FURTHER OBSERVATIONS ON THE USE OF ORGAN CULTURES IN THE STUDY OF ACUTE RESPIRATORY-TRACT INFECTIONS

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ALTHOUGH many viruses are known to cause acute respiratory-tract infections, the proportion of illnesses that can be diagnosed in the laboratory remains disappointingly small—about 30 per cent. The introduction of organ cultures of human embryonic trachea and nose (Tyrrell and Bynoe, 1965; Hoorn, 1966) and the discovery of the human respiratory coronaviruses (Tyrrell and Bynoe; Hamre and Procknow, 1966) renewed hope that the virological investigation of such infections would be more profitable and the conclusions drawn from epidemiological studies less speculative.

A comparison of the standard tissue-culture methods with organ cultures for the isolation of viruses from patients with acute respiratory infections (Higgins, Ellis and Woolley, 1969) showed that, although some viruses undetected by the standard methods could be isolated in organ cultures, both systems were necessary for maximum efficiency. Furthermore, this and other studies in which organ cultures were used (Tyrrell and Bynoe, 1966; Higgins, 1966; McIntosh *et al.*, 1967; Higgins *et al.*, 1970; Roome, Dickinson and Caul, 1971; Higgins and Ellis, 1972) were limited by the scarcity of human embryonic material and the laborious nature of the technique. The purpose of this paper is to report on the use of organ cultures in the study of a larger number of cases of acute infection of the respiratory tract.

MATERIALS AND METHODS

Specimens were collected and examined by standard methods, as described previously (Higgins, Ellis and Boston, 1963; Higgins, Boston and Ellis, 1964). A nose and a throat swab were taken from each patient, placed in the same bottle of transport medium and conveyed to the laboratory on melting ice.

The standard method of examination included inoculation onto a blood agar plate and into tissue cultures of monkey kidney, the Bristol line of HeLa cells, human-embryo diploid fibroblasts (W1-38) and human-embryo kidney. The specimens were also inoculated subcutaneously and intracranially into suckling mice.

Organ cultures of human-embryo nose and trachea were prepared initially as described by Hoorn but the method was later modified as recommended by Tyrrell and Blamire (1967). For the last 2 yr of the study all organ cultures were maintained in $6 \times \frac{5}{8}$ -in. (15×1.5 -cm) test tubes, instead of in 6-cm petri dishes, and transverse sections of trachea were used in preference to longitudinal sections (Higgins and Ellis). The fluids, harvested at intervals,

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from each organ culture were pooled and tested for virus in tissue cultures of monkey kidney, human-embryo kidney and human-embryo diploid fibroblasts as previously described (Higgins); for a limited period L132 cells (Davis and Bolin, 1960) were also inoculated. Organ-culture fluid for electron microscopy was centrifuged at 60,000*g* for 30 min. after which the pellet was resuspended in a few drops of distilled water, applied to a formvar-carbon coated grid, stained with 2 per cent. phosphotungstic acid and examined in a Phillips 200 electron microscope.

RESULTS

Advantages of organ cultures in the study of acute infections of the respiratory tract

An earlier study in which standard tissue-culture methods and organ culture were used in parallel (Higgins, Ellis and Woolley) showed that organ cultures could be employed most profitably by keeping them for the examination of specimens that were negative by the standard methods. Such specimens from 466 patients with respiratory-tract illnesses, which occurred between May 1966 and Jan. 1971, were examined in organ cultures; 442 of them (95 per cent.) were further passaged once, 230 (49 per cent.) twice and 223 (48 per cent.) three times in organ culture. Eighteen of the 24 specimens that were not passaged yielded a virus on initial organ culture. Pooled organ-culture fluids from 147 (66 per cent.) of specimens that had received three or more passes in organ culture were examined by electron microscopy.

