Resistance of Respiratory Ciliated Epithelium to Action of Polio and Adeno Viruses *in vitro*. (23485)

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It has been observed that a tissue entirely resistant to a viral infection *in vivo* may become highly sensitive to the same infection after explantation *in vitro*. This phenomenon is well illustrated by the behavior of the kidney of the monkey in which no multiplication of poliovirus occurs when the agent is inoculated directly in the organ *in vivo* but which supports a rapid multiplication of the same virus when separated from the organism and maintained in tissue culture(1). Since the dominant feature of the evolution of *in vitro* explanted tissues is their histomorphological disorganization and their functional dedifferentiation, we undertook to investigate with certain particular tissues in which the cellular differentiation may be easily recognized *in vitro*, the relation between the degree of differentiation and the sensitivity to viruses. The ciliated epithelium of the respiratory mucosa appeared to us as a particularly suitable material for this study. We used in this work *in vitro* cultures of human (adult and embryo) and simian (adult) tissues of the bronchial and tracheal lining. To some of these cultures were added other cellular implants remarkable for their dedifferentiated, astructural growth, such as kidney epithelium and the human carcinoma HeLa cell strain(2). Type I poliovirus and type I adenovirus were used as viral agents.

Material and methods. Tissue cultures. All of our chicken plasma embedded cultures were prepared on 12 x 32 mm cover slips in 16 x 160 mm flattened tubes(3). Fragments of human bronchial lining tissue were excised by biopsy at the intersection of the bronchus of the right middle lobe and the main bronchus in adults of both sexes with a normal bronchoscopic aspect of their mucosa. The fragments (1-2 mm size) were directly transferred into cultures in an entirely heterologous medium containing 0.5% lactalbumin hydrolysate in Earle's solution supplemented with 0.1% yeast extract, 5% bovine embryo extract and 10 to 15% horse serum. The same medium was also used for the cultures of human embryo and monkey tissues (*c.f.* later). Human tracheal tissue was obtained from a premature newborn child (5½ months *in utero*) 5 hours after death. 0.5 to 1.0 mm thick fragments were cut perpendicularly to the long axis of the trachea and placed for culture with their cut face applied to the cover slips. This disposition is essential for the observation of the ciliary movement. Fragments of monkey trachea were prepared in the same way. The sensitivity to poliomyelitis virus of the *in vitro* cultivated tissues from the African monkeys *Erythrocebus patas* used in this work had been previously demonstrated(4) and confirmed(5). In the present work the virus sensitivity was tested additionally for each individual monkey on cultures prepared with one of its kidneys previously removed by nephrectomy. It appeared that the sensitivity of *Erythrocebus* kidney cell cultures to poliovirus was essentially similar to that of the Asiatic Cynomolgus kidney cultures,* and their sensitivity to the type I adenovirus was comparable to that of the HeLa strain cultures. Further, plasma imbedded or trypsin dispersed cell cultures of human embryo kidneys, monkey kidneys and the HeLa strain were employed separately, or associated in the same tubes with tissues of the respiratory tract. Observation of the cultures was performed either directly in the tubes or after mounting the culture bearing cover slips in special perfusion chambers(6). For more detailed observation the cultures were fixed with Bouin fixative and the growth area stained with hematoxylin-eosin directly on the culture cover slip. The tissue fragments after removal were sectioned for

* Obtained from Microbiological Associates.

slide preparations by the usual histological technics. *Virus.* The Mahoney poliovirus type I and the adenovirus Ad. 71 type I strain† were used. The virus titrations and neutralization tests were performed in the usual way in tube cultures of trypsinized monkey kidney cells for poliovirus and in similar cultures of the HeLa strain for the adenovirus.

Results. 1. *Human bronchial tissue and poliovirus.* Of the total number of 8 biopsied patients (31 to 62 years old) 6 furnished viable tissue material showing after explantation *in vitro,* ciliary movement and cell proliferation.

The cilia were particularly active at the edges of the fragments where they could be observed immediately after explantation and over a period of two to three weeks. With isolated cells or cell agglomerations this vibratile activity ceased after 5 to 6 days.

Growth, beginning on the 3rd or 4th days, was predominantly epithelial. Isolated spots of ciliated cells were observed in the growth area close to the fragments until the 22nd day after explantation.

