Cultivation of a Novel Type of Common-cold Virus in Organ Cultures

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In recent years it has become evident that the common cold and similar minor upper respiratory diseases are due to infection with viruses belonging to a number of different groups, including adenoviruses, myxoviruses—such as the influenza, para-influenza, and respiratory syncytial viruses—enteroviruses, and rhinoviruses. When tests adequate to detect all these are used a virus or a β-haemolytic streptococcus can be isolated from about one-third of patients suffering from colds and related diseases (Working Party, 1965). The failures might occur because no virus or bacteria were present in the respiratory secretions tested, but in one study (Kendall *et al.*, 1962) two out of four such specimens which apparently contained no virus were administered to volunteers and produced colds ; it is therefore likely that some failures are due to the presence of viruses which cannot be cultivated by present methods.

In the past four years efforts have therefore been made to discover something of the nature of such viruses and to devise methods of cultivating them in the laboratory. Some success has been achieved and is reported in this paper.

Materials and Methods

New Viruses.—The primary sources of these were nasal washings in phosphate-buffered saline which were collected at the height of a cold, mixed with an equal volume of bacteriological nutrient broth, and stored at −70° C.

Other viruses were propagated in chick embryos or in tissue cultures as appropriate, and were handled by standard methods.

Organ cultures were prepared mainly from the tracheas of 14- to 22-week-old human embryos obtained at hysterotomy from cases in which there was no clinical suspicion of an infection in the mother or foetus. Four to six tissue fragments were planted with the ciliated surface uppermost on the scratched surface of a 6-cm. plastic Petri dish (Falcon), and 1.25 ml. of Medium 199 (Glaxo) containing 0.035 g. of sodium bicarbonate per litre was added. The dish was incubated at 33° C. in a humidified box, and the medium was changed daily for two days. Cultures were then inoculated by dripping 0.3 ml. of inoculum on to the fragments ; thereafter the medium removed each day was mixed with broth and stored at −70° C. After about 10 days some cultures were fixed in Bouin's solution, embedded, sectioned, and stained with haematoxylin and eosin.

Haemagglutination-inhibition tests were performed, using four agglutinating doses of virus and sera treated with cholera filtrate (Philips) ; tests were done in 0.025-ml. volumes, using the Takatsy apparatus as modified by Sever (1962). Human group O cells were used in almost all tests.

Volunteers.—The methods of obtaining, housing, and observing volunteers have been described elsewhere (Andrewes, 1949). Sera were collected from some volunteers at the time of developing symptoms of a cold, and again about two weeks later after leaving the unit.

Results

Most work has been done with a nasal swab and washing number B814, obtained from a boy with a typical common cold in 1960 (Kendall *et al.*, 1962). Further infectious secretions were obtained from volunteers who developed colds after intranasal inoculation of the original specimen. In this way three serial passages of the cold-producing agent were made in man, and it was concluded that it must be self-propagating. In over 20 experiments washings were tested by inoculation into a variety of test systems for known viruses. These are outlined in Table I, and should have revealed the presence of influenza A, B, or C, para-influenza 1, 2, 3, or 4, respiratory syncytial viruses, herpes simplex virus, and adenoviruses, cytopathic enteroviruses or rhinoviruses, or mycoplasma, particularly *Mycoplasma pneumoniae*. None was found. Further tests with a limited number of techniques showed that there was no evidence that the cold-producing agent was propagated even for a few days to a sufficient extent to produce the small amount of virus usually needed to cause a cold in a volunteer (Table II).

TABLE I.—*Tests for the Presence of Known Respiratory Viruses and Other Agents in Specimens Containing B814 Agent*

Test System	Condition of Incubation	Assessment	No. of Tests Done
Tissue cultures of rhesus, patas, or vervet kidney cells in medium 199	Rolled at 33° C. for 10 days	Cytopathic effect, haem-adsorption and inter-ference test (E.C.H.O. 11)	6
Human-embryo-kidney cells or diploid fibro-blast cell strains (WI-26 or WI-38) in medium with 2% calf serum	Rolled at 33° C. up to 21 days	Cytopathic effect	5
HeLa cells	Stationary or rolled	Cytopathic effect	5
Solid media and monkey-kidney cultures suitable for *M. pneumoniae*	At 36° C. for 1 week or more	Examination for colo-nies. Cultures sub-inoculated into chick embryos and tested for specific antigen in lung	3
6-, 10-, or 12-13-day-old chick embryos inocu-lated amniotically	33° C for 6 days	Test for chicken and human red-cell agglu-tinins at bench temp. and 4° C.	7

No virus was isolated in any test.

