation test for avian leukosis (COFAL test; 17). Both strains of IBV were neutralized by specific antisera against the Wachtel strain and Massachusetts strains 41 and 82828. Virus pools contained titers of 10^7 median egg infective doses 50 (EID_{50})/ml.

Antisera. Hyperimmune serum against strain 229E was prepared in guinea pigs by six weekly or biweekly intraperitoneal injections of virus grown in WI-38 cells. The titer of neutralizing antibodies was 1:160.

Immune serum prepared in leukosis-free chickens against LVF IBV (strain Massachusetts 82828) was purchased from SPAFAS, Inc., Storrs, Conn. Immune sera against the Wachtel and Massachusetts 41 strains were obtained from Harold DeVolt and Charles Cunningham, respectively.

Preparation of infected tissues for electron microscopy. WI-38 roller tube cultures were obtained from commercial sources and maintained with a mixture of equal amounts of Medium 199 and Eagle's Minimal Essential Medium, supplemented with 2% fetal calf serum (inactivated at 56 C for 30 min), 250 units/ml of penicillin, 250 μg/ml of streptomycin, and 2 mM glutamine. Replicate tubes were inoculated at a multiplicity of 1 to 4 PFU of strain 229E per cell and incubated on a roller drum at 33 C. Every 6 hr four infected and two uninfected control tubes were removed for study. Cells from two infected tubes were washed, removed from the glass surface by scraping, and pooled. Intracellular infectious virus was measured in WI-38 monolayers by the method of Hamre et al. (9). In the remaining two infected tubes and in the two control tubes, the cells were washed with buffered saline, fixed in 6.5% glutaraldehyde at *p*H 7.2 for 1 hr at room temperature, and postfixed in Palade's 1% osmium tetroxide (14). The cells were then scraped from the glass and the tubes were centrifuged lightly. The pellets were dehydrated through graded concentrations of ethyl alcohol and embedded in Epon 812.

LFV embryonated hens' eggs, 10 or 11 days old, were inoculated allantoically with 10^3 or 10^7 EID_{50} of IBV (Beaudette strains 42 and 66579) and incubated at 37 C. Every 6 hr, portions of the CAM of infected and control uninfected eggs were harvested and washed with isotonic saline. Fixation and embedding procedures were identical to those described above.

Sections were cut on a Porter-Blum MT2 ultramicrotome by use of glass knives. Tissues were stained before embedding with saturated uranyl acetate in 70% ethyl alcohol and after sectioning with Reynolds' lead citrate (15). The preparation of negatively stained virus has been described previously (11). Photomicrographs were taken with a Siemens Elmiskop 1A electron microscope at 80 kv and magnifications of 20,000 to 60,000.

Neutralization tests. Virus suspensions were mixed with an equal volume of a 1:10 dilution of control or hyperimmune serum and incubated for 2 hr at room temperature. Embryonated eggs or roller tube cultures were inoculated with the virus-serum mixture and incubated as described above. After 43 hr (strain 229E) or 24 hr (IBV), replicate tissues were harvested and prepared for ultrathin sections and for titration of intracellular infectious virus. At the same time, nutrient medium or allantoic fluid was examined for the presence of characteristic particles in negatively stained preparations.