HeLa cells implanted in the human bronchial cultures proliferated but did not visibly disturb the development and the activity of these cultures during a period of observation of 15-20 days.

When 10^5 $I.D._{.50}$ of poliovirus were introduced into this type of, 5 to 7 days old, mixed cultures, a complete destruction of the HeLa cells occurred after 48 hours whereas in the growth area of the bronchial epithelium the cytopathogenic process was progressing only slowly from the periphery of the growth area towards the edges of the fragments; healthy areas could be seen in the vicinity of the fragments as late as 10 days after introduction of the virus. High degree of resistance of the ciliated cells to virus action could be shown in all 6 series of human bronchial cultures. Distinct activity of the cilia was observed, 5, 10, and in one series, 19 days after introduction of the virus. This activity was particularly tenacious on the explants but was also observed in the proximate growth area. During the observation period the virus titers were constantly high. This may be illustrated by the example of one series (bronchial cultures associated with HeLa cells) in which the viral titers were $10^{6.8}$ 24 h., $10^{7.5}$ 48 h. and $10^{6.8}$ $I.D._{.50}$ per ml 5 days after infection.

2. *Human embryo trachea cultures and poliovirus.* Explants of human embryo trachea have shown a mixed, chiefly epithelial growth which started from the 3-4th day and became quite extensive after one week. The ciliary movement was intense and constant on the interior borders of the fragments.

When 10^5 $I.D._{.50}$ of poliovirus were added to the mixed trachea-kidney cultures whether on the 3rd or on the 7th day, the growth areas were entirely destroyed after 48 hours around the kidney and after 3 days around the trachea fragments. Nevertheless uninterrupted ciliary activity could be observed 4 days after introduction of virus when it was present in a titer of $10^7 ID_{50}$ per ml.

In sections, the lining epithelial sheet appeared flattened but composed of entirely normal cells, the underlying connective tissue was necrotic while the glandular epithelium and the cartilage seemed normal.

3. *Monkey trachea cultures and poliovirus.* The ciliary activity of the explanted monkey tracheal fragments was evident during a period of 45 days *in vitro.* In the growth area, predominantly epithelial islands of ciliated cells were visible until the 20th day. Monkey kidney tissue fragments or HeLa cells if added to these cultures were growing abundantly, apparently without disturbing the tracheal cultures. 10^5 ID_{50} of virus added to these combined cultures induced after 48 hours a complete destruction of the growing HeLa and monkey kidney cells. This was followed by a rise in virus titers to 10^6-10^7 ID_{50} per ml. Titrations on the 5th day still gave titers of $10^{6.5}$ and on the 11th day of $10^{5.8}$ per ml.

These high virus concentrations did not appear to influence the vibratile activity of the ciliary cells which was clearly seen as long as 10 days after the infection. During this period the growth area of the tracheal epithelium outside the fragments was progressively attacked by the virus but uninjured narrow epithelial sheets with beating cilia

† Received from Dr. R. Huebner, (N.I.H.)

