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Rhinoviruses: The Isolation and Characterization of Three New Serologic Types* (32682)

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The increasing number of reports concerning the isolation, characterization and epidemiology of a new group of viruses, the rhinoviruses, attests to the growing interest in them as causative agents of a significant proportion of mild illnesses affecting the upper respiratory tract of man. Rhinoviruses have been classified as a subgroup of human picornaviruses (1) and as such they posses an internal RNA component, are approximately 15-30 mμ in diameter, are ether stabile, and inactivated at low pH. On initial isolation rhinoviruses grow optimally at 33°C. The definition of the cultural conditions necessary for the detection of rhinoviruses in tissue cultures (2) and the concomitant use of human diploid cells (3) has led to the isolation of a large number of serologically distinct virus types. Through the efforts of a collaborative program conducted under the auspices of the Committee for Vaccine Development, National Institute of Allergy and Infectious Disease, and of the World Health Organization, a classification system was established (4) for those rhinoviruses which have been adequately characterized. Type numbers have been assigned to 55 serologically distinct rhinoviruses.

Three rhinoviruses, now established as new immunotypes, were isolated in this laboratory in 1962. They have been submitted as prototype strains to the Reference Laboratory of the Collaborative Study Program for rhinoviruses, and their isolation and characterization is the subject of this report.

Materials and Methods. Cell cultures. Diploid cell cultures of human fetal lung (HFD) were initiated and passaged according to the method of Hayflick and Moorhead (5). Tube cultures of HFD cells were seeded with 10^5 cells in Eagle's minimum essential medium with 10% fetal bovine serum and outgrowth was usually complete in 3–4 days.

Primary rhesus monkey kidney (MK) cell cultures were prepared by a modification of the method of Bodian (6). Outgrowth medium for MK cells consisted of 85 ml of Hanks' balanced salt solution, 10 ml of 5.0% lactalbumin hydrolyzate and 2 ml fetal bovine serum.

For maintenance of both cell types, Leibovitz Medium no. 15 with 2% fetal bovine

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serum was used (7). All media contained 100 units of penicillin, 100 μg of streptomycin and 10 μg of amphotericin B per ml.

Clinical specimens and virus isolation. The methods used for the collection and treatment of specimens prior to the inoculation of cell cultures for virus isolation attempts have been described in detail (8).

Immune serum. In this laboratory, infectious tissue culture fluids were centrifuged at high speed (30,000 rpm 12–18 hours) and the pelleted virus was resuspended in 1/10 of the original volume. Such concentrated antigens were treated with genetron and then homogenized with an equal volume of complete Freund's adjuvant prior to injection. Eight guinea pigs were given 2 intramusclar injections of 1.0 ml of the virus-adjuvant mixture at 6-week intervals, and were bled 2 weeks after the second injection. Certain rhinovirus immune sera were obtained from Drs. V. Hamparian and R. Conant of the Rhinovirus Reference Laboratory.

Neutralization test. Serum neutralization tests were performed in tube cultures of HFD cells. All test sera were inactivated at 56°C for 30 min. Equal volumes of serum dilutions and virus (approximately 100 TCD_{50}) were mixed and incubated at room temperature for 1–2 hours prior to inoculation into cell cultures. Virus titrations were carried out for each test and, except where indicated, the serum neutralization titers were read when the titration indicated the test virus dose to be between 32 and 100 TCD_{50}.

Nucleic acid determination. Indirect evidence for the type of nucleic acid present in the candidate viruses was obtained by use of 5-iododeoxyuridine (IDU). Parallel titrations of the candidate viruses, a known RNA virus (poliovirus type 1) and a known DNA virus (vaccinia virus), were made in HFD cell cultures containing 10 μg per ml of IDU and in cultures without IDU. All cultures were incubated in a roller drum at 33°C and observed 7–10 days for cytopathic effects (CPE).

The test was considered satisfactory if IDU completely inhibited the growth of vaccinia virus and did not interfere with the growth of poliovirus type 1. The specificity of the action of IDU was determined by the capacity of 50 μg per ml of thymidine to suppress the inhibitory action of IDU on the multiplication of vaccinia virus.