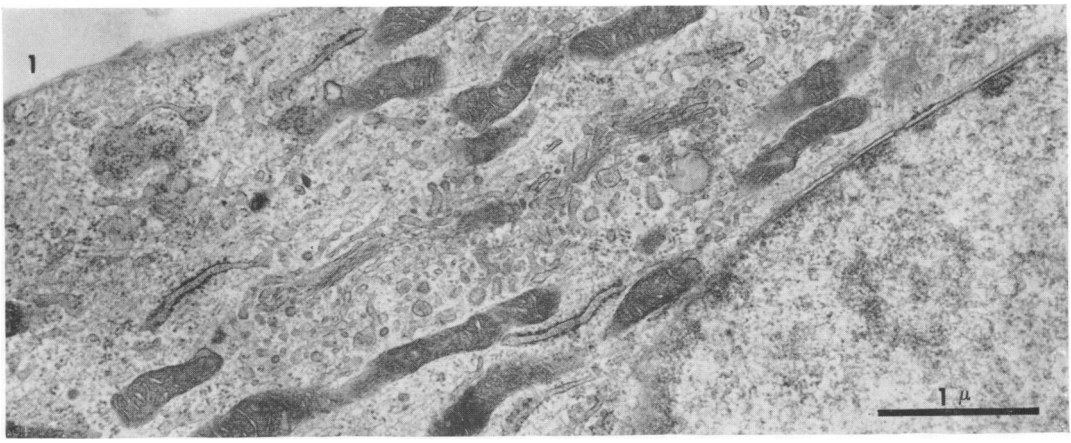
RESULTS

Observations of virus strain 229E in infected WI-38 cells. Because the low multiplicity of infection (1 to 4 PFU/cell) did not assure synchronous infection of all cells, a precise temporal assessment of virus development was not possible. Certain individual samples clearly contained cells in many stages of infection. However, some general observations on virus morphogenesis could be made. Round electron-dense particles approximately 82 mμ in diameter, regarded as virions, appeared for the first time in cells harvested 12 hr after inoculation. In cells harvested 12 and 18 hr after inoculation, the particles appeared individually or in short rows in the space between membranes of rough or smooth endoplasmic reticulum or within the Golgi apparatus. These cytoplasmic membranes often formed small irregular cisternae. Uninfected cells are shown in Fig. 1 and 2, whereas the cell depicted in Fig. 3 represents an early stage of infection.

In the samples fixed 24 to 36 hr after inoculation (Fig. 4), rows of particles were, in general, more numerous than in earlier samples, and clusters appeared for the first time. The cisternae enlarged to accommodate larger numbers of virions and in some areas had the appearance of small vesicles. At this stage, virions were seen in extracellular spaces, indicating that particles had been liberated from some cells into the medium. There was little visible phagocytosis of viral particles at the cell surface, although some of the "vesicles" may represent phagosomes. In the 42- and 48-hr samples (Fig. 5), numerous virions were grouped within vesicles and in many cells occupied most of the cytoplasmic space. In certain crowded cells, vesicle walls showed breaks. Although clusters of amorphous tubular material appeared in the cytoplasm of some infected cells (Fig. 5), reticular or helical "internal component" was not seen. Virus particles were not observed in the nuclei of infected cells nor in any sections prepared from control uninoculated tissue.

Observations of IBV in infected embryonated eggs. The inoculum of 10^7 EID_{50} was not large enough to achieve synchronous infection of the cells lining the chorioallantoic cavity. Virus was first detected in these cells at 12 or 18 hr after inoculation, and the embryos died within 24 hr. With the small inoculum (10^3 EID_{50}), the appearance of viral particles and death of the embryo were delayed for 6 to 12 hr. However, the developmental sequence was similar with both inocula.

In eggs inoculated with 10^7 EID_{50} of IBV strain 66579, characteristic particles approximately 82 mμ in diameter, regarded as virions, first appeared 12 hr after infection. The particles appeared singly, in chains, and in small groups in the endoplasmic reticulum of the cells lining the chorioallantoic cavity. At 18 hr after inoculation, the cytoplasm of these cells contained many dilated cisternae and vesicles enclosing large numbers of



