Studies on Parainfluenza Type 2 and 4 Viruses Obtained from Patients with Common Colds

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Summary : Four agents which were obtained from adults with common colds were cultivated and identified—one was influenza type B and the others were parainfluenza viruses of types 2 and 4. Their cultivation was assisted by the use of organ cultures of human embryo tracheal or nasal epithelium. They infected and caused typical common colds in volunteers.

Introduction

Four viruses which caused haemadsorption in tissue cultures of monkey kidney cells have been recovered from a total of 31 nasal washings obtained from patients who were suffering from common colds (Tyrrell, Bynoe, and Hoorn, 1968). Two of these viruses were detected by haemadsorption of tissue cultures inoculated with washings. Two other specimens which were negative on this test were inoculated into organ cultures in which typical "large" myxovirus particles were seen on electron microscopy (Tyrrell and Almeida, 1967). Fluids from these cultures produced haemadsorption in tissue cultures and were identified as parainfluenza viruses. This paper describes some points of interest from further work with these organisms.

Materials and Methods

Volunteers, organ cultures, and *tissue cultures* were described earlier (Tyrrell *et al.*, 1968). In most cases a "modified" technique of organ cultures was used and serial cultures were made with 0.1 ml. of organ culture fluid collected four days after inoculation (Tyrrell and Blamire, 1967). Cultures were all incubated at 33° C.

Haemagglutination Inhibition.—The Takátsy (1955) micromethod as modified by Sever (1962) was used. The haemagglutinating antigens for prototype strains of parainfluenza viruses were kindly supplied by Dr. L. Zakstelskaya, Ivanovsky Institute of Virology, Moscow. The sera were treated overnight at 37° C. with 4 volumes of cholera filtrate (Philips Duphar) and then heated at 56° C. for 30 minutes. The serum dilutions in saline were allowed to react with 4 units of virus at room temperature for one hour and then one drop of 1% guinea-pig red cells was added and the test was read after about one hour at 4° C.

Neutralization Tests.—These were performed in rhesus or patas monkey kidney cells by using serial dilutions of inactivated serum to which an equal volume of virus dilution was added ; the mixtures were held at room temperature for 20 minutes. Two or three cultures were inoculated with 0·2 ml. of such mixture, which was calculated to contain about 100 TCD_{50} of virus before neutralization. Cultures were tested by haemadsorption after incubation in a roller drum at 33° C. for five days. End-points were interpolated when necessary.

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Results

On the initial tests two specimens—F.E.B. 10/12/64 and D.T. 2/4/65—were inoculated into rhesus monkey cultures which were rolled at 33° C. for 10 days. The haemadsorption with human red cells was definite but incomplete. The isolation could not be repeated regularly, presumably owing to variations in the sensitivity of the monkey kidney cells. Nasal washings of F.E.B. 10/12/64 were inoculated into organ cultures and the fluids from these produced the signs and symptoms of a common cold in volunteers (Table I) though they showed more constitutional symptoms than are usually seen in rhinovirus infections. Virus was recovered from volunteers and identified as influenza B virus. Infected volunteers showed rising antibody titres against the same virus. The virus from D.T. 2/4/65 also produced colds when propagated in serial passages in organ cultures and then inoculated into volunteers ; in later passages it became easier to detect by inoculation into tissue culture. It was shown by neutralization test to be a parainfluenza 2 virus. This shows that, like influenza B, a human parainfluenza 2 virus unadapted to tissue culture can multiply in organ cultures of human respiratory epithelium and produce a mild coryzal illness. Previously only tissue-culture-adapted strains of parainfluenza 2 virus had been grown in organ culture (Tyrrell and Hoorn, 1965) and inoculated into man (Taylor-Robinson and Bynoe, 1963).

TABLE I.—*Recovery of Four Haemadsorbing Viruses*

Inoculum Nasal Washings Obtained from	Result of Test in Tissue Culture	Test of Fluids from Organ Cultures Inoculated with Washings	
		In Tissue Culture	In Volunteers*
D.T. 2/4/65	Positive	Positive	1/4
F.E.B. 10/12/64 ..	Positive	Positive	4/6
Volunteers given H.W. 20/1/64 washings ..	Negative	Negative	4/6
F.A.L. 26/2/65 ..	Negative	Negative	3/6†

* The numerator indicates the number of volunteers who developed colds and the denominator the number who were inoculated.
† Washings treated with ether produced no colds in seven volunteers. Untreated washings produced colds in six out of seven.

