

## **Morphogenesis of Avian Infectious Bronchitis Virus in Primary Chick Kidney Cells**

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With 10 Figures

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### **Summary**

Primary chick kidney cells were infected with avian infectious bronchitis virus (IBV) and examined by electron microscopy. Virus particles entered the cells by viropexis and distinction could be made between engulfment by cell processes (phagocytosis) and entry by micropinocytosis in coated transport vesicles.

Virus maturation occurred by budding into either the cisternae of the endoplasmic reticulum or cytoplasmic vacuoles, and evidence was obtained to suggest that the viral surface projections could be attached during the budding process. Late in infection large numbers of virus particles were present, mainly in cytoplasmic vacuoles, and the majority were released by cell lysis. Release by fusion of vacuoles with the plasma membrane was also observed, and individual virions could be transported from the endoplasmic reticulum to the surface within coated vesicles.

### **Introduction**

Studies on the morphogenesis of coronaviruses have shown that replication takes place in the cytoplasm of the host cell by a budding process from either the membranes of the endoplasmic reticulum or cytoplasmic vacuoles (11). This method of development has been demonstrated for human coronaviruses in WI-38 cells (3, 8) and human embryo lung cells (7), and avian infectious bronchitis virus (IBV) in chorioallantoic membrane cells (3), chick embryo fibroblasts (10), VERO cells (6) and the trachea of infected fowls (15). The budding mechanisms of virus particles in infected cells have been recorded in detail but little is known of the modes of entry and release although it has been generally reported that infection and release occur by viropexis and cell lysis respectively.

In the present study we have examined the morphogenesis of two IBV strains in primary chick kidney (CK) cells with particular emphasis on aspects of entry and release.

## Materials and Methods

### *Virus*

The Beaudette strain of IBV was used as described previously (1). Tissue culture adapted 'T' strain of IBV was obtained from C. D. Bracewell, Central Veterinary Laboratory, Weybridge, U.K. and had been passaged six times in CK cells. A stock was grown and this virus was used for infecting monolayer tissue cultures.

Virus infectivity was estimated as plaque forming units (PFU) as described (4).

### *Cell Culture*

Primary CK cells were prepared from 4-week-old chicks as described (4) and the medium was maintained at pH 7.0—7.2 with 15 mM HEPES buffer.

Cells were grown on 7.5 cm<sup>2</sup> surfaces in 30 ml plastic culture bottles and inoculated at confluency with 2—5 PFU of virus per cell. Cultures that were to be examined at times up to one hour after infection were inoculated with approximately 500 PFU of virus per cell. After an adsorption period of one hour, the inoculum was removed and the monolayers were washed prior to overlay and incubation at 37° C. At specified times after inoculation cell cultures, including uninfected controls, were prepared for electron microscopy.

### *Electron Microscopy*

The medium was removed from the cells and the monolayers were rinsed briefly in 1 per cent glutaraldehyde in 0.05 M sodium phosphate buffer pH 7.2 (SPB). Further glutaraldehyde was added and the cells fixed for 30—60 minutes at room temperature before washing in SPB and post-fixation in 1 per cent osmium tetroxide for 30—60 minutes. After dehydration in graded ethanol solutions, the monolayers were covered with araldite and left for approximately one hour. The mixture of resin and residual alcohol was replaced with fresh araldite which was allowed to harden. The araldite blocks containing the monolayers were finally prised from the plastic substrate and small chips were re-embedded in capsules. Thin sections of the sandwiched monolayers were cut on an LKB ultratome, using glass knives, and stained in 25 per cent methanolic uranyl acetate for 30 minutes at room temperature followed by lead citrate for one minute (16). Grids were examined at 80 kV in either a Philips EM 300 or a JEM 100B electron microscope.

## Results

When the confluent monolayers of CK cells were inoculated with 2—5 PFU of IBV per cell infection was to some extent asynchronous and at any particular stage different cells showed virus in different phases of the replication cycle, and varying degrees of cytopathic effect. However, a sequence of infection, virus production, and release of progeny could be established over a time scale of approximately 24 hours for strain Beaudette.

