Vol. XLVI, No. 1 (February, 1965), was issued on 22.2.65.

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XLVI APRIL, 1965 NO. 2

ON THE GROWTH OF CERTAIN " NEWER " RESPIRATORY VIRUSES IN ORGAN CULTURES

B. HOORN AND D. A. J. TYRRELL

From the Medical Research Council, Common Cold Research Unit, Salisbury, and the Bacteriology Department, University of Lund

Received for publication June 26, 1964

THE principal object of organ culture is to retain the various constituents of a tissue in normal relationship to each other and functioning in a normal way, while they are outside the body in an artificial culture medium. Various techniques have been used for this purpose, particularly for experiments in physiology and experimental embryology (Fell, 1957), but organ cultures have also been used a little in virology. For example, Bang and Niven (1958) placed fragments of ferret trachea on small pieces of rayon net and transferred them every 5–7 days to fresh liquid or solid medium. Dedifferentiated cells grew out. Bang and Niven observed the activity of the cilia, which is a valuable measure of the functional activity of the cells, and washed the fragments to recover virus from them. Influenza virus was shown to grow in such cultures. Attempts were also made to cultivate common cold viruses in similar cultures of human embryonic trachea ; washings of the cultures were tested in volunteers to see if they produced colds, but no definitely positive results were obtained (Bang and Niven, 1958 ; Pereira, personal communication).

Barski, Kourilski and Cornefert (1957) and Barski, Cornefert and Wallace (1959) on the other hand, added poliovirus and adenovirus to fragments of human ciliated epithelium, and found that the cilia continued to beat for up to 19 days although outgrowth from the explant or HeLa cells and monkey kidney cells added to the cultures were completely destroyed.

One of us developed a method of organ culture which is particularly useful for the study of viruses which multiply in the cells of mucous membranes (Hoorn, 1964). Because the explants in this technique are surrounded with ample fluid it is easy to collect numerous samples each day for virus assay. Because the fragments are stuck to a scratched surface without the use of plasma and because serum is not needed in the maintenance medium, it is possible to eliminate potential virus inhibitors from the culture system. Furthermore, because neither

fibroblast nor epithelial cell outgrowth occurs these can be eliminated as a source of new virus and of confusion in interpreting the results.

It has been shown that egg-passaged influenza virus, adenovirus and herpes simplex virus can grow in and damage the epithelium of human foetal tracheal explants cultivated in this way (Hoorn, 1964). This report describes further experiments which were done in order to test the ability of organ cultures of mucous membranes to support the growth of some recently discovered viruses of the respiratory tract.

MATERIALS AND METHODS

Human tissue was obtained by dissecting, within 1 or 2 hr. of operation, foetuses obtained at hysterotomy. The foetuses ranged in crown-heel length from 8–18 cm. Rhesus monkey material was prepared from animals killed less than an hour previously. The preparation of the cultures is described below. All cultures were maintained in a warm room at 33° in a humidified plastic box. In the absence of a CO_2 incubator formerly used for these organ cultures, a smaller amount of bicarbonate was used in order to maintain a suitable pH of 7·0–7·2, viz. 0·035 per cent instead of 0·088 per cent.

The viruses used had in general been passed little or not at all in cultures. They are listed below.

Echovirus type 11.—U virus (Philipson and Wesslén, 1958). A pool of virus passed 3 times in cultures of human embryo fibroblasts and previously used for inoculation of human volunteers (Buckland, Bynoe, Philipson and Tyrrell, 1959).

Poliovirus type 1.—Strain LSc. Sabin vaccine strain seed virus.

*Coxsackie type A*21 (*Coe*).—A pool of nasal washings obtained from volunteers inoculated with secretions obtained from an infected Serviceman (Parsons, Bynoe, Pereira and Tyrrell, 1960).

*Monkey adenovirus SV*17.—Fluid from infected monkey kidney cultures representing the 3rd passage *in vitro* of a virus obtained from Patas monkeys with an upper respiratory infection (Tyrrell, Buckland, Lancaster and Valentine, 1960).

Rhinoviruses.—Strain P.K. Nasal washings from volunteers infected with a strain of M rhinovirus which is indistinguishable from the strain H.G.P. Both these strains were also used after tissue culture passage.

Strain P. A similar pool of nasal washings from volunteers given a virus indistinguishable from the H rhinovirus strain 16/60 and similarly capable of producing experimental colds (Taylor-Robinson and Bynoe, 1964).

