Cultivation of "Difficult" Viruses from Patients with Common Colds

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Viruses can often be isolated from patients with common colds by inoculating their nasal secretions into tissue cultures, but more viruses can be propagated by inoculating such secretions into organ cultures of human embryo nasal or tracheal epithelium (Tyrrell and Bynoe, 1965; Hoorn and Tyrrell, 1966). Many of the viruses which grow in organ cultures can be recognized in the laboratory. We have made preliminary reports (Tyrrell and Bynoe, 1966; Tyrrell, 1967) of studies on a collection of nasal washings. We now wish to make a full report, and in this paper we describe the cultivation of still more viruses by further experiments in which a modified technique of organ culture was used.

Methods and Material

The volunteers were aged between 18 and 50 years and were of both sexes. They were cared for and examined as described elsewhere (Tyrrell, 1963). They received 1 ml. of washings or organ culture fluid as intranasal drops which were usually diluted 1:10 in saline. Paired sera and nasal washings were collected from a proportion of those who developed colds.

The patients were mainly adults with colds and similar respiratory illnesses who belonged to the laboratory staff or who were visiting the unit as volunteers; but all the patients had apparently contracted upper respiratory infections outside the unit, the volunteers before arrival. The illnesses were usually graded as typical common colds and were usually afebrile. A few children aged 11 to 13 were tested; one of these had a severe pharyngitis with meningism and was found to be infected with adenovirus type 3. Nasal washings were collected within four days of onset and paired sera were sometimes obtained also.

Organ cultures were prepared and handled by the basic technique described by Hoorn (1966), with modifications referred to below. The tissue was obtained from human embryos removed at hysterotomy at the 14th to 24th weeks of gestation. Nasal and tracheal cultures were used almost interchangeably, but in later experiments with "difficult" viruses both nasal and tracheal cultures were inoculated at each passage and pooled fluids were used. For virus isolation attempts 0.2 ml. of nasal washings in 50% broth-saline were inoculated into cultures. Organ culture fluids were mixed with an equal volume of bacteriological nutrient broth and held at about -65° C. until needed. During these studies 116 volunteers were given fluids from uninoculated organ cultures and three developed colds; 120 were given saline and none developed colds.

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Tissue cultures were prepared and handled basically as described by the Medical Research Council Working Party (1965). When viruses are said to be "tested in tissue culture" this means that they were inoculated into cultures of monkey kidney, human diploid, HeLa, and often human embryo kidney cells, and examined in order to detect upper respiratory viruses, including influenza and parainfluenza viruses, adenoviruses, rhinoviruses, and enteroviruses.

Propagation of Previously Uncultivated Viruses

We reported earlier that certain viruses apparently present in nasal secretions could be propagated in organ cultures while others could not (Tyrrell and Bynoe, 1966). Since then we have investigated further the growth of influenza and rhinoviruses in organ cultures (Tyrrell and Blamire, 1967) and have modified and improved our previous technique, the O method, in which we used medium 199 and an atmosphere of air. The main differences are outlined in Table I. We have used the modified (M) technique in attempts to propagate viruses which were present in nasal washings, but which we had failed to grow before. Other features, such as the technique of preparing the tissue and the incubation temperature of 33° C., were unchanged. The cultures were observed for ciliary activity for up to 10 days.

Some of the results summarized earlier and others obtained recently are presented in Table II, which shows that viruses which could not previously be propagated can now be grown; further, the proportion of colds produced suggests that with the improved methods they are growing as freely as those others cultivated by the "original" method. It should be noted that the strain H.G.P. 26/7/57, is not the prototype rhinovirus 2; it is a virus which was previously reported to be neither an M nor an H rhinovirus and to be unable to grow in tissue cultures (Tyrrell and Bynoe, 1961). The strain F.E.B. 22/12/64 is likewise distinct from the prototype rhinovirus strain with the same initials, and represents a washing collected from the same subject when suffering from a later cold.

The cultivation of some of these more "difficult" viruses was performed in a series of experiments which may now be summarized.