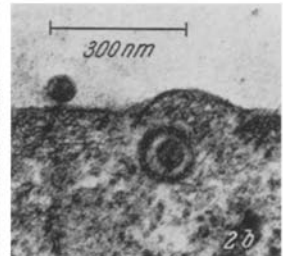
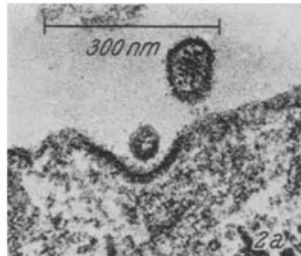
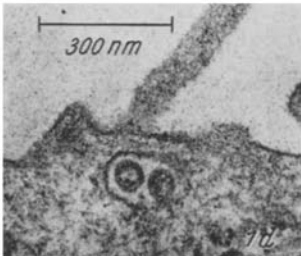
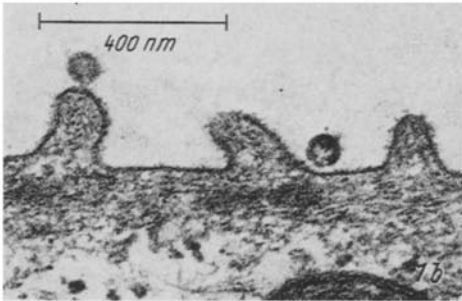
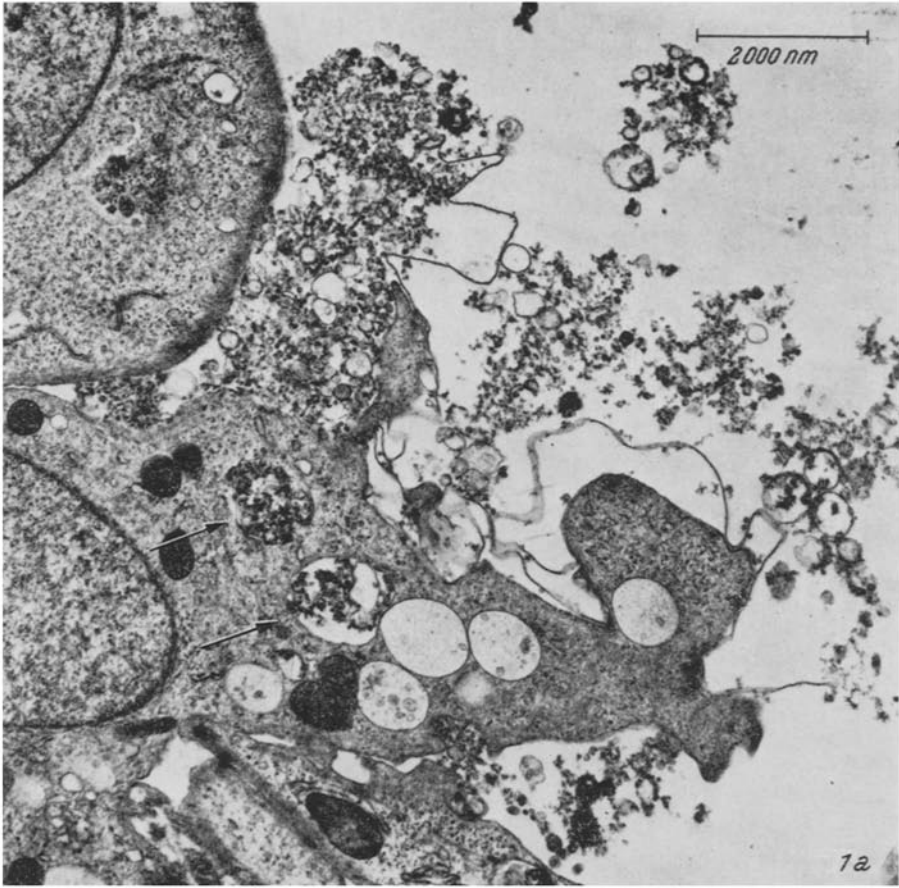
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Fig. 1. Infection of CK cells by IBV strain Beaudette. CK cells were inoculated with approximately 500 PFU virus per cell and examined one hour later