Influenza A virus.—A pool of allantoic fluid representing the 6th passage *in ovo* of the A.Scot/49/57, a strain which was known to multiply in the upper respiratory tract of man.

Viruses were assayed by standard methods, inoculating groups of two cultures or eggs with 10-fold dilutions and incubating at 33°.

Echovirus 11 and poliovirus were stored at 4° and titrated in monkey kidney cell cultures. M rhinoviruses were handled similarly and microplaques were counted to give increased precision to the assay (Parsons and Tyrrell, 1961). 16/60 strain was similarly assayed in human embryo kidney cell cultures. SV17 was stored at −20° and assayed in monkey kidney cultures and Coxsackie A21 was stored similarly and assayed in human diploid cells. The influenza virus was stored at −70° and assayed by allantoic inoculation of 10–11 day eggs.

Organ cultures

Strict asepsis was observed throughout the manipulations. The trachea, nasal epithelium or palate was cut into roughly square pieces 1–3 mm. across. Very sharp knives were used and care was taken never to touch the ciliated surface. The piece of mucous membrane and the underlying cartilage or bone was put on the bottom of a 60 × 15 mm. plastic petri dish (Falcon or Nunc brand) where it had been scratched with a scalpel blade. It adhered to this surface and 4–6 such fragments were planted in each dish and Parker medium 199 was added until the tops of the fragments, which were always formed by the ciliated surface, were level with or slightly above the level of the fluid. Provided enough tissue was added and the medium contained 3·8 g./l. of sodium bicarbonate the pH remained between about 7·0 and 7·2 during the next 24 hr. The medium was completely replaced each day.

The surface of the membrane was examined daily under the low power of the microscope by reflected light. Cultures were only inoculated if the cilia were seen to be beating vigorously over the whole surface. Cultures were inoculated by adding the virus to the medium. Cultures were washed with medium 3 times after an adsorption period of 6–12 hr. The medium removed each day was mixed with an equal volume of broth saline before being stored. Medium was also collected from " dummy " cultures which contained no cells and were also not washed after inoculation. The presence or absence of ciliary activity on each fragment was recorded daily and uninoculated control dishes were included in most experiments. Ciliary activity continued for up to 3 weeks in most of these. Tissue fragments were recovered and fixed in Bouin's fluid for histological examination when ciliary activity ceased and sometimes before.

A number of experiments, particularly with monkey tissue, were terminated by contamination with moulds or bacteria ; in the remainder we attempted to titrate virus daily or almost daily for up to 3 weeks. It was possible to recognise virus multiplication by the following criteria : the virus titre of the medium rose at a certain time higher than that of the inoculum ; the titre of the culture medium was higher than that of the control fluid ; and it was possible to detect virus for long periods despite repeated medium change and in some cases serial passage in organ cultures, so that the total amount of virus recovered was much greater than the amount put in.

RESULTS

Using these methods 62 dishes were inoculated and adequately tested.

Growth of viruses in rhesus monkey tissues

The results obtained are illustrated in Fig. 1 and summarised in Table I. The growth curves in Fig. 1 show that on first passage in organ cultures the virus

TABLE I.—*Growth of Viruses in Various Tissues of M. Rhesus*

Type of tissue	Frequency of virus growth in organ cultures inoculated with indicated virus				
	M rhino	Adeno SV17	Influenza A2	Echo 11	Coxsackie A21
Nasal	0/1†	1/1	2/2	..	..
Tracheal	0/4	7/7	?1*/5	0/1	0/1

† Numerator = number of organ cultures in which virus multiplication was detected, and Denominator = number of cultures tested.

* No increase in titre observed, but virus persisted and was much higher in titre than in control dishes.

was present in the culture medium at a high and slightly rising titre ; in a control dish inoculated with virus but containing no tissue, the titre fell 100-fold in 4 days. In cultures inoculated with virus passed in organ cultures the titre rose from $10^{1.5}$ — 10^{4} between 1 and 10 days after inoculation. Virus was still found to a titre of 10^{2} in the 15 days after inoculation in one experiment. Four serial passages were successfully performed. It was concluded that the SV17 virus multiplied in the culture system. Cultures inoculated with SV17 virus lost their ciliary activity between 8–13 days after inoculation. The cytopathic effect observed in sections is described below. It was noted that virus production continued long after the cilia had ceased to beat. We thought that there might be much more virus in the explant than was released into the medium and therefore we attempted to release this by freezing and thawing or by grinding the tissue. No more virus was released than had already been found in the medium.

