Cytopathic Effects of Parainfluenza Virus Type 3 in Organ Cultures of Human Respiratory Tract Tissue

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PARAINFLUENZA TYPE 3 is one of the many viruses responsible for respiratory disease in man. It is etiologically associated with a variety of syndromes involving both the upper and lower respiratory tracts.[1] Although infection occurs commonly in the general population, little information is available on the cytopathology of the virus in human tissues.

We, as well as others,[2-7] have found that tracheal and bronchial tissues from fetuses and surgical specimens can be maintained as organ cultures for extended periods in vitro. Several groups of workers have shown that cultures of human fetal trachea support the multiplication of a variety of respiratory pathogens.[2,3,5-7] The technique of organ culture not only makes possible studies on virus growth in differentiated respiratory tissues, but also permits documentation of virus cytopathology independent of host defenses.

Recorded here are studies with parainfluenza virus Type 3 using tracheobronchial cultures prepared from fetuses and adults with pulmonary disease. This work was carried out in conjunction with our attempts to identify covert agents in tissues from persons with neoplastic and non-neoplastic respiratory tract disease.

Materials and Methods

Source and Preparation of Tissues

Specimens of adult bronchus or trachea were obtained, using sterile precautions, after surgical removal of lung tissue or at necropsy. Fetal tracheal tissue was obtained after therapeutic abortion. The source of the tissues and approximate interval between death or surgery and receipt of the tissue in the laboratory are recorded in Table 1.

Adult respiratory mucosa with subjacent connective tissue and cartilage was cut into blocks having a surface area of approximately 15–35 sq.mm. Segments of fetal trachea were cut into somewhat smaller rectangles, and the side opposite the mucosa was allowed to adhere to pieces of sterile filter paper. The tissues were washed repeatedly with balanced salt solution and then placed in 35-mm. plastic culture dishes (Falcon) containing Eagle's medium with 5% chicken serum, heated at 56° C. for 30 min., and antibiotics. Specimens were maintained routinely in fluids containing 200 U. of penicillin, 200 μg. of streptomycin, 100 μg. of kanamycin, and 100 U. of nystatin

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per milliliter. The volume for individual cultures was adjusted so that the fluid moistened but failed to flood the mucosal surface (2–3.5 ml.). All cultures were incubated in a humidified atmosphere of 5% CO_2 and 95% O_2 at 36° C. The final pH of the medium under these circumstances was approximately 7.4.

Infection and Testing for Virus

The parainfluenza virus used had been grown in monkey heart and Hep-2 cell cultures before being passaged six times in HeLa cells in this laboratory.

After overnight incubation, individual cultures were inoculated with virus diluted in 2- to 3.5-ml. aliquots of balanced salt solution containing 5% heated chicken serum. Adsorption was allowed at 36° C. for 1 hr. The specimen was then transferred to a clean dish, washed thoroughly, and placed in a second dish containing medium. Controls were handled in an identical fashion except that virus was not used.

Virus multiplication was documented at twice weekly intervals. On the day of harvest, tissues were transferred to a new dish and washed repeatedly. They then were placed in fresh medium and returned to the incubator. After 6 hr., the culture fluid was removed and replaced with new medium. Virus titrations were carried out in tube cultures of HeLa cells using the culture medium, which had been stored at —65° C. Cytopathic effect or hemadsorption or both methods were used to determine endpoints. Titers (ID_{50}) were calculated by the method of Reed and Muench [8] and are expressed here as the virus released from individual cultures over a 6-hr. period.

Table 1. Source and Experimental use of Respiratory Tract Tissue

Specimen	Age (yr.)	Pulmonary disease	Tissue	Interval * (hr.)	Dose (log_{10})	Duration of Experiment (hr.)	
						Virus yield	Tissue cultured
1	Fetus	—	Trachea	<2	4.5	750	918
2	Fetus	—	Trachea	<2	3.5	390	390
3	52	Emboli	Trachea	3.3	4.5	1110	1182
4	57	Broncho-pneumonia	Trachea	4.8	4.5	294	294
5	80	Emphysema	Trachea	8.8	3.5	510	510
6	40	Metastatic carcinoma	Bronchus	<2	5.5	270	270
7	31	Metastatic carcinoma	Bronchus	<2	5.5	534	630
8	48	Primary adenocarcinoma	Bronchus	<2	3.5	270	270
9	68	Primary epidermoid carcinoma	Bronchus	<2	5.5	582	822
10	49	Primary epidermoid carcinoma	Bronchus	<2	6	294	294

* Time-lapse between death or surgery and receipt of tissue in laboratory.