a) The inoculum contained large amounts of cell debris which was ingested with virus particles and could be seen in phagocytic vacuoles (arrowed)  $\times 11,500$ . b—d) Stages in the entry of virus by phagocytosis. b) Two particles lying close to the plasma membrane;  $\times 62,500$ . c) Particles almost engulfed by a cell process  $\times 61,500$ . d) Two virions within a small phagocytic vacuole;  $\times 61,500$

Fig. 2. Entry of virus, strain Beaudette, by micropinocytosis

a) Virus particle lying within an invagination of the cell plasma membrane. The membrane in the region of the invagination is thickened;  $\times 77,800$ . b) A small cytoplasmic vesicle with thickened membrane enclosing a virus particle;  $\times 60,000$



*Entry of Strain Beaudette Virus*

CK cells were inoculated with approximately 500 PFU of IBV strain Beaudette per cell and examined 10, 20 and 60 minutes later. The inoculum, which was concentrated by centrifugation, contained large amounts of cell debris visible with virus particles outside the cells. One hour after inoculation particles and cell debris were also seen within the cells in cytoplasmic vacuoles (Fig. 1a).

Virus particles appeared to enter CK cells by one of two methods, phagocytosis or micropinocytosis, both of which may be termed viropexis. Phagocytosis occurred with the engulfment of virus particles and cell debris by surface processes to produce phagocytic vacuoles (Fig. 1a—d). Entry by micropinocytosis occurred with the formation of small invaginations, each containing a single virion, which were distinctive in that the membrane enveloping the virus appeared thickened (Fig. 2a). One hour after inoculation small virus-containing vesicles with thickened membranes could be seen within the cells (Fig. 2b).

*Production of Strain Beaudette Virus*

The uncoating of ingested virions and subsequent core replication was not evident in our preparations. Six to eight hours after infection with strain Beaudette, virus was visible within the cisternae of the endoplasmic reticulum. Virus particles could not be seen in large numbers until about ten hours after inoculation by which time extracellular particles were also observed. Evidence was obtained which showed that virus particles were formed by budding from the membranes of the smooth endoplasmic reticulum into the cisternae (Fig. 3).

Electron microscopy of cells infected with strain Beaudette indicated that virus release occurred by at least two distinct mechanisms. A small number of virus particles were enveloped individually by thickened portions of the endoplasmic reticulum which formed rounded vesicles (Fig. 4a, b). These vesicles, with a diameter in the range 90—180 nm, resembled those associated with incoming particles and often exhibited small exterior spicules. Vesicles with thickened membranes were seen in infected cells throughout the cytoplasm both empty and containing virus particles, and empty vesicles were also present in uninfected cells. The observations of vesicles at cell boundaries suggested that, after formation in the endoplasmic reticulum, the vesicles moved to the cell surface where they could be seen fused with the plasma membrane (Fig. 4c, d). Since this mechanism is the reverse of micropinocytosis it was important to establish in which direction the vesicles at the cell surface were moving in order to distinguish outgoing particles from already released progeny virus re-entering the monolayer in a secondary cycle of infection. That infection or re-infection of cells occurred for a considerable time (at least ten hours) after inoculation was evident from the continued phagocytosis of virus particles (Fig. 5). As a rule it was not possible to judge the direction of movement for vesicles opening onto the exposed cell surface (i.e. away from the substrate) since the large amount of extracellular virus seen close to the surface prevented unambiguous interpretation. However, virus release by reverse micropinocytosis could be demonstrated for thickened vesicles opening into regions of the monolayer in which different cells were in close contact. In this case virus particles could be seen to have been effectively trapped in the narrow