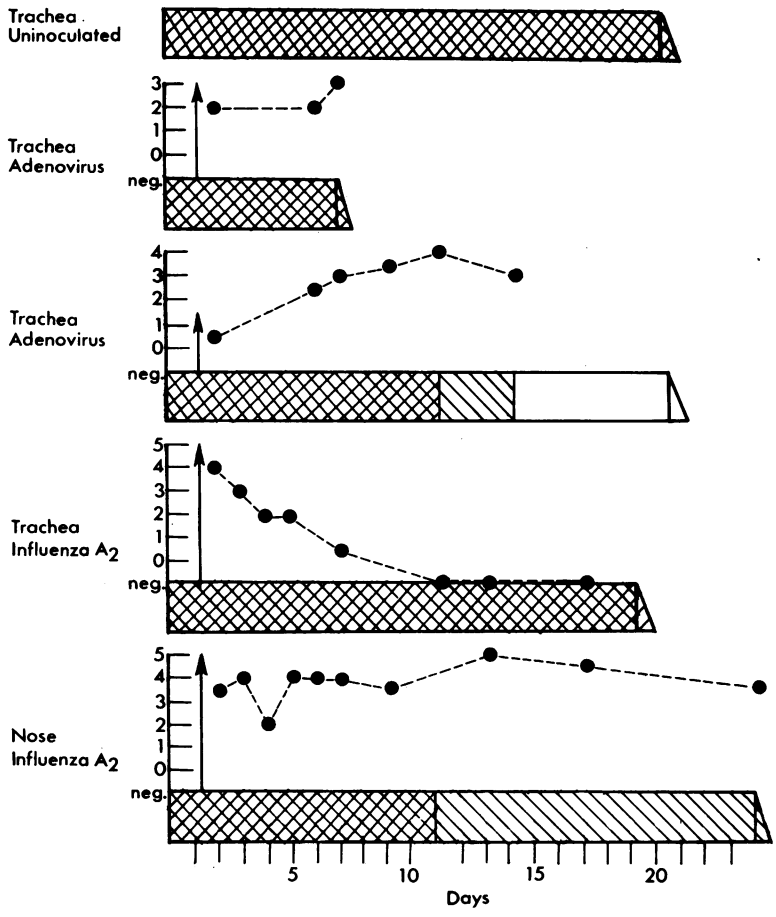
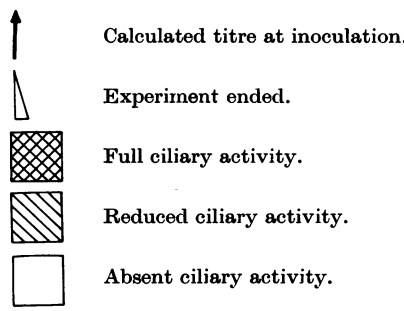


FIG. 1.—A representative experiment on rhesus monkey cultures. The ciliary activity of the uninoculated tracheal explants (top) continued for 20 days, when the tissue was fixed. Two dishes were inoculated with SV17 virus. The first with the original virus and the second with virus passed twice in organ cultures. In both the virus apparently multiplied and in the second dish ciliary activity was reduced and finally abolished for a week before the end of the experiment. The lower two sections show that Influenza A2 virus multiplied poorly, if at all, in tracheal epithelium; in control experiments no virus was found in dishes without tissue which were incubated for 3 days. The virus apparently grew well in cultures of nasal epithelium, but there was still ciliary activity at the end of the experiment.

Ordinate units are $\log_{10}$ $1D_{50}$ of virus per unit volume of medium.



Although influenza A2 may have multiplied in 1 of 5 cultures of trachea it did apparently multiply quite well in nasal epithelium. There was no evidence that M rhinoviruses multiplied whether or not they were adapted to monkey kidney cells ; similarly, echovirus 11, which grows readily in monkey kidney cells, did not grow in differentiated epithelial cells.

Growth of viruses in human tissues

Table II summarizes the results obtained with fragments from 4 sites in the human foetus—the nasal septum and turbinates, trachea, oesophagus or palate. As in the case of monkey tissue, similar results were obtained with tissue from different embryos. Every virus tested grew in at least one type of organ culture.

