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Isolation of Rhinoviruses and Coronaviruses From 38 Colds in Adults

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Nasal washings were collected from 27 normal adults during 38 naturally acquired colds. The washings were exhaustively tested using tissue cultures, organ cultures and electron microscopy. Washings yielding no identifiable agent were inoculated into human volunteers, and further specimens obtained from the latter were examined by the same techniques in vitro. Viruses were identified in association with 25 of the original 38 colds (65.7%). Fifteen were rhinoviruses (39.5%), seven coronaviruses (18.4%), two were parainfluenza viruses, and one was influenza virus. Use of organ cultures and of volunteers significantly increased the isolation rate. No agent was cultivated from the remaining 13 specimens, although tests in volunteers showed that cold-producing agents were present in five of them (13%). Three specimens gave doubtful results in volunteers, and five others, all collected within a period of six weeks in December and January, apparently contained no infectious agent.

Key words: common cold, rhinovirus, coronavirus, volunteers, organ culture

INTRODUCTION

Although a variety of viruses are known to cause respiratory infections, in clinical practice, rates of virus isolation from the respiratory tract remain obstinately low and normally do not exceed 30%–40% of specimens tested [Higgins, 1974]. Isolation of many of the agents responsible, such as coronaviruses, some rhinoviruses, and some parainfluenza viruses is known to present technical difficulties, and the use of human embryonic nasal or tracheal organ culture, although laborious, has been shown to increase isolation rates [Tyrrell and Bynoe, 1966; Higgins and Ellis, 1973]. It has remained uncertain whether available techniques, including organ culture and electron microscopy, are capable of identifying the large majority of upper respiratory infections, or whether a significant proportion of such infections is associated with agents not cultivable even by these elaborate methods. The present study was undertaken to elucidate this point. In clinical practice negative results from cultures may not infrequently be attributable to less-than-

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optimal methods of collection, transport, or storage of specimens. This study was therefore confined to subjects in the acute stages of upper respiratory infections from whom good samples could be obtained.

METHODS

Study Period, Subjects, and Specimens

Following advertisement, employees of the Clinical Research Centre or Northwick Park Hospital, Harrow, England, who had recently noted symptoms of a cold volunteered for study. A short medical history was obtained, and a simple questionnaire regarding 17 possible symptoms was completed. The nasal cavity was washed with 10–15 ml of 0.85% saline, which was then mixed with an equal volume of nutrient broth and stored in replicate 2-ml quantities at −70°C. A serum sample was obtained at the same time and again 3–4 weeks later.

Collection of specimens, inoculation of volunteers, and use of foetal tissues were approved for this study by the Ethical Committee of Northwick Park Hospital. All specimens were obtained between December, 1974, and July, 1976.

Virological Methods

Duplicate roller-tube cultures of human embryo lung fibroblasts (MRC-5), "Ohio" strain rhinovirus-sensitive HeLa cells [Stott and Tyrrell 1968], and primary or secondary rhesus monkey kidney cells were each inoculated with 0.2 ml of undiluted nasal washings and incubated at 33°C. MRC-5 and monkey kidney cells were maintained as confluent cultures with Eagle's minimal essential medium (MEM) and 2% foetal calf serum; whereas HeLa cells were inoculated before they were confluent, 24 hours after seeding, using MEM with 4% tryptose phosphate broth, 2% foetal calf serum, and 0.03M magnesium chloride.

The continuous human cell line MRC-C (obtained from Dr. A.F. Bradburne, Wellcome Research Laboratories, Beckenham, Kent) was used for culture of nasal washings collected from Common Cold Unit volunteers. The cells were grown in Eagle's basal medium with 10% newborn calf serum and maintained in L-15 medium with 2% foetal calf serum and 20 μg/ml DEAE dextran.

All tubes were examined thrice weekly for cytopathic effect, and monkey kidney tubes were tested for haemadsorption using 1% human group O cells at 25° and at 4°C. Blind passages were not carried out. Rhinoviruses were identified by the characteristics of their cytopathic effect (cpe) and by their acid lability [Grist et al, 1974]. Haemadsorbing agents were identified by neutralization with typing antisera, and coronaviruses by electron microscopy.

Organ cultures of human foetal trachea and nasal mucosa were prepared by methods based on those of Tyrrell and Blamire [1967]. The cultures were maintained in L-15 medium without serum.

For electron microscopy, culture fluids were first clarified by centrifugation at 3,000 rpm, and were then centrifuged at 100,000g for 1 h. Three-hundred-mesh parlodion/carbon grids were applied to pellets resuspended in phosphate buffered saline (PBSA), negatively stained and examined in a Philips EM 300 electron microscope.