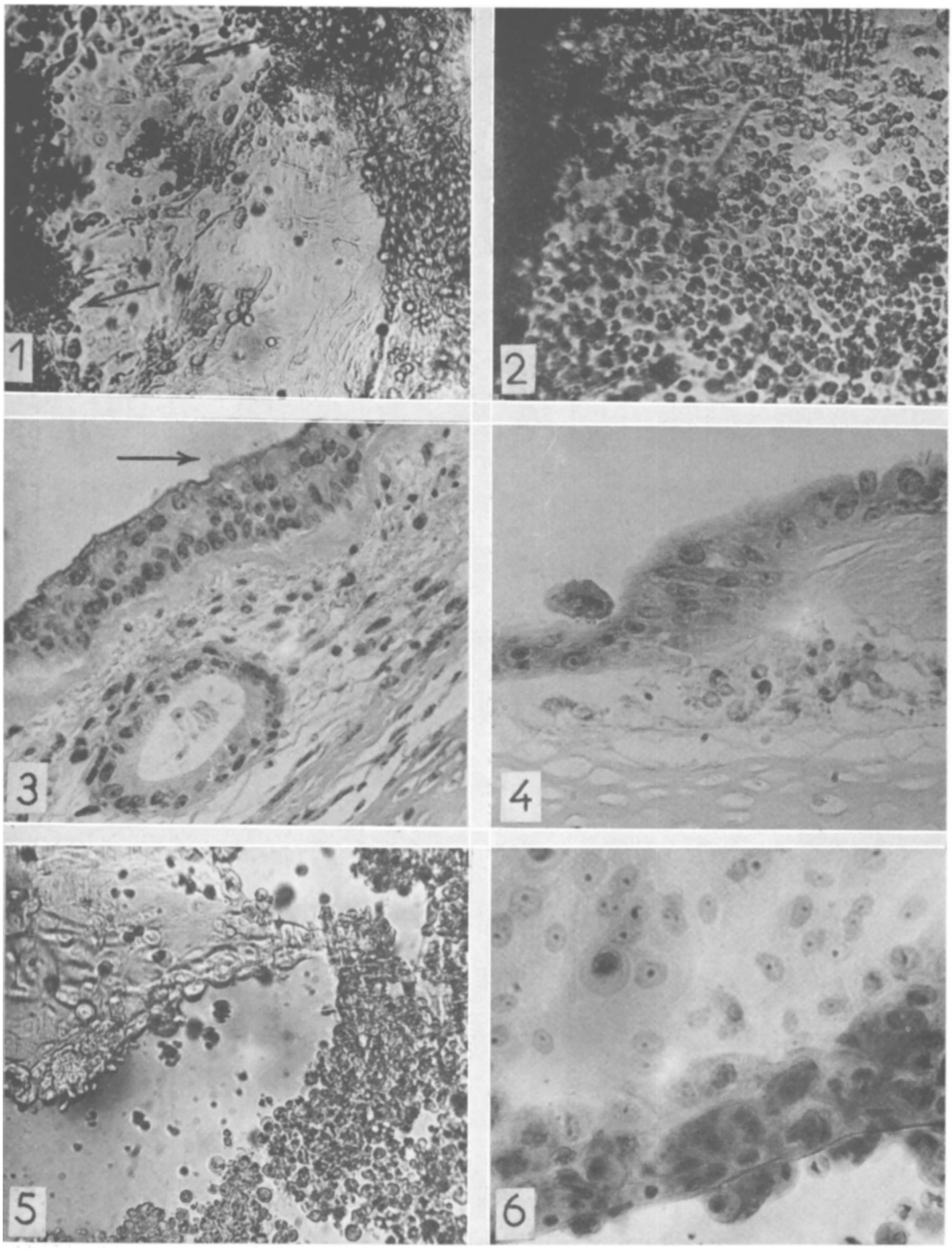

FIG. 1. 9 days' human bronchial culture; 3 days with poliovirus. Active ciliary cells (arrows).
FIG. 2. 9 days' monkey kidney culture; 3 days with poliovirus.
FIG. 3. 9 days' monkey tracheal culture; 6 days with poliovirus. Nearly normal glandular and lining epithelium with active cilia. Connective tissue cells destroyed.
FIG. 4. 11 days' monkey tracheal culture; 8 days with poliovirus. Normal lining epithelium.
FIG. 5. 14 days' monkey tracheal culture associated with HeLa cells; 4 days with adenovirus. HeLa cells (at right) completely destroyed.
FIG. 6. 16 days' monkey tracheal culture; 6 days with adenovirus. Virus lesions on the border of the growth area.
(Figs. 1, 2, 5, living cultures. Figs. 3, 4, 7, Bouin fixed and Hem. eosine colored sections. Fig. 6 colored directly on the cover slip.)

could be seen near the explants until the 6th day after introduction of the virus.

In sections of tracheal fragments from infected cultures (5, 8 and 11 days after addition of virus) a continuous sheet of lining columnar epithelium was seen. In older cultures, it was often reduced to a monolayer of flattened but otherwise normal cells regularly dotted with cilia. On the other hand the underlying connective tissue and smooth muscle cells were heavily damaged, distinctly more than in the noninfected controls while the cells of the columnar epithelium, at different levels of the mucosa, retained a practically normal aspect.