Seventy-three viruses were detected, giving an isolation rate for material negative by the standard methods of examination of 15.7 per cent. (table I). Rhinoviruses accounted for 59 (81 per cent.) of the isolates and there were four parainfluenza viruses (two each of types 1 and 3). The most rewarding finding was the isolation of seven coronaviruses. Four of them were recognised by their cytopathic effect on WI-38 cells, although none of the original specimens had produced any cytopathic effect when inoculated directly into these cells. The four strains resembled the prototype 229E strain of coronavirus in ease of adaptation to standard tissue cultures, and an antiserum to the 229E strain (kindly supplied by Dr E. J. Stott) had the same neutralising-antibody titre when tested against one of these isolates as against the prototype virus. The other three coronaviruses were "organ-culture strains" and failed to adapt to the standard tissue cultures; they were detected by electron microscopy. The only other viruses isolated were two strains of echovirus type 23 and one of Coxsackie virus type B5. The latter virus was isolated from a specimen that also yielded a rhinovirus in organ culture.

No virus was isolated in L132 cells, although organ-culture fluids from 159 specimens were tested in these cells between the beginning of Mar. 1968 and the end of Jan. 1970. However, this cell line was not in use at the time when the seven coronaviruses were isolated; it was shown later that the four 229E-like virus strains would grow in L132 cells while the organ-culture strains did not.

The isolation rates for children under 5 yr old (11.2 per cent.) and older children (12.9 per cent.) were very similar, but the isolation rate for all children (11.9 per cent.) was only two-thirds of that for adults (18.0 per cent.) (table I).

TABLE I
Viruses isolated in organ cultures from 466 respiratory specimens negative by standard isolation methods

Illness	Number, type (and percentage) of viruses isolated from patients aged					Total number (and percentage) of isolations
	0-4 yr	5-15 yr	16 yr+			
Cold	4 rhinovirus H 1 parainfluenza virus, type 3	5/30* 1 rhinovirus H	1/11 3 rhinovirus H 1 echovirus, type 23 2 coronavirus	25/78		31/119 (26.1)
Sore throat	—	0/12 2 rhinovirus H 1 Cocksackie virus B5	4 rhinovirus H 4 rhinovirus M	8/88		11/128 (8.6)
Laryngitis and croup	—	0/3	1 rhinovirus H 1 parainfluenza virus, type 1	2/17		2/22 (9.1)
Tracheitis	1 rhinovirus H	1/6 1 rhinovirus H 1 229E-like virus	2 rhinovirus H 2 rhinovirus M	4/19		7/30 (23.3)
Bronchitis	—	0/7	1 rhinovirus H	1/6		1/13 (7.7)
Bronchiolitis and pneumonia	1 rhinovirus H 1 parainfluenza virus, type 3	2/14	—	0/3		2/18 (11.1)
"Influenza-like" illness	2 rhinovirus H 1 echovirus, type 23 1 coronavirus	4/35 2 rhinovirus H 1 229E-like virus	3/23 7 rhinovirus H 2 rhinovirus M 1 parainfluenza virus, type 1 2 229E-like virus	12/78		19/136 (14.0)
Any	12/107 (11.2)	9/70 (12.9)	52/289 (18.0)			73/466 (15.7)

* Number of specimens positive out of number examined.

The isolation rates for patients with colds and tracheitis were 26.1 per cent. and 23.3 per cent. respectively, while that for patients with all other diagnoses combined was 11.0 per cent., ranging from 7.7 per cent. for bronchitis to 14.0 per cent. for "influenza-like" illnesses.

Effect of the number of passages in organ culture on the virus isolation rate

Between May 1966 and Jan. 1970 the majority of specimens were passaged several times through organ cultures and the culture fluids were tested for the presence of virus after each passage (table II). Seventy-one viruses were isolated from 562 specimens (12.6 per cent.) after initial organ culture. An additional 15 viruses were obtained from 411 (3.6 per cent.) of the remaining negative specimens after a further passage in organ culture but subsequent passages produced few isolations. McIntosh *et al.* have shown that, in the case of those coronaviruses that grow only in organ culture, at least three blind passages are needed to ensure the presence of enough virus for detection by electron microscopy. It was decided, therefore, that the most satisfactory scheme for the examination of specimens negative in standard tissue cultures was to inoculate them into organ culture and to follow this with a blind passage. The fluids from the initial and the passage cultures were then pooled and tested for virus in standard tissue cultures. If negative, two further blind passages were carried out in organ cultures. The pooled fluids from these were tested, as before, for virus and if still negative were then examined by electron microscopy. Examination in this way of 124 specimens, collected between Feb. 1970 and July 1971, yielded 33 viruses (26.6 per cent.) at the first step, including three strains of 229E-like virus. Another four viruses were isolated from 52 of the specimens (7.7 per cent.) that were carried through second and third passages; three of these were organ-culture strains of coronavirus and were detected by electron microscopy.