The results with the other two specimens (H.W. 20/1/64 and F.A.L. 26/2/65) were of greater interest. Both washings were repeatedly tested for their ability to produce haemadsorption in secondary tissue culture of monkey kidney cells, which were maintained for periods of at least 10 days. The results were always negative (Table I). However, as shown in the Diagram, the specimens were inoculated into organ culture and the fluids from these induced colds in volunteers. Serial passages were made without difficulty and virus particles were detected by electron microscopy of the tissue (Tyrrell and Almeida, 1967) ; fluids from organ cultures were inoculated into tissue cultures which developed haemadsorption in the second or third organ culture only after 10 days but by the fifth to ninth culture after five days. The haemadsorption was feebler than in the case of cultures inoculated with the other two viruses except in fluids obtained from later organ culture passages. The virus produced little or no cytopathic effect at first, and infectivity titres were low, about $10^2 TCD_{50}$/ml. After five serial passages in monkey kidney F.A.L. produced moderate-sized syncytia, and after four passages H.W. produced a dubious slight diffuse cytopathic effect. Culture fluids did not haem-

agglutinate, and though 1 ml. of each was inoculated into a rabbit, only that receiving H.W. produced a homologous antibody response. The viruses therefore appeared to grow poorly in tissue cultures.

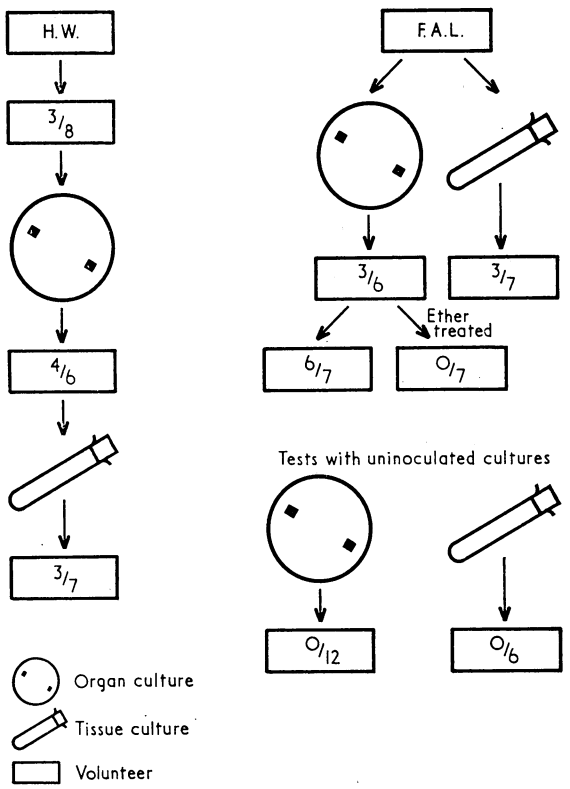
Serological Identification of Viruses

The viruses were titrated in neutralization tests with a number of W.H.O. typing sera. The patients' sera were also titrated either with the virus isolated or with a laboratory strain of the same serotype. The results are shown in Table II and indicate that the viruses belong to known serotypes, and that the patients had been infected with viruses antigenically related to those which had apparently been recovered from them. The viruses F.A.L. and H.W. were thought to be of particular interest and were studied further. Infected nasal secretions were inoculated into tissue cultures of primary human embryo kidney cells maintained at 33° C. with Eagle's medium containing 0.2% bovine plasma albumin. After five days the culture cells did not haemadsorb but the fluids induced colds in volunteers (see Diagram).

TABLE II.—*Serological Identification of Viruses*

Source of Serum	Type of Test*	Virus Used		
		F.A.L.	D.T.	H.W.
Typing serum:				
Parainfluenza type 1	N	<5	<5	<5
Parainfluenza type 2	N	80	160	<5
Parainfluenza type 3	N	<5	<5	<5
Parainfluenza type 4A	N	—	<5	40
Parainfluenza type 4B	N	—	<5	<20
Patients from whom virus was isolated	N	<10/20	<4/128	20/160(4A)
	HI	5/30(2)	10/160(2)	160/320(4B)

Number in parentheses shows that the test was done with a prototype parainfluenza virus of the indicated serotype.
* N = Neutralization. HI = Haemagglutination inhibition.



