

Pathogenesis of Influenza Virus Infection in Mouse Tracheal Organ Cultures¹ (36565)

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(Introduced by G. E. Cartwright)

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The pathogenesis of influenza virus infection has usually been studied at the cellular level (1) or in the intact animal (2). Studies utilizing tracheal organ cultures offer advantages over the monolayer cell culture system in that the cells infected by the virus remain differentiated and similar to the *in vivo* condition. Tracheal organ cultures have advantages over the intact animal in that they permit study of the effect of virus infection on such host defenses as ciliary activity under conditions where the effects of host immunity or bacterial superinfection can be eliminated.

The present study describes the effects of influenza A virus infection on ciliary activity and histological structure of mouse trachea in organ culture.

Materials and Methods. Preparation of organ cultures. Organ cultures were prepared by an adaptation of the technique of Hoorn and Tyrrell (3). Tracheas from 5-week-old Swiss-Webster mice were excised in bloc by sterile technique and stripped of excess connective tissue. The tracheas were placed in Hanks' balanced salt solution plus 1000 units/ml of penicillin G for 30 min, after which they were cut with razor blades into square pieces of tissue measuring 2 mm on an edge. Each trachea yielded from 8 to 10 squares of tissue. Tracheal tissue was then placed with the ciliary side upright into 16 × 150 mm roller tubes which were slightly moistened so that the tissue would adhere to the glass surface. Each roller tube contained approximately 30 pieces of tissue. Once the trachea explants were attached to the glass, 1 ml of L-15 medium (4) containing 100

units/ml of penicillin G and 0.2% bovine serum albumin was added. The cultures were incubated on a roller drum at 33°.

The ciliary action was observed with a Zeiss Universal microscope employing 63× or 160× total magnification. Only explants showing strong ciliary activity after 24 hr were marked and subsequently evaluated for changes in ciliary activity. Each piece of tissue was rated as either full activity, reduced activity, or absence of activity.

Virus. The PR-8 strain of influenza A virus used in this study was obtained from the Research Reference Reagent Laboratory of the National Institutes of Health, Bethesda, MD. The virus had a history of 8 passages in ferrets, 593 passages in mice and 168 passages in chick embryos before it was received in this laboratory. The virus pool was prepared by one passage in the allantoic cavity of embryonated hen eggs. Allantoic fluid was harvested after 48 hr and stored at -70°.

Virus quantitation. Virus samples were assayed using 50% end-point titrations in a continuous line of African green monkey cells (Vero). Evidence of virus infection was obtained by demonstrating hemadsorption employing a 0.4% suspension of guinea pig erythrocytes (5). The technique for calculating the tissue culture infective dose (TCID₅₀) was that of Reed and Muench (6).

Inoculation of organ cultures. Organ cultures were inoculated with virus after 24 hr of incubation and this was designated as day 0 of the experiment. Twenty-four hours after virus inoculation (day 1), the medium was completely removed for virus titrations, tracheal tissue was washed once with L-15 media and fresh medium was then added.

¹ Supported by U.S. Public Health Service Grants Nos. 5 T01 AI0039 and 9 R01 HE14703.

Media was subsequently harvested for titrations, tissues were washed, and fresh medium was replaced at 48-hr intervals. Ciliary activity was also assessed at 48-hr intervals just before each medium change. Pieces of tracheal tissue were removed at 48-hr intervals and fixed for histological and electron microscopic studies.

Electron microscopy. The procedure for the preparation of the tracheal tissue for examination with the electron microscope was based on the technique described by Pease (7). Mouse tracheal explants were placed in 3% glutaraldehyde overnight and then transferred to cold Millers buffer (pH 7.4) for primary fixation. The tissue was postfixed in 2% osmium tetroxide. Samples were dehydrated in graded alcohols and embedded in epoxy resin (Epon 812). The embedded tissue was then sectioned with diamond knives on an MT-1 Porter-Blum ultramicrotome, placed on grids, and stained with aqueous uranyl acetate and Reynolds' lead citrate (8). Sections were examined and photographed employing a Zeiss EM 9 A electron microscope at magnifications ranging from 1650 to 20,000.