During the whole period of the study, May 1966 to July 1971, a total of 147 organ-culture fluids, harvested after three or more blind passages and negative on testing in tissue cultures, were actually examined by electron microscopy and only three (2.0 per cent.) showed the presence of virus particles, the coronaviruses already mentioned.

Ciliary activity and the growth of viruses

In a previous investigation (Higgins), it was observed that loss of ciliary activity was a poor indicator of the growth of viruses in organ cultures, because the cilia continued to beat in 13 of 16 infected cultures. In the present study, 27 of the 92 rhinoviruses (29.3 per cent.) isolated in organ culture were associated with cessation of ciliary activity within the duration of the test (8 days). With 13 of them absence of ciliary activity was first noted only after 7 or 8 days. Thus, only 15.2 per cent. of all the cultures infected with rhinoviruses had lost their normal ciliary activity within the first 6 days. This was in contrast to cultures infected with influenza viruses; 10 of 14 cultures (71.4 per cent.)

TABLE II
The effect of passage in organ culture on the isolation of viruses

Examination for virus after	Number and type of viruses isolated after			
	initial organ culture	one passage	two passages	three passages
individual passages				four or more passages
	44 rhinovirus H 12 rhinovirus M 11 influenza A 2 parainfluenza virus, type 3 1 Cocksackie virus B3 1 poliovirus, type 1	71/562* 7 rhinovirus H 4 rhinovirus M 1 parainfluenza virus, type 3 1 adenovirus, type 5 1 echovirus, type 9 1 229E-like virus	15/411 1 rhinovirus H 1/177	3 rhinovirus H 3/171 2 rhinovirus H 2/53
double passages	Type and number of viruses isolated from pools of			
	<div> <div>initial organ culture + 1st passage culture</div> <div>2nd + 3rd passage cultures</div> </div>			
	22 rhinovirus H 3 parainfluenza virus, type 1 1 parainfluenza virus, type 3 2 echovirus, type 23 1 Cocksackie virus B5 3 229E-like virus 1 influenza B	33/124	3 coronavirus 1 rhinovirus H	4/52

* Number of specimens positive out of number examined.

infected with influenza A virus and 8 of 12 cultures (66·7 per cent.) infected with influenza B virus had lost ciliary activity by the end of 6 days and by the 8th day the cilia had ceased to beat in 23 of these 26 cultures (88·5 per cent.). Seven coronaviruses grew without any apparent effect on the ciliary activity of the cultures.

No correlation was found between the type of clinical illness and loss of ciliary activity in organ cultures infected with rhinoviruses, the only group of viruses isolated in sufficient number to allow a comparison to be made. Ten of 38 rhinoviruses (26·3 per cent.) isolated from patients with colds were associated with cessation of ciliary activity, compared with 5 of 15 (33·3 per cent.), 5 of 15 (33·3 per cent.), and 4 of 16 (25·0 per cent.) from cases of tonsillitis, tracheitis and "influenza-like" illness, respectively.

DISCUSSION

This study confirms the findings of earlier workers (Higgins, 1966; Tyrrell and Bynoe, 1966; Higgins, Ellis and Woolley, 1969; Higgins *et al.*, 1970) that rhinoviruses account for most of the additional viruses that can be isolated from patients with acute respiratory-tract infections by the use of organ cultures. All but a few of these can be detected in the initial organ-culture fluids. The main indication for repeated blind passage of specimens through organ cultures is to detect organ-culture strains of coronaviruses, i.e., strains that do not readily grow in or adapt to standard tissue cultures. The three strains reported here, together with those described by McIntosh and his co-workers (1967) and by Tyrrell, Bynoe and Hoorn (1968), bring the total of such organ-culture strains of coronaviruses, isolated from patients with naturally occurring acute respiratory illnesses, to 12.

