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Electron Microscopic Studies of Coronavirus

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SUMMARY

Electron-microscopic studies of the morphology and development of a coronavirus (LINDER strain), isolated in human foetal diploid lung cells from a case of upper respiratory illness, revealed virus particles of diameter 80 to 160 nm. They were initially observed 12 hr after infection. Extracellular and intracytoplasmic virus concentration increased sharply at 18 hr and reached a maximum at 24 hr. The number of particles decreased slightly at 48 hr and by 72 hr many cells were lysed. The particles formed by budding into the cisternae of the endoplasmic reticulum or into an inclusion composed of tubular structures resembling part of the complete virus particle. There were cytoplasmic inclusions of dense particles within a granular matrix and surrounded by a double membrane. The release of particles by lysis is illustrated. Extracellular virus was specifically tagged with ferritin-labelled antibody.

INTRODUCTION

Coronaviruses or infectious bronchitis virus-like agents have been isolated in human embryonal tracheal or nasal organ cultures and in monolayer tissue culture cells from cases of mild upper respiratory illness of man (Tyrrell & Bynoe, 1965; Hamre & Procknow, 1966; Bradburne, Bynoe & Tyrrell, 1967; McIntosh *et al.* 1967; Kapikian *et al.* 1969). The virus group is characterized morphologically by widely spaced club-shaped projections surrounding the virus envelope, thus forming what appears in negative contrast as a 'corona' (Almeida & Tyrrell, 1967; Tyrrell & Almeida, 1967; McIntosh *et al.* 1967; Parker, Cross & Rowe, 1970). A virus (LINDER strain) isolated in human diploid lung cells in this laboratory from a military recruit at Fort Ord, California, U.S.A., was subsequently shown to be antigenically identical to the 229 E coronavirus strain isolated by Hamre & Procknow (1966). The 229 E strain was later characterized morphologically by thin-section electron microscopy (Hamre, Kindig & Mann, 1967; Becker *et al.* 1967). In this report we present additional studies, including ferritin-tagging, on the morphology and development of this agent.

METHODS

Human foetal diploid lung cells (HFDL) grown in fortified Eagle's in Earle's supplemented with 10 % foetal bovine serum were infected with approximately 5×10^4 TCD 50 of a coronavirus (LINDER strain). After adsorbing for 2 hr, 10 ml. of maintenance medium consisting of Leibovitz Medium no. 15 and 2 % foetal bovine serum was added to each bottle. Cultures were examined at 12, 18, 24, 48 and 72 hr after infection; the maintenance medium was removed, the cell layer was washed once with Tyrode's solution, then fixed for 10 min. with

a 1 % solution of glutaraldehyde in 0·1 M-phosphate buffer, pH 7·2. The cells were washed with several changes of phosphate buffer over a 2 hr period, scraped off the glass and pelleted. The pellet was fixed with 1 % osmium tetroxide for 30 min., washed, dehydrated with a graded series of ethanol concentrations and embedded in Epon for thin sectioning. Sections were stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop I.

Ferritin-labelled antibody was prepared by coupling goat anti-rabbit gamma globulins to horse-spleen ferritin using xylylene di-isocyanate as the coupling agent (Singer, 1959; Rifkind, Hsu & Morgan, 1964). Ferritin-tagging experiments were performed by the indirect method on HFDL cells grown on coverglasses and infected with 0·04 ml. (5×10^5 TCD 50/ml.) of coronavirus (LINDER strain). After a 2 hr adsorption period, the infected cells were incubated for 24 hr. The infected coverglass cell cultures were rinsed with phosphate buffered saline and treated with a 1:20 dilution of rabbit anti-LINDER virus serum. The coverglass preparations were washed by flooding with 0·1 M-phosphate buffer followed by immersion and gentle agitation in four different containers of buffer. The cells were then covered with ferritin-labelled goat anti-rabbit gamma globulin conjugate and incubated 20 min. at room temperature. The cells were once again washed by flooding with phosphate buffer followed by immersion and gentle agitation in several changes of buffer. The washed cells were fixed in 1 % glutaraldehyde and processed for electron microscopy.

