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Viruses from the common cold. A survey in Royal Air Force recruits on arrival from civilian life

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The isolation of cytopathic agents in monkey kidney and human embryo kidney cultures from nasal washings of patients with common colds (Tyrrell & Parsons, 1960) prompted us to study common colds in R.A.F. recruits. These recruits come from many parts of the country to a central recruiting unit and any colds they have on arrival constitute a sample of infection current in different parts of the British Isles. Our aim was to assess the frequency of infection with these agents in epidemiologically unrelated colds over a period of several months from as wide an area of the country as possible.

Since this study began several papers have been published which describe the isolation of cytopathic agents from common colds. Hobson & Schild (1960) in Sheffield isolated eight strains from twenty-five patients; one strain grew in both monkey and human embryo kidney (M strain), whereas the others grew only in human kidney cultures (H strains). Tyrrell & Bynoe (1961) report the isolation of twenty-five strains from nasal washings of 110 children and adults from London, Salisbury, Sheffield, Cirencester and Epsom; Kendall, Bynoe & Tyrrell (1962) isolated eighteen agents from fifty-nine boys at an Epsom boarding school, and Higgins, Ellis & Boston (1963) isolated twenty-three strains from 428 patients in general practice in the West of England.

In the U.S.A. Hamre & Procknow (1961) isolated fifty-three strains from 199 specimens from 101 medical students with common colds. Hamparian, Ketler & Hilleman (1961) described the isolation of eighteen strains from 110 patients; and, more recently, Ketler, Hamparian & Hilleman (1962) reported the isolation of thirty-five strains from 403 adults and children.

The M strains most frequently isolated have proved similar to ECHO virus type 28 of which the JH strain of Price (1956), and strain 2060 of Pelon, Mogabgab, Phillips & Pierce (1956) were prototypes. A few other strains have been isolated which were different from ECHO 28; two such strains were reported by Taylor-Robinson & Tyrrell (1962) and one by Ketler *et al.* (1962).

H strains have been isolated more frequently than M strains but their identification has been made difficult by the fact that there are many antigenic varieties. In England, Taylor-Robinson & Tyrrell (1962) separated their strains into four different serological groups. In the U.S.A., the strains of Hamparian *et al.* (1961) belonged to six different groups; and these authors quoted the unpublished findings

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of Johnson *et al.* of at least six different serotypes among strains isolated in Washington. The thirty-four H strains described by Ketler *et al.* (1962) fell into nineteen different groups. Together these different common cold viruses, or rhinoviruses, appear to form a group with many properties similar to enteroviruses.

It has been suggested that in addition to the rhinoviruses some agents such as adenovirus, influenza and para-influenza virus, and respiratory syncytial virus, which cause acute respiratory disease in children, may be responsible for colds in adults. The role of these viruses in the common cold was also included in our study.

MATERIALS AND METHODS

Field methods

The study began at the end of May 1960 and continued for 6 months. A total of 3610 recruits were interviewed in groups of 20–30 men within 36 hr. of their arrival. All men with symptoms of acute respiratory infection were questioned and 104 with a history of onset of symptoms on the morning when seen or during the previous two days (i.e. within 56 hr.) were examined clinically. Ninety patients with typical coryzal symptoms who were afebrile at the time of examination (maximum temperature 98·4° F.) were asked if they would allow specimens to be taken for laboratory examination and all but three agreed. In addition, during visits to three R.A.F. units the opportunity was taken to obtain specimens from fifteen men who fulfilled similar clinical criteria.

Specimens

The specimens taken from each patient for laboratory study consisted of a nose or per-nasal swab and a throat swab or washing and two blood samples, one at the time and the second 2 weeks later. The swabs were broken off into screw-capped bottles containing 2 ml. of Hanks's balanced salt solution with 0·5 % lactalbumin hydrolysate (LAH) and 0·02 % sodium bicarbonate. The bottles were placed immediately in a vacuum flask containing solid carbon dioxide for transport to the laboratory, where they were transferred to an electric refrigerator and stored at −65° C. until tested. Throat washings were obtained by giving the patient 10 ml. of 10 % nutrient broth-saline and asking him to gargle for 1 min. The washings were collected into sterile 20 ml. screw-capped bottles and transported on solid carbon dioxide to the laboratory where they also were stored at −65° C.