Results. Growth of influenza A virus. Organ cultures of mouse trachea appeared to support the growth of influenza A/PR-8 virus for 15 days (Fig. 1). Following an inoculum of $10^{3.3}$ TCID₅₀/ml (and per tube), the titer of influenza virus in media just before each 48-hr change of media persisted between $10^{1.8}$ and 10^3 TCID₅₀/ml. In contrast, infectious virus was not found after 48 hr in control tubes which did not contain tracheal tissues. As Hoorn and Tyrrell (3) pointed out, maintenance of virus titers in

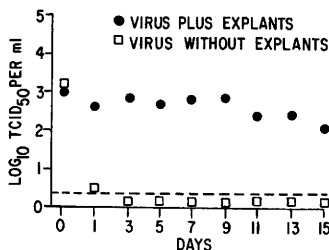


FIG. 1. Titer of influenza A/PR-8 virus from supernatant medium of mouse tracheal organ cultures. (---) Limit of sensitivity of the assay system.

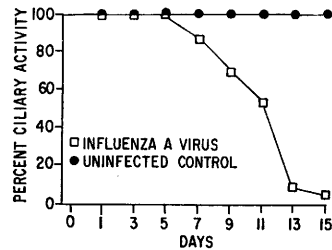


FIG. 2. Percentage of mouse tracheal explants showing ciliary activity after infection with influenza A/PR-8 virus. Percentage ciliary activity was calculated by dividing the number of explants that showed full or reduced ciliary activity by the total number of explants observed.

the supernatant media of organ cultures after several changes of media suggests virus multiplication. The new viruses produced are balanced by the losses of thermal inactivation and medium changing.

Ciliary activity. A reduction in the frequency of coordinated ciliary beating in the tubes infected with influenza virus was first apparent by day 3. Total cessation of ciliary activity of some virus infected explants was first seen on day 7 (Fig. 2). The number of inactive tissues gradually increased in the virus infected tubes through day 15 when only 6% of the tracheal explants showed any ciliary activity. In contrast, all tracheal explants in uninfected control tubes had actively beating cilia after 15 days in culture.

Histopathology. The histological structure of tissues from uninfected control cultures was well maintained through day 15. Cilia remained clearly distinguishable and the only change in the ciliated cells after culturing was a tendency for nuclei to be found throughout the cytoplasm rather than in their initial location at the base of the cells. The histological sections of infected explants first revealed damage to the epithelial layer 5 days after infection (Fig. 3B). When compared to the control tissue (Fig. 3A), the epithelial layer of infected tissues showed the presence of pyknotic nuclei and extensive vacuolization. The epithelium had lost its characteristic columnar appearance and appeared more flattened. Although cilia could still be seen on the surface of some infected

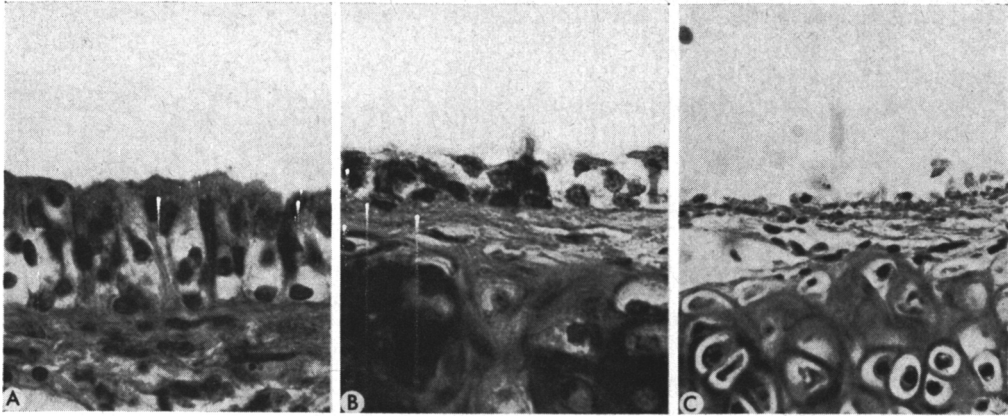


FIG. 3. Appearance of mouse tracheal explants after culturing. (A) Uninfected control after 5 days; (B) infected with influenza A/PR-8 virus after 5 days; (C) infected with influenza A/PR-8 virus after 11 days. H and E stain $\times 400$.