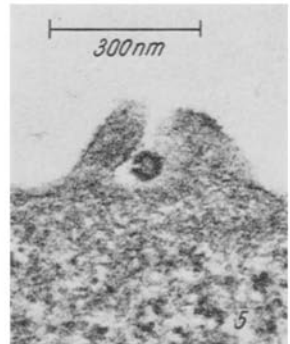
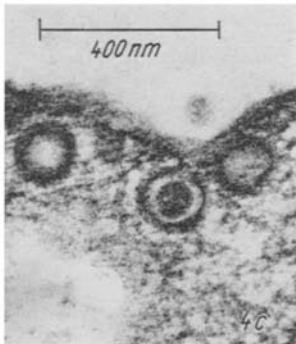
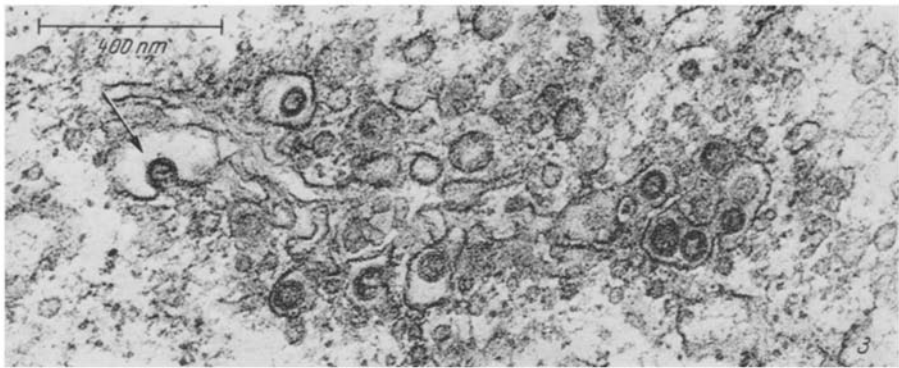


Fig. 3. Virus particles within the cisternae of the smooth endoplasmic reticulum 21 hours after inoculation with strain Beaudette. One particle (arrowed) is in the process of budding from the membrane and a small number of surface projections are visible attached to the incomplete envelope;  $\times 61,500$

Fig. 4. Release of virus, strain Beaudette, by reverse micropinocytosis  
 a) and b) Intracellular virus within the cisternae of the endoplasmic reticulum 10 hours after inoculation. Thickened regions of the membrane (arrowed) envelope individual particles;  $\times 61,500$ . c) A thickened vesicle containing a virus particle is fused with the cell plasma membrane, 21 hours after inoculation. Two empty thickened vesicles are also present;  $\times 61,500$ . d) The thickened membrane of a vesicle incorporated into the cell plasma membrane, 24 hours after inoculation;  $\times 75,000$

Fig. 5. A virus particle, strain Beaudette, entering a cell by phagocytosis 10 hours after inoculation;  $\times 64,000$

extracellular spaces and their previous association with the vesicles could be inferred (Fig. 6a, b).

The majority of virus particles remained within the endoplasmic reticulum and were seen later in infection in large cytoplasmic vacuoles, many of which were apparently formed by dilation of the cisternae. The size of the vacuoles was not related to the number of enclosed virus particles and many vacuoles were empty (Fig. 7); control cells often contained large numbers of empty vacuoles. There was evidence to suggest that the vacuoles fused with each other (Fig. 7) but with strain Beaudette there was no observation of fusion with the plasma membranes.