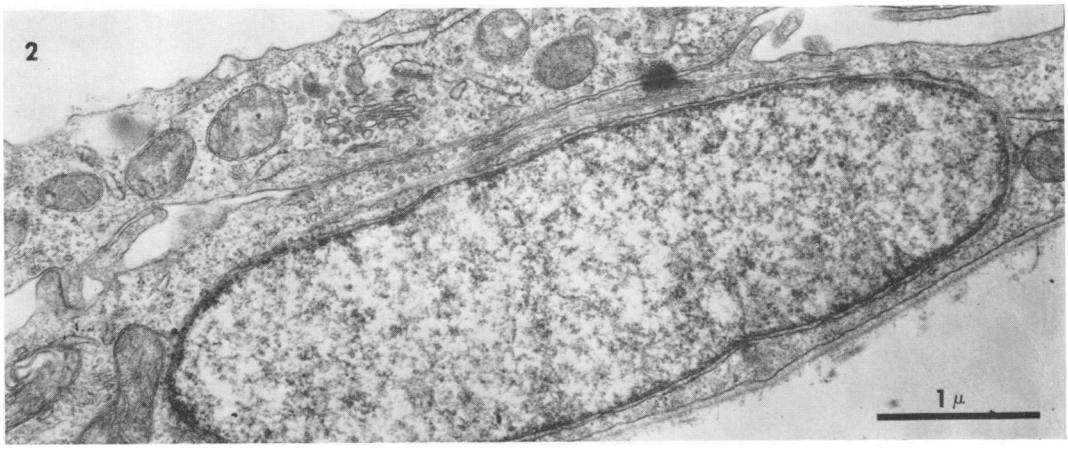


FIG. 1. *Normal uninfected WI-38 cell. The numerous mitochondria and extensive system of cytoplasmic membranes are characteristic.* × *21,000.*

FIG. 2. *Normal chorioallantoic membrane. The cell at the top of the picture borders the allantoic cavity.* × *21,000.*

virions. This progression of the infection is illustrated by Fig. 6–8. At 24 hr after inoculation, the superficial cells of the CAM had degenerated, and particles were seen for the first time in the cells of the subjacent layer.

In eggs infected with IBV strain 42, virus particles were not seen until 18 hr after inoculation. The subsequent progression of infection was identical to that seen with strain 66579.

Viral structures were never seen in the nuclei of infected cells. Moreover, control tissues showed no signs of viral infection. Neither control nor infected tissues showed electron microscopic evidence of latent infection with members of the avian leukosis virus group. As in 229E-infected cells, phagocytosis of viral particles was rarely seen at the plasma membrane. The possibility that some virus-filled cytoplasmic vesicles represented phagosomes, however, could not be excluded.

Postulated scheme of virus development. The mechanism of development of individual particles appeared to be that of budding from intracytoplasmic cellular membranes. However, in cells harvested in the early stages of infection (at 12 and 18 hr in strain 229E-infected cells and at 12 hr in IBV-infected cells), examples of this budding process were infrequent. Only later in infection when particle production appeared to be occurring at a maximal rate was budding common. In these samples, we observed what appeared to be various stages of virus development, and these observations form the basis of a postulated scheme of particle maturation.

In this scheme, the earliest event appeared to be the development of a crescent (Fig. 9 and 11);