A sample of blood was obtained from all the patients in the acute stage of illness, but convalescent specimens were taken from only eighty-eight patients, the remaining fourteen having left the Service. At the time of the second bleeding details were recorded of the duration of symptoms, the presence of any complications and history of other respiratory illnesses since the first interview. Serum specimens were stored in the laboratory at −30° C. until they were tested.

Laboratory methods

Isolation of virus

Monkey kidney and human embryo kidneys were trypsinized and grown in Hanks's saline with 0·5 % LAH and 5 % ox serum. Before inoculation the medium was altered to contain 2 % calf serum and 0·03 % bicarbonate.

HeLa cells were grown in Gey's solution with 0·5 % LAH and 10 % human serum and tube cultures were maintained with 0·25 % LAH + 5 % rabbit serum.

Tubes of each tissue were inoculated with 0·1 ml. amounts of the materials to be tested, the HeLa cell cultures were incubated without rolling at 37° C., whereas monkey and human embryo kidney cells were kept rolling at 33° C.

Cultures were examined on alternate days for evidence of cytopathic change. Fluids were replaced with fresh medium when necessary and incubation was continued for a period of 3 weeks or longer if the cell sheets were still healthy. Once cytopathic changes were noted, subcultures were made and pools of infected fluids prepared and titrated in readiness for neutralization tests.

Preparation of rabbit antisera

Rabbit antisera were prepared with the prototype H strain FEB (Sal/1/58/H) and the prototype M strain HGP (Sal/1/57/M) by repeated twice-weekly intravenous inoculations. The first H strains isolated were tested with the H prototype antiserum and, as only one was neutralized by it, rabbit antisera were prepared with some of them. These antisera were prepared by twice-monthly intramuscular injections of virus with a Bayol–Arlacel adjuvant for a period of 3 months. Rabbits were bled after a further 3 weeks. Sera were inactivated by heating at 56° C. for $\frac{1}{2}$ hr. before use.

Neutralization tests

Rhinovirus M strains were identified by serum neutralization tests. Equal volumes of serum dilutions and virus suspensions containing 200 TCD 50/ml. were kept at room temperature for 1 hr. and then transferred to monkey kidney cultures and incubated at 33° C. Results were read 2 days after the virus controls showed a definite cytopathic effect. Neutralization end-points were recorded as the highest serum dilution preventing a cytopathic effect.

For the estimation of antibodies to ECHO 28 and HGP viruses, acute and convalescent sera were tested first in a dilution of 1/5 followed by titration of any pairs showing antibody.

Rhinovirus H strains were identified by a method based on the reduction of microplaques as described by Taylor-Robinson & Tyrrell (1962) with a dose of virus estimated to produce between 10 and 50 microplaques per tube in 2–3 days.

For the estimation of antibodies to FEB virus, acute and convalescent sera were tested in a similar manner with a dilution of 1/10. Paired sera from patients from whom strains of virus were isolated were tested for antibody response to the infecting strain.

For the detection of antibodies to Coxsackie A 21 (Coe virus) paired sera were tested by neutralization in HeLa cells as described by Pereira & Pereira (1959).

Complement-fixation tests

These were done in plastic haemagglutination trays with 0·1 ml. unit volumes and overnight fixation at 4° C. Antigens were standardized by chess-board titration with human convalescent sera and used at optimal dilution. The antigens used were: influenza A, B and C, para-influenza 1, 2 and 3, adenovirus and respiratory syncytial virus. An antigen prepared with Eaton agent was kindly supplied by Dr B. E. Andrews.