4. *Cultures of monkey trachea and human bronchial tissues infected with adenovirus.* 10^4 ID_{50} (for HeLa cells) of the adenovirus added to the HeLa cell or monkey kidney cell cultures (whether trypsin dispersed or plasma imbedded) induced specific cell lesions within 48 hours and after 3 days nearly complete destruction of both kinds of cultures. A similar inoculation of the same amount of virus into 10-day-old cultures of monkey trachea did not appear to influence the ciliary beat which was still observed, on the periphery of the explants, 11 and in some cases 16 days after the infection. Relatively high virus titers were maintained throughout this period. This may be illustrated by the following figures (ID_{50}/ml of supernatant fluid in trachea epithelium cultures associated with HeLa cells): $10^{4.8}$ on the 6th day, $10^{4.2}$ on the 8th day, $10^{2.8}$ on the 11th day.

The epithelial sheets growing out from the tracheal fragments were attacked by the virus very slowly. The first specific cellular lesions (rounding of cells, intranuclear inclusions) began to appear at the periphery of the epithelial growth areas only 3 to 4 days after infection, and healthy cells could be seen near the fragments as late as 9 days after infection.

In sections of tracheal fragments after 26 days in culture and 16 days in presence of the adenovirus, normal lining epithelial ciliated cells were seen along with typically altered cells in the growth area.

A series of human bronchial tissue cultures were prepared from a patient without antibodies for the adenovirus type I and im-

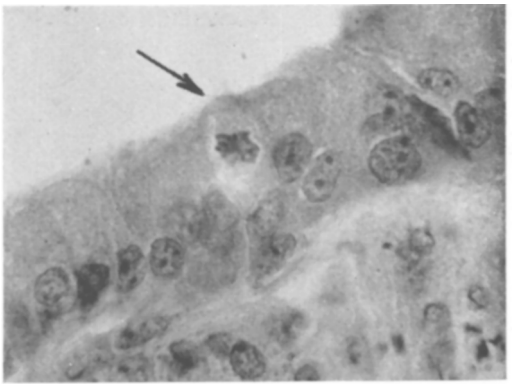

FIG. 7. 15 days' human bronchial culture; 5 days with adenovirus. Normal lining epithelium. One cell in mitosis (arrow).

planted additionally with HeLa cells. After inoculation of 10^4 ID_{50} per culture, all HeLa cells were destroyed, in 3 to 4 days the bronchial epithelial outgrowth also reacted to the virus but more slowly, whereas the epithelial sheets lining the fragments and inside the numerous glandular ducts, tubes or villi maintained a normal appearance during at least 6 days (Fig. 7).

Discussion. 1. The *in vitro* culture of the tracheal and bronchial lining tissue from different mammalian species was attempted by several authors(7,8,9,10). Improvements in tissue culture technics made it possible for us to maintain *in vitro* in an entirely heterologous medium adult human or simian respiratory tract tissues for a period of 30 days or more in a state of specific functional activity which was accompanied by a certain degree of proliferation.

2. The epithelial sheets extending from the explanted bronchial or tracheal fragments exceed considerably the surface of the original lining epithelium, mitotic figures are frequent and consequently these sheets may be considered as real growth areas. Thus, the ciliated cells appearing in these areas should be regarded as formed and differentiated *in vitro*. Nevertheless the general tendency of these growth areas which may be compared to cicatricial epithelium(11) is towards dedifferentiation and the ciliated cells definitely disappear from the growth zone between the 10th and the 14th days.

3. The reaction of the tracheal and bron-

chial epithelium to virus infection *in vitro* parallels this development. The periphery of the growth area, where the differentiated ciliated cells never appear, is the first to be attacked by the virus. On the other hand, the original lining epithelium which, more or less, retains its primary histological structure most efficiently resists the viral action. It must be emphasized that owing to the liquefaction of the plasma clot near the explants and also to the movement of the cilia the surfaces at the lining epithelium are in continuous contact with viral particles abundantly present in the fluid medium. Though the resistance to virus may be most easily demonstrated with the ciliated epithelium it appears to extend to other epithelial elements of the respiratory tract and particularly to the glandular epithelium at the different levels of the mucosa.