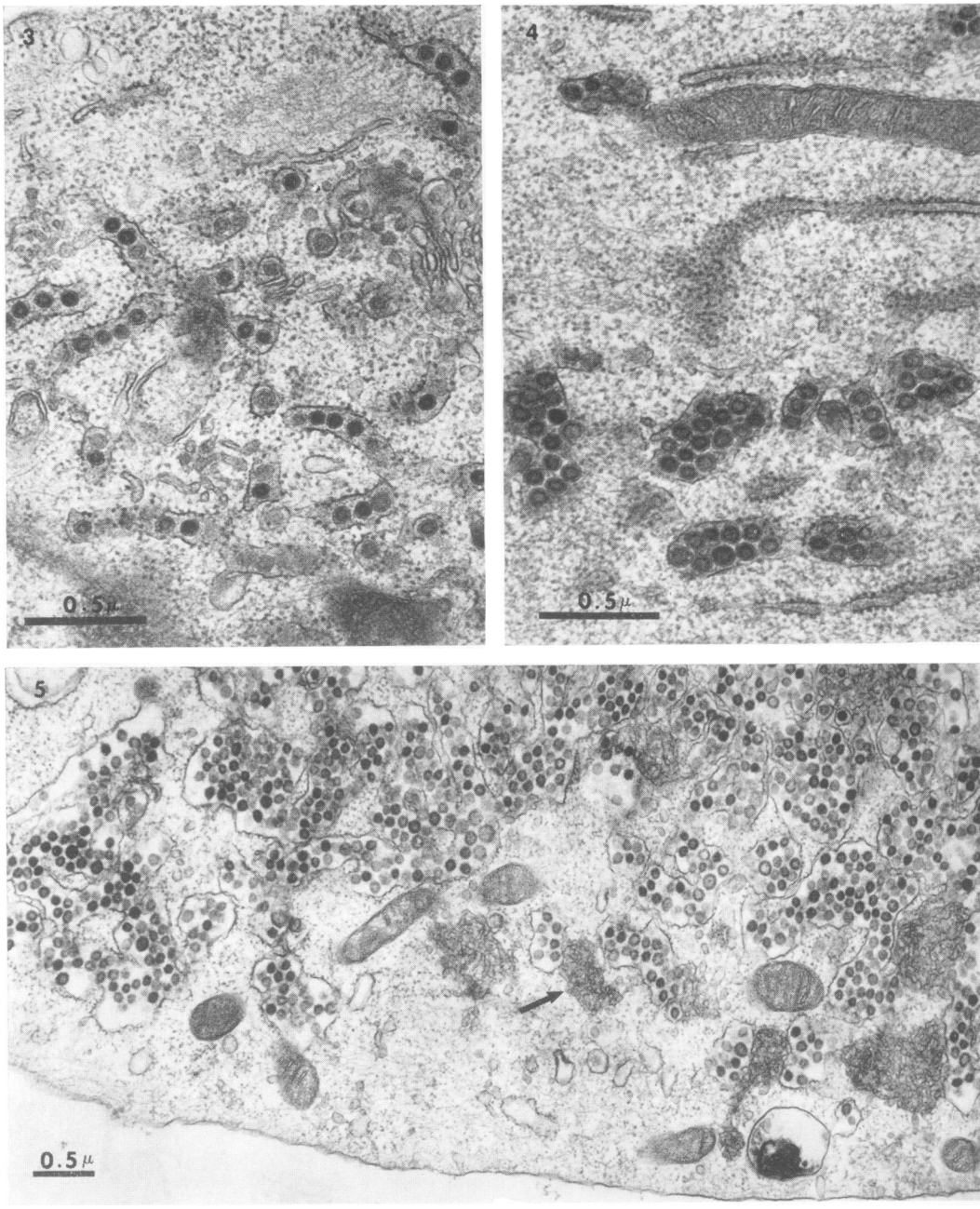
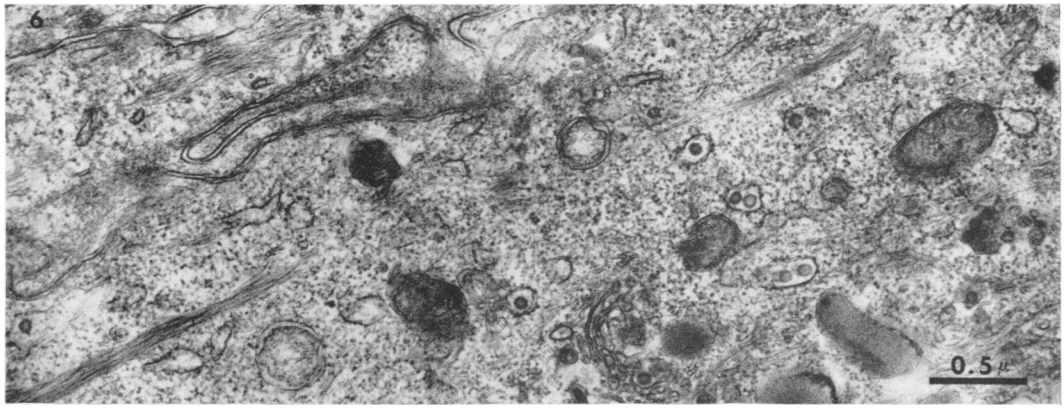
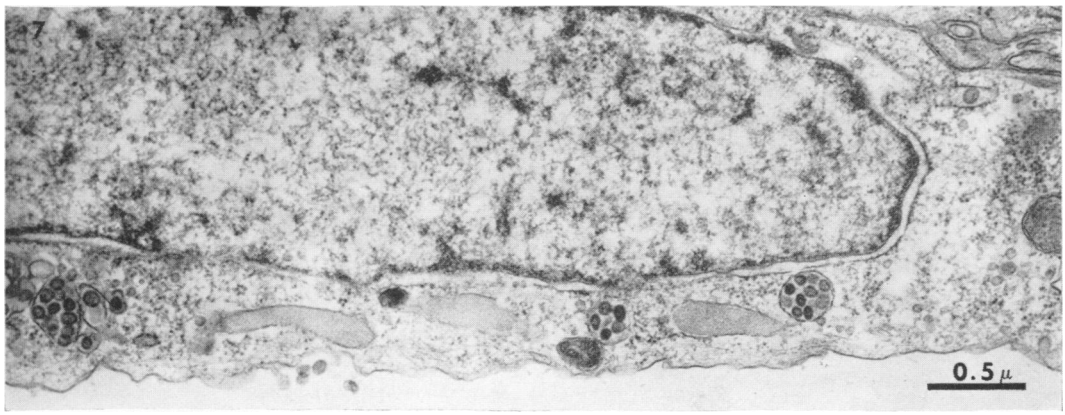