TABLE II.—*Growth of Virus in Various Human Tissues*

	Frequency of virus growth in organ cultures inoculated with indicated virus					
	M rhino					
Type of tissue	Tissue culture fluids	washings	Echo 11	Coxsackie A21	Polio 1	Influenza A2
Nasal	2/3	3/3	2/2	1/2	1/1	1/1
Tracheal	2/3	1/2	2/2	1/1	1/1	2/2
Oesophageal	0/3	0/1	2/2	1/2	1/1	0/1
Palatal	0/1	—	1/2	0/1	—	—

Fig. 2 illustrates a result obtained with rhinoviruses. The PK strain of M rhinovirus multiplied in nasal epithelium as judged by the rise in titre, after a short latent period. The virus disappeared from the control dishes within 24 hr. In addition it was passed in series 3 times. PK virus was found at lower titres and for a shorter time in cultures of trachea and in certain experiments it failed to grow in these cultures, although cultures from the same embryo supported the growth of influenza virus and echovirus 11 as well as did nasal tissue. It was noted in several cases that cultures produced fluctuating amounts of M rhinovirus and continued to produce virus as long as the experiment was continued. In a single experiment it was shown that the H rhinovirus used also multipled in nasal epithelium ; the virus was present in low titre and could be detected only on titration in human embryo kidney and not in a human diploid cell strain. It seemed that the organ cultures were relatively sensitive to infection since the washings used for inoculation contained low titres of virus. There was little, if any, effect on ciliary activity in the infected cultures.

Coxsackievirus A21 (Coe virus) is regarded as an enterovirus, and yet it causes typical colds in man. It was therefore of interest that it multiplied best in human nasal and tracheal epithelium, rather like the rhinoviruses, and did not seem to destroy the epithelium. An enterovirus, echovirus 11, grew rapidly and to very high titre in cultures of nasal epithelium, trachea and oesophagus and feebly or not at all in the squamous epithelium of the palate (Fig. 3 and Table II). The poliovirus behaved much like echovirus 11 in nasal, tracheal and oesophageal epithelium—this was unexpected but while these experiments were in progress it was reported that virus may be recovered in quite high titre from the nasal secretions of subjects naturally infected with poliovirus (Meenan and Hillary, 1963).

Ciliary activity was destroyed by echovirus 11, but not by the strain of poliovirus used, which was a fully attenuated vaccine strain. The attenuated strain of influenza A2 virus used multiplied as well as the other strains used in earlier experiments (Hoorn, 1964) and it also grew to a similar titre in trachea, nasal epithelium and oesophagus, although it failed to grow in palatal epithelium (or in

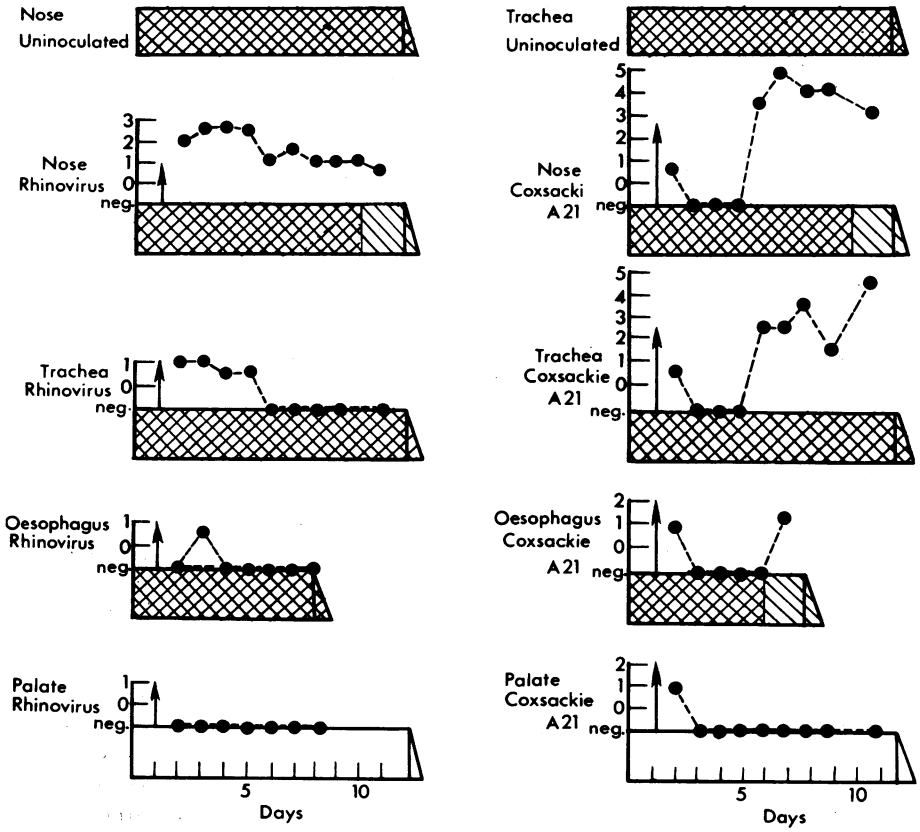


FIG. 2.—An experiment on human embryo cultures inoculated with a rhinovirus and coxsackievirus A21. The M rhinovirus, P.K., multiplied in nasal and tracheal epithelium, but not in oesophagus or in palate. Coxsackievirus A21 also grew in nasal and tracheal epithelium, but appeared later after inoculation and reached higher titre than P.K. There was a possible reduction in ciliary activity in the nasal epithelium. (See Fig. 1 for key.)