RESULTS

At 12 hr after infection only an occasional particle with typical coronavirus morphology was seen. The number of virus particles observed increased rapidly after 18 hr and reached a maximum at 24 hr after infection. At this time period the cisternae of the endoplasmic reticulum in many of the cells were filled with virus particles; the cells were, however, still intact and in good condition. The number of particles observable by thin-section electron microscopy began to diminish at 48 hr and at 72 hr few cell-associated particles were visible.

Fig. 1 and 2 illustrate various developmental stages observed in cells 24 hr after infection. Numerous particles may be seen in the cisternae of the endoplasmic reticulum (Fig. 1*a*) and in the perinuclear spaces (Fig. 1*b*). The spherical particles which ranged in size from 80 to 160 nm. are composed of an outer double membrane and a core composed of a dense inner ring, the centres of which appeared to contain material of different grades of density. Particle formation by budding is illustrated in Fig. 1(*c*). The 6 particles are in various stages of the budding process and the largest measures 150 nm.

Cytoplasmic inclusions composed of tubular structures were occasionally seen (Fig. 1*d*). The tubular structures contained a dense material similar in appearance to the dense core material noted in the virus particles. These structures may represent another stage in the formation of mature virus particles or an accumulation of virus precursors (Fig. 1*e*). Arrows point to structures of developing particles which also resemble the tubular structures of the inclusion. Fig. 1(*f*) shows a similar inclusion with densely staining material around the tubular membrane.

Another structure which was frequently seen 24 hr after infection was composed of densely stained particles within a granular matrix and surrounded by a double membrane (Fig. 2*a*). The particles ranged in size from 60 to 100 nm. and were surrounded by a double membrane. Structures which resembled membranous debris were frequently included within the granular matrix.

Large numbers of extracellular particles were present 18, 24 and 48 hr after infection. These particles were seen in large clusters on or near the surface of the cell (Fig. 2*b*) or lined

the surface of the cell (Fig. 2*c*). The characteristic club-like structures around the particles which were readily apparent by negative staining were at times also visible in thin sections. Intranuclear particles or structures associated with infection were not observed.