Attempts to Recover Covert Viruses

The medium bathing uninfected control cultures was collected at intervals and aliquots were introduced into tube cultures of diploid human embryonic fibroblast (WI-38) and human embryonic kidney cell cultures. Tubes were observed for cytopathic effect, and hemadsorption was carried out at intervals. These studies failed to yield isolates and will not be considered further here.

Histologic Preparation of Tissues

Infected and uninfected cultures were fixed in Bouin's solution at intervals after inoculation or at the termination of an experiment. Tissue blocks were sectioned serially or at levels, and selected sections were stained with hematoxylin and eosin or by the periodic acid-Schiff technique.

Results

Features of Uninfected Cultures

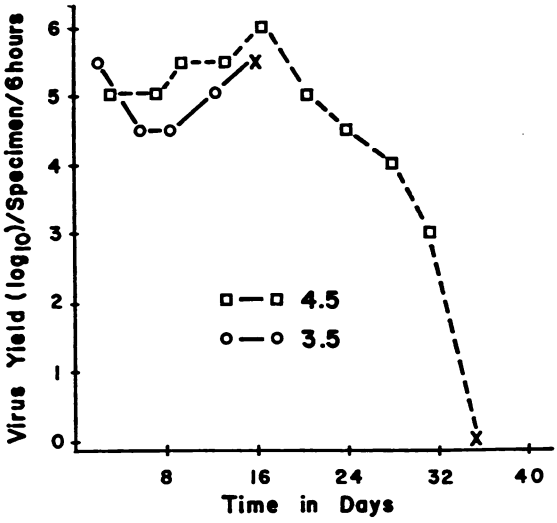
Viable cultures of human respiratory tract mucosa were maintained for periods of as long as 50 days. Tissues from fetuses appeared to retain a differentiated morphology longer than those from adults. The bronchial mucosa of surgical specimens deteriorated more rapidly than the mucosa of the trachea obtained from cadavers.

Nonspecific morphologic changes of varying extent were found in uninfected control specimens fixed for histologic examination at intervals during the course of our experiments. Foci of necrosis occasionally were seen in tissues from both adults and fetuses. Often the epithelium of the adult specimens developed a pseudostratified cuboidal appearance. Although mucous secretion by submucosal glands persisted indefinitely, only a rare goblet cell was found in the mucosa after prolonged maintenance in vitro. Our microscopic observations on long-term cultures are similar to those of previous investigators.[2-6]

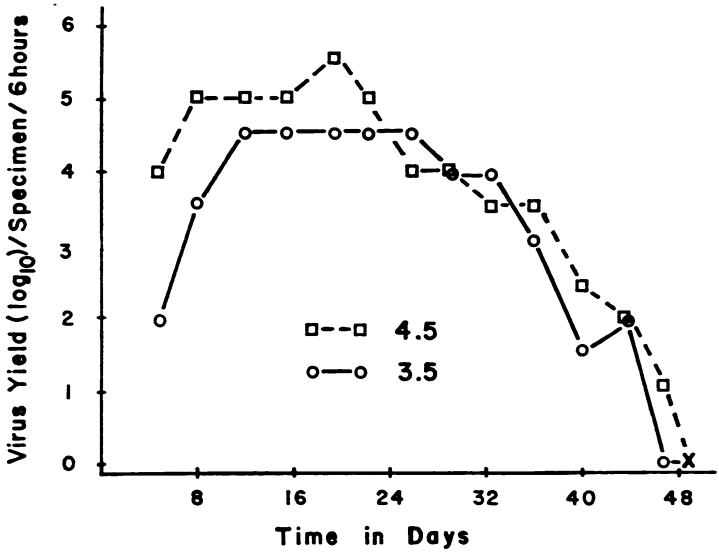
No attempt was made to document the activity of cilia in this study, since histologic sections showed that few of the structures survived the washing routinely administered to the cultures. A simple squamoid epithelium grew over the cut surfaces of fetal tissue after short periods of incubation. It seems likely that these cells contributed to the virus yield of the specimens (Text-fig. 1) for they often exhibited the cytopathic changes described in detail below. Similar outgrowths of undifferentiated epithelium were infrequently observed in adult tissues. They were never prominent nor did they compare in extent to those seen in specimens from fetuses.

Virus Growth

Organ cultures of both fetal and adult respiratory tract tissue supported growth of parainfluenza virus Type 3 for extended periods. Virus was re-



TEXT-FIG. 1. Virus recovered from cultures of fetal human trachea at intervals after inoculation of $10^{4.5}$ or $10^{3.5}$ ID_{50} per specimen of parainfluenza virus Type 3. Each point indicates virus yield over 6-hr. test period. X = time experiment terminated.