The overall density of the cytoplasm increased during the last stages of infection, cells fused, and individual organelles tended to lose their identity

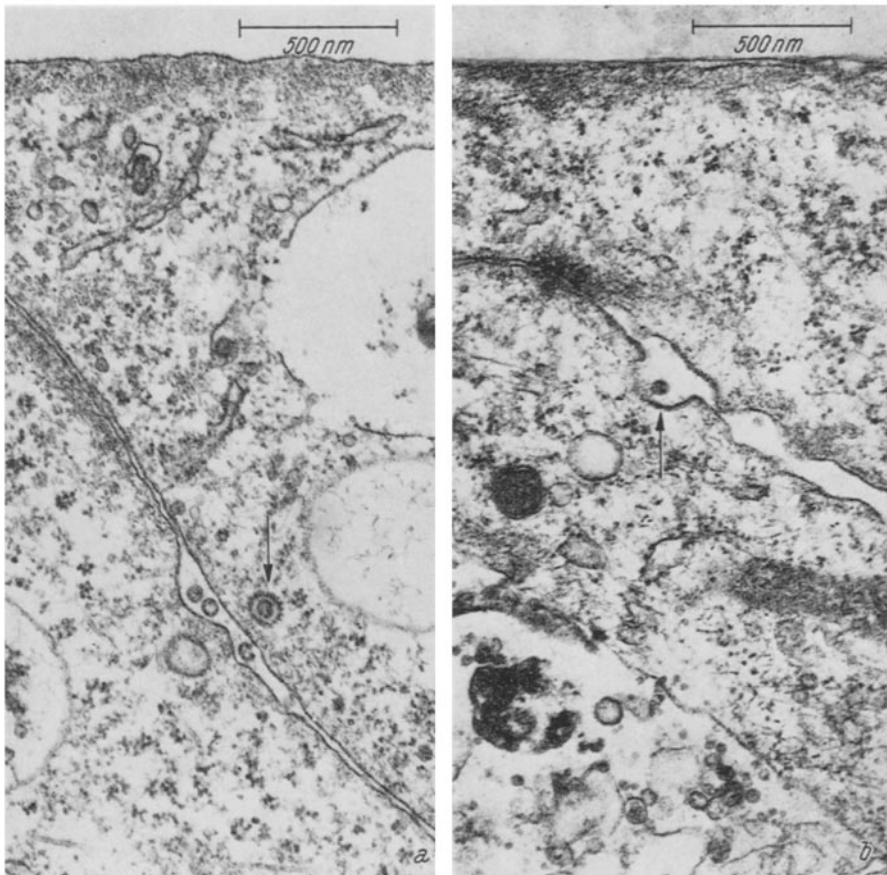


Fig. 6. Release of virus, strain Beaudette, by reverse micropinocytosis  
 a) A thickened vesicle (arrowed) enclosing a virus particle close to the cell surface, 10 hours after inoculation. The confined extracellular space already contains released particles. Note the 'spicules' on the vesicle membrane  $\times 41,000$ . b) Virus particle released (arrowed) from a vesicle into a confined extracellular space, 14 hours after inoculation;  $\times 41,000$

in a granular mass; the nuclei were fragmented and appeared as small areas of densely stained material, while regions of endoplasmic reticulum remained electron transparent. The cell masses rounded-up and detached from the substrate prior to lysis and, from the beginning of the stage at which cells were seen dying, large numbers of extracellular virus particles were present in the sections. Disintegrating cells were observed and progeny virus within the cytoplasmic vacuoles was seen to be released by membrane rupture. Virus particles released into confined extracellular spaces tended to lie in close-packed arrays with hexagonal configuration.