4. We have previously reported a similar relative resistance *in vitro* of the human adult tonsil epithelium(12) to poliovirus infection and proved that it was independent of the state of specific immunity of the tissue donor. This fact is confirmed by the present data obtained with the monkeys and at least one human case in which sera were free of specific viral antibodies. Moreover, in experiments with the monkey and the human embryo tissues the resistance of the differentiated respiratory epithelium to viruses could be contrasted with the sensitivity of the dedifferentiated cells originating from the same individual.

5. It may be mentioned that the same kind of resistance to virus action we are reporting here for human and monkey respiratory mucosa culture was observed in similar conditions in mouse trachea cultures and Mengo encephalomyelitis virus, an agent highly virulent and cytopathogenic for mouse tissue *in vitro*.‡ Thus the refractory state of the differentiated epithelium of the respiratory tract to cytopathogenic viruses *in vitro* may well represent a general phenomenon. It should also be noted here that Harford *et al.*(13,14) observed in living mice that the ciliated bronchial epithelium retained its full activity despite heavy and even lethal infection with a virulent influenza virus strain, though Francis and Stuart-Harris have shown in ferrets(15) as did Panthier *et al.* in mice(16) that this virus may cause necrosis in the respiratory mucosa cells.

6. The lining epithelium of the respiratory tract is considered by many not only as a way of entry for a number of viral infections but also for some of them as the site of their primary multiplication. However several attempts to use *in vitro* tissue cultures of the respiratory mucosa for the multiplications of respiratory viruses(17,18) gave rather unexpectedly negative results. These are easier to explain in the light of the reported data.

7. The *in vitro* resistance of the organized tissues of the respiratory tract to the cytopathogenic action of viruses obviously cannot exclude the possibility of latent infection or even virus multiplication and release from apparently normal cells(19). Closer investigations are here needed. Anyhow, our observations disclose that the well known general protective role of the ciliated epithelium ascribed to its mechanical and secretory functions may be extended to include a high degree of cellular resistance to virus action demonstrable even a long time after their explantation *in vitro*. Further work is necessary to understand the mechanism of this resistance and to determine whether it is or is not shared with other tissues which are able to maintain *in vitro* their functional differentiation.

Summary. 1. Fragments of human adult or embryonic bronchial mucosa and of monkey trachea cultivated *in vitro* in heterologous media maintain during several weeks their morphological and functional differentiation. 2. High concentrations of poliovirus or of adenovirus which rapidly destroyed the dedifferentiated growth of monkey kidney and HeLa strain cells in parallel or in mixed cultures had little or no destructive action on the differentiated respiratory epithelium and did not impair its ciliary activity.

‡ Unpublished data.

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Cuff for Use with Endpoint Devices for Estimation of Arterial Blood Pressure of the Rat.* (23486)

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Over the last several years a number of endpoint devices have been described, employing diverse principles, for estimation of the systolic or mean arterial blood pressure of the rat. Each of these has been designed to be used with an occlusive cuff by means of which the applied pressure may be varied. In our experience, most of the available cuffs have been unsatisfactory and have limited the confidence which one may place in data so obtained. After some experience with a commercially available inflatable cuff† designed to occlude circulation in the hind limb of the rat, it became apparent that, to avoid error introduced by the manner of placement, tightness of wrapping, etc., a cuff of fixed circumference is essential. Fig. 1 describes the preparation of such a cuff from the commercially available model, adhesive tape and a pair of snap fasteners.

In using the cuff, a few cautions should be observed. The width of the tape is critical; it should be just wide enough to reach from the foot to the thigh, thus accurately repositioning the inflatable portion of the cuff in subsequent measurements. Rats of different sizes require cuffs of proportionate size, *i.e.*, of different distances between the snap fasteners. A graded series, marked for identification, should be kept in stock and care taken that the same cuff is used for successive measurements with the same animal. When used on the tail, rather than on a limb, care should be taken that the cuff is replaced each time in the same position.

With these precautions, much of the subjective error of such measurements is reduced. Thus in a series of normal and renal hypertensive rats with systolic blood pressures ranging from 87-160 mm the following results were obtained: In 204 instances in which the blood pressure of a single animal was determined by more than one operator the mean deviation from the mean was within 6 mm, in 432 determinations in which a single operator performed repeated determinations on a single rat the mean deviation from the mean was

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† Metro Industries, Long Island City, N. Y.