cells after 5 days, in most sections it was difficult to find cilia. After 11 days in culture, the epithelial layer of infected explants was usually completely destroyed (Fig. 3C). The destructive effect of the virus was confined to the epithelial layers of the tracheal tissue, since subepithelial connective tissue cells and structures remained normal in appearance.

Electron microscopy. Electron microscopic changes in infected tissues were first seen after three days in culture. Most notable was the observation that virus particles could be seen adhering to the cilia (Fig. 4). Often the virus particles acted as bridges causing the cilia to clump together (Fig. 5). This sticking together of the cilia was noted in all the electron photomicrographs of the virus infected tissues examined between days 1 and 5. Ciliary clumping was not noted in any of the uninfected control tissue sections. Structures resembling budding virions were seen at the surface of infected cells; however, these could not be distinguished from microvilli seen in control cultures. Viral cytoplasmic inclusions could not be identified in any of the preparations.

Discussion. In addition to the direct pathologic effect of influenza virus infection in the lung, infection with the virus may also lead to increased morbidity and mortality from secondary bacterial infections. Staphylococcal pneumonia, in particular, has been de-

scribed as a serious secondary infection following influenza in man (9). An established infection with influenza virus in mice also promotes the capacity of *Haemophilus influenzae* organisms to establish themselves and add to the severity of the disease (10).

One possible mechanism whereby influenza virus infection might predispose to secondary bacterial pneumonia is the damaging effect of the influenza virus infection on the ciliary clearance mechanism. In the present study, influenza virus infection led to a reduction of ciliary activity by day 3 and ciliostasis by day 5 in mouse tracheal explants. It is also possible that damage to other tracheal epithelial cells, such as mucus secreting cells, may decrease resistance to bacterial infection.

Influenza virus has been reported to cause inhibition of ciliary activity in human tracheal organ cultures after 7 days (11) and cause reduced ciliary activity in monkey nasal explants after 11 days (3). The reduced frequency of ciliary beat observed in this study may be a manifestation of the clumping of the cilia as seen in the electron photomicrographs. Dourmashkin and Tyrrell (12) recently reported the attachment of influenza virus to cilia of guinea pig tracheal epithelial cells grown in organ culture but did not mention clumping of the cilia or effects on ciliary activities.