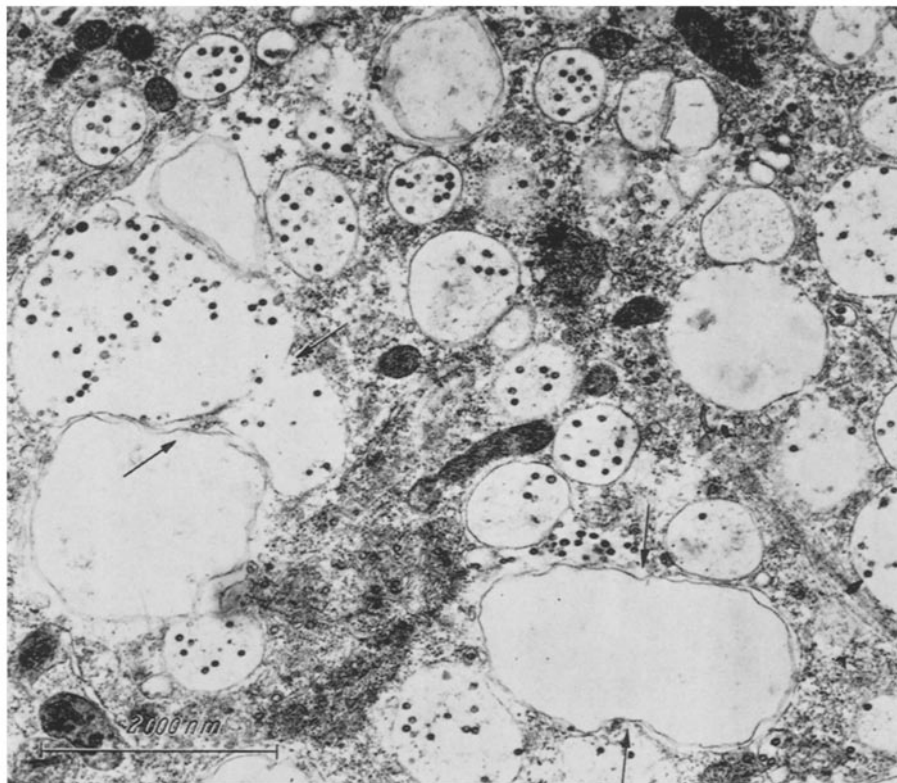


Fig. 7. Virus particles, strain Beaudette, in large vacuoles 24 hours after inoculation. The numerous vacuoles show no relationship between size and the number of enclosed virus particles. There is evidence that the vacuoles are able to fuse with each other (arrows)  $\times 16,000$

#### *Morphogenesis of T Strain Virus*

The replication of the 'T' strain of IBV in CK cells infected with 2—5 PFU of virus per cell occurs over a time scale approximately twice as long as that of strain Beaudette. (ALEXANDER, unpublished.) In monolayers examined 46 hours after infection progeny virus was seen in large numbers and cells were observed rounding-up and detaching from the substrate as described above for strain Beaudette infections.

Virus particles were seen in the process of budding from internal membranes but, unlike strain Beaudette, appeared to form predominantly within pre-existing cytoplasmic vacuoles (Fig. 8). However, because of the difficulty in tracing membrane continuity in thin sections it was not always possible to distinguish between vacuoles and endoplasmic reticulum. The majority of progeny virus particles appeared to be released by cell lysis. Virus release by reverse micropinocytosis was not observed with 'T' strain but there was evidence that the large virus-containing cytoplasmic vacuoles could fuse with the plasma membranes and release virus particles before gross degenerative changes had taken place in the cytoplasm (Fig. 9).