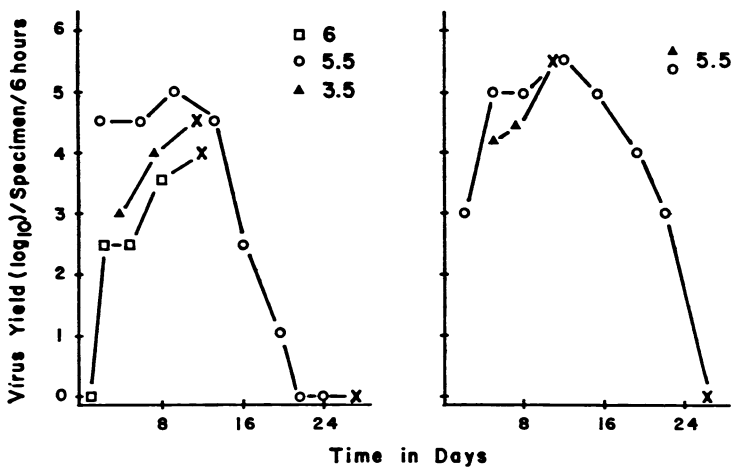
TEXT-FIG. 2. Virus recovered from cultures of adult human trachea at intervals after inoculation of $10^{4.5}$ or $10^{3.5}$ ID_{50} per specimen of parainfluenza virus Type 3. Each point indicates virus yield over 6-hr. test period. Tissue was obtained at necropsy 3.3 hr. after death. X = time experiment terminated.

covered from the medium bathing the fetal trachea for as long as 31 days after inoculation (Text-fig. 1). Cytopathic changes in the epithelial cells which are believed to be virus-specific appeared during the late stages of the infection and virus multiplication subsided as the mucosal surface gradually was destroyed. Cultures of trachea prepared from adult cadavers yielded virus for as long as 46 days (Text-fig. 2). The quantity of virus released was roughly equivalent to the amount produced by fetal tissues. Although cytopathic effects first became evident 7–15 days after inoculation, the epithelium was capable of supporting virus growth for additional periods of as long as one month.

As can be seen in Text-fig. 3, cultures of the bronchi from persons with carcinoma of the lung and pulmonary metastatic disease supported replication of parainfluenza virus Type 3. The virus produced by these tissues was comparable in quantity to that fabricated by fetal and cadaver specimens, at least during the initial 2 weeks of maintenance in vitro. Because the amount of tissue available from individual surgical specimens was small, systematic documentation of cytopathic changes at regular intervals after inoculation could not be accomplished. Nonetheless, it appeared that alterations attributable to the virus resulted in early destruction of the mucosa.

Cytologic Changes Associated with Infection

Multinucleate cells formed in the respiratory mucosa at scattered sites during the course of parainfluenza virus Type 3 infection (Fig. 1–8). The



TEXT-FIG. 3. Virus recovered from cultures of adult human bronchus at intervals after inoculation of 10^6, $10^{5.5}$, or $10^{3.5}$ ID_{50} per specimen of parainfluenza virus Type 3. Each point indicates virus yield over 6-hr. test period. Tissue was obtained surgically from patients with primary (*left*) or metastatic (*right*) neoplasms of lung. X = time experiment terminated.

multinucleate structures, as well as the individual epithelial cells of the mucosa, exhibited a variety of nuclear and cytoplasmic changes attributable to the virus. The characteristic alterations were: (1) rounding of cells associated with apparent increase in cytoplasmic mass (Fig. 14 and 16); (2) enlargement of the nucleus with an increase in size of the nucleolus and aggregation of nuclear chromatin (Fig. 2, 8, 14 and 16); (3) formation of unilocular and multilocular cytoplasmic vacuoles of varying size and configuration (Fig. 4, 6, 8, and 14–16); and (4) development of intracytoplasmic inclusions of two types. One form of inclusion was found exclusively in multinucleate cells; it consisted of distinct basophilic or eosinophilic pyknotic nuclei (Fig. 3, 4, and 6–8). The second form, a granular eosinophilic aggregation of cytoplasm, appeared in both individual and multinucleate cells (Fig. 2).

Cytopathic changes in the mucous membranes usually were focal. Only late in the course of an experiment were they distributed diffusely over the expanse of the respiratory mucosa (Fig. 11). Formation of multinucleate cells from intact elements of the pseudostratified epithelium was prominent (Fig. 16). These structures contained from 2 to 7 nuclei and exhibited vacuoles of varying size or intracytoplasmic inclusions or both (Fig. 1–8). The multinucleate cells appeared to develop from superficial layers of the epithelium (Fig. 16); they separated with maturation, leaving scattered basal cells or a denuded surface. Often several closely associated cells were enlarged and exhibited vacuoles and inclusions (Fig. 14 and 15). The superficial elements of these collections frequently were necrotic and appeared to peel from the surface. Focal aggregations of altered cells occasionally formed prominent excrescences on the mucosa.