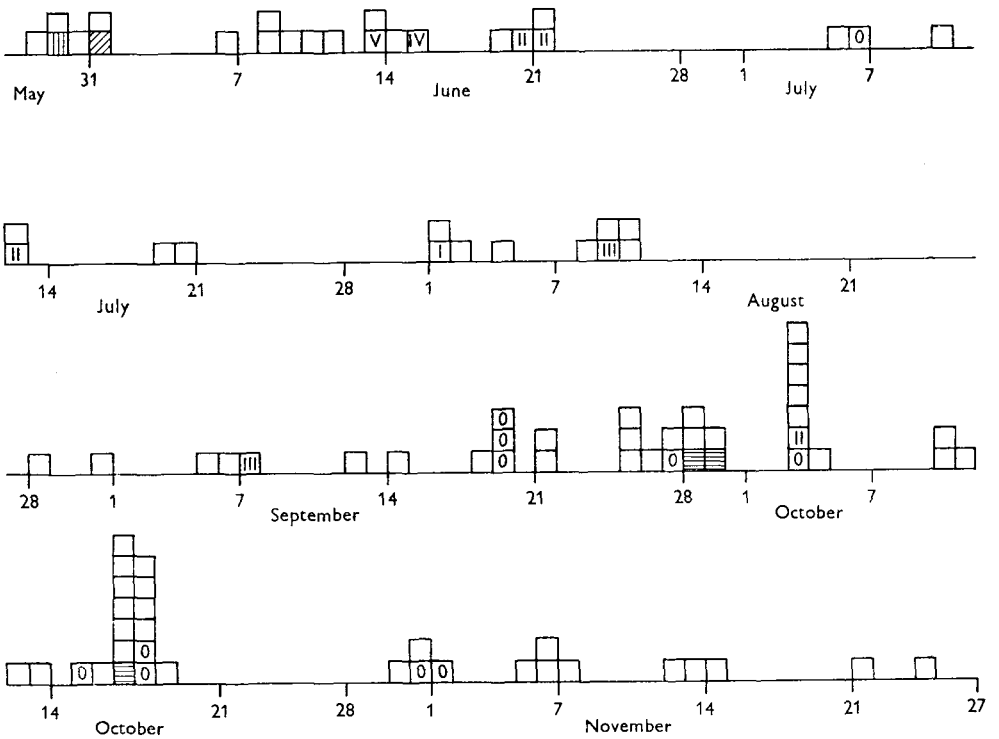

Fig. 1. Common colds in an R.A.F. recruit centre, June–November, 1960. Each square indicates a cold from which specimens were tested. Virus infections are shown as follows: ECHO 28, RS, Influenza C. Rhinovirus H by numbers I–V or 0 (unclassified) corresponding to serological groups in Table 1.

RESULTS

Virus isolation

Altogether twenty-three strains of virus were isolated from the 102 patients examined. All these agents produced a cytopathic effect in human embryo kidney cultures and three of them in monkey kidney cultures also. None of the specimens, including those that yielded these twenty-three strains, caused detectable effects in HeLa or human amnion cultures.

Virus strains were isolated from 19/99 (19 %) nasal or per-nasal swabs and from 10/102 (10 %) throat swabs or washings. Strains were isolated from 3/12 (25 %) specimens taken within 24 hr. of the onset of illness, from 11/43 (26 %) specimens

taken between 24 and 48 hr., and from 9/47 (19 %) specimens taken between 48 and 56 hr.

The seasonal distribution of the colds from which agents were obtained is shown in Fig. 1. The H strains were isolated from material from four patients with typical common colds in June, two patients in July, two in August, five in September, six in October and one in November. Six strains were from patients coming from the south of England and Wales, seven from the Midlands, one from the north of England, and five from Scotland and Ireland. One man had just arrived from Fiji. No particular geographical pattern was evident.

Table 1. *Cross-neutralization tests with rabbit antisera to 'H' strains of rhinovirus*

	Rabbit antiserum						
	I		II		III	IV	V
Rhinovirus strain	Sal/1/58/H FEB	T	W	Mor	Md	Mu	L
Sal/1/58/H FEB	+	+	–	–	–	–	–
T	+	+	–	–	–	–	–
Sheffield/1/60/H 16/60	–	–	+	+	–	–	–
W	–	–	+	+	–	–	–
Mor	–	–	+	+	–	–	–
P	–	–	+	+	–	–	–
B	–	–	+	+	–	–	–
Md	–	–	–	–	+	–	–
F	–	–	–	–	+	–	–
Mu	–	–	–	–	–	+	–
L	–	–	–	–	–	–	+
Sal/1/59/H Th	–	–	–	–	–	–	–
Sal/1/51/H No	–	–	–	–	–	–	–
N, Mur, O'L, S, Mg, C, D, Bo, R, Ru, Br	–	–	–	–	–	–	–

+ = K value > 0·2. – = K value < 0·2.

Identity of virus strains

The three M strains were identified as ECHO 28 virus on the basis of neutralization by a monkey antiserum to this virus. Virus was isolated from all the specimens (nasal or per-nasal swabs, throat swabs and gargles) obtained from the three patients in question. All three men came from the south of England but they were not apparently associated in any way; their colds occurred between the end of September and the middle of October.