Cultivation of Some "Difficult" Viruses

S.T. 28/3/65 (Fig. 2)

This virus was originally passed in O cultures at first in Salisbury and then in Lund. It soon produced obvious ciliary destruction and continued to grow and to produce colds when

TABLE I.—Technical Details of "Original" and "Modified" Methods of Organ Culture

	No. of Fragments	Volume of Medium	Medium	Concentration of			Frequency of Changing Medium	
				Bicarbonate (g./100 ml.)	CO ₂	Bovine Plasma Albumin (g./100 ml.)		
Original or O	{ Tracheal Nasal	{ 5 or 6 2 " 3	{ 1.25 2.5-3.5	199	0.035	Ambient	0	Daily
Modified or M	{ Tracheal Nasal	{ 2 1	{ 1.25 2.5-3.5					
			Eagle's (Gibco)	0.1	5%	0.2	4 days	

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inoculated into volunteers after serial passages in M cultures. Even after the final passage it had no effect when inoculated into tissue cultures, but, using arrest of cilia as an index of the presence of virus, we showed it to be ether-stable, acid-labile and to pass a filter of A.P.D. about 50 mμ. It is therefore presumably a fastidious rhinovirus like the H.S. virus previously reported (Hoorn and Tyrrell, 1966). However, it was not neutralized by antiserum against H.S. virus.

M.R.

This agent has been under study at the unit since 1956, when a volunteer, M.R., was given material from an infected tracheal culture and developed a cold three days later. As the series of experiments was otherwise negative it is thought likely that she was infected outside the unit, particularly as the incubation period of colds due to this agent has proved to be

unusually long. This is shown in Table III, from which it will be seen that the colds were of long duration. Table III also shows that colds due to avian-infectious bronchitis-like viruses (A.I.B.-like viruses) have a long incubation period, but in these the duration was short. Colds due to M.R. showed more fever and malaise, were graded as severe more often than were colds due to rhinoviruses, and were much less likely to be followed by a cough.

In a series of experiments with the virus some years ago it was shown that volunteers given a second inoculation of wash-

TABLE II						
Donor*	Date	Inoculum Patients Washing (P) or Pool of Washings from Infected Volunteers (V)	Results of Inoculation of Fluid from Organ Cultures Maintained by			
			“Original” Method into		“Modified” Method into	
			Volunteers	Tissue Culture	Volunteers	Tissue Culture
H.G.P.	26/7/57	V	1/6†	Rhinovirus		
J.E.D.	24/4/64	P	2/6	”		
F.T.	30/3/64	P	4/10	”		
M.T.	12/62	P	4/15	”		
B.R.	31/12/63	P	—	”		
P.D.B.	4/5/65	P	—	”		
P.K.B.	1/6/65	P	—	”		
D.T.	11/6/65	P	—	”		
J.E.D.	12/8/61	P	—	”		
B814		V		None		
H.W.	20/1/64	V	4/6	Para- influenza		
F.A.L.	26/2/65	P	3/6	”		
D.T.	2/4/65	P	1/4	”		
E.V.S.	2/3/65	P	4/6	None		
D.L.J.	2/1/64	P	1/7	None	—	Rhinovirus
M.R.		V	0/12	”	6/13	None
L.P.	11/3/65	P and V	0/6	”	3/6	”
E.W.G.	26/4/65	P	0/6	”	2/9	Rhinovirus
D.T.R.	16/10/60	P	0/6	”		
S.T.	28/3/65	P	2/6	”	4/7	None
F.E.B.	22/12/64	P	3/5	”	1/8	”
D.T.R.	19/12/60	P	0/6	”	1/5	”
A.	3/3/65	P	0/6	”		
D.	2/1/64	P	1/7	”	0/8	”

* In addition 9 specimens yielded viruses on direct inoculation into tissue cultures—for example, 2 strains of haemadsorbing virus, 1 of adenovirus, 2 enteroviruses, and 4 rhinoviruses.
† In these and subsequent tables the numerator denotes the number of colds observed and the denominator the number of volunteers inoculated.
Studies on strain B814, F.T., and M.T. were reported in more detail previously (Tyrrell and Bynoe, 1965).