Cytopathic changes attributable to parainfluenza virus Type 3 first became apparent in both fetal and adult tissues approximately 175 hr. after inoculation. These alterations were increasingly obvious with time and persisted either until the epithelium was destroyed or the tissue underwent frank necrosis due to nonspecific causes. Thus, development of the cytologic changes followed the onset of virus replication by a considerable duration. An association between the amounts of virus produced and the appearance of virus-specific alterations was not established since most specimens yielded large amounts of virus over much of the period of maintenance in vitro.

Discussion

Parainfluenza virus Type 3 possesses the ability to multiply in monolayers of a variety of cell types.[9] In addition, it grows in organ cultures of mouse,[10] guinea pig,[11] ferret,[6] and human fetal[6] respiratory epithelium.

In monolayers, most new isolates of the virus produce few cytopathic changes, whereas established strains cause both focal cell necrosis and formation of multinucleate syncytia.[1] Virus growth in cultured, trypsin-dispersed cells also is associated with the appearance of cytoplasmic inclusions.[1,12,13] Cytopathic changes in organ cultures of human or animal respiratory tissue attributable to parainfluenza Type 3 have not been reported.

Clinical, epidemiologic, and experimental studies have shown that man and a variety of animals are susceptible to parainfluenza virus Type 3.[14] Although many species are naturally infected, cytopathic changes in the respiratory epithelium have been observed rarely and only under experimental circumstances. Craighead showed that multinucleate cells develop at scattered foci in the nasal mucosa of suckling mice a few days after the intranasal introduction of virus.[15] Multinucleate cells and areas of necrosis were found in the bronchi of experimentally inoculated hamsters by Buthala and Soret[16] and in calves by Dawson *et al.*[17] The histopathologic observations of these workers suggest that the cytologic alterations induced by parainfluenza virus Type 3 in vivo characteristically are focal rather than diffuse. Moreover, it seems likely that virus-altered cells are replaced readily by regenerating epithelium in the intact animal.

The multinucleate cells formed in our organ cultures were similar to those observed in intranasally inoculated animals. The lesions initially were focal but became more prominent with prolonged maintenance. Although virus replication appeared promptly in vitro, the development of histologically recognizable changes was delayed for periods of as long as 15 days. It may be that formation of multinucleate cells is not dependent upon the occurrence of virus replication in the infected cell. Several groups of workers have demonstrated syncytia formation in monolayers shortly after exposure of the cultures to large inoculums of parainfluenza Type 3 and before the onset of virus replication.[12,18]

Previous investigations have documented the appearance of two types of inclusion bodies in the cytoplasm of parainfluenza virus Type 3 infected cells. Love and Suskind[12] showed by histochemical studies that the inclusions formed in HeLa cell syncytia represent degenerate nuclei or nuclear debris. On the other hand, less well-defined eosinophilic cytoplasmic inclusions were observed in several other cytologic studies of myxovirus-infected cells.[1,9,12,13,19] These latter inclusions appear to represent in large part aggregates of viral nucleoprotein.[20,21] We found inclusion bodies of both types in organ cultures of human respiratory tissues. The first form was located only in multinucleate cells, whereas the second occurred in both multinucleate and individual cells.

Unilocular and multilocular vacuoles were a prominent feature in cultures infected with parainfluenza Type 3. These alterations have not been described previously in association with this virus but are characteristically observed in cultured cells infected with two other closely related myxoviruses, mumps, and parainfluenza virus Type 2.[22–24] The cytologic basis for the vacuoles thus far has not been defined satisfactorily.

A variety of respiratory tract syndromes in infants and young children are associated with parainfluenza Type 3 infection.[1] In contrast, illness in adults occurs infrequently and is usually mild and transitory.[25] The data reported here show that the respiratory epithelium from fetuses and persons over a wide range of ages supports the growth of parainfluenza virus Type 3. Thus, it seems likely that factors other than an age difference in susceptibility of respiratory mucosa determine the nature of the clinical illness in man.

The cytopathic changes described here appear to be caused by parainfluenza virus Type 3. Additional studies with other viruses would seem indicated to determine their morphologic specificity. No evidence was found to suggest that covert agents were responsible for the lesions. Attempts to isolate viruses from uninfected tissues in human embryonic cell cultures were negative and there was no apparent interference by unrecognized viruses with parainfluenza Type 3 growth.