TABLE II.—*Properties of B814 Agent in Nasal Washings*

Inoculum Given	Proportion of Volunteers Developing Colds
Untreated washing inoculated	5/11
Washings filtered through Gradocol membrane 0·59μ A.P.D. inoculated	3/7
Washings held overnight at 4° C. with 20% diethyl ether	0/12
Volunteers given 150 mg. q.i.d. of demethylchlortetracycline from 2 days before inoculation of washing to 4 days after	8/10
Volunteers given similar capsules containing lactose and same washings	5/8
Washings inoculated into : (*a*) Human and rhesus kidney cultures, WI-38 and WI-26 diploid cells incubated at 33° C. and harvested at intervals up to 21 days. Pooled culture fluids inoculated into volunteers	0/14
(*b*) 6- and 10-day-old chick embryos inoculated by amniotic route and incubated at 33° C for 6 or 10 days. Pooled amniotic fluids inoculated into volunteers	1/22

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We therefore attempted to determine by experiments in volunteers a few basic properties which would confirm that we were indeed dealing with a virus. These experiments, also shown in Table II, indicate that the infectivity of B814 can pass a bacteria-tight filter, is inactivated by ether, and can induce colds in volunteers given sufficient antibiotics to cure a fully developed infection with the Eaton agent (*M. pneumoniae*). These results showed that B814 is a virus, not a mycoplasma, and that it is not an adenovirus, enterovirus, or rhinovirus because it is ether-labile. Another uncultivable agent produced colds in two out of six volunteers after ether treatment. This was the agent recovered from the subject H. G. P. on 26 July 1957 (Tyrrell and Bynoe, 1961). It was concluded that there must be at least two biologically different viruses among these "uncultivable" viruses.

Cultivation of B814 in Organ Cultures

It was thought that the failure to cultivate the virus in tissue culture probably arose because the virus was unable to propagate in the highly modified dedifferentiated cells used so far. It had been shown that human nasal or tracheal ciliated epithelium maintained in organ culture would support the growth of representative strains of influenza A and herpes virus (Hoorn, 1963), adenovirus, poliovirus, coxsackievirus A21, echovirus 11, H and M rhinoviruses (Hoorn and Tyrrell, 1965), and also myxoviruses such as influenza B and C, parainfluenza virus types 1, 2, 3, and 4, and respiratory syncytial virus (Tyrrell and Hoorn, 1965). Several viruses were successfully grown from nasal washings without passage in any other culture system. It was therefore thought reasonable to attempt to propagate the B814 virus in cultures of this type.

The experiments done so far are summarized in Table III. There was some trouble with bacterial and fungal contamination at first, but later the technique outlined under "Methods" was found to be trouble-free. It was regularly possible to produce colds in volunteers who were given culture fluids from the first or later passages. For several reasons it is believed that the B814 virus was multiplying and causing these colds. Firstly, no colds were produced by fluids from "dummy" cultures containing no tissue and inoculated one or two days before with nasal washings. Similarly, no colds were produced by fluids from numerous uninoculated parallel cultures set up from the same embryos, changes at the same time in the same cabinet, and using the same medium as those used for the virus-infected cultures; no colds were produced by medium from inoculated cultures in which ferret trachea was used instead of human tissue. On the other hand, colds were produced with fluids coming from cultures which had been changed up to eight times after inoculation, involving a lapse of at least a week in culture and a probable dilution of the order of 10^8 of the original inoculum.