The twenty H strains isolated were tested with a rabbit antiserum prepared with the FEB virus (Sal/1/58/H), the only named or known H strain available to us. As only one strain was found to be neutralized, further antisera were prepared in rabbits with some of the other strains. The results of cross-neutralization tests with all available antisera are shown in Table 1.

From the absence of antigenic crossing in these tests it can be seen that there were at least six different antigenic types prevalent during the survey. The group numbered II contained the largest number of similar strains. Of the four strains

in this group two were from colds during the same week in June, one from a cold in July 3 weeks later and one from a cold in October. The four patients came from widely separated places.

Serological tests

All three of the patients from whom ECHO 28 virus was isolated responded to infection with a fourfold or greater increase in neutralizing antibody to this virus. None had detectable antibody in a 1/5 dilution of the acute-stage sample of serum.

Paired sera were obtained from fourteen of the men from whom H strains were isolated. Of these only six showed an increase in neutralizing antibody to the virus isolated. The possibility of accidental infection of cultures in the laboratory was investigated by returning to the original throat or nose washings and attempting re-isolation. In every case this was successful.

The proportions of patients with neutralizing antibodies in their acute-stage specimens to the prototype M strain HGP (Sal/1/57/M), to the H strain FEB (Sal/1/58/H), and to ECHO 28 were 89, 45 and 35 %, respectively. There was no appreciable difference in the proportion with antibody against these viruses in men under or over 20 years of age. However, the age range in population studied was narrow and all but two were aged between 17 and 28 years.

The association of rhinoviruses with twenty-three common colds still left a large number from which no agent was isolated.

All the results of serological tests with other antigens were negative except for one pair showing a fourfold increase to influenza C and one pair to respiratory syncytial virus. Both these patients had complement-fixing antibody in the acute-stage serum, suggesting either that infection occurred despite the presence of antibody or that it was coincidental and not the cause of the cold.

Table 2. *Clinical picture on the first day of illness*

Symptoms	Virus isolated (23 patients) (%)	No virus isolated (79 patients) (%)
Coryza (clear nasal discharge)	74	89
Blocked nose	61	41
Dry sensation in throat or slight sore throat	43	39
Slight hoarseness	22	8
Dry cough	4	6
Sweating	9	4
Shivering	9	1

Clinical findings

The symptoms present in the 102 patients who took part in this investigation were fairly typical of the mild afebrile upper respiratory illness usually associated with the common cold. The distribution of symptoms in patients from whom viruses were and were not isolated is shown in Table 2; there appeared to be no important differences. The three illnesses associated with ECHO 28 infection were also similar to the rest.

DISCUSSION

The role of respiratory viruses other than rhinoviruses in the causation of common colds was found to be almost negligible in this group of R.A.F. recruits studied during the summer and autumn months. These findings presumably reflect the fact that during these seasons most other respiratory viruses are seldom epidemic and contrast with results obtained in a preliminary survey made during the previous winter. During that period febrile respiratory illness was prevalent and associated with infection with adenovirus type 3, Coxsackie A 21 (Coe virus) and, to a lesser extent, para-influenza 3 virus. One or other of these viruses was isolated from 7/23 afebrile common colds which were concurrently investigated, but no rhinoviruses were isolated. The results of this survey do not allow one to compare the prevalence of rhinoviruses in autumn and winter, but it has been our experience that rhinoviruses are infrequently found associated with common colds in winter and it may be that this group of viruses tends to follow the seasonal pattern of other enteroviruses.

The rhinoviruses isolated in the present study were a heterogeneous collection. The ECHO 28 strains grew relatively quickly to a high titre in monkey kidney cultures and the patients developed antibody. The twenty H strains grew rather slowly and needed several passages before workable titres of virus were obtained. Titres above 10^4 were exceptional. Less than half the patients developed antibody against the strains isolated but the convalescent sample of serum was taken 2 weeks after the onset of illness and it is possible that specimens taken later would have contained antibody.