The strains of 229E-like virus are the first wild strains to be isolated in organ culture. The original strains of 229E virus were isolated in secondary cultures of human embryo kidney (Hamre and Procknow, 1966). The only other recorded isolations were made in cultures of diploid fibroblasts derived from foetal gut (Kapikian *et al.*, 1969), and the authors were unable to propagate their strains in organ cultures of human embryonic trachea.

Also of interest was the isolation of two strains of echovirus type 23, a serotype that often grows poorly in tissue cultures (Wigand and Sabin, 1961). However, both strains grew readily in tissue culture after their preliminary passage in organ culture.

The virus-isolation rates for the various age groups and clinical conditions reported here reflect essentially the incidence of rhinovirus infections that are diagnosed by this technique, accounting for the highest isolation rate being achieved in patients with colds. The difference in isolation rates between children and adults may be a function of the amount of infective virus excreted by the patient. Primary infection in a young child is likely to lead to the production in the respiratory tract of a larger amount of virus than in the adult and would therefore be more readily diagnosed by the standard tissue culture methods. This would account for the higher isolation rate in adults than in

children when the specimens negative in standard tissue cultures are tested in the more sensitive organ cultures. However, the ready passage to tissue culture of the four strains of 229E-like virus and the two strains of echovirus type 23 suggests that an adaptive process occurs in organ culture in addition to an increase in virus concentration.

Loss of ciliary activity was confirmed as a poor indicator of the presence of rhinoviruses and coronaviruses in organ culture. On the other hand, the growth of influenza virus in organ culture was usually associated with cessation of ciliary activity, commonly within 6 days of inoculation. This finding supports the suggestion of Mostow and Tyrrell (1972) that reduction of ciliary activity is related to the virulence of the infecting virus.

Although foetal material is scarce and the technique is laborious and expensive in detector tissue cultures, organ culture can provide a diagnosis in about 15 per cent. of cases of acute respiratory-tract infection where the infective agent is not detected by the standard methods of investigation. This is an underestimate, because organ-culture strains of coronaviruses were sought in only one-third of the specimens studied. The lack of any further evidence that L132 cells, or any other tissue cultures, are suitable for the primary isolation of organ-culture strains of coronaviruses (Bradburne and Tyrrell, 1969) leaves organ culture as the only confirmed method for the detection of infection with these agents. At present, to determine even such basic facts as the significance of the known viruses in acute respiratory-tract illnesses the method of organ culture must be employed.

Although organ cultures are necessary to achieve the isolation of the maximum number of infecting agents in acute infections of the respiratory tract, the standard methods of virus isolation cannot be dispensed with. Rhinoviruses, herpes simplex virus, mumps virus and a number of enteroviruses have been isolated by the standard methods, but remained undetected when organ cultures were inoculated with the same material (Higgins, Ellis and Woolley). The Hong Kong variant of influenza A2 virus has been isolated more frequently in eggs and tissue cultures than in organ cultures (Higgins and Ellis, 1972) and similar results have been recorded for respiratory syncytial virus (Roome, Dickinson and Caul, 1971) and certain avian respiratory viruses (Butler, Ellaway and Hall, 1972). The reason for the inability of organ cultures to permit the multiplication of these viruses may have a clinical significance in the development and transmission of infections.

SUMMARY

Specimens from 466 patients with acute respiratory-tract illnesses that failed to yield a causative agent by the standard methods of virus isolation were examined in organ cultures. Seventy-three viruses (15.7 per cent.) were isolated. Most of them were rhinoviruses (81.0 per cent.) but seven coronaviruses, four of which were 229E-like, were also isolated. The majority of the rhinoviruses were detected in the initial organ-culture fluids, but several blind passages were needed for the organ-culture strains of coronaviruses. Although laborious,

organ culture must remain an integral part of any detailed study of acute infections of the respiratory tract.

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