the trachea of rhesus monkeys, as mentioned above). Although it was an attenuated strain it destroyed the ciliated epithelium of the cultures in which it multiplied.

Pathological changes in infected cultures

The tissue from practically every experiment was fixed and stained with haematoxylin and eosin or by Papanicolau's technique. It was observed that the cells of uninfected cultures spread rapidly to cover the cut ends of the explants

with an epithelial layer one or two cells thick of flattened or cuboidal cells. The ciliated cells of the original epithelium and the submucous layer and cartilage were well preserved for the first 2 weeks after preparation of the cultures and were probably functioning well, for in one experiment influenza virus grew to high titre in cultures which had been set up 14 days before inoculation. There were occa-

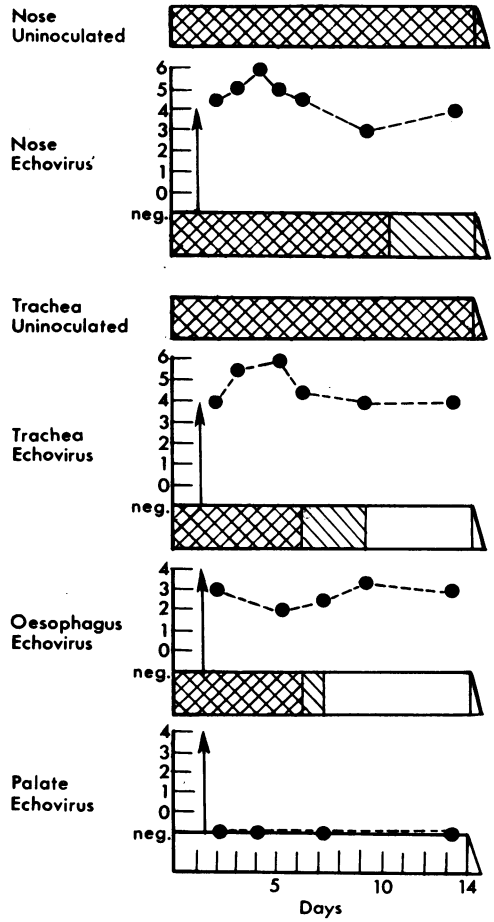


FIG. 3.—An experiment on human embryo cultures inoculated with echovirus 11. Virus grew to high titre and ciliary activity disappeared completely from tracheal and oesophageal cultures—virus was still produced after ciliary activity had ceased. (See Fig. 1 for key.)

sional mitoses in the epithelial cells for the first 2 weeks. By the end of 3 weeks the ciliated cells were being shed and degenerated cells could be seen in the epithelium. The ciliated cells lining the human oesophagus are shed spontaneously after about 1 week in culture as they are during normal development in the intact embryo ; therefore it is almost impossible to recognize changes due to viruses in cultures of this tissue. The main changes observed following infection with various viruses are given below.

Rhesus monkey cultures infected with adenovirus SV17.—The uninoculated tracheal cultures show an epithelial layer lying on a thick basement membrane (Fig. 4). Eight to 10 days after inoculation degenerated cells are seen in most parts of the epithelium (Fig. 5). The lesions give the impression in some areas of penetrating towards the basal cell layers and by 20 days most of the epithelial cells are shed and only the basal cells remain (Fig. 6). Under high power there are prominent changes in the nuclei many of which show appearances which seem to be identical with those produced by the same virus in monkey kidney cells (Tyrrell *et al.*, 1960). Typical large central basophilic intranuclear masses are readily seen (Fig. 5*a*). There are no distinctive changes in the submucosa.