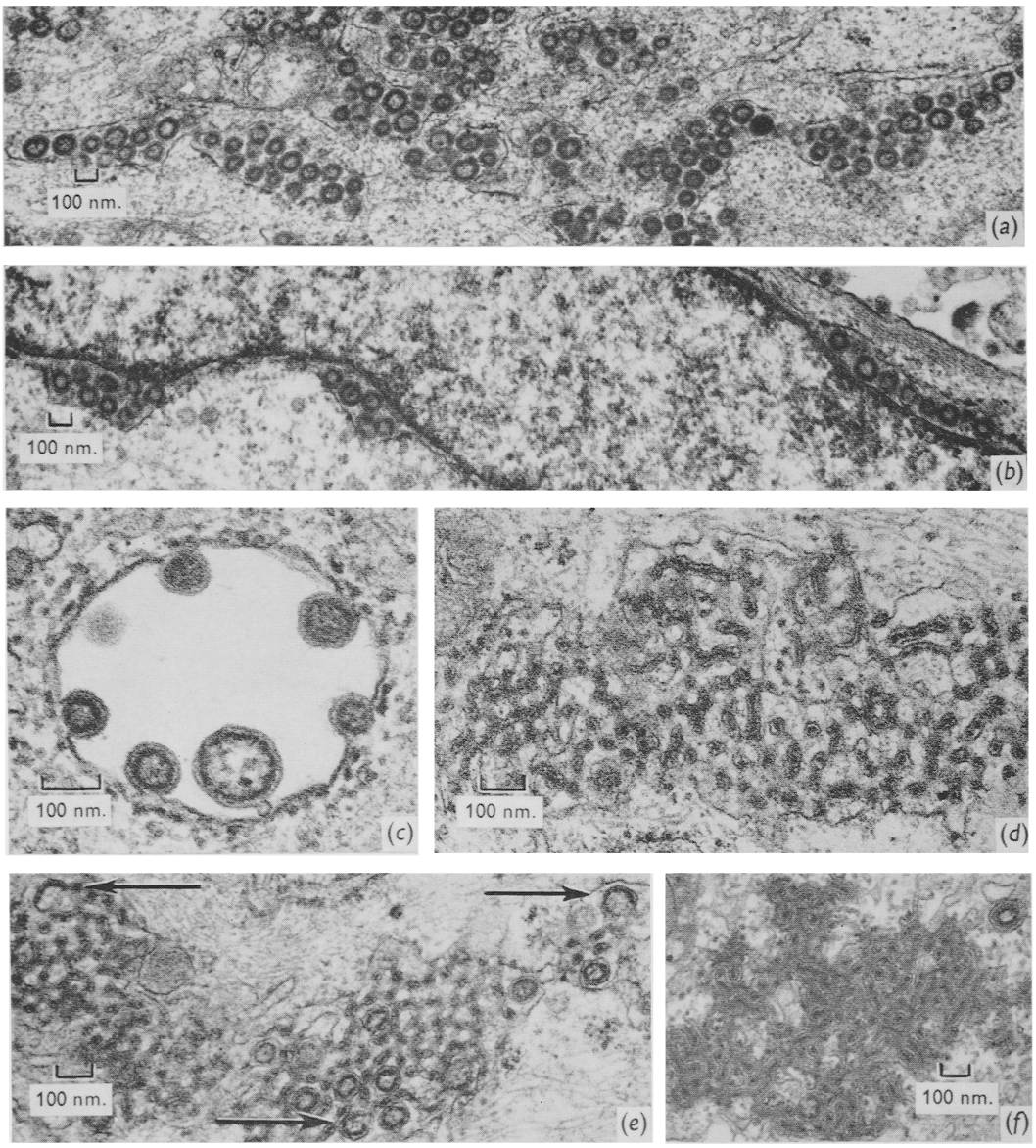

Fig. 1. (*a*) Part of the cytoplasm of a cell showing coronaviruses in the cisternae of the endoplasmic reticulum. (*b*) Part of a cell showing coronaviruses in the perinuclear spaces. (*c*) A vacuole in the cytoplasm showing six virus particles in various stages of bud formation. (*d*) A cytoplasmic inclusion composed of tubular structures containing a dense inner core. (*e*) The relationship of virus particles to a cytoplasmic inclusion composed of tubular structures is illustrated. Note the resemblance between the tubular structures and the structures which make up the virus particle (arrows). (*f*) A cytoplasmic inclusion composed of tubular structures containing a dense inner core and a densely staining material around the tubular membrane.

Two routes of egress were indicated by the observation made 24 hr after infection. A primary mode of virus extrusion was suggested by the presence of lysed cells (Fig. 3*a*). There were also occasional cells which exhibited a bulging of the plasma membrane adjacent to a cisterna of the endoplasmic reticulum containing virus particles (Fig. 3*b*), suggesting another mode of release of virus from certain areas of the cell.