FIG. 3, 4, and 5. *Strain 229E in WI-38 cells. Figure 3 shows the cytoplasm of a cell in an early stage of infection where electron-dense particles appear singly or in short rows between membranes of endoplasmic reticulum. In Fig. 4 cisternae have formed. In Fig. 5 irregular vesicles contain large numbers of particles. The arrow points to amorphous tubular material seen occasionally in late stages of infection. Figure 3, × 34,000, 30 hr after inoculation; Fig. 4, × 34,000, 24 hr after inoculation; Fig. 5, × 17,000, 48 hr after inoculation.*

an altered segment of cisternal or vesicular membrane bulged away from the cytoplasm into the cisternal or vesicular lumen and developed at the same time a dense underlying layer on the cytoplasmic side. The membrane itself was double, with two electron-dense layers, each 30 A thick, separated by a narrow 20 A electron-transparent zone. The dense underlying layer in the IBV-in-





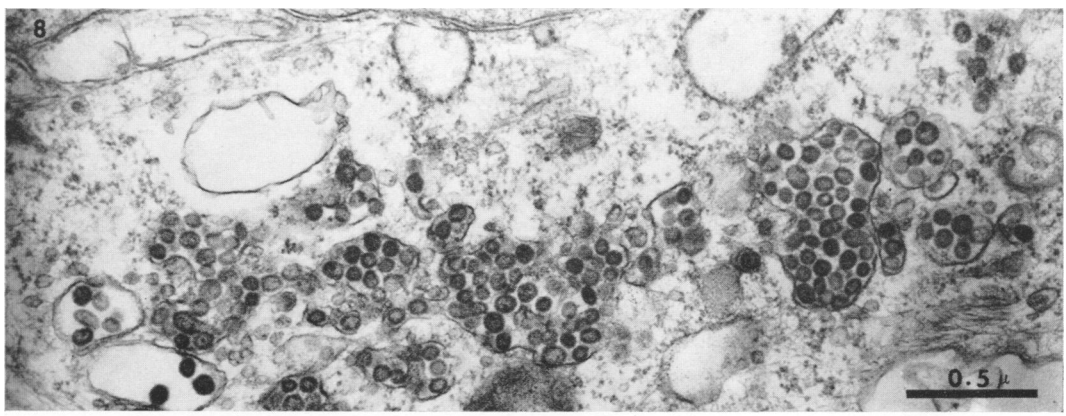


FIG. 6, 7, and 8. *IBV strain 42 in the chorioallantoic membrane. Figure 6 shows a cell in an early stage of infection. Single particles appear in small cisternae of endoplasmic reticulum. In Fig. 7, vesicles have formed. Figure 8 represents a cell in a late stage of infection. Figure 6, × 25,000, 24 hr after inoculation, 10^3 EID_{50} inoculum; Fig. 7, × 25,000, 18 hr after inoculation, 10^7 EID_{50} inoculum; Fig. 8, × 34,000, 18 hr after inoculation, 10^7 EID_{50} inoculum.*

fected cells (Fig. 11 and 12) appeared closely apposed to the vesicular membrane with no clear intermediate zone visible. In the 229E-infected cells (Fig. 9), there was always an electron-transparent zone separating the dense underlying layer from the double vesicular membrane.