Human embryo cultures infected with rhinoviruses and coxsackievirus A21.—The uninoculated nasal epithelium is completely covered with ciliated cells (Fig. 7). The epithelium is largely intact in cultures in which P.K. virus had multiplied. However here and there are areas in which the superficial cells have become rounded up and apparently shed. A well-marked example of this is shown in Fig. 8. Some degenerated cells contain vacuoles and the nuclei are pyknotic. The deeper cell layers are not affected. Several small foci of vacuolated cells are seen in the epithelium of one explant infected with H rhinovirus strain 16/60 ; because there was too little material to repeat the experiment it is not certain that the changes were due to infection with the virus, although it was certainly multiplying in the culture. No definite changes are seen in cultures infected with coxsackievirus A21.

Human embryo cultures infected with echovirus 11.—Nasal and tracheal cultures infected with echovirus 11 showed extensive degenerative changes within a week of inoculation (Fig. 9). The changes extended throughout the epithelium. Cells disintegrated and the nuclei became fragmented, and cells and debris were shed from the epithelium until by 20 days after inoculation only the basal cell layer remained. The submucosa showed little or no change. No fixed material was available from the cultures described above which were inoculated with poliovirus, but other experiments have been performed since which show that several different strains of poliovirus multiply well in organ cultures of human trachea and nasal epithelium, but while some produce extensive cell destruction, like that produced by echovirus 11, others produce no obvious cell damage.

Human embryo cultures infected with influenza virus A2.—The superficial ciliated cells are completely lost from cultures infected with A/Scot/49/57 within 1 week of inoculation. The intermediate epithelial cells in the tracheal epithelium

EXPLANATION OF PLATES

FIG. 4.—Uninoculated monkey tracheal epithelium 13 days after preparation of the culture. Stained haematoxylin and eosin (×370).

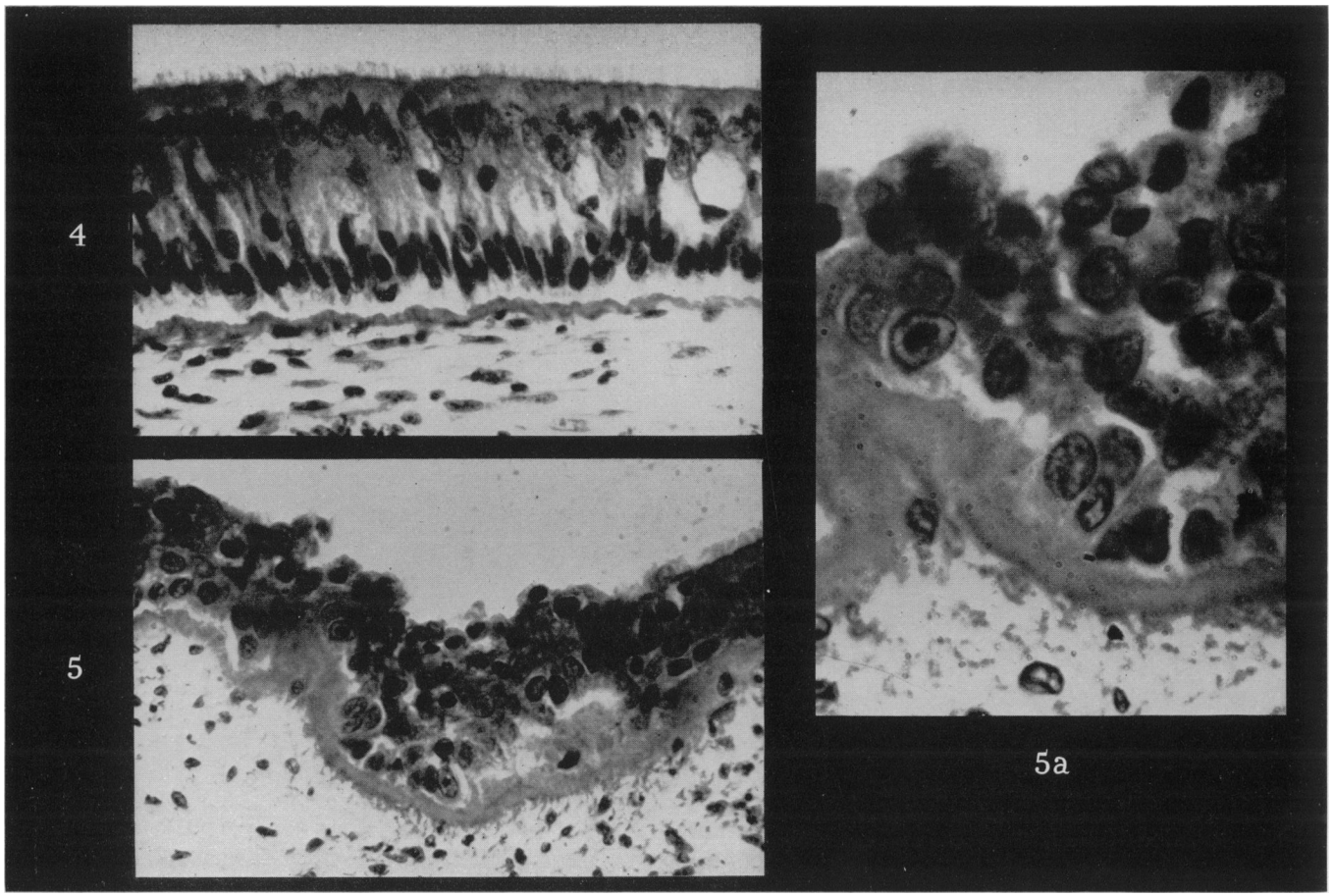
FIG. 5.—Monkey tracheal epithelium 10 days after inoculation of adenovirus SV17. Cytopathic changes in many cells (H. and E. ×370). Fig. 5a shows a higher powered view of cells some of which show nuclear changes resembling those seen in monolayer cultures (H. and E. ×730).

