AN ELECTRON-MICROSCOPE STUDY OF THE TRACHEA OF THE FOWL INFECTED WITH AVIAN INFECTION BRONCHITIS VIRUS

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Early electron-microscope studies of metal-shadowed preparations of avian infectious bronchitis virus (IBV) revealed that the virus particle had a diameter of 60–120 nm (Reagan et al., 1948; Reagan and Brueckner, 1952). Later, negative staining showed it to differ from Newcastle disease virus in having less densely packed and more loosely attached surface projections that gave a strawberry-like appearance (Chu, 1964).

Berry et al. (1964) confirmed that the projections of IBV are morphologically distinct from those of the myxoviruses, and reported that the diameter of the virus particle was 80–120 nm. Domermuth and Edwards (1957) examined ultrathin sections of IBV-infected chorio-allantoic membranes and observed intracytoplasmic particles having a doughnut-like appearance and measuring 178–200 nm in diameter. Viruses isolated from the respiratory tracts of persons with common colds have been described as “avian infectious bronchitis-like” on account of their morphology in negatively stained preparations (Almeida and Tyrrell, 1967). However, differences in internal structure have been reported between the avian and human viruses present in ultrathin sections of infected chorio-allantoic membrane and human embryo kidney tissue culture cells respectively (Becker et al., 1967).

In this paper we present an electron-microscope study of the multiplication of avian-IBV in its natural host, the domestic fowl. This does not appear to have been previously described. The structure of the virus grown in the fowl is also compared with that of virus grown in the embryonated hen egg.

MATERIALS AND METHODS

Infectious bronchitis virus (IBV), strain A 163 (isolated by Dr H. P. Chu) was used. It was propagated in the allantoic cavity of embryonated hen eggs. All fowls and eggs were obtained from a respiratory disease-free flock maintained at the School of Veterinary Medicine, Cambridge.

Fowls, 4 wk of age, were infected intratracheally with 0·25 ml of allantoic fluid-virus suspension (titre 10^6·5 EID50 per ml). Two birds were then killed after 6, 12, 18, 24, 48 and 72 hr. Control uninfected birds were killed at the same times. Freshly harvested tissues were fixed in Dalton’s chrome-osmium fixative (Dalton, 1955), dehydrated through ascending

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concentrations of ethyl alcohol and embedded in Araldite (Richardson, Jarett and Finke, 1960). 0.1 ml of a 1 in 100 dilution of the virus (titre 10^8 EID50 per ml) was injected allantoically into 9-day embryonated hen eggs. Eggs dying within 24 hr were discarded. After 36 hr the allantoic fluid was collected and pooled, and pellets were obtained by centrifugation in a refrigerated centrifuge at 19,000 r.p.m. for 1 hr. Part of each pellet was resuspended in 1 per cent. ammonium acetate, mixed with an equal volume of 2 per cent. potassium phosphotungstate (adjusted to pH 6.8 with 1 per cent. KOH) and spread on collodion-coated copper grids for electron microscopy. The remainder of the pellet was fixed and embedded in Araldite as described above.

Sections, 2 µm thick, were first cut from each Araldite block, stained with methylene blue and examined by light microscopy to provide orientation. At the selected sites ultrathin sections, 60–70 nm thick, were then cut, with an A. F. Huxley ultramicrotome, stained with uranyl acetate and lead citrate (Echlin, 1964) and examined in a Siemens Elmiskop I electron microscope. Mean viral diameters were always calculated from measurements of 100 virus particles.

RESULTS

Normal uninfected fowl trachea

Ultrathin sections of uninfected fowl trachea show that the epithelial layer is formed of ciliated and mucus-secreting cells (fig. 1). Each cilium is covered with a triple-layered plasma membrane and contains longitudinally arranged filaments (fig. 2). Microvilli, which are much shorter than the cilia, are also covered by a triple-layered plasma membrane, but do not possess filaments. There was no evidence of viral infection in any of the control specimens.

Infected fowl trachea

The first definite changes, at the ultrastructural level, in the epithelial cells of the trachea are discernible 6 hr after infection. Cilia and microvilli are either lost or degenerate. In some areas the epithelial cells show distortion of their plasma membranes and changes in their internal structure. There is swelling of mitochondria with vesiculation of the cristae, and increase in the amount of endoplasmic reticulum and of the membranes of the Golgi apparatus (fig. 3). In addition, electron-dense areas with well-defined but incomplete margins are present in the cytoplasm. These are frequently seen in the early stage of infection (12–24 hr) but rarely later (48–72 hr). Electron-dense particles, thought to be immature viral particles, are visible within these areas, usually peripherally arranged (fig. 4). Similar particles are present, singly and in small groups, in the spaces between the membranes of the endoplasmic reticulum and within the Golgi apparatus (figs. 5 and 6).

Between 48 and 72 hr the virus particles increase in number within the endoplasmic reticulum and are also visible within cisternae and vesicles, which have probably formed by dilatation of the intracellular membranes (figs. 7 and 8). At 72 hr extracellular virus particles are seen, probably liberated from the distended cisternae and vesicles. Viral particles are never observed in the nucleus at any stage of the infection.

The viral particles present in the later stages of infection possess distinct outer and inner coats or shells separated by an electron-transparent zone (figs. 8
**Fig. 1.**—Electron micrograph (EM) of uninfected epithelial cells of chicken trachea showing ciliated and mucous-secreting cells. N = Nucleus; M = mitochondria. Double staining with uranyl acetate and lead citrate (UA-LC). × 5100.