TABLE III.—Clinical Features of Colds Produced by Four Unidentified Viruses Compared with those of Rhinovirus Type 2, and Avian-Infectious Bronchitis-like Viruses

	M.R.	L.P.	E.V.S.	F.E.B. 22/12/64	Rhinovirus Type 2	A.I.B.-like 229E and B814
No. of volunteers inoculated	207	68	15	27	213	153
No. getting colds	61	37	11	6	78	78
	29	54	73	22	37	51
Incubation period in days	Mean 3.8 Range 2-6	Mean 2.7 Range 2-4	Mean 3.5 Range 3-4	Mean 2.8 Range 3-4	Mean 2.1 Range 1-5	Mean 3.0 Range 2-5
Duration of colds in days	Mean 10 Range 3-18	Mean 6 Range 3-11	Mean 5 Range 2-12	Mean 7 Range 2-19	Mean 9 Range 3-19	Mean 7 Range 2-19
Maximum No. of handkerchiefs used daily	Mean 17 Range 4-60	Mean 20 Range 6-60	Mean 8 Range 5-14	Mean 12 Range 4-32	Mean 14 Range 3-38	Mean 20 Range 4-120
Malaise (%)	49	73	36	66	28	55
Headache (%)	47	65	64	66	56	67
Chill (%)	21	13	27	33	28	22
Pyrexia	29	27	36	16	22	19
Mucopurulent nasal discharge (%)	Range 99.2-101.8°	Range 99.2-100.4°	Range 99.2-100.6°	Range 99.2-100.6°	Range 99.2-100.4°	Range 99.2-103
Sore throat (%)	70	27	27	33	83	54
Cough (%)	69	35	64	16	87	64
	26	19	18	0	68	54
Severity of colds	Mild 44-72% Moderate 8-13% Severe 9-15%	Mild 25-68% Moderate 9-24% Severe 3-8%	Mild 9-82% Moderate 1-9% Severe 1-9%	Mild 5-84% Moderate 1-16% Severe —	Mild 63-81% Moderate 12-15% Severe 3-4%	Mild 52-67% Moderate 17-22% Severe 9-11%
Colds in controls	1/137	1/51	1/14	0/25	2/88	2/103
Hanks's saline	0/72	0/20	7	0	2-3	1-9
Uninoculated organ culture fluids	1/65	1/31	0/6	0/13	2/88	2/67
			1/8	0/12	—	0/36

FIGS. 1-4.—The diagrams summarize the experiments with four agents. Each passage is indicated by an arrow; organ cultures are shown by circles, open if the O technique was used and filled if the M technique. Reduction in ciliary activity is shown as + and doubtful reduction as ±. The fractions indicate the outcome of a volunteer experiment—the numerator the number of colds observed and the denominator the number of volunteers infected. Some test results and experimental conditions are also shown.

TABLE VII.—Results of Attempts to Isolate and Classify Viruses

	Entero	Rhino	Parainfluenza	Adeno
Viruses isolated and studied in tissue culture	C.J.B. 12/4/65 (Coxsackie A21) N.J.D. 29/4/65 (? Echovirus untyped)	E.M.B. 10/11/60 N.J.D. 24/4/64 G.T. 16/12/63 M.McM. 29/12/61	F.E.B. 10/12/64 D.T. 2/4/65	S.C.T. 24/7/65 (Type 3)
	A.I.B.-like	Rhino	Parainfluenza	Unidentified Ether-labile
Viruses cultivated in organ culture and grown and studied in tissue culture or organ culture	B814	H.G.P. 26/7/57 J.E.D. 12/8/61 F.T. 30/3/64 M.T. 00/12/62 B.R. 31/12/63 J.E.D. 24/4/64 P.D.B. 4/5/65 P.K.B. 1/6/65 D.L.J. 21/1/64 E.W.G. 26/4/65 D.T. 11/6/65 S.T. 28/3/65 (grows only in organ culture)	F.A.L. 26/2/65 H.W. 20/1/64	M.R. E.V.S. 2/3/65 L.P. 11/3/65
Partially successful cultivation and no properties determined	F.E.B. 22/12/64 D.T.R. 19/12/60			
No virus in specimen and no virus grown	A. 3/3/65 D. 2/1/64			