Colds were also produced after four serial passages in which the fluids collected daily between one (sometimes three) and 10 days after inoculation were pooled and used to inoculate further batches of cultures. This method of serial passage was adopted in order to ensure that some infectious virus was passed, because in some experiments with rhinoviruses and respiratory syncytial virus the viruses had been shed into the medium for only a few days and at rather unpredictable intervals after the inoculation of the cultures. Fluid for this fourth serial passage was also inoculated after overnight treatment with ether; it caused colds in none of six volunteers. Another aliquot was filtered through a membrane of A.P.D. 0.59 μ and produced colds in three out of six volunteers who were treated with demethylchlortetracycline. It was concluded that these colds were due to the presence of an ether-labile virus, as were those produced by the washings used to initiate the serial passage. Similar culture fluids failed to cause disease when inoculated intramuscularly and intranasally into adult white mice, intracerebrally and intraperitoneally into suckling white mice, and intracerebrally and intranasally into guinea-pigs; and no virus was isolated by amniotic inoculation of 10-day-old chick embryos.

TABLE III.—*Results of Attempts to Passage B814 Virus in Organ Cultures of Respiratory Epithelium*

Serial Passage No.	Tissue	No. of Passages	Days of Harvest	Frequency of Colds in Volunteers Receiving	
				Test Material	Control Material*
1	Human trachea	1	3–10	4/6	0/13
	" "	1	3 and 4	4/5	0/6
		1	5–7	3/5	
		1	8–9	4/7	
	None	1	2	0/6	
	"	1	1	0/7	
	Ferret trachea	1	3–10	0/6	0/6
	Ferret	3 human, 1 ferret	1–10	0/5	
2	Ferret trachea	1 human, 1 ferret	1–10	0/5	1/5
	Human trachea	2 human	1–10	4/6	0/6†
	Ferret trachea	2 human, 1 ferret	1–10	0/5	
	Human trachea	4 human	1–10	4/4	0/9
3	Human trachea	1	1–9	6/6	0/6†

* In first passages control fluids were collected from uninoculated cultures harvested and stored exactly as the test cultures. For later passages the control fluids were passed serially in parallel with the test cultures.
† Identical volunteers

Studies of Volunteers' Sera

A number of paired sera were available from volunteers who had developed colds after receiving the B814 virus either as washings or as tissue-culture fluid. It was thought possible that the sera might manifest a rise in antibody titre against some known myxovirus because the B814 virus might be either a myxovirus of a known serotype which was more difficult to cultivate than any previously encountered, or a myxovirus of a new serotype with a distant antigenic relationship to one or more known types. The sera were therefore titrated by haemagglutination-inhibition test in this laboratory, and by complement-fixation tests by Dr. L. Hatch in the Portsmouth Laboratory of the Public Health Laboratory Service. The results are summarized in Table IV. A few rising titres were detected against influenza C and para-influenza viruses; the rises detected by complement-fixation were observed in two pairs with two or three antigens. It was concluded that B814 did not belong to any of the serotypes of myxovirus used, but might be distantly related to influenza C or Sendai viruses. Paired sera from six volunteers were tested for their ability to fix complement with culture fluids collected from the fifth serial passage and concentrated fifteenfold with polyethylene glycol. No fixation was observed.

TABLE IV.—*Results of Serological Tests on Sera from 22 Volunteers Who Developed Colds After Receiving B814 Virus*

Inoculum	Proportion of Pairs of Sera Showing Rising Titres* Detected by	
	Haemagglutination-Inhibition	Complement-fixation
Washings	Influenza C, 1/8	0/5
Organ culture fluid	Para-influenza 1, 1/14	Influenza C, adenovirus, and mumps, 1/14
	Influenza C, 2/14	Influenza C and Sendai, 1/14

* Fourfold or eightfold rise.
All sera were tested against influenza A, B, and C, para-influenza 1 and 3 by haemagglutination-inhibition test and against these plus para-influenza 2, mumps V and S antigen, respiratory syncytial virus, herpes simplex, adenoviruses, psittacosis, *Rickettsia burnetii*, *Mycoplasma pneumoniae* by complement-fixation test.