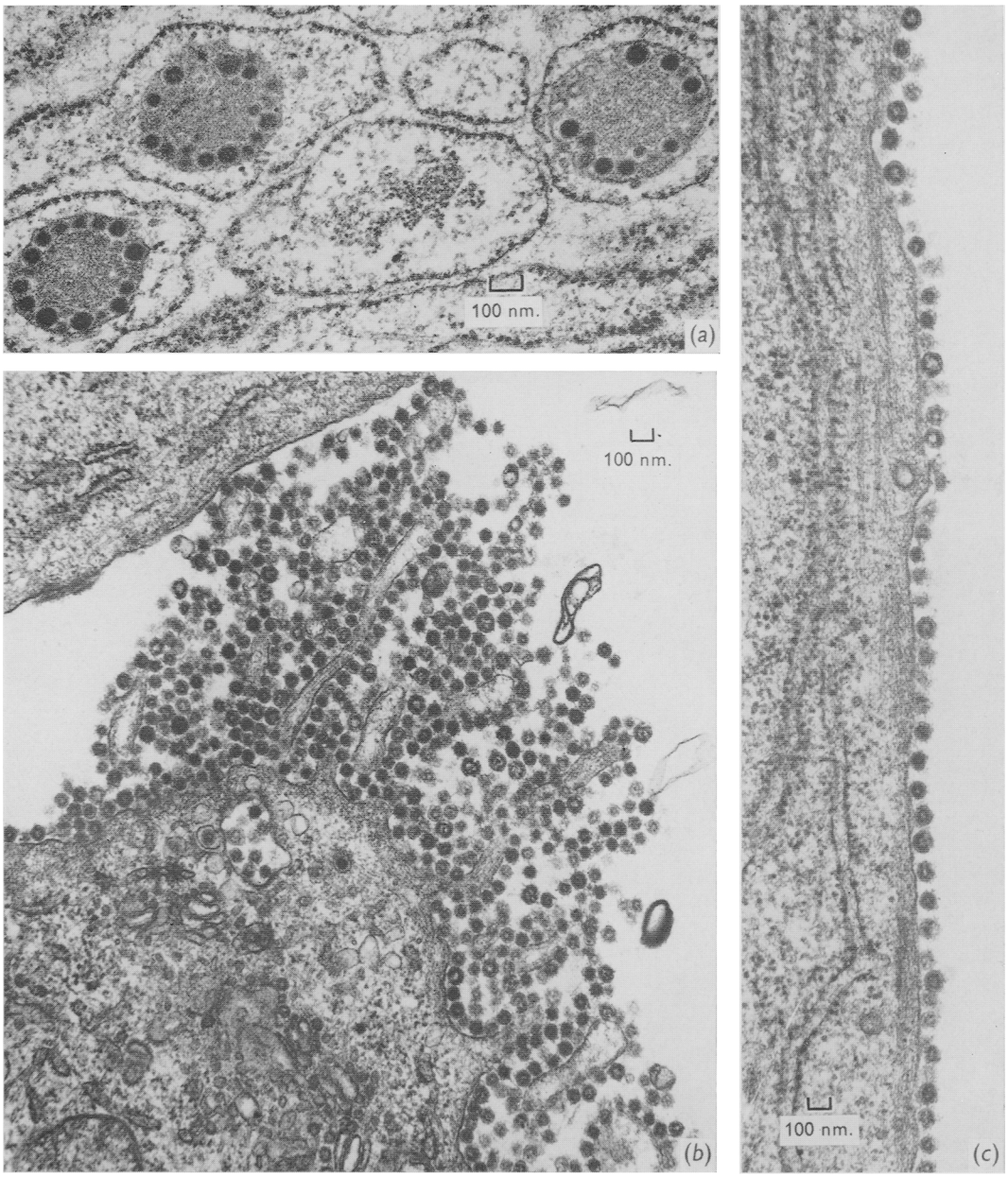

Fig. 2. (*a*) Cytoplasmic inclusions composed of densely staining particles within a granular matrix and surrounded by a double membrane. (*b*) Numerous coronavirus particles on the cell surface and within the extracellular spaces. (*c*) Coronaviruses lining the surface of a cell.

To demonstrate that the particles observed represented LINDER virus, ferritin-labelled goat anti-rabbit gamma globulin was applied to monolayer cultures of HFDL cells infected with LINDER virus and treated with either normal rabbit serum or anti-LINDER virus serum. The use of normal rabbit serum as the intermediate serum did not result in virus or membrane tagging (Fig. 3*c*). Treatment of infected cells with rabbit anti-LINDER virus serum resulted in virus-tagging (Fig. 4). No membrane-tagging was observed.