Summary of some of the experiments on the passage of viruses in organ cultures of human respiratory epithelium and in roller tube tissue cultures of human embryo kidney cells. The fractions within the oblong symbols represent the number of volunteers who developed colds and the total number inoculated. The virus from patient H. W. was passed successfully through organ and tissue cultures, and produced colds in man thereafter. The virus from F. A. L. was also passed through these two cultures and was also shown to lose its capacity to produce colds after ether treatment—additional evidence that the illnesses were not produced by a contaminating undetected ether-stable virus, such as a rhinovirus.

Serological Studies on Infected Volunteers

Serum specimens were available from many volunteers, and those were titrated by haemagglutination inhibition and neutralization tests as indicated in Table III. There were rising titres of antibody against at least one of the two type 4 viruses in all 10 volunteers who developed colds after inoculation of parainfluenza 4 virus either as culture fluid or as nasal washings. There was also an antibody rise in two of the three volunteers who did not develop a cold. There was no rise in antibody titre against types 1 to 3 viruses but there were rises against type 4B virus as well as 4A; it was thought that this might be because the neutralization test used would not distinguish between antibody directed against type 4A and type 4B virus, but one individual showed a large rise in titre against 4B virus and not against 4A and the titre rises in the sera of H.W. were quite different with the two viruses (Table II).

TABLE III.—*Serological Responses in Certain Volunteers*

Virus Administered	Symptoms	Rising Titre of Antibody by					
		Haemagglutination Inhibition against Parainfluenza Type			Neutralization Against Type		
		1	2	3	2 F.A.L.	4A	4B
F.A.L.	Common cold	0/11	2/11	0/11	3/11	0/9	—
	None	0/5	0/5	0/5	1/5	0/2	—
H.W.	Common cold	0/10	0/10	0/10	0/3	9/10	7/8
	None	0/3	0/3	0/3	—	2/3	1/3

Attempts were made to isolate viruses from volunteers by testing nasal washings in cultures. Initial attempts were made to recover viruses from 11 volunteers given types H.W. or F.A.L. by inoculating washings into rhesus monkey kidney cells maintained in Eagle's medium. Haemadsorption was negative at 10 days. Another group of specimens was tested both in patas monkey kidney cultures and in organ cultures. These were harvested at four and eight days and inoculated into tissue cultures, which were tested by haemadsorption 5 and 10 days after inoculation. The results are shown in Table IV. In each system virus was isolated from volunteers with colds and not from those without and the isolation rates were similar whether organ cultures were used or not. We think that the failure to isolate virus in monkey kidney previously was due to insensitivity of the cells which sometimes gave low titres with prototype virus for periods of months. The organ cultures were always sensitive and passage from these enabled haemadsorption to be detected in somewhat unsatisfactory cells such as human embryo kidney.

TABLE IV.—*Recovery of Virus from Nine Volunteers Infected with H. W. Strain of Parainfluenza 4A*

Clinical Status of Volunteers	Haemadsorption Present in				
	Monkey Kidney Cultures Inoculated with			Human Embryo Kidney Cultures Inoculated with	
	Nasal Washings	Organ Culture Fluid from		Organ Culture Fluid from	
		1st Pass	2nd Pass	1st Pass	2nd Pass
No cold	0/2	0/2	0/2	0/2	0/2
Mild cold	3/4	3/5	4/5	2/5	4/5
Moderate or severe ..	2/2	2/2	2/2	0/2	2/2
Total ..	5/8	5/9	6/9	2/9	6/9

All volunteers showed an antibody rise against the virus used.

Clinical Features

The clinical features of the diseases produced in volunteers were recorded in detail and are summarized and compared with those of colds due to rhinoviruses. The illnesses were in general compatible with the diagnosis of a common cold; the colds due to F.E.B. were accompanied by pronounced constitutional symptoms with relatively little nasal discharge and were rather severe. Apart from this group all illnesses were afebrile (Table V). The incubation period was longer than that seen with rhinoviruses; it was noted by Reichelderfer *et al.* (1958) that colds following infection with parainfluenza virus type 1 showed a long incubation period. There was typical nasal discharge which often became mucopurulent and lasted for one to two weeks. A few patients developed a mild cough, fewer than those with rhinovirus infections, and there was no sign of the severe lower respiratory tract involvement found in children with croup cause by parainfluenza 2 virus. There was no special prominence of pharyngitis in patients given parainfluenza 2 as there was in previous studies with naturally infected students (Mogabgab *et al.*, 1961) and with volunteers (Taylor-Robinson and Bynoe, 1963).