This study was carried out during the course of a comprehensive investigation to identify covert virus infections in persons with neoplastic and non-neoplastic respiratory tract disease. The work reported here was undertaken in part (1) to document interference by an unrecognized virus with growth of parainfluenza Type 3, (2) to determine whether or not parainfluenza Type 3 might enhance the replication of an unrecognized virus which is suppressed by autointerference,[26] and (3) to explore the effect on cytopathology of synergism between parainfluenza Type 3 and a covert agent. Our observations, although preliminary, suggest that organ culture is a feasible system for future exploration of these theoretic considerations.

Summary

Organ cultures of human fetal and adult respiratory tract tissue supported growth of parainfluenza virus Type 3 for extended periods of time. A variety of cytologic alterations attributable to the virus appeared during the course of the infection. These cytopathic changes are described and correlated with the virologic observations.

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Legends for Figures

All photomicrographs were prepared from tissues which were fixed in Bouin's solution, sectioned at 5 μ, and stained with hematoxylin and eosin.

Figures 1–8 depict selected cells found in infected cultures of adult tissue at the indicated interval after virus inoculation. Originals × 400.

Fig. 1. Focus of multinucleate cells forming in tracheal mucosa at 294 hr. Similar cells were located in another isolated focus in section. Epithelium otherwise was comprised of pseudostratified cuboidal cells.

Fig. 2. Well-defined eosinophilic inclusion in cytoplasm of bronchial epithelial cell at 294 hr. Less distinct inclusions often were found scattered in mucosal cells.

Fig. 3. Multinucleate cell in bronchial mucosa at 318 hr. Note beaded basophilic inclusion in cytoplasm.

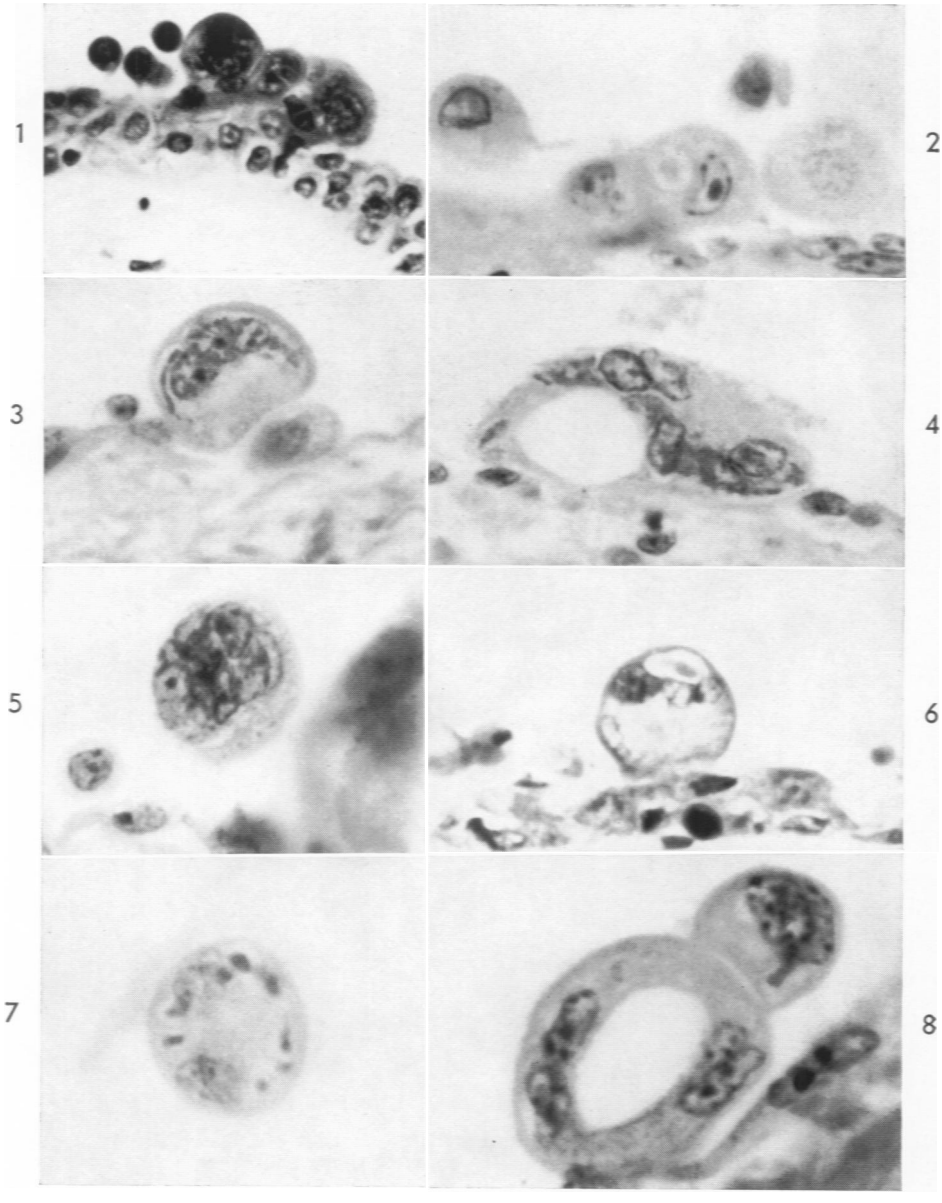
Fig. 4. Vacuolated multinucleate cell in bronchial mucosa at 294 hr. Note pyknotic nuclei which appear to be forming inclusions similar to those seen in Fig. 3, 6, 7, and 8.