Various paired pre- and postinfection serum specimens were tested by complement fixation for antibody rises to respiratory syncytial virus, influenza A and influenza B viruses, parainfluenza viruses 1, 2, and 3, and Mycoplasma pneumoniae, courtesy of Dr. Philip Sanderson (Consultant Microbiologist, Northwick Park Hospital).

Methods in Volunteers

The methods were generally as previously described [Tyrrell, 1965; Beare and Reed, 1976]. Volunteers were isolated singly or in groups of 2 or 3, and were quarantined for three days before administration of the test inoculum as nasal drops. Each specimen was tested in between nine and 22 volunteers. Volunteers sharing accommodation received the same inoculum. Nasal washings were collected from all subjects on at least two days between the second and fifth days after inoculation and were stored at −70°C after addition of nutrient broth as above. The washings were tested in rhinovirus-sensitive HeLa cells, MRC-5 cells, and MRC-C cells, and some were also examined in human embryo nasal organ culture and by electron microscopy. The volunteers provided one serum specimen before they received the virus inoculum and another about three weeks later.

The chloroform sensitivity of unidentified agents found to produce colds in volunteers was tested by dividing a freshly thawed portion of the specimen into two halves. An equal volume of chloroform was added to one half. Both halves were shaken for 10 min at room temperature and centrifuged at 2,500 rpm for 10 min at 4°C. The aqueous layer of the chloroform-treated half and the control half were each inoculated into volunteers.

RESULTS

Subjects and Symptoms

Nasal washings were collected from 38 colds occuring in 14 male and 13 female subjects. Figure 1 shows how the collection of specimens was distributed over the study period. Twenty-three or 61% of the specimens were collected in the first four months. The modal interval between onset of symptoms and collection was one to two days, and 79% of the specimens were collected within three days of onset. The longest intervals were six and eight days, and both these specimens yielded agents.

Only two subjects had no symptoms relating to their nares; one complained of sore throat and the other of cough with sputum. Many subjects complained of constitutional symptoms, and a quarter had symptoms that might suggest influenza. Four subjects noted nausea, and three had had loose bowel motions.

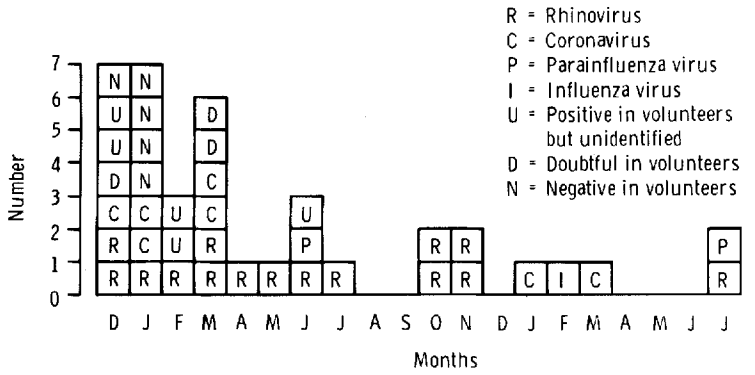

Fig. 1. Temporal distribution of specimens collected and viruses isolated during the months December, 1974, to July, 1976. Each square represents one specimen collected from a naturally occurring cold.

TABLE I. Agents Identified in 38 Nasal Washings Collected From Adults With Colds

Method	Rhino-virus	Para-influenza virus	Influenza virus	Corona-virus	Total
Tissue culture	11	2	1	0	14
Tracheal organ culture and tissue culture	3	0	0	0	3
Tracheal organ culture and electron microscopy	0	0	0	1	1
Inoculation of volunteers[a]	1	0	0	6	7
Total	15	2	1	7	25

[a]Followed by examination of their nasal washings in tissue cultures, nasal organ cultures, and by electron microscopy.

Viruses Isolated

Fourteen or 37% of the nasal washings yielded viruses on inoculation into tissue culture (Table I). Four additional agents were recovered after two serial passages in human embryonic tracheal organ cultures, giving an initial isolation rate of 45%. One of these viruses was a coronavirus. Only one influenza virus was identified; this resembled A/Victoria/3/75. The parainfluenza viruses were serotypes 1 and 3. All rhinoviruses except one were isolated in HeLa cells, the exception showing cytopathic effect only in MRC-5 cells.

Studies in Volunteers

The 20 washings that did not yield viruses after initial inoculation of tissue and organ cultures were tested in volunteers. Twelve of these washings were judged to be positive, ie, colds developed in at least 25% of inoculated subjects, and at least one subject rated a clinical score of 20 or more. In seven of the twelve, the causal agent was eventually identified, and in five it was not. Three specimens gave doubtful results in volunteers, and five were negative (Table II).