Estimation of size. An estimation of the size of the candidate viruses was made by filtration through gradocol membranes. Prior to filtration of the virus suspension, 20–25 ml of tryptose phosphate broth was passed through each membrane. Virus suspensions were first clarified by filtration through a gradocol membrane with an average pore diameter (APD) of 450 mμ, and then 20–40 ml of the clarified candidate virus suspension was filtered through membranes of 51 mμ and 27 mμ APD. The infectivity titer of each of the filtrates was determined by preparing 10-fold dilutions and inoculating 0.5 ml of each dilution into 4–8 HFD cell cultures. The inoculated cultures were incubated in a roller drum at 33°C and observed for 7–10 days for specific viral induced CPE.

Acid lability and ether sensitivity. The methods used for determination of acid lability and ether sensitivity of the candidate viruses have been described (8).

Results. Virus isolation and human origin. The 3 new rhinovirus candidates were isolated from nasopharyngeal swabs of military recruits at Fort Ord, California. The pertinent data relating to the isolations of these viruses are shown in Table I. Each of the strains was isolated in HFD cell cultures and no evidence of virus growth was observed in MK cell cultures. The CPE in HFD cells began as foci of small round, highly refractile degenerating cells scattered throughout the cell monolayer. Infection eventually spread to the entire culture and the infected cells ultimately detached from the glass surface.

The acute- and convalescent-phase serum specimens from each of the 3 patients from whom virus was isolated were tested for neutralizing antibodies to their homologous virus. A 4-fold or greater rise in neutralizing antibody titer was demonstrated (Table I) with 2 of the 3 paired serum specimens but antibody was not detectable in the paired sera from patient Treganza (FO2-2547). However, 14 additional rhinoviruses immunologically indistinguishable from the Treganza strain (FO2-2547) were recovered from other recruits. Paired serum specimens from 8 of these 14

TABLE I. Evidence of Human Origin of 3 Rhinovirus Candidates.

Rhinovirus candidates	Clinical diagnosis	Age (year)	Sex	Date of spec. collection	Neut. antibody titer[a] Acute serum	Neut. antibody titer[a] Conval. serum	Additional virus isolates
FO2-2317 (Wood)	URI	24	M	4/26/62	<1:8	1:16	0
FO2-2513 (Mitchinson)	Non-URI	23	M	5/10/62	<1:8	1:128	2
FO2-2547 (Treganza)	URI	19	M	5/14/62	<1:8	<1:8	14

[a] Neutralizing antibody titer determined against 10–50 TCD_{50} of homologous virus.

patients were also examined for neutralizing antibody to the Treganza (FO2-2547) virus; 7 of the 8 serum pairs tested showed neutralizing antibody, with 5 showing a 4-fold or greater rise in titer.

Serologic relationship among the candidate rhinoviruses. Immune serum against each of the rhinovirus candidates was made in guinea pigs. The viruses used as immunizing antigens were purified by three successive terminal dilutions and extraneous proteins were partially removed by treatment with genetron. The immunizing schedule is given in the section of "Materials and Methods."

The results of reciprocal neutralization tests with the three candidate rhinoviruses (Table II) demonstrated the antigenic distinctness of the viruses.

Serologic relationship of candidate rhinoviruses to previously known rhinoviruses. Serum neutralization tests employing 20 antibody units of immune serum to rhinovirus prototypes 1A through 55 (4) were tested against approximately 100 TCD_{50} of each of the terminally diluted rhinovirus candidates. Similarly approximately 100 TCD_{50} of each of the prototype rhinoviruses (types 1A through 55) were tested against immune serum to the 3 candidate viruses diluted to contain 20 antibody units. The results of these tests clearly indicated that the candidate viruses are seologically distinct from the prototype rhinoviruses.

TABLE II. Immunologic Distinctness of 3 Rhinovirus Candidate Strains.

Rhinovirus candidates[a]	Neutralizing antibody titer[b] [immune serum (guinea pig) to virus strain] FO2-2317	FO2-2513	FO2-2547
FO2-2317 (Wood)	1:512	<1:16	<1:16
FO2-2513 (Mitchinson)	<1:16	1:2048	<1:16
FO2-2547 (Treganza)	<1:16	<1:16	1:2048

[a] Viruses used in these tests and for the immunization of guinea pigs were purified by 3 successive terminal dilutions.