As the crescent bulged further into the vesicle (Fig. 9 and 12), the inner electron-dense shell was

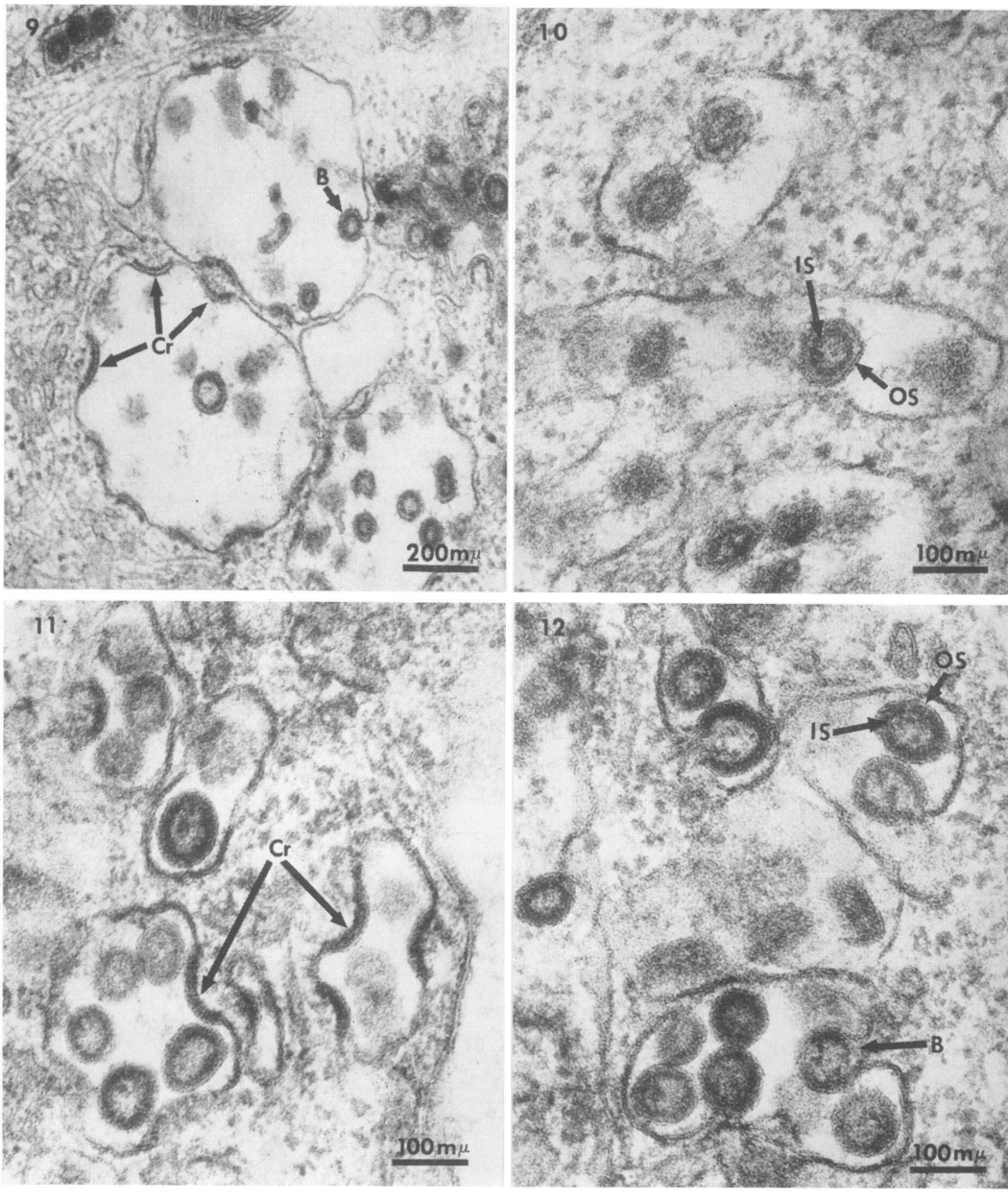
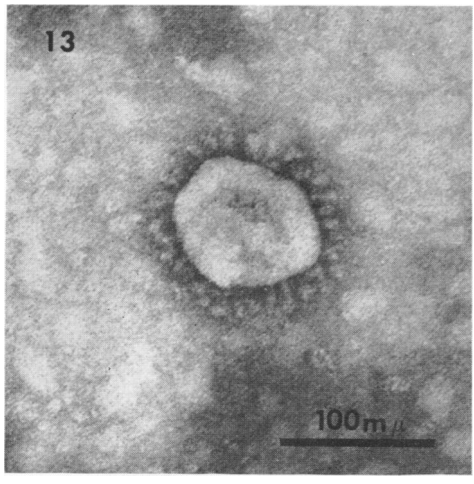