Nasal washings from groups of volunteers who developed colds were extensively tested in tissue cultures and nasal organ cultures; a rhinovirus and six coronaviruses were identified as causal agents. The rhinovirus was a slowly growing strain isolated initially from three of 14 volunteers inoculated, who developed definite colds. The virus was also later recovered from the original specimen. In the case of two specimens, nasal washings from more than half of the inoculated volunteers were found to produce 229E-like cytopathic effect in MRC-C cells. Coronaviruses were identified by electron microscopy in the tissue culture fluids, and two agents serologically related to 229E were eventually established in tissue culture. The cytopathic effect was slight during the first few passages, becoming clearer later. In the case of four other "symptom positive" specimens, no cytopathic effect could be obtained in tissue culture using volunteer's nasal washings,

but electron microscopy on harvests from human embryo nasal organ cultures inoculated with volunteers' nasal washings revealed typical coronavirus particles. In all six cases the original specimen used to inoculate the volunteers was reexamined by passage in nasal (instead of tracheal) organ culture, or in MRC-C cells, and coronaviruses were identified by electron microscopy in five of the six cases.

Five inocula gave positive results in volunteers, although the causal agents were not identified (Table II). In two such cases the agent appeared not to be highly pathogenic and produced few, generally mild colds. In three cases in which the symptoms were more definite, attempts were made to assess the chloroform sensitivity of the agent using volunteers as indicators: two agents appeared to be stable to chloroform and one labile. Harvests from human embryo nasal organ cultures inoculated with these three "symptom positive" specimens were examined both by the standard electron microscopic method and also by immune electron microscopy, using paired sera from volunteers who had experienced colds due to these agents. No agent was seen. Paired sera from similar volunteers who developed colds after inoculation of these unidentified agents were tested for rises in complement-fixing antibody to respiratory syncytial virus, parainfluenza viruses 1, 2, and 3, and Mycoplasma pneumoniae, but no rises were found.

Three specimens, two of which came from the same donor during different episodes of illness, gave no clear result in volunteers, and five of the original 38 specimens were negative in volunteers (13%) (Table II). Two of the specimens giving doubtful results in volunteers contained Streptococcus pneumoniae, but this organism was recovered from only one of the inoculated subjects. Paired sera from the donors of the doubtful and negative specimens were tested for rises in complement-fixing antibody against influenza A and B and respiratory syncytial virus, but no rises were found.

TABLE II. Results Obtained in Volunteers Using Specimens From Which No Cold-Producing Agent Was Cultured

Specimen	Proportion of colds	Mean score	Maximum score	Probable chloroform sensitivity	Result
C.S. 24.12	7/14	13.3	53	stable	
M.L. 30.12	7/13	10.0	50	labile	positive
R.B. 10.2	8/13	6.8	25	stable	(unidentified
J.P. 4.6	3/12	5.9	29	N.D.	agents)
M.P. 28.2	3/11	4.5	33	N.D.	
C.G. 12.12	2/10	4.4	10	N.D.	
T.M. 4.3	4/22	2.3	16	N.D.	doubtful
T.M. 25.3	1/12	3.2	8	N.D.	
J.C. 19.12	0/12	0.5	2	N.D.	
D.R. 2.1	0/14	0.0	0	N.D.	
T.M. 31.1	0/11	0.8	4	N.D.	negative
E.L. 17.1	1/11	1.0	7	N.D.	
P.S. 17.1	0/9	0.3	3	N.D.	
Controls	2/59	1.1	10	N.D.	

N.D. – Not determined.

Four viruses thought to be irrelevant to the inocula were isolated from inoculated volunteers' nasal washings. One (a rhinovirus) was isolated from a symptomless control subject who had received a saline inoculum. Two more rhinoviruses and one herpes simplex virus were isolated, each from a single volunteer in groups of 6–8 inoculated subjects. Each of the groups had received a different inoculum. The two subjects carrying these rhinoviruses had no symptoms; the herpes simplex isolate came from a subject with mild coryzal symptoms. These volunteers were retrospectively excluded from the assessment.

Symptoms Associated With Various Viruses

The symptoms reported by the donors of the original specimens were examined in relation to the viruses eventually identified (Table III). There were no striking associations, although constitutional symptoms were more often associated with parainfluenza viruses and influenza viruses and perhaps corona-viruses than with rhinoviruses. None of the "volunteer doubtful or negative" group experienced hoarseness (when this was evaluated separately from sore throat); although hoarseness was common in all other groups. Likewise, the "volunteer doubtful or negative" group had significantly fewer total symptoms ($P = 0.002$) and constitutional symptoms ($P = 0.056$, two sample t-test).

In inoculated volunteers it was noted that the coronaviruses produced colds in over 50% of subjects, the total proportion for the six coronaviruses tested being 57 of 75 subjects (76%). The mean clinical score in volunteers given the various coronaviruses ranged from 12.4–31.7, the symptoms being typically coryzal.