Clinical Features of Colds Produced by B814 Virus

The frequency of some symptoms, clinical signs, and certain other data regarding volunteers who developed colds after inoculation with B814 material are listed in Table V. This shows that the illnesses were slightly more frequent, were more

severe, and had a shorter incubation period when they followed the inoculation of organ-culture fluid than after the inoculation of nasal washings. This could all have been the result of the administration of a larger dose of virus. In both groups the illness was a typical common cold. Fever was rare, but there was often considerable malaise, and the nose often streamed with watery secretion—one volunteer used 120 paper handkerchiefs in one day—but there was little cough and no sputum, and on the average the disease cleared up in less than a week. The clinical picture in 213 volunteers infected with an M rhinovirus is also shown in Table V; this was significantly different in that malaise and constitutional upset were less common, and sore throat, cough, and sputum were more so—the respiratory symptoms were less strictly confined to the nose and lasted longer, but there was less nasal discharge at the height of the illness.

TABLE V.—*Symptoms and Signs in Volunteers Developing Colds After Inoculation With B814 Virus*

	Frequency of Indicated Clinical Features and other Information on Volunteers Inoculated With		
	B 814 Nasal Washings (%)	B 814 Organ Culture Fluids (%)	Rhinovirus M H.G.P. or P.K. (%)
Malaise	47	64	28
Headache	53	71	52
Chill	18	25	28
Aching back or limbs	12	18	4
Sneezing	91	89	78
Nose blocked	79	89	82
Sore throat	38	57	87
Cough	44	28	68
Sputum	0	3	21
Fever	12	25	22
Nasal discharge	100	100	100
Mucopurulent nasal discharge	62	75	83
Mean peak No. of handkerchiefs used	21	15·6	14
Percentage of colds graded as Mild	70·5	61	81
Percentage of colds graded as Moderate	20·5	25	15
Percentage of colds graded as Severe	9	14	4
Mean incubation period	3·2	2·8	2·2
Duration	6·3	6·7	9·6
Percentage of inoculated volunteers developing colds	45	60	37
No. Studied	34	28	213

Preliminary Attempts to Propagate More Otherwise Uncultivable Viruses in Organ Cultures

A number of other nasal washings were collected from patients with colds and tested for respiratory viruses. From certain of these no virus was recovered, and so they were inoculated into organ cultures. Some results obtained are outlined in Table VI. This shows that the viruses contained in these specimens were apparently propagated in organ cultures, because culture fluids caused colds. The organ-culture fluids were tested further by inoculating them into a range of tissue cultures. In the case of the M.T. strain the virus apparently grew, as judged by inoculation of volunteers, but was obviously different from B814, because tests in volunteers showed that it was ether-stable. When organ-culture fluids were inoculated into other cultures they produced a cytopathic effect which resembled that due to rhinoviruses.

TABLE VI.—*Use of Organ Culture of Human-embryo Trachea in Attempts to Propagate and Detect Viruses from Nasal Washings from Patients with Common Colds*

Strain	No. of Passages	Reduction in Ciliary Activity	Day of Harvest	Frequency of Colds in Volunteers Receiving		Other Tests
				Test Material	Control Material	
B 814	2 (series 2)	—	1–10	4/6	0/6	Sections show doubtful degenerative changes
	3 „	? +12	1–10	—	—	
	4 „	+10	1–10	—	—	
	1 (series 3)	? +10	3–10	6/6		Degeneration on section
M.T.	1	+4	7 and 8	2/5	—	No virus detected in monkey kidney, human kidney, HeLa cells. Effect like rhinovirus in some cultures of human diploid fibroblasts.
	2	?+2	1–10	2/5*	0/4	
	3, 4, 5	+4	1–10	—	—	
F.T.	1	0	3–10	2/6	0/6	No virus isolated in above cells. Rhinovirus-like effect on inoculation into human diploid cells
	2	0	1–10	2/4	0/3	
	3, 4, 5	−, +10, +4	1–10	—	—	
	Repeat 2	0	1–10	—	—	

* Inoculum treated with ether.