The histopathologic effects of influenza vi-

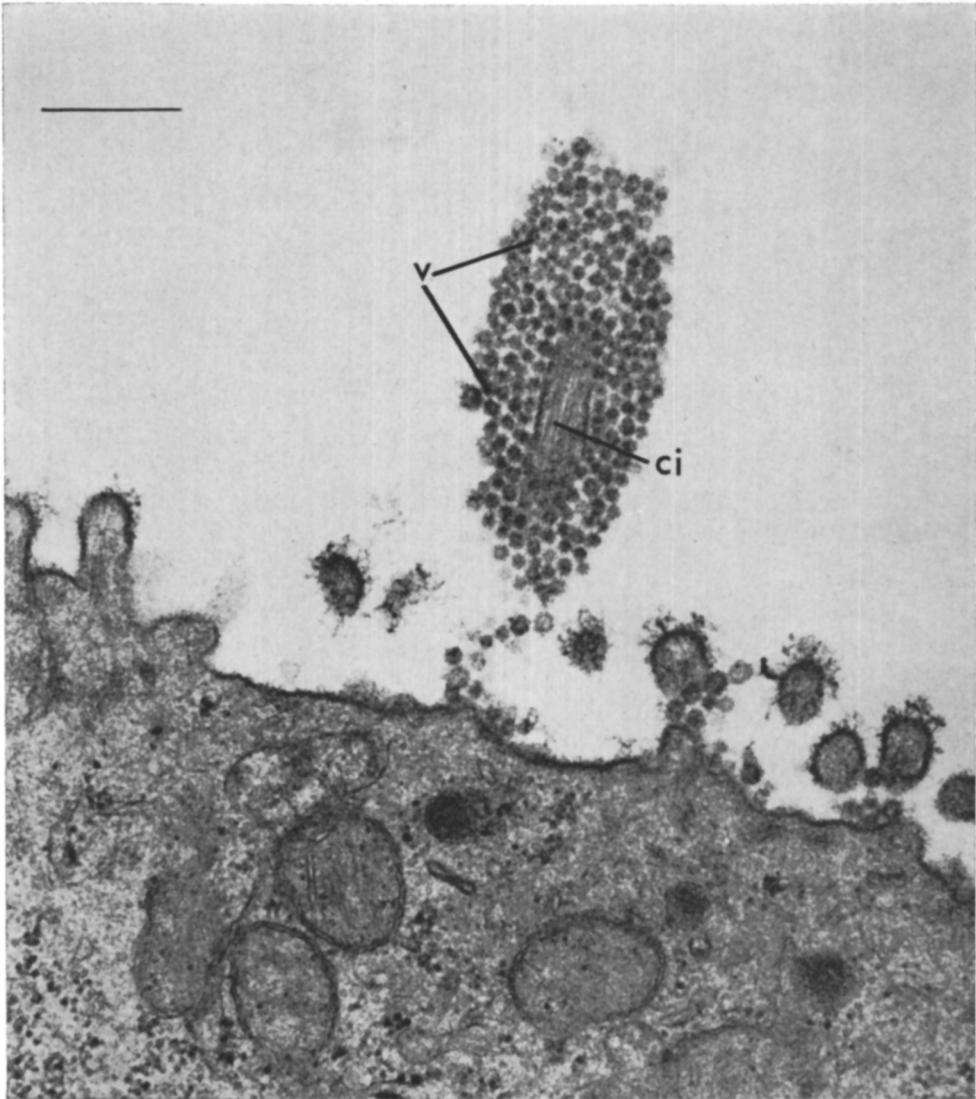


FIG. 4. Electron photomicrograph of a cluster of influenza A/PR-8 virus particles attached to a cilium. (v) Virus particles, (ci) cilium. Bar indicates 0.5 μ m.

rus infection on mouse trachea explants described in this study were similar to changes described by Reed (13) for parainfluenza virus infection of calf tracheal organ cultures. She observed severe damage to the superficial layers of the epithelium characterized by cellular vacuolization leading to complete desquamation of the epithelial layer. Changes seen in organ cultures of mouse tracheas were also similar to those seen *in vivo* following influenza virus infection. Nelson and

Oliphant (14) in their studies of influenza A/PR-8 virus infected mice reported that 4 days after infection, bronchial and tracheal epithelium in some areas was markedly flattened, irregular, or completely denuded. Hers (15) reported that the influenza virus lesions produced in humans was characterized by degeneration and desquamation of the ciliated epithelium of the trachea and bronchi. In later stages of the infection the epithelium had become undifferentiated and