Fig. 5. Unattached multinucleate cell in bronchial culture at 294 hr. Tiny inclusions which may represent nuclear fragments are seen near periphery.

Fig. 6. Vacuolated multinucleate cell with cytoplasmic inclusion in tracheal culture at 366 hr.

Fig. 7. Unattached multinucleate cell in bronchial culture at 318 hr. Note irregular basophilic cytoplasmic inclusions.

Fig. 8. Vacuolated multinucleate cell with cytoplasmic inclusion in bronchial culture at 270 hr.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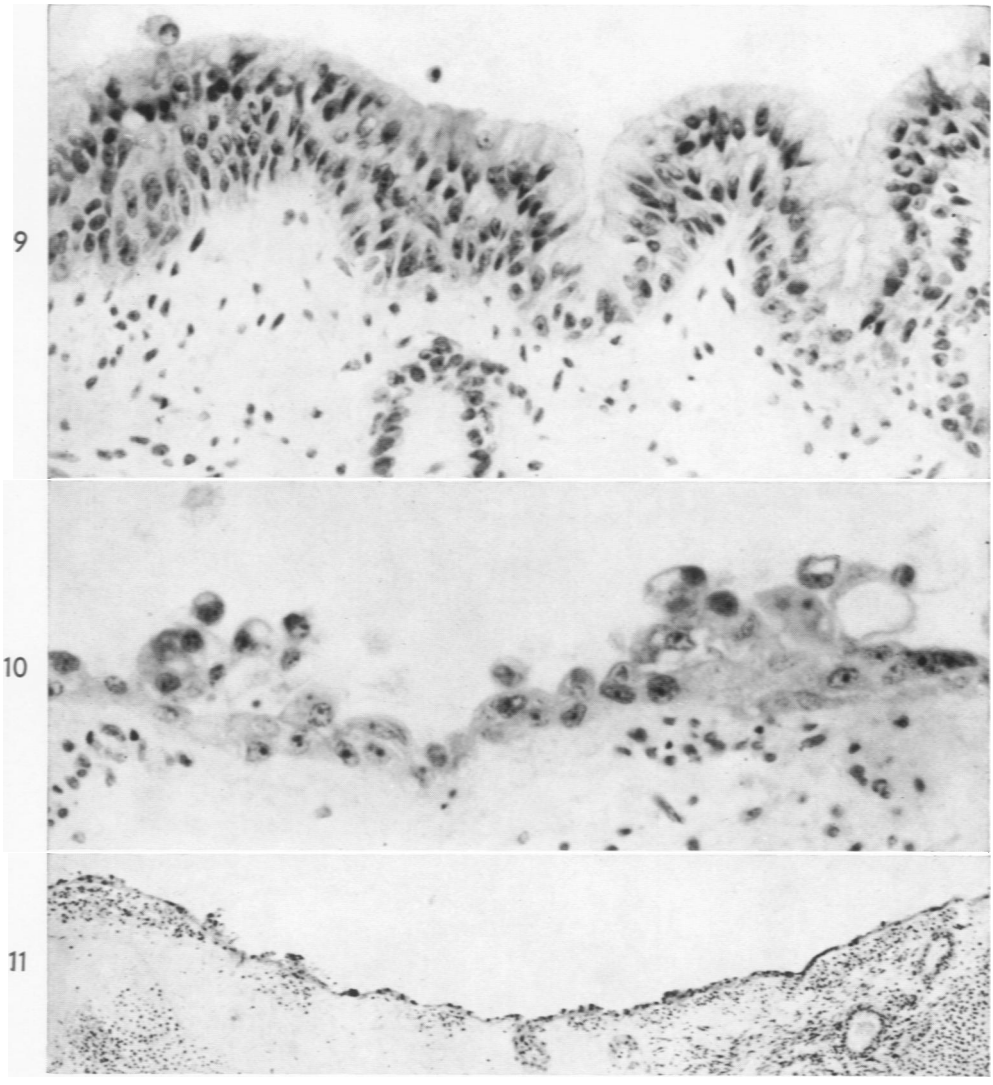