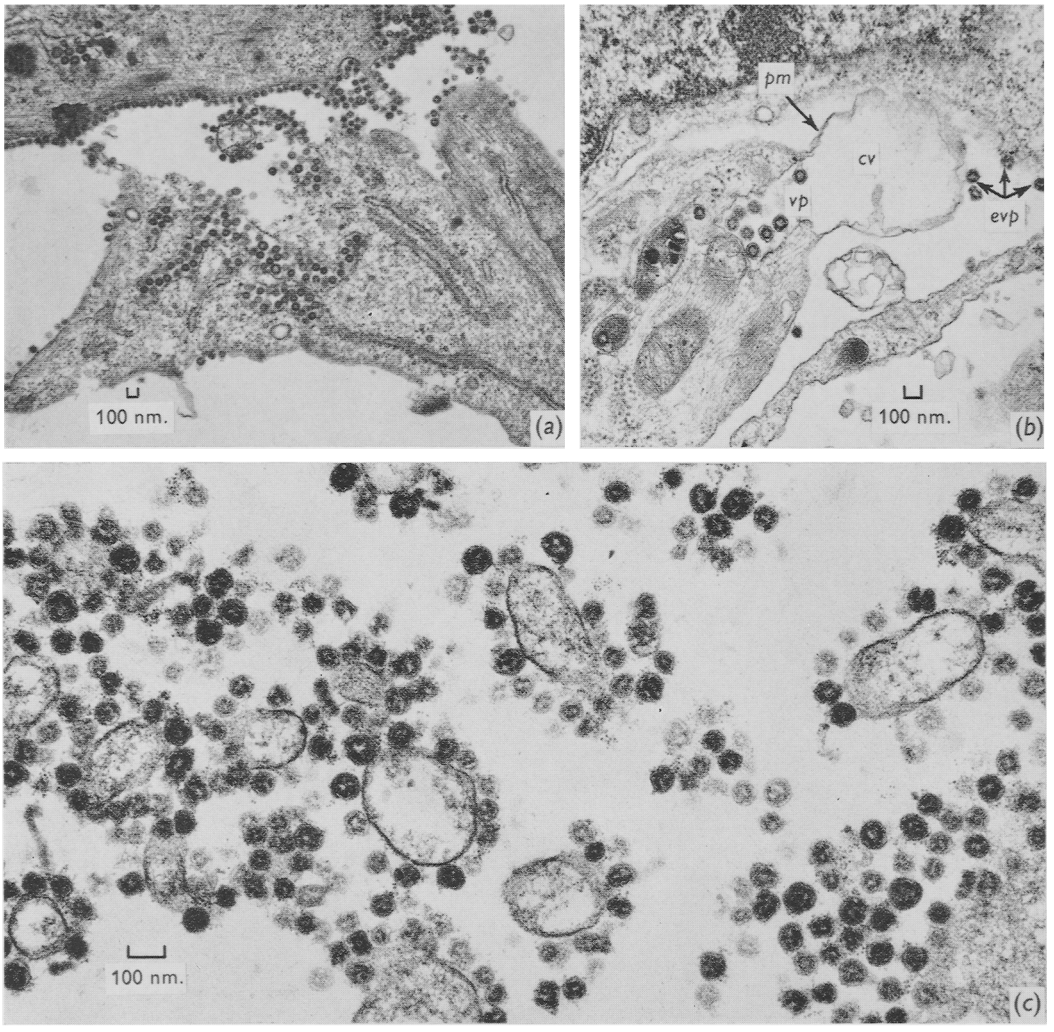

Fig. 3. (*a*) A late stage in lytic process showing virus particles leaving the cisternae of the endoplasmic reticulum via a large break in the cell surface membrane. (*b*) A possible alternative route of virus egress is illustrated by a bleb on the plasma membrane (*pm*) adjacent to a cisterna of the endoplasmic reticulum resulting in a cytoplasmic vesicle (*cv*) containing virus particles (*vp*). Extracellular virus particles (*evp*) are also shown. (*c*) Normal serum control for the indirect ferritin-labelled antibody tagging experiments. Note few background ferritin granules.