We have excluded from this Table the specimen D.T.R. 16/10, from which no virus was grown, but which could not be tested in volunteers or in modified cultures, and B, from which a virus was apparently grown in tissue culture but the specimen was exhausted and the virus was lost on passage.

to be parainfluenza viruses, and a detailed account of serological and clinical studies with these organisms will also be published. The first two of these strains were originally suspected to be paramyxoviruses because the virus particles were visualized with the electronmicroscope (Tyrrell and Almeida, 1968). For completeness we should also mention here the strain B814, which was shown to be an avian-infectious-bronchitis-like virus by electronmicroscopy. However, the ether-labile viruses, M.R., L.P., and E.V.S., could not be identified either by tissue culture or by electronmicroscopy. The remaining agents were propagated with such difficulty that it has not been possible to study their properties.

We have summarized the results of virus isolation and classification so far in Table VII. This shows that a definite or presumed virus was grown from 29 out of 31 specimens and that the remaining two did not produce colds in volunteers. One and possibly two viruses could not be propagated serially for certain. Fourteen viruses, 11 of them rhinoviruses, which could not be detected in tissue culture, were grown in organ cultures and detected and classified either by adapting them to tissue cultures or in one instance by electronmicroscopy and in another by observing reduction in the ciliary activity of organ cultures.

Three further viruses were certainly grown in organ cultures; they appear to be ether-labile, but have not been shown to be any of the known ether-labile viruses of the respiratory tract.

Discussion

It is clear that the techniques used were successful in propagating viruses from colds which had occurred over a long period of time, and included some which had been tested repeatedly by earlier methods without success. Some of the experiments with M.R. are mentioned here and, with B814, have been described before (Tyrrell and Bynoe, 1965), but the strain H.W. and some others were also repeatedly tested.

Relatively few patients have been studied, but we believe we obtained a representative sample of the colds of adults in our area. If anything, the sample is somewhat loaded in favour of "difficult" viruses, since it includes strains, like H.G.P., M.R., H.W., and B814, which were included because after intensive study they had failed to grow in tissue cultures. On the other hand, we did take pains to collect nasal washings from quite definite and typical cases. Our rate of isolating rhinoviruses in tissue culture is lower than that in an earlier study from this laboratory (Kendall *et al.*, 1962) and elsewhere (Hamre *et al.*, 1966), but the numbers are rather small and so the difference is not statistically significant. Phillips *et al.* (1965) recovered

rhinoviruses from about a quarter to a half of three series of students with colds by the use of a number of different strains of cells. Some of our specimens containing rhinoviruses—for example, H.G.P. 26/7/57—had been tested in human embryo kidney and several different strains of fibroblasts, and failed to produce a recognizable cytopathic effect. It therefore seems to us unlikely that the apparent improvement in the isolation of rhinoviruses when organ cultures were used was an artifact due to inefficient testing in tissue cultures. The improvement has in fact already been confirmed independently (Higgins, 1966).

The aetiology and epidemiology of colds do not vary greatly in most centres of population, and it is therefore likely that it will be found elsewhere that almost all common cold viruses can be grown in organ cultures of human ciliated epithelium and that most viruses can be identified by the combination of organ culture with other techniques. On the other hand, it is probable that the distribution of viruses between the different groups and serotypes will be found to vary considerably from time to time and from place to place. The unidentified ether-labile viruses should be studied further.

Though we still await further studies on the serotyping of viruses, it is clear already that the use of organ cultures has not greatly changed the aetiological pattern revealed by the use of tissue cultures. Thus rhinoviruses are still the predominant organisms recovered, they belong to various serotypes, and, just as some rhinoviruses may be recovered in organ culture but not directly in tissue culture, so some parainfluenza viruses were detected in organ cultures and not in tissue cultures. However, organ cultures are apparently needed to grow the A.I.B.-like viruses.