TABLE V.—*Clinical Features of Colds Produced by Parainfluenza Viruses*

	Parainfluenza			Rhinovirus
Seratype	4	2	2	Type 2
Strain	H.W.	F.A.L.	D.T.*	H.G.P. and P.K.
No. of volunteers inoculated	14	20	37	213
No. getting colds	7 (50%)	9 (45%)	11 (30%)	79 (37%)
Incubation period in days:				
Mean	3·4	2·8	4·4	2·1
Range	2–5	1–4	2–6	1–5
Duration of colds in days:				
Mean	13	6	12	9
Range	9–16	3–11	4–17	3–19
Maximum no. of handkerchiefs used daily:				
Mean	8	22	10	14
Range	4–13	8–29	4–20	3–38
Malaise (%)	14	44	36	28
Headache (%)	28	66	64	56
Chill (%)	14	22	9	28
Pyrexia (over 99·2 °F.) (%)	0	0	0	14
Mucopurulent nasal discharge (%)	57	33	20	83
Sore throat (%)	57	44	73	87
Cough (%)	14	33	9	68
Volunteers with colds of indicated severity:				
Mild	6 (86%)	5 (56%)	10 (91%)	63 (80%)
Moderate	1 (14%)	1 (11%)	1 (9%)	12 (15%)
Severe		3 (33%)		4 (5%)

* Additional data from volunteers inoculated with virus passed in cultures of human embryo kidney.

Discussion

Parainfluenza type 2 viruses were first isolated from children with serious respiratory disease, croup, or acute laryngo-tracheo-bronchitis (Chanock, 1956; Beale *et al.*, 1958), and it has been proved that the virus causes the disease (Kim *et al.*, 1961). The virus has also been found to cause mild afebrile respiratory disease in children (Kapikian *et al.*, 1963; Harris *et al.*, 1968). It has also been reported that infection of adults may occur (Craighead *et al.*, 1961), but it appears that in these cases the virus may be isolated only with great difficulty (Bloom *et al.*, 1961). In one study parainfluenza 2 virus infections were diagnosed only by serology (Mogabgab *et al.*, 1961) and in another virus isolation required serial blind passage of material in monkey kidney cells (Bloom *et al.*, 1961). Parainfluenza 4A and 4B viruses are also generally isolated from children (Johnson *et al.*, 1960; Canchola *et al.*, 1964), and propagated with considerable difficulty in the laboratory. Our failure to isolate these viruses by direct inoculation of tissue culture is therefore not out of line with previous experience, though had we been able to obtain specially sensitive monkey kidney cells and maintain them for several weeks we might have made our primary isolations in tissue cultures.

The fact that these difficult myxoviruses grow readily in organ cultures, retain their virulence for man, but acquire the ability to grow more readily in tissue cultures is exactly parallel to our experience with the isolation of many rhinoviruses (Tyrrell *et al.*, 1968). We therefore think it may be of value to use organ cultures in cases where it seems particularly important to try to recover a parainfluenza virus. It is possibly because of the relative difficulty of cultivating parainfluenza viruses of types 2 and 4 that there have been so few reports of infections of adults with these organisms. Organ cultures of human respiratory epithelium seem to be highly sensitive to these viruses, but there seem to be gross variations in the sensitivity of apparently satisfactory monkey kidney cultures, especially to type 4, though from time to time batches of kidney may be used which are about as effective in detecting virus as organ cultures. In our studies one source of patas kidney was particularly satisfactory, though others have found rhesus cells best for detecting parainfluenza viruses (Chanock, personal communication).

The inoculation of parainfluenza type 4 viruses into human volunteers has not been reported so far. Our data seem to us to indicate that the virus we recovered causes typical common colds when administered to man. We recovered this virus from the original patient and from volunteers by the use of organ cultures from several batches. This experiment thus fulfils the final clause of Koch's postulates and provides final proof that parainfluenza type 4 is a cause of human respiratory disease and that it can cause common colds in adults. There was good serological evidence that volunteers were infected with a virus antigenically related to the strain used. H.W. seemed to belong to type 4A, giving identical titres to those obtained with the prototype virus against typing sera and several human sera; however, it induced antibodies to type 4B in volunteers and also in an experimentally inoculated rabbit. We found, like Taylor-Robinson and Bynoe (1963), that volunteers may develop symptoms after inoculation of parainfluenza type 2 virus and yet develop no measurable increase in circulating antibody. This may add to the difficulty of detecting natural infections.