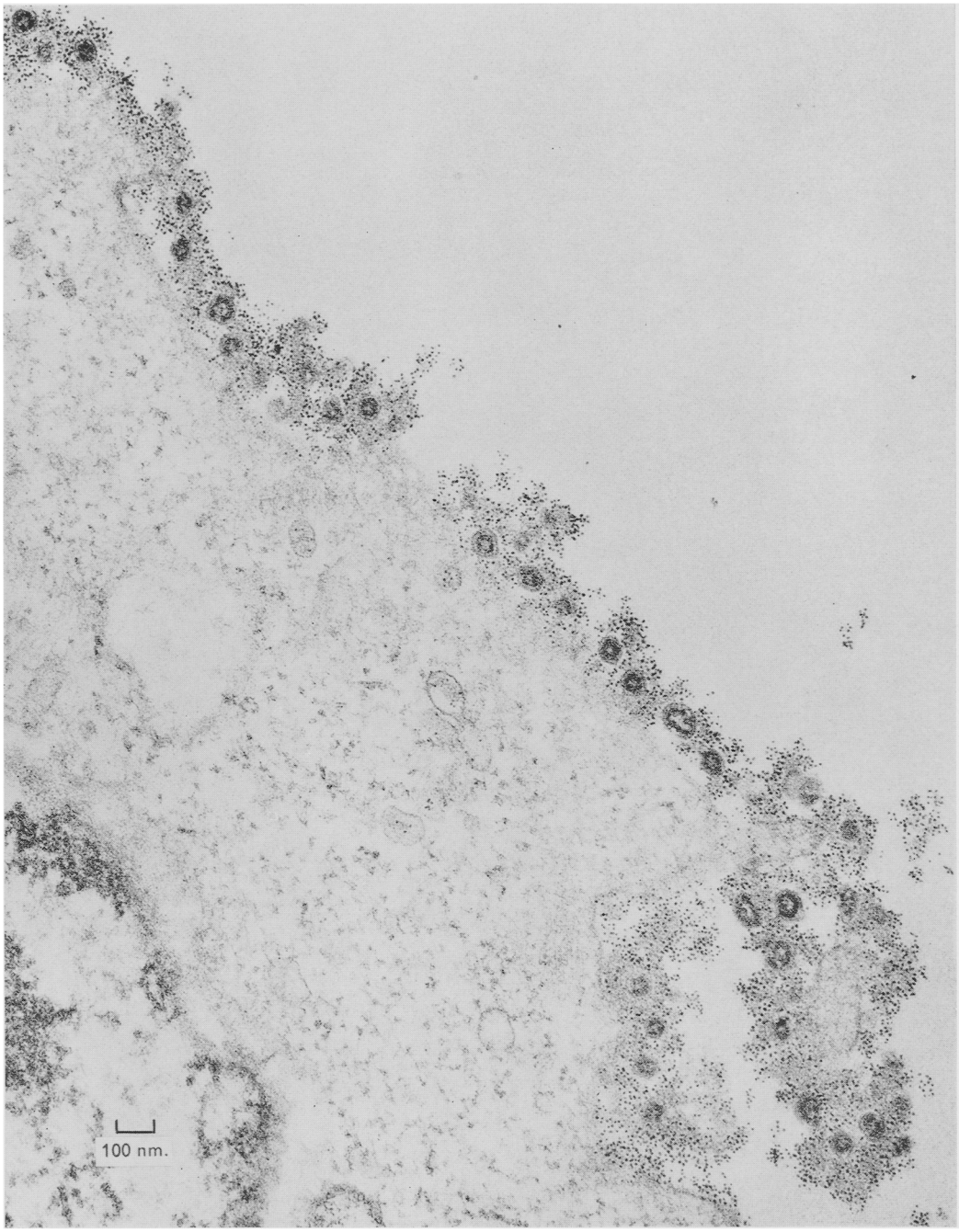

Fig. 4. Ferritin-labelled antibody tagging of coronaviruses lining the surface of a cell.

DISCUSSION

We have described some new features of virus development, in addition to those described earlier by Hamre *et al.* (1967) and by Becker *et al.* (1967).

The frequent occurrence of particles budding into the cisternae of the endoplasmic reticulum, together with the observation of numerous particles in the dilated cisternae, suggested bud formation as the principal means of virus reproduction. The occasional observation of a condensed tubular network of agranular endoplasmic reticulum consisting of particles in various stages of maturation (Fig. 1 *d* to *f*) indicated an alternative mode of virus reproduction. These structures resembled those found by David-Ferreira & Manaker (1965) in cells infected with mouse hepatitis virus.