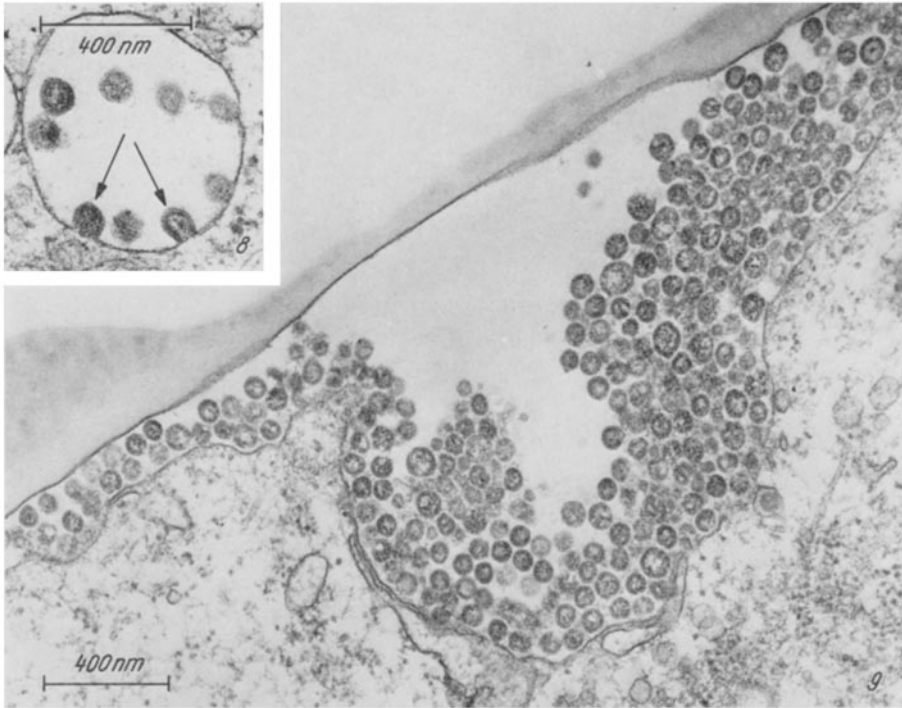


Fig. 8. A cytoplasmic vacuole containing progeny virus in a cell infected with T strain, 46 hours after inoculation. Some of the particles are in the process of budding (arrowed)  $\times 48,000$

Fig. 9. A large number of T strain virus particles released from a vacuole which has fused with the plasma membrane. The virus particles are trapped between the monolayer and the substrate;  $\times 41,000$

#### *Morphology of Virus Particles*

Virus particles of both strains were circular in cross-section with diameters of 60–100 nm although some larger particles were also observed. Individual virions consisted of an outer envelope, approximately 20 nm thick, surrounding a pale central region. Areas of more densely stained material were often seen at the periphery of the central region close to the envelope and apparently 'empty' particles were also present.



Many virus particles, especially those seen outside the cells after release, possessed well-defined surface projections or spikes. These could be seen clearly on both strains of virus, and were particularly distinctive on 'T' strain particles (Fig. 10 a, b), but the number of spikes visible varied considerably from one particle to another. Each spike was 15–20 nm in length and approximately 5 nm across with a small thickened knob at the distal end.

Although projections were most numerous and clearly visible on released virions they could also be identified on particles still in the process of budding; generally no more than one or two spikes were seen attached to the incomplete envelope (Figs. 10 c, 3).

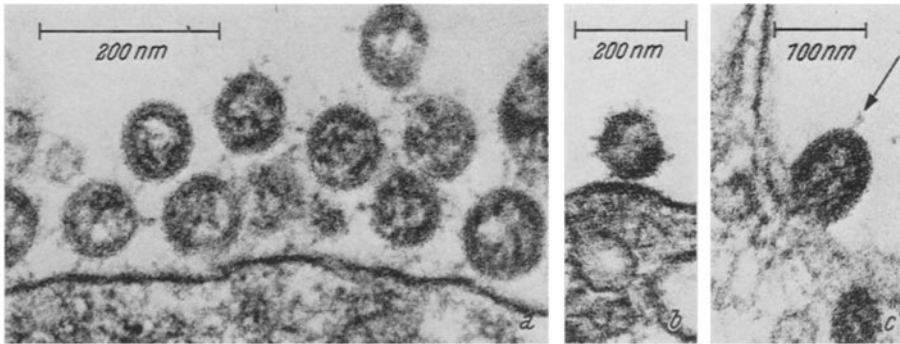


Fig. 10. Morphology of IBV particles

a) Extracellular T strain particles 46 hours after inoculation. Note the well-defined surface projections  $\times 120,000$ . b) Extracellular strain Beaudette virus 21 hours after inoculation also exhibiting surface spikes  $\times 75,000$ . c) A strain T virus particle budding into a cytoplasmic vacuole 46 hours after infection. A surface spike can be seen (arrowed) on the immature particle;  $\times 120,000$

### Discussion

The general features of IBV development in primary CK cells observed in this study are consistent with those reported in previous investigations of coronavirus morphogenesis (11). In common with other IBV studies the intracellular inclusions seen in cells infected with mouse hepatitis or human respiratory viruses (7, 12) were not observed in our material.