Early experiments at this unit suggested, though the results were not conclusive, that the agents that caused common colds might be ether-labile (Andrewes, 1951). This tended to be overlooked in recent years when attention was being focused on the rhinoviruses, which are ether-stable. It has now become clear that several ether-labile viruses can cause colds. They include influenza B virus, parainfluenza viruses, and the viruses related to avian infectious bronchitis (Tyrrell and Bynoe, 1965; Hamre and Procknow, 1966; Tyrrell and Almeida, 1967; McIntosh *et al.*, 1967). Such viruses may well have been included by chance in the washings selected for study in the early days.

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Coumarin Therapy and Platelet Aggregation*

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Summary: Platelet aggregation has been related to blood coagulation studies in patients on nicoumalone, a coumarin anticoagulant. Aggregation studies were performed by means of Chandler's tube and the adenosine diphosphate (A.D.P.)-induced optical density method. Platelet aggregation in Chandler's tube has been shown to be quite different from A.D.P. aggregation and to be dependent on the "intrinsic" (blood) clotting system. When the intrinsic system was depressed by coumarin anticoagulant, aggregation was delayed in Chandler's tube, but patients with a predominantly "extrinsic" (tissue) system defect gave normal results even when their prothrombin time was excessively prolonged. In contrast there was an increased response to A.D.P. in the anticoagulated patients.

The study emphasizes the different mechanisms of platelet aggregation, which we have referred to as coagulation-induced and A.D.P.-induced aggregation. It also shows the limitations of routine control of oral anticoagulants by prothrombin time alone, as the coagulation-induced platelet aggregation appears to be quantitatively related to the overall level of clotting factors in the intrinsic system and independent of the extrinsic system.

Introduction

As well as their action on blood clotting, oral anticoagulants may have additional effects which might contribute to or even explain their role in the prevention of thrombosis. One possible important action may be on platelets.

Anticoagulant treatment reduces both "extrinsic" (tissue) and "intrinsic" (blood) systems of prothrombin conversion. The observation of abnormal platelet function in haemophilia (Hellem and Owren, 1964) suggested to us that platelet aggregation may be abnormal in patients on anticoagulants when their intrinsic system is depressed. We therefore studied the coagulation system in a group of patients receiving a coumarin drug, and tried to correlate these results with platelet aggregation.

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Method of Study

A group of 30 patients on nicoumalone (Sinthrome) was studied. Platelet aggregation studies and coagulation factor assays were performed on the same blood specimens. A parallel group of healthy adults was also studied.

The following coagulation tests were performed: prothrombin time (Quick test), cephalin time, and factor II, VII, VIII, IX, and X assays. Platelet aggregation was studied by Chandler's tube and adenosine diphosphate (A.D.P.)-induced platelet aggregation by an optical density method. A normal range was obtained for Chandler's tube aggregation from 53 normal adults (24 males, 29 females) and for the A.D.P.-induced aggregation from 63 adults (27 males, 36 females).

Technique

Coagulation Studies.—Prothrombin time (Quick test) (Poller, 1964), cephalin time (Hjort *et al.*, 1955) activated by the addition of kaolin, factor II (Pechet, 1964), factor VII (Poller and Thomson, 1964), factors VIII and IX (Egeberg, 1961) with the addition of kaolin, and factor X (Denson, 1961). The results were expressed as seconds and compared with the parallel normal controls, except for factor VIII assays. Results of factor VIII were expressed as a percentage (Poller and Thomson, 1964).

Platelet Aggregation Studies.—(1) Chandler's tube method (Cunningham *et al.*, 1965, modified). Platelet-rich plasma, prepared by centrifugation at 105 *g* for 10 minutes, was rotated in a tube (internal diameter, 1 cm., length 60.8 cm.) at an angle of 44° at 34 r.p.m. in a 37° C. incubator. Then 0.5 ml. of M/4 calcium chloride and 10 ml. of 0.9% sodium chloride were mixed and 5 ml. of platelet-rich plasma was added. (2) A.D.P.-induced aggregation (O'Brien *et al.*, 1966, slightly modified). The difference between the initial and final optical density readings was measured. This figure was adjusted to give the change corrected for a platelet count of 400,000/cu. mm.

Results

Three of the 30 patients were not sufficiently anticoagulated —that is, were not in the therapeutic range on the prothrombin scale (15–30% activity with the Manchester reagent)—and were therefore excluded. Six patients were over-anticoagulated and were also eliminated. One patient with very lipaemic plasma had to be excluded because of difficulty in interpretation. Three