Fig. 2*b*, *c* and 3*a* show that the extracellular particles were not produced in the cells to which they are adherent, but from an adjacent lysed cell. There was great disparity between tremendous numbers of particles illustrated in Fig. 2*b* and the relatively low infectious titre of such tissue culture material (1×10^5 TCD 50/ml.).

We agree with Hamre *et al.* (1967) and Becker *et al.* (1967) that virus particles are formed by budding into the cisternae of the endoplasmic reticulum but have not been seen budding at the cell surface membrane. Hamre *et al.* (1967) presumed that particles were liberated from intracytoplasmic vesicles by rupture, while Becker *et al.* (1967) suggested communication of cytoplasmic vesicles directly with the extracellular spaces. While neither of these groups of investigators had seen direct evidence for this, our observations show the two modes of virus release. The occasional appearance of cells in the lytic state, 24 hr after infection, indicated that coronaviruses were mostly released by extrusion. There were no gross cytoplasmic changes at this time, but many cells were in the early lytic stages and tremendous numbers of particles were gradually being released from these cells.

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REFERENCES

ALMEIDA, J. D. & TYRRELL, D. A. J. (1967). The morphology of three previously uncharacterized human respiratory viruses that grow in organ culture. *Journal of General Virology* **1**, 175.

BECKER, W. B., McINTOSH, K., DEES, J. H. & CHANOCK, R. M. (1967). Morphogenesis of avian infectious bronchitis virus and a related human virus (strain 229E). *Journal of Virology* **1**, 1019.

BRADBURNE, A. F., BYNOE, M. L. & TYRRELL, D. A. J. (1967). Effects of a 'new' human respiratory virus in volunteers. *British Medical Journal* **3**, 767.

DAVID-FERREIRA, J. F. & MANAKER, R. A. (1965). An electron microscope study of the development of a mouse hepatitis virus in tissue culture cells. *Journal of Cell Biology* **24**, 57.

HAMRE, D. & PROCKNOW, J. J. (1966). A new virus isolated from the human respiratory tract. *Proceedings of the Society for Experimental Biology and Medicine* **121**, 190.

HAMRE, D., KINDIG, D. A. & MANN, J. 1967). Growth and intracellular development of a new respiratory virus. *Journal of Virology* **1**, 810.

KAPIKIAN, A. Z., JAMES, H. D. JUN., KELLY, S. J., DEES, J. H., TURNER, H. C., McINTOSH, K., KIM, H. W., PARROTT, R. H., VINCENT, M. M. & CHANOCK, R. M. (1969). Isolation from man of 'avian infectious bronchitis virus-like' viruses (coronaviruses) similar to 229E virus, with some epidemiological observations. *Journal of Infectious Diseases* **119**, 282.

McINTOSH, K., DEES, J. H., BECKER, W. B., KAPIKIAN, A. Z. & CHANOCK, R. M. (1967). Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. *Proceedings of the National Academy of Sciences of the United States of America* **57**, 933.

PARKER, J. C., CROSS, S. S. & ROWE, W. P. (1970). Rat coronavirus (RCV): A prevalent, naturally occurring pneumotropic virus of rats. *Archiv für die gesamte Virusforschung* **31**, 293.

RIFKIND, R. A., HSU, K. C. & MORGAN, C. (1964). Immunochemical staining for electron microscopy. *Journal of Histochemistry and Cytochemistry* **12**, 131.

SINGER, S. J. (1959). Preparation of an electron-dense antibody conjugate. *Nature, London* **183**, 197.

TYRRELL, D. A. J. & ALMEIDA, J. D. (1967). Direct electron microscopy of organ cultures for the detection and characterization of viruses. *Archiv für die gesamte Virusforschung* **22**, 417.

TYRRELL, D. A. J. & BYNOE, M. L. (1965). Cultivation of a novel type of common-cold virus in organ cultures. *British Medical Journal* **1**, 1467.

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