The specimens from another organ-culture experiment with strain G.T. were titrated in human diploid cells and the data are plotted on Fig. 1, which shows that a rhinovirus had grown freely and could be readily detected after three passages in organ culture. The original nasal washing had produced some doubtful cytopathic effect when tested in four batches of human-embryo-kidney cells, and a more definite effect in one strain of human-embryo fibroblasts, although the latter was not successfully passaged. Finally, the virus F.T. was apparently a rhinovirus that failed to produce any cytopathic effect in the form in which it was present in the nasal secretions and in the first passage in organ cultures, although it could produce colds. It nevertheless multiplied in organ culture, and became adapted so that it rapidly damaged the cilia (see below), and also was able to produce a cytopathic effect in cells of a human fibroblast strain.

We report these experiments in order to illustrate that organ cultures may be of value in cultivating and recognizing other "difficult" respiratory viruses, and also to emphasize that because a virus is grown in organ cultures it is not safe to conclude that a new type of virus has been cultivated.

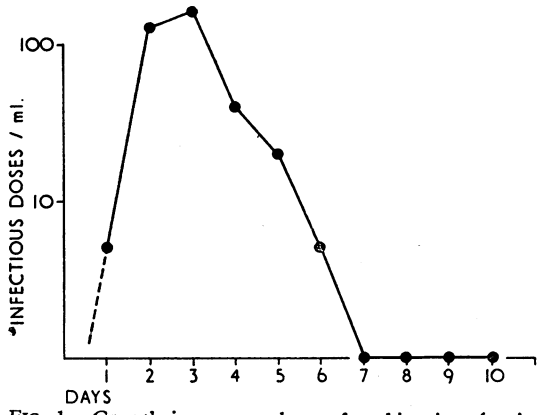

FIG. 1.—Growth in organ culture of a rhinovirus (strain G.T.) which was not successfully isolated in human-embryo-kidney or human diploid cells. The virus was passed twice in organ cultures, and the medium collected on each day of the third pass was titrated in diploid cells. There was a reduction in ciliary activity.

Detection of Infection in Organ Cultures

It has been shown earlier that the ciliary activity of infected cultures may be reduced or absent compared with that in uninoculated cultures. Fixed and stained sections may also show degeneration or shedding of some or all of the surface epithelium (Hoorn, 1963; Hoorn and Tyrrell, 1965). Attempts were therefore made to detect these effects in the cultures used in the experiments described above. The results are outlined in Table VI. The activity of the cilia on each tissue fragment was examined by reflected light daily or on alternate days. In experiments in which ciliary activity continued vigorously in the controls it was sometimes noted that the ciliary activity of all or most of the virus-infected fragments had ceased. This is recorded (as +) in Table VI, as is also the day on which a reduction in ciliary activity was first recognized. It can be seen that this effect was observed in only the later passages of B814 and was never clear-cut; possible

or definite degenerative changes, particularly in the tracheal ciliated epithelial cells, were seen in fixed and stained tissue in which during life the cilia were thought to be beating less well than in the control.

We also attempted to detect the presence of B814 by using the phenomenon of virus interference as we had done in earlier studies on rhinoviruses (Hitchcock and Tyrrell, 1960). A set of human-embryo-trachea organ cultures was inoculated with fluid from the fourth passage of the virus (see Table III). The cultures were incubated as usual for five days, and then, although the cilia were beating freely, they were challenged with Sendai virus, the U strain of echovirus 11, or a human strain of para-influenza 3. The media were harvested daily and titrated for infectivity by inoculation of serial dilutions into monkey-kidney-cell cultures. The results were generally the same with each virus, and may be illustrated by those obtained with Sendai virus (Fig. 2). The titre was tenfold or more