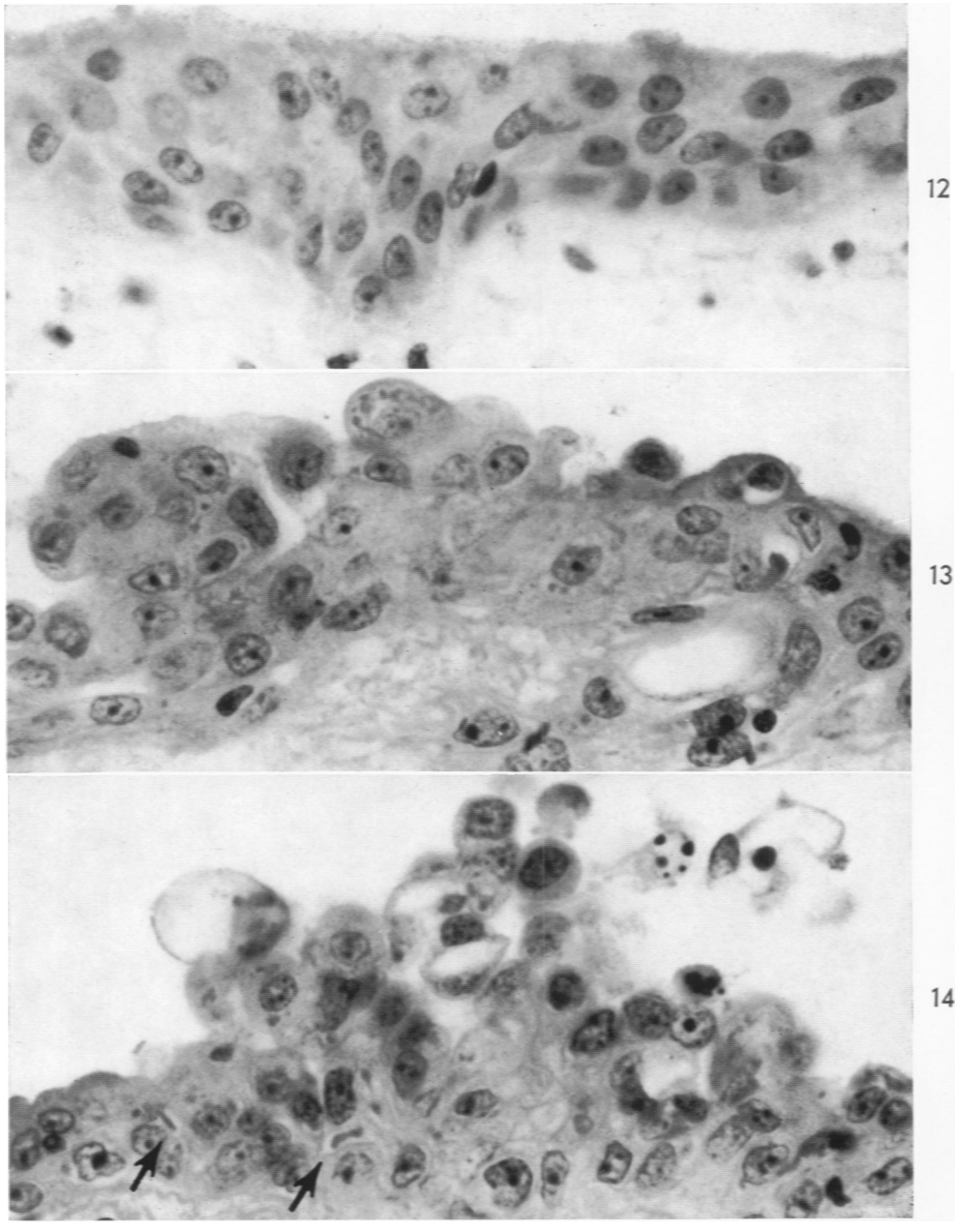


Figures 9–11 were prepared from cultures of fetal tracheal tissue fixed at 390 hr.

Fig. 9. Uninfected culture. Mucosa is well differentiated but cilia are absent. Original × 400. **Fig. 10.** Infected culture. Foci of altered cells similar to those shown were scattered widely over mucosal surface. Original × 400. **Fig. 11.** Infected culture. Note extensive destruction of epithelium. Original × 25.



Figures 12–14 show cultures of adult bronchial tissue fixed at 270 hr. Originals × 400.