**Fig. 2.**—Longitudinal section of cillum (CI) and microvillus (MI). FR = Fibrillar rootlet; PM = plasma membrane; F = filaments. EM. UA–LC. × 48,000.

**Fig. 3.**—Infected epithelial cell of chicken trachea 12 hr after infection, showing loss of cilia, degenerated and swollen microvilli, distorted mitochondria (M) and well-defined electron-dense areas (ED). EM. UA–LC. × 22,800.

**Fig. 4.**—Infected epithelial cell of chicken trachea 24 hr after infection, showing viral particles (VP) within electron-dense areas (ED). EM. UA–LC. × 60,000.
Fig. 5.—Viral particles in epithelial cells of chicken trachea 18 hr after infection. They are seen in the Golgi apparatus, in cisternae and in electron-dense areas. N = Nucleus; NUC = nucleolus; NM = nuclear membrane. EM. UA–LC. ×9000.

Fig. 6.—Enlargement of squared area in fig. 5. EM. UA–LC. ×52,000.

Fig. 7.—Infected cell 48 hr after infection, showing large numbers of virus particles in a vesicle (V) and in the endoplasmic reticulum. EM. UA–LC. ×48,000.
Fig. 8.—Infected cell 72 hr after infection, showing dilatation of endoplasmic reticulum owing to presence of many virus particles. EM. UA-LC. ×40,000.

Fig. 9.—Enlargement of squared area in fig. 8. Virus particles possess outer (OS) and inner (IS) shells or coats separated by an electron-transparent zone. EM. UA-LC. ×160,000.

Fig. 10.—Ultrathin section of viral pellet prepared from allantoic fluid of embryonated eggs infected with IBV. The virus particles have outer and inner shells separated by an electron-transparent zone. EM. UA-LC. ×72,000.

Fig. 11.—IBV particles negatively stained with potassium phosphotungstate. EM. ×164,000.
and 9); this is not obvious in viral particles present during the early stage of infection. The mean diameter of the mature virus particles is 81 ± 1 nm.

Structure of virus particles harvested from allantoic fluid

Ultrathin sections of the pelleted virus show some degree of pleomorphism of the particles. Most particles, however, are round in shape; some of them are still contained within vesicles, and many of them clearly show inner and outer shells separated by an electron-transparent zone (fig. 10). The mean diameter of the virus particles is 86 ± 2 nm.

Negatively stained preparations also show some degree of pleomorphism of the particles, but the majority of these are round and they possess distinct, well-spaced spikes on the surface (fig. 11). Their mean diameter is 90 ± 6 nm.

DISCUSSION

In recent years human foetal tracheal organ cultures have been used for the isolation of IBV-like viruses from the respiratory tract of man (Tyrrell and Bynoe, 1965; McIntosh et al., 1967). Loss of ciliary activity is used as an indicator of virus isolations (Tyrrell, Bynoe and Hoorn, 1968). Our studies of chickens infected with avian-IBV demonstrated extensive loss of cilia by the epithelial cells of the trachea. There was also distortion and decrease in number and size of the microvilli, together with intracellular disorganisation, such as swelling of the mitochondria, enlargement of the endoplasmic reticulum, increase in the size of the Golgi apparatus membranes and the appearance in the cytoplasm of electron-dense areas of varying sizes. These are all changes that are generally considered to be associated with viral infections.

The early occurrence of large numbers of the electron-dense areas, often containing virus particles, suggests that they are sites of virus production. Somewhat similar structures have been reported in association with the development of other viruses (Murphy et al., 1968; Lecatsas, 1968; Lascano, Berria and Barrerora, 1969). Becker et al. (1967) postulated a mechanism of replication of IBV involving budding from intracellular membranes and incorporation of cellular membranes into the outer coat of the virus. This was not observed in any of our specimens taken within 6–24 hr of infection, and in all the later sections prepared 48–72 hr after infection only two or three probable sites of budding into cytoplasmic vesicles or cisternae were detected. We, therefore, consider that the development of IBV by budding requires further clarification. However, the formation of virus particles within cisternae and vesicles of the endoplasmic reticulum is in agreement with the findings of Becker et al. and of Nazerian and Cunningham (1968).

The avian-IBV particles in the ultrathin sections of the infected chicken trachea closely resembled the virus particles in the embryonated-egg allantoic fluid in both size and morphology. The majority of the viral particles present in the tracheal cells 48–72 hr after infection possessed outer and inner membranes or shells separated by an electron-transparent zone. Becker et al. described a similar structure for the human IBV-like virus in ultrathin sections of infected...
cells, but not for the avian-IBV itself, although both viruses had the same double-shell morphology in negatively stained preparations. Possibly, these differences are dependent on the host cell, the strain of IBV or the time of harvesting of specimens after infection.

**SUMMARY**

Fowls were infected with avian-IBV and, subsequently, their tracheas were sectioned and examined in the electron microscope. The lining epithelial cells showed extensive loss of cilia, degeneration of microvilli, swelling of mitochondria, increased amount of endoplasmic reticulum and of other intracellular membranes, and the presence of electron-dense areas in the cytoplasm. Virus particles were first observed about 12 hr after infection within the electron-dense areas and within spaces between the membranes of the endoplasmic reticulum. By 48–72 hr they were considerably more numerous and were present mainly within cisternae and vesicles formed from the membranes of the endoplasmic reticulum. There was little evidence of budding. The mature virus particle had a mean diameter of $81 \pm 1$ nm and possessed both inner and outer electron-dense coats or shells separated by an electron-transparent layer. Virus particles grown in the embryonated hen egg and harvested from the allantoic fluid had a similar size and structure.

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