FIG. 9 and 10. *Strain 229E in WI-38 cells. Figure 9 shows several cytoplasmic vesicles in which the process of budding is illustrated. The double vesicular membrane bulges inward to form a crescent (Cr); at the same time a dense layer forms underneath, separated from the vesicular membrane by an electron-lucent space. A particle in a later stage of budding (B) shows the completion of the inner shell. In Fig. 10, a completed particle shows the double outer shell (OS), the inner shell (IS), and the electron-lucent zone between them. Figure 9, × 50,000; Fig. 10, × 100,000.*

FIG. 11 and 12. *IBV in the chorioallantoic membrane. Crescents (Cr) are formed by the bulging of cisternal membranes and the close apposition of a dense underlying layer. In Fig. 11, continuity of the vesicular membrane with the outer shell of a budding particle (B) is visible. A completed particle in Fig. 12 shows the double outer shell (OS) and the dense inner shell (IS), surrounding a core of amorphous material. Figure 11, × 100,000; Fig. 12, × 100,000.*

completed (Fig. 9); the outer double membrane was gradually pinched off, appearing first as a short stalk and finally fusing around the particle. The budding process was never seen at the cell surface, although it is possible that some of the cytoplasmic vesicles communicated directly with the extracellular space.

The completed particles in 229E-infected cells (Fig. 10) were round, 66 to 112 mμ (mean, 82 mμ) in diameter, bounded by a double membrane 7 to 8 mμ thick. Within this outer shell was an electron-dense inner shell 9 to 17 mμ thick, separated from the outer shell by an electron-transparent zone 4 to 8 mμ wide. The central portion of the particle contained amorphous material of variable density.



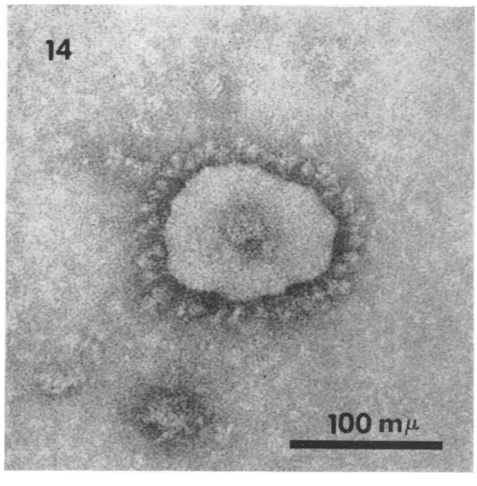


FIG. 13 and 14. *Single virus particles of strain 229E (Fig. 13) and IBV strain 42 (Fig. 14) negatively stained. The club-shaped projections are evident. No internal structure is visible in these preparations.* × *200,000.*

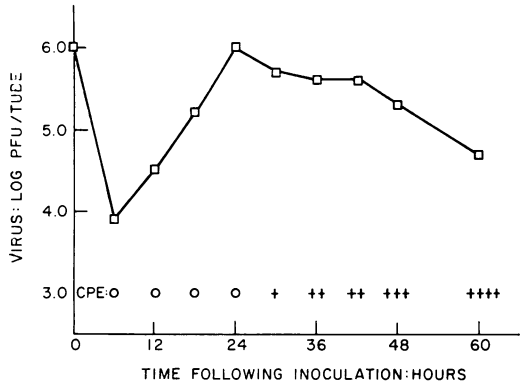


FIG. 15. *Growth of virus strain 229E in WI-38 cells. Roller tubes were inoculated at 0 hr and incubated at 33 C on a roller drum. Every 6 hr intracellular virus was titrated in two duplicate tubes. Final titers of virus were calculated as plaque-forming units per roller tube.*

The overall diameter of completed particles in IBV-infected cells (Fig. 12) was 67 to 110 mμ (mean 82 mμ), identical to that of strain 229E; the dimensions of the outer (7 to 8 mμ) and inner (9 to 17 mμ) electron-dense shells were also similar. However, IBV consistently lacked the electron-transparent zone between the outer and inner shells. For this reason, the amorphous central core appeared larger than that of strain 229E.

The club-shaped projections, so characteristic of this group of viruses in negatively stained preparations (Fig. 13 and 14), were never definitely seen in tissue sections.