The IBV particles seen in this investigation showed morphological characteristics similar to those already described for the coronaviruses (11). A distinctive feature, however, was the presence on many particles of well-defined surface projections which have not previously been clearly observed in sections of cells infected with IBV (2) or other coronaviruses. The general shape of the individual spikes was similar to that seen in negatively-stained preparations (3, 9). The presence of spikes on budding particles was also established and this observation demonstrates the early attachment of at least some of these subunits during virus formation. The apparent absence of surface projections on many particles, particularly those within large vacuoles, could represent fundamental differences due to different methods of maturation or, alternatively, be merely an artifact of specimen preparation.

Further information was obtained concerning the mode of virus entry and release. The electron micrographs indicate that IBV was able to enter CK cells by two distinct mechanisms both of which may be classified as viropexis. Although we did not observe any morphological evidence of uncoating, no other means of entry was detected and we assume that viropexis initiates infection. The mechanism by which the majority of particles entered the cells involved engulfment of the virus, and cell debris present in the inoculum, by surface processes or pseudopodia to form phagocytic vacuoles. The second method involved the uptake of individual particles by micropinocytosis. The thickened membrane forming the enveloping invagination can be identified with the modified or 'coated' membrane characteristic of the transport vesicles present in many cell types (13, 7). Virus entry by micropinocytosis and subsequent association with coated vesicles has not been described previously for coronaviruses but has been observed in L cells infected with a rhabdovirus, vesicular stomatitis virus (14), and in HeLa cells infected with type 5 adenovirus (5).

The budding of particles from the endoplasmic reticulum and the release of progeny virus took place by processes which may be general for all coronaviruses (11). However, the envelopment of virus particles by coated vesicles and their subsequent release from the cells by reverse micropinocytosis are phenomena not previously described for any virus. This mechanism may be peculiar to those members of the coronavirus group which bud into the cisternae of the endoplasmic reticulum since the particles are formed within the membranes from which the coated transport vesicles are produced (14). In this respect our failure to observe any association of 'T' strain virus particles with coated vesicles may be significant since this strain was seen to bud predominantly into cytoplasmic vacuoles. The method of release of virus particles by fusion of vacuoles with the cell plasma membranes seen in 'T' strain infected cells appears similar to the process described for coronaviruses from human respiratory infections (12).

One of the more conspicuous aspects of the morphogenesis of IBV in CK cells was the large number of virus particles seen in the cytoplasmic vacuoles and outside the cells late in infection. Although infectious particle release was not measured in the present study, ALEXANDER and COLLINS (1) have demonstrated release of infectious virus, measured as PFU, from CK cells infected with approximately 5 PFU of IBV strain Beaudette per cell. The maximum titre of released virus was reached 12—14 hours after inoculation, at a time when light microscopy of the infected cells revealed cytopathic effect in the form of small syncytia, but no obvious cell death or lysis had occurred. In their study the maximum titre represented a total infectious particle production of 10—20 PFU per cell (ALEXANDER, unpublished). This low total and the failure to detect an increase in the rate of infectious particle release late in infection would suggest that the majority of particles seen in our thin sections were non-infectious. Similarly, observations on human coronaviruses grown in tissue culture have shown a lack of correlation between the infectivity titres and the large numbers of particles seen by electron microscopy (12). While it is tempting, in the case of strain Beaudette, to correlate the number of released infectious particles with the frequency of virus release from coated vesicles in our work, clearly further study

is required to show more exactly the relationships between the different methods of particle release and the production of infectious virus.

### Acknowledgments

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