FIG. 6.—As Fig. 5, 20 days after inoculation. The epithelial cells are almost completely destroyed. H. and E. (×370).

FIG. 7.—Uninoculated human nasal epithelium. H. and E. (×230).

FIG. 8.—A culture parallel to that in Fig. 7 and fixed at the same time. It was inoculated 13 days previously with P.K. virus. A focus of degenerative change is shown. H. and E. (×230).

FIG. 9.—Human nasal epithelium inoculated 7 days previously with echovirus 11. Epithelial cells are being damaged and shed. H. and E. (×370).



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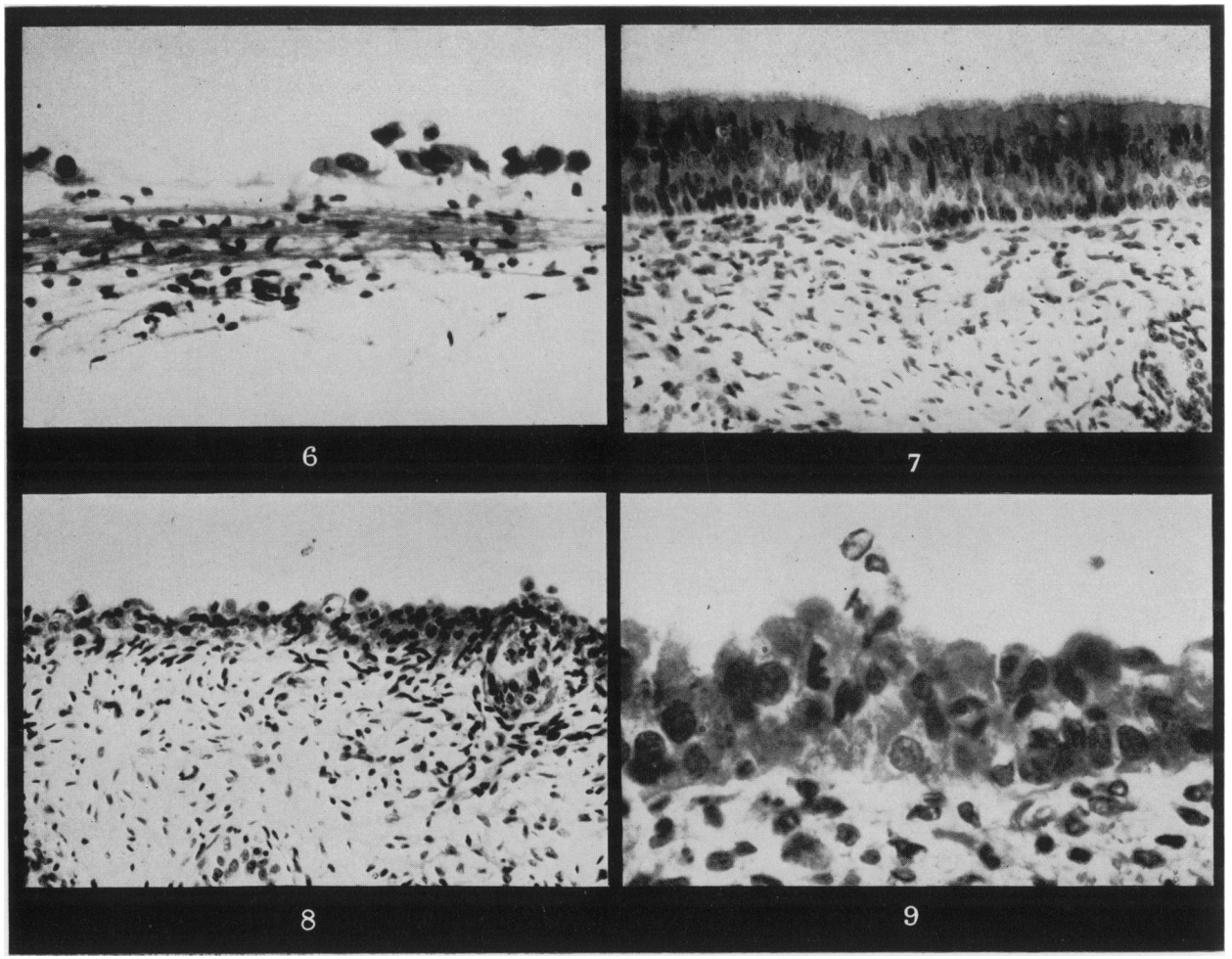