Temporal Distribution of Agents

The distribution of both identified and unidentified agents in the study period is shown in Figure 1. Although the majority of specimens were collected during the first four months, these colds were not caused by any single group of agents. Rhinoviruses were isolated throughout the entire period except when very few specimens were collected. Although five of the seven coronavirus infections occurred during the first four months, this was not significantly more often than in the rest of the study. The two coronavirus strains adapted to tissue culture originated a year apart in March.

All of the volunteer-negative specimens originated in the first two months of the study. These come close to being a significant cluster ($P = 0.08$, Fisher's exact test). Symptoms of these subjects did not differ qualitatively from those of the entire group, and all of the specimens were collected early in the illness.

DISCUSSION

The study confirms that a large part of the difficulty in isolating viruses from respiratory illness lies in the fastidious nature of many of these agents on cultivation: the number of isolates increased with the increasing number and variety of techniques used for identification. This difficulty is certainly relevant to isolation of rhinoviruses: maximum isolation rates for these viruses were only achieved by using more than one cell line, supplemented by passage in organ culture. Rhinoviruses accounted for 39% of the colds studied, although two chloroform-stable unidentified agents, which have so far resisted attempts at cultivation in tissue culture, may also be rhinoviruses.

The use of organ cultures increased the rate of isolation of rhinoviruses in the manner expected [Tyrrell and Bynoe, 1966; Higgins and Ellis, 1973]. Organ culture techniques also allowed identification of five coronaviruses: nasal organ cultures produced

TABLE III. Prevalence of Various Symptoms in the 38 Colds From Which the Original Specimens Were Collected

Agent isolated	Number of specimens	Number of symptoms reported			Number of colds associated with				
			Mean per cold						
		total	local	general	Local symptoms	Consti-tutional symptoms	Either sore throat or hoarseness	Cough or sputum	Gastro-intestinal[a]
Rhinovirus	15	7.5	3.0	1.8	15	12	12	10	2
Coronavirus	7	8.4	3.0	2.7	7	7	6	3	2
Parainfluenza or influenza	3	8.3	2.3	3.7	3	3	2	2	1
Unidentified "volunteer positive"	5	6.2	2.2	1.6	4	3	5	2	1
?None "volunteer doubtful or negative"	8	5.0	2.6	1.3	7	6	3	3	0

[a]Nausea, vomiting, diarrhoea or abdominal pain.

four of these after tracheal cultures had proved negative. In general, nasal mucosa cultures offer a larger ciliated surface for virus growth than do tracheal cultures and they may also be more sensitive. Two other coronaviruses were 229E-like strains cultivable with difficulty directly from volunteers' nasal washings using the human coronavirus-sensitive cell line, MRC-C.

Coronaviruses were associated with 18% of the colds studied. Although isolations of human coronavirus are seldom reported, this study confirms that they may commonly be identified if suitable techniques are used. The present result agrees well with the suggestion of McIntosh [1974] that coronaviruses may account for about 15% of colds in adults. The slightly higher proportion found in our study may be related to the fact that few of our specimens were collected in the autumn months when rhinoviruses are known to be particularly prevalent [Gwaltney, 1975]. McIntosh et al [1970] and Cavallaro and Monto [1970] also observed that coronavirus infections occur in the winter months and early spring.

Five agents producing colds in volunteers were not identified despite repeated attempts to do so. Variable sensitivity to chloroform indicated that they were not a homogeneous group. Their temporal distribution also was not uniform. For example, chloroform-stable agents occurred in the first and third months of the study and another unidentified agent in the seventh month. It is possible that although these strains are particularly difficult to cultivate, they may be members of virus groups already known. In addition to agents identified in this study, parainfluenza 4 viruses [Tyrrell and Bynoe, 1969; Gardner, 1969] and influenza C virus [Joosting et al, 1968] are also known to produce colds.

The reason for the rather high proportion (13%) of specimens which appeared to contain no agent infectious for volunteers is not known. The specimens were collected by the same technique as the others, no later in the illness than the average, and were stored under identical conditions. It is noteworthy that these five specimens were all collected within a period of six weeks. This raises the possibility that the cluster of cases was due to an agent labile to freezing and storage at −70°C. Other possibilities include an outbreak of coryzal symptoms occuring in the absence of an infectious agent in nasal secretions, or an infectious agent to which the volunteer population had, in the intervening 2–3 years, become totally immune.

The overall rate of virus identification is the study was 25 of 38 specimens (65.7%). The five unidentified agents which caused symptoms in volunteers raise the total of infectious agents to 30 (78.9%). It is uncertain what caused symptoms in the remaining cases. Apart from the apparent clustering of five cases yielding no infectious agent on examination of frozen specimens, there was no clear suggestion that groups of agents as yet unknown cause respiratory infection.

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