Fig. 12. Uninfected tissue. Cilia are absent and vertical orientation of cells is lost. Goblet cells are no longer present. **Fig. 13.** Infected tissue. Note superficial, enlarged cell containing several irregular basophilic inclusions. **Fig. 14.** Infected tissue. Scattered linear bodies (arrow) are found between cells deep in mucosa. Similar structures were observed frequently in previous study with parainfluenza virus Type 3 using guinea pig nasal mucosa.[11] They were not common in cultures of human tissue. Note irregular basophilic inclusions and vacuoles in scattered cells elsewhere in mucosa.

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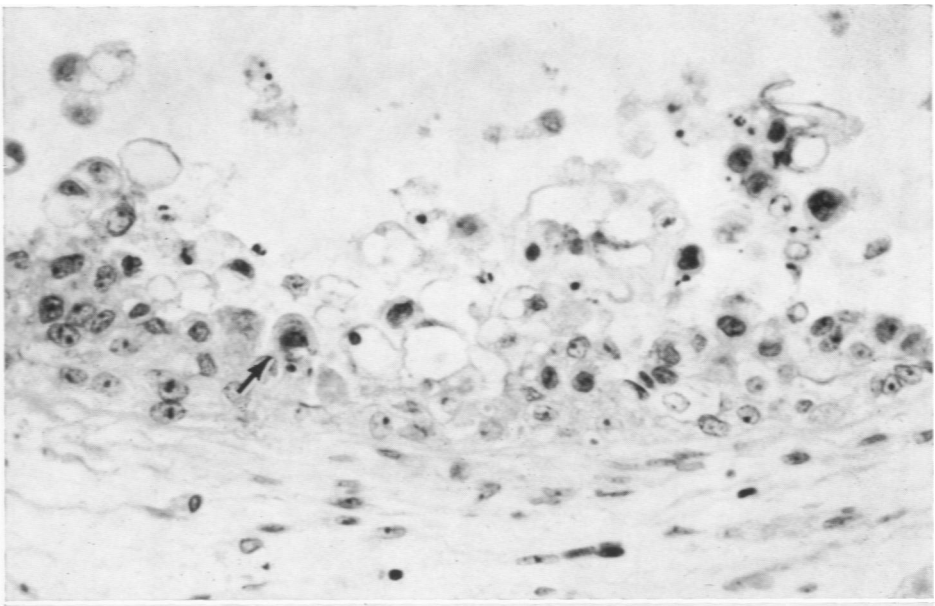

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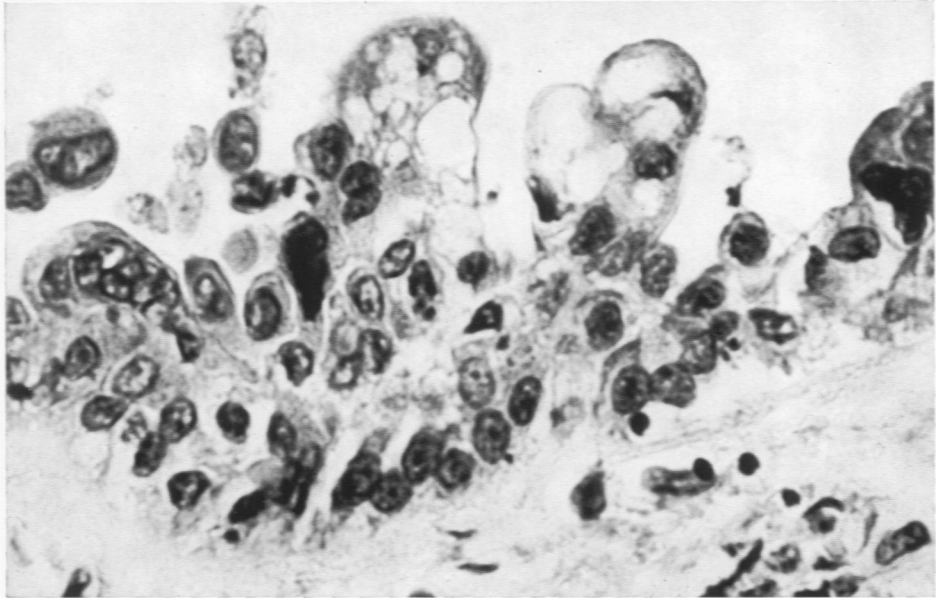

Fig. 15. Infected bronchial tissue at 270 hr. after inoculation. Numerous unilocular and multilocular vacuoles are seen. Single enlarged cell with irregular, basophilic inclusions is located among vacuolated cells (arrow). Original × 400. **Fig. 16.** Infected tracheal tissue at 294 hr. after inoculation. Multiloculate cells and single multinucleate cell are forming on surface of mucosa. Note individual cells with enlarged irregular nuclei. Original × 400.