The prevalence of antibody against the three prototype rhinoviruses HGP, FEB, and ECHO 28 as judged by results of tests on acute-stage sera suggest that a high proportion of people have been infected with these agents and possibly reinfected. It is remarkable that 45 % had antibody to the H strain FEB having regard to the fact that antibody was not always detected even after the isolation of a strain of virus. The M strains appear to be better antigens so that the figure of 89 % for HGP reflects a high prevalence of this strain at some time in the past. The figure of 35 % for ECHO 28 is of the same order as that found by Price, Emerson, Ibler, Lachaine & Terrell (1959) who reported the presence of neutralizing antibody in 5 % of children under 5 years of age rising to 35 % in the 6–12 age group and to 63 % in the 25–35 age group.

The number of distinct serological types of rhinovirus found associated with this small group of colds indicates the complexity of the disease.

The prevalence of certain types may vary in time and it is significant that the group containing the largest number of similar strains was one in which the prototype strain was isolated in the same year.

At least three different antigenic types of virus were defined in addition to those already identified in this country. Neutralization tests with rabbit antisera to these three, designated Md, Mu and L, against five different serotypes from the National Institutes of Health, U.S.A., designated 33342, 353, 1059, 11757 and 1734 (Johnson & Rosen, 1963), have shown them to be antigenically distinct.

SUMMARY

Twenty-three strains of rhinovirus were isolated from 102 patients who had common colds on arrival at a Royal Air Force recruit centre during a 6-month period from June to November, 1960. Three of these strains were M type rhinoviruses similar to ECHO 28 virus. Twenty strains were H type rhinoviruses which fell into six or more different antigenic types. Two of these types were similar to the prototypes Sal/1/58/H and Sheffield/1/60/H. Three types were antigenically distinct from those previously reported in this country and several strains are still unclassified. Other human respiratory viruses were not isolated from common colds occurring at this time among the population studied.

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REFERENCES

HAMPARIAN, V. V., KETLER, A. & HILLEMAN, M. R. (1961). Recovery of new viruses (coryza virus) from cases of common cold in human adults. *Proc. Soc. exp. Biol., N.Y.*, **108**, 444.

HAMRE, D. & PROCKNOW, J. J. (1961). Viruses isolated from natural common colds in the U.S.A. *Brit. med. J.* ii, 1382.

HIGGINS, P. G., ELLIS, E. M. & BOSTON, D. G. (1963). The isolation of viruses from acute respiratory infection. A study of the isolations made from cases occurring in general practice in 1962. *Mon. Bull. Minist. Hlth Lab. Serv.* **22**, 71.

HOBSON, D. & SCHILD, G. C. (1960). Virological studies in natural common colds in Sheffield in 1960. *Brit. med. J.* ii, 1414.

JOHNSON, K. M. & ROSEN, L. (1963). Five newly recognized entero-viruses recovered from the human oropharynx. *Amer. J. Hyg.* **77**, 15.

KENDALL, E. J. C., BYNOE, M. L. & TYRRELL, D. A. J. (1962). Virus isolations from common colds occurring in a residential school. *Brit. med. J.* ii, 82.

KETLER, A., HAMPARIAN, V. V. & HILLEMAN, M. R. (1962). Characterization and classification of ECHO 28—rhinovirus—coryzavirus agents. *Proc. Soc. exp. Biol., N.Y.*, **110**, 821.

PELON, W., MOGABGAB, W. J., PHILLIPS, I. A. & PIERCE, W. E. (1956). Cytopathic agent isolated from recruits with mild respiratory illnesses. *Bact. Proc.* p. 67.

PEREIRA, M. S. & PEREIRA, H. G. (1959). Coe virus. Properties and prevalence in Great Britain. *Lancet*, i, 539.

PRICE, W. H. (1956). The isolation of a new virus associated with respiratory clinical disease in humans. *Proc. nat. Acad. Sci., Wash.* **42**, 892.

PRICE, W. K., EMERSON, H., IBLER, I., LACHAINE, R. & TERRELL, A. (1959). Studies of the JH and 2060 viruses and their relationship to mild upper respiratory disease in humans. *Amer. J. Hyg.* **69**, 224.

TAYLOR-ROBINSON, D. & TYRRELL, D. A. J. (1962). Serotypes of viruses (rhinoviruses) isolated from common colds. *Lancet*, i, 452.

TYRRELL, D. A. J. & BYNOE, M. L. (1961). Some further virus isolations from common colds. *Brit. med. J.* i, 393.

TYRRELL, D. A. J. & PARSONS, R. (1960). Some virus isolations from common colds. *Lancet*, i, 239.