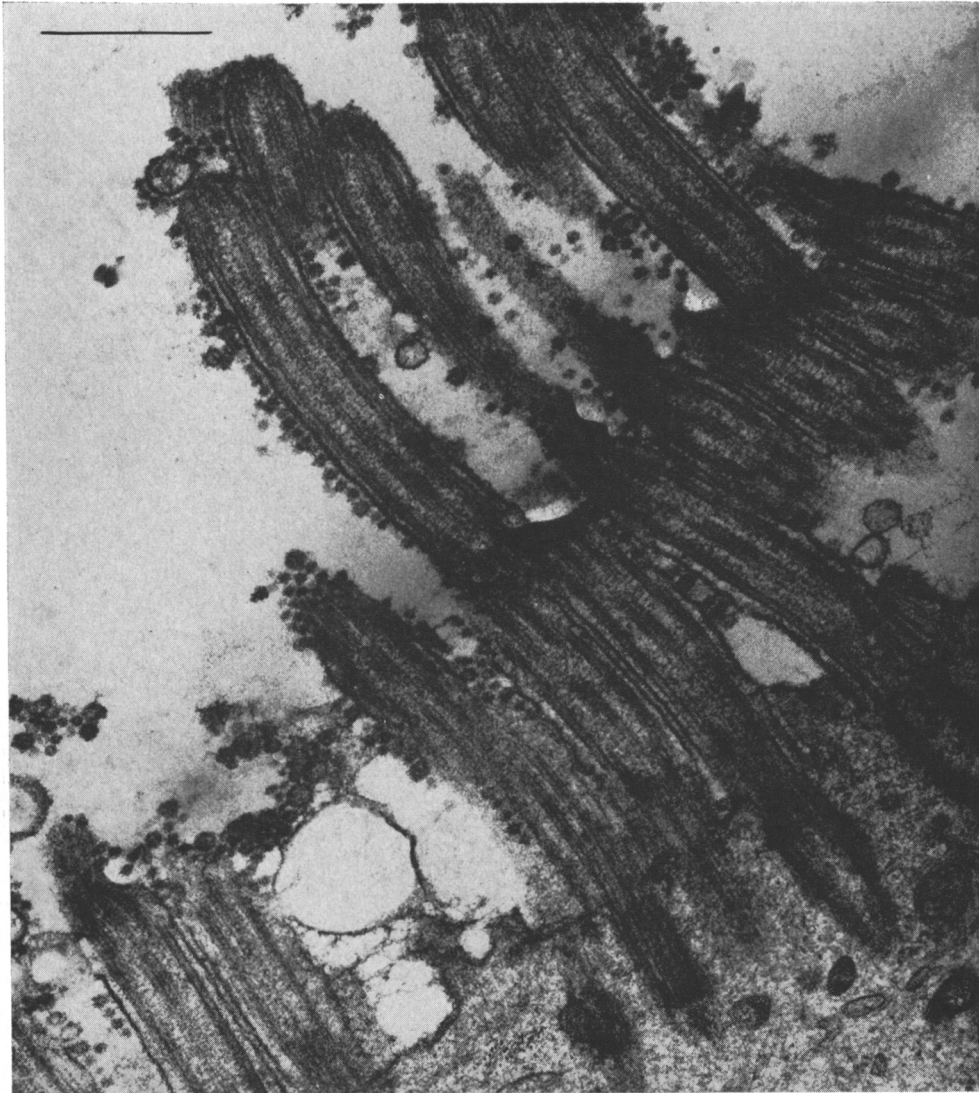


FIG. 5. Electron photomicrograph of influenza A/PR-8 virus particles causing cilia to clump together. Bar indicates 0.5 μ m.

the normal differentiated ciliated cells could rarely be found.

The similarity in pathologic changes seen in tracheal organ cultures and *in vivo* suggest that the mouse tracheal organ culture served as a good experimental model of influenza virus infection of respiratory epithelium. In addition they permitted assessment of the effect of the virus infection on the ciliary clearance mechanism.

Summary. Influenza A/PR-8 virus was

shown to replicate and produce ciliostasis in mouse tracheal organ cultures. Histological sections of infected tissue revealed progressing damage to the epithelium resulting in complete desquamation of the epithelial surface of the tracheal explant. Electron microscopy studies showed that the virion attached to and caused clumping of the cilia.

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- Received Nov. 29, 1972. P.S.E.B.M., 1972, Vol. 140.