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remain practically normal while all the cells of the nasal epithelium down to the basal cells are completely destroyed. These changes differ in detail only from those described earlier (Hoorn, 1964) and these differences may be due to small variations in experimental methods such as the use of different strains of virus.

DISCUSSION

The experiments reported here have convinced us that several respiratory viruses can be cultivated quite readily in ciliated epithelium maintained as organ cultures. This method may be of value in several ways. For example, the technique may help in working out the pathology of human infection by viruses which cause non-fatal mild disease in man even though organ cultures are not strictly comparable with intact organs, since their constituent cells are slowly dying, and secondary vascular reactions cannot take place. The sections we obtained bear many resemblances to those published by Hilding (1944) who took biopsies from the noses of patients with colds of unknown aetiology.

Organ cultures enabled us to study the tissue tropisms of viruses in an *in vitro* system. Our results in this connection suggest that the cells are reacting very much as they do in the intact host ; for example, monkeys are apparently insusceptible to human colds and the nasal and tracheal epithelium will not support the growth of M rhinovirus although trypsinized kidney cell cultures will do so. It is also striking that although nasal epithelium and tracheal epithelium look much alike they reacted differently to certain viruses ; for example, influenza virus grew in the nasal epithelium of the monkey and not in the trachea, while the rhinoviruses grew much more readily in human nasal epithelium than in trachea from the same embryo. Finally, it seems that viruses which in the intact host invade a wide range of tissues of the respiratory and alimentary tract, echovirus 11 for example, will also infect a wide range of tissues *in vitro*, while those like rhinoviruses, which infect primarily the nose, will infect this tissue most successfully *in vitro* also. In the intact host other factors such as the pH and temperature of the surroundings, the presence of antibodies, and the way in which the virus reaches the organism can also influence where and to what extent the virus multiplies ; in organ culture, however, it is possible to show that under standard experimental conditions there are real differences between the sensitivity of different epithelia to infection with viruses, and real differences in the amount of virus which they produce. It was also noted that viruses varied in the amount of damage they caused to epithelial cells ; it is possible that this can be correlated to some extent with the way the viruses behave in man, but at the moment it is not clear how this may be done.

We have not attempted to grow in organ culture the important viruses of the parainfluenza virus group or respiratory syncytial virus. We think this should be possible, but we are more interested in the possibility of using the technique to cultivate, and if possible to detect, the various viruses which we have under study which cause colds and yet cannot be cultivated in any known system.

SUMMARY

Organ cultures of the nasal and tracheal epithelium of the rhesus monkey have been prepared and kept healthy for up to 3 weeks. A simian adenovirus

(SV17) multiplied in these cultures and destroyed the ciliated epithelium. Influenza A2 virus multiplied in nasal, but not in tracheal cells.

Similar cultures of human foetal tissue supported the growth of influenza A2, a strain of M rhinovirus and another of H rhinovirus, echovirus 11, coxsackievirus A21 and poliovirus 1. The epithelium was completely destroyed by echovirus 11, rhinoviruses caused mild focal changes and coxsackievirus A21 caused no detectable changes in living or stained epithelium. Cultures of human oesophagus supported the growth of poliovirus 1, coxsackievirus A21, echovirus 11 and, to lesser extent of influenza A2. The M rhinovirus did not grow in this tissue. Cultures of human palate supported the growth of echovirus 11 in one of two experiments and not of rhinovirus or coxsackievirus A21.

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