Summary

Specimens were collected during 31 colds in 27 patients, mainly adults. The specimens were tested for the presence of viruses by the combined use of tissue culture, organ culture, and inoculation of volunteers. Known or presumed viruses were grown from 29 specimens. These included 16 rhinoviruses, 2 enteroviruses, 4 parainfluenza viruses, 1 virus resembling avian infectious bronchitis virus, 1 adenovirus, and 5 unclassified agents, probably viruses. Of these, 4 rhinoviruses, 2 enteroviruses, 2 parainfluenza viruses, and 1 adenovirus were detected by the use of ordinary tissue cultures only, organ cultures being unnecessary.

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experiments, and Miss C. J. Blamire and Mrs. P. K. Brown in the laboratory work. Dr. P. J. Chapple carried out some of the preliminary experiments on strain H.W., and Drs. H. G. Pereira and A. T. Roden on strain M.R.

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Necrotic Cervicitis Due to Primary Infection with the Virus of Herpes Simplex

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[WITH SPECIAL PLATE FACING PAGE 602]

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Since July 1965 I have encountered in London six cases of extremely severe and necrotic cervicitis, the whitish appearances of which were strikingly similar. The condition in the first case was unfamiliar, but with subsequent cases evidence has been assembled which strongly suggests that the cervicitis is due to the virus of herpes simplex.

Present Series

Case Material.—The basic data concerning the six cases are given in Table I. The women were aged 20-28 years (average 23.3 years), four were white and two Negro, and three were single and three married. All were attending the venereal diseases clinic for the first time and none gave a history of any previous venereal infection.

TABLE I.—Basic Data

Case No.	Age in Years	Race	Married or Single	Occupation	Previous History of Venereal Disease
1	21	White	S	Receptionist	No
2	20	White	S	Sales promotion	No
3	23	Negro	M	Canteen assistant	No
4	21	Negro	S	Machinist	No
5	28	White	M	Housewife	No
6	27	White	M	Public relations	No

Symptoms.—The presenting symptoms are given in Table II. All six patients complained of vaginal discharge, four of dysuria, three of local soreness, and four of pain in the lower abdomen, right iliac fossa, or the genitals. In Case 1 the dysuria was severe, and twice daily catheterization had been performed in Spain before the patient decided to fly home to take further advice. The onset was acute or subacute, symptoms having been present for from one to nine days before they attended the clinic.

Findings on Examination.—These are shown in Table III and in the Special Plate. In all cases there was gross cervicitis, from which pieces of dead tissue might separate, leaving a white necrotic core around the os. Pain on touching or moving the

cervix was elicited in four cases, and in all six the cervix bled easily. The inguinal lymph nodes were enlarged and tender in only one case. There was some known fever in four cases, but in all except one this was not severe at the time of recording, though it may well have subsided by the time the patients were first seen. In one case it was known to have reached 104° F. (40° C.). The presumptive or alternative diagnosis on these cases at the first cervical inspection ranged from gonorrhoea (two cases), salpingitis (two cases), carcinoma of the cervix, leucoplakia, and the effects of cautery.

TABLE II.—Symptoms

Case No.	Vaginal Discharge	Dysuria	Local Soreness	Pain	Duration of Symptoms
1	Yes	Yes*	Yes	Yes, lower abdomen	10 days†
2	"	"	"	" R.I.F.	2 "
3	"	"	"	" "	9 "
4	"	No	No	" genital	3 "
5	"	"	"	No	4 "
6	"	"	"	"	1 "

* Required repeated catheterization. † Had been treated elsewhere after only a few days of discharge and dysuria.

TABLE III.—Findings on Examination

Case No.	Cervi- citis	Pain on Touching or Moving Cervix	Tender Inguinal Nodes	Fever (° F.)	Presumptive or Alternative Diagnosis at First Clinical Examination
1	++	Yes	No	99°, ? earlier more so	Gonorrhoea, salpingitis, effects of cautery
2	++	"	Yes	99°	Salpingitis
3	++	"	No	No	Carcinoma of cervix
4	++	"	"	"	"
5	++	No	"	98.6°, earlier probably more so	Leucoplakia of cervix
6	++	"	"	104°	Gonorrhoea

Investigations

The results of some of the investigations made on these patients are shown in Table IV. The tests for venereal disease, which included urethral and cervical smears and cultures for gonococci, an examination of a wet specimen of vaginal exudate for trichomonads, a dark-field examination of a cervical speci-

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