Identity of electron-dense particles in infected cells. The evidence that the particles seen in infected cells represent virions was indirect. Attempts were made to identify the particles as virions by correlating the development of particles in ultrathin sections with the production of infective virus in timed growth curves. In addition, virus inocula were neutralized with specific immune sera, and a similar correlation was made between the yield of infective virus and the development of electron-dense particles.

In growth curves of strain 229E in WI-38 cells, intracellular infective virus was measured in roller tubes harvested in parallel with those prepared for ultrathin sections. One such growth curve is represented in Fig. 15. Virus production was first detected at 12 hr, increased over the following 12 hr, and then remained fairly constant until 42 hr after inoculation. Cytopathic effect was evident at 36 hr. This growth curve roughly parallels the development of particles in ultrathin sections.

Neutralization tests were carried out with both

viruses. Virus yield in tissues inoculated with unneutralized or neutralized virus was examined at a uniform time of harvest. It was found that suppression by specific immune serum of infective virus production was paralleled by the absence of detectable characteristic particles in ultrathin tissue sections and in negatively stained preparations of tissue fluids.

DISCUSSION

The evidence obtained in this study suggests that the particles observed in infected cells represent the virion. Conclusive identification of these particles, however, may require application of ferritin-conjugated antibody techniques. The diameter of intracellular particles (82 mμ) was less than that of negatively stained virus measured without surface projections (120 mμ; 11). However, this degree of disparity has been observed with a number of viruses (3). It is probably due to the collapse and flattening of unfixed virus particles in negatively stained preparations and to minor contractions of tissues during the embedding procedure (13).

The similarity of particle morphogenesis in tissues infected with IBV and strain 229E is striking. They both appear to develop in the cytoplasm by a process of budding into cisternae or vesicles, incorporating cellular membrane material into their outer coat. The completed particles are identical in size and shape. This similarity reflects their similar appearance in negatively stained preparations and provides further support for the assertion that, along with the "IBV-like" viruses and strain B814, they form a distinct virus group.

On the other hand, although IBV and strain 229E could not be distinguished by the negative staining technique, examination after positive staining in ultrathin sections revealed a difference in their internal structure. There was an electron-transparent zone separating the inner and outer shells of strain 229E which was not present in IBV. It is unclear whether this difference is host determined or intrinsic to the viruses themselves. This question cannot be answered finally until both viruses can be grown in the same host tissue under identical conditions.

Similar studies on the morphogenesis of the organ culture grown "IBV-like" viruses and strain B814 will contribute to the further understanding and classification of this group. In our search for other RNA-containing viruses which might have properties in common with IBV and strain 229E, it was pointed out to us by Wallace Rowe that the mouse hepatitis virus (MHV), another ether-labile (4, 8), RNA-containing (12) virus, appeared similar to strain 229E in ultrathin sections (5). Preliminary evidence indicates that the virion of MHV is indistinguishable from those of IBV, strain 229E, and the "IBV-like" viruses in negatively stained preparations. Moreover, MHV appears to develop by budding into cytoplasmic "cisternae" (5). It is likely that the "IBV-like" viruses will prove to have a similar morphogenesis in ultrathin sections.

The relationship of IBV and strain 229E to the myxoviruses can now be more clearly delineated. In size, nucleic acid type, and ether lability they are similar to the myxovirus group. To these similar qualities may now be added the presence of an outer shell which appears to be at least partly made up of altered host cell membrane. On the other hand, these studies have added several new distinctions. Collections of helical nucleoprotein were not seen in the nuclei or cytoplasm of infected cells. Particles were never seen to bud from the plasma membrane. The virions showed a remarkable uniformity in their round shape and only limited variation in size, in contrast to the marked pleomorphism, even in thin sections, of the myxoviruses.

The developing avian leukosis viruses have certain characteristics which distinguish them from the particles seen in IBV infected cells. They bud primarily from the plasma membrane. The early crescents and the A-type particles show clear outer and inner electron-dense shells with a less well-defined intermediate shell between. The mature particles contain an electron-dense nucleoid (6). Such particles were not seen in this study in either the control or the infected CAM. In addition, we were unable to demonstrate the presence of avian leukosis virus in our materials by the COFAL test (17). It is unlikely, therefore, that the morphogenesis of IBV as described in this study was complicated by the presence of latent tumor viruses.

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