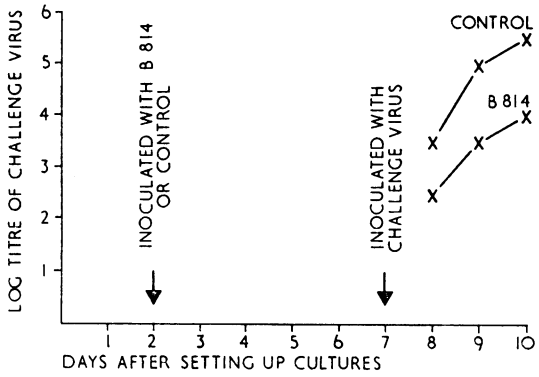

FIG. 2.—Interference in an organ culture inoculated with B814. Human-embryo-trachea cultures were inoculated with fifth-passage virus or control fluids and then challenged with Sendai virus. The titre of challenge virus in the daily medium harvest is shown. The titres are consistently lower in the dish first inoculated with B814 than in that given control fluids.

lower in cultures previously infected with B814 than in those previously inoculated with media from uninfected organ cultures. This was noted in all eight comparative titrations, using the three challenge viruses mentioned. It was concluded that infected cultures could be definitely detected by virus interference or reduction in ciliary activity.

Discussion

These experiments seem to show that organ cultures of human tracheal epithelium can support the growth of at least one respiratory virus which we have been unable to grow by any other laboratory technique. After considerable initial doubts we now believe that the B814 strain is a virus virtually unrelated to any other known virus of the human respiratory tract, although, since it is ether-labile, it may be a myxovirus. It is disappointing that so far no satisfactory serological test is available, but there are many possibilities still to be explored; for instance, we have not yet tried to use the fluorescent antibody technique with sections of organ cultures, because model experiments with cultures infected with influenza virus were not encouraging. Further, we are testing in organ cultures specimens from other patients with respiratory disease in order to find out how frequently hitherto unrecognized viruses can be isolated. These results will be reported later, but it seems likely that some of these may be fastidious rhinoviruses. However, it also seems possible that organ cultures of other tissues might be useful in propagating other viruses which have not so far been grown in dedifferentiated cells—for example, the viruses of molluscum contagiosum, and gastro-enteritis. In order to increase the chances of success in such experiments it is also necessary to look for further improvements in the techniques of organ culture, in particular a substitute for human foetal cells and improvements in the medium and other conditions used.

Summary

Volunteers developed colds after the intranasal inoculation of secretions derived from a boy with a common cold. Colds developed, although the secretions were passed through a filter of A.P.D. 0.59 μ and the volunteers were treated with demethylchlortetracycline. No colds developed if the washings were treated with ether.

The virus thus demonstrated would not grow in tissue cultures and eggs which would support the multiplication of known viruses of the upper respiratory tract. It multiplied and was serially propagated in organ cultures of human foetal tracheal epithelium. The colds produced by washings and tissue cultures were clinically similar, and in the aggregate distinct, from those produced by M rhinoviruses.

Sera of infected volunteers were tested by haemagglutination-inhibition and complement-fixation tests; a small proportion showed slight rises against influenza C and Sendai viruses.

Infection of organ cultures with B814 was detected with difficulty by a decline in ciliary activity and by degenerative changes in sections of the tissue, but there was a tenfold to a hundredfold reduction in titre on challenge of the cultures with other viruses—virus interference.

Other viruses distinct from B814 have been recognized and similarly cultivated, including uncharacterized ether-stable viruses which may be rhinoviruses.

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REFERENCES

Andrewes, C. H. (1949). *Lancet*, **1**, 71.
Hitchcock, G., and Tyrrell, D. A. J. (1960). Ibid., **1**, 237.
Hoorn, B. (1963). *Acta otolaryng. scand.*, Suppl., No. 188, p. 138.
—— and Tyrrell, D. A. J. (1965). *Brit. J. exp. Path.*, **46**, 109.
Kendall, E. J. C., Bynoe, M. L., and Tyrrell, D. A. J. (1962). *Brit. med. J.*, **2**, 82.
Sever, J. L. (1962). *J. Immunol.*, 88, 320.
Tyrrell, D. A. J., and Bynoe, M. L. (1961). *Brit. med. J.*, **1**, 393.
—— and Hoorn, B. (1965). *Brit. J. exp. Path.* In press.
Working Party on Acute Respiratory Virus Infections (1965). *Brit. med. J.* In press.