[b] Neutralizing antibody titer determined against 32–100 TCD_{50} of virus.

In addition 20 antibody units of immune serum to the 3 rhinovirus candidates (FO2-2318, FO2-2513 and FO2-2547) failed to neutralize approximately 100 TCD_{50} of rhinovirus candidates CV-34 through 53, described by Hamparian *et al.* (9).

Size estimation. Each of the 3 candidate rhinoviruses passed through gradocol membranes with an average pore diameter (APD) of 51 mμ (Table III) but no infectious virus could be detected in the filtrates from the 27 mμ membranes. Using Black's conversion factor (10) the size of each virus was estimated to be between 17 and 30 mμ in diameter.

Nucleic acid type. Growth of the 3 candidate rhinoviruses was not significantly suppressed by 10 μg per ml of IDU. In the same experiment it was demonstrated that 10 μg per ml of IDU completely inhibited the growth of vaccinia virus (a known DNA virus) while poliovirus (a known RNA virus) was unaffected by IDU. This finding provides indirect evidence that the candidate rhinoviruses are RNA viruses.

Acid lability. After incubation in an acid medium (pH 3.5) for approximately 3 hours at room temperature the infectivity of each

TABLE III. Physical and Chemical Characteristics of New Candidate Rhinoviruses.

Virus strain	Virus titer[a] after filtration through gradocol membranes			Nucleic acid determination			Acid lability (virus titer[a])		Ether sensitivity (virus titer[a])	
				Virus titer[a]						
	450 mμ	51 mμ	27 mμ	With IDU[b]	Without IDU	Nucleic acid type	pH 7.0	pH 3.5	Untreated	Ether treated
FO2-2317 (Wood)	4.0	1.7	NV[c]	4.0	3.5	RNA	3.0	1.0	4.3	3.7
FO2-2513 (Mitchinson)	3.0	1.0	NV	5.0	5.0	RNA	4.8	2.7	4.0	3.5
FO2-2547 (Treganza)	4.4	1.5	NV	4.0	4.0	RNA	2.3	<1.0	4.0	4.0

[a] Virus titers expressed as $\log_{10}$ TCD_{50} per 0.1 ml.

[b] IDU-5-iododeoxyuridine 10 μg per ml final concentration.

[c] NV = No virus detected in 4.0 ml of the undiluted filtrate.

of the candidate rhinoviruses was reduced 100-fold or more when compared to control samples incubated at pH 7.0 for a similar period (Table III).

Ether sensitivity. Following exposure to fresh 20% diethyl ether for 18 hours at 4°C the infectivity titer of each of the 3 candidate rhinoviruses was not significantly different from that of the untreated controls (Table III).

Discussion. The 3 new rhinoviruses described in this report were isolated from young adult males at Forth Ord, California. Each of the viruses was isolated in HFD cells and did not produce a cytopathic effect in MK cells.

Two of the 3 patients from whom the viruses were recovered developed a 4-fold or greater rise in neutralizing antibody titer. Although no titer rise was demonstrated for the third patient, 14 identical virus strains were recovered from other patients and a 4-fold or greater rise in neutralizing antibody titer was demonstrable with the sera of certain of these. This establishes the human origin of the viruses isolated.

Rhinoviruses are small 15–30 mμ, have an RNA core, are acid labile and do not contain essential lipids (1, 2). The candidate viruses were shown to possess each of the characteristics and thus can be considered rhinoviruses.

The new rhinoviruses are serologically distinct from each other and from all of the known prototype rhinoviruses. In addition they also appear to be distinct from certain other previously described (9) candidate rhinoviruses. However the lack of relationship to these candidate rhinoviruses is based upon the results of "one-way" neutralization tests, since antisera to the other candidate viruses (CV34 through 53) were not available for testing.

Summary. Three new rhinovirus immunotypes (FO2-2317, FO2-2513 and FO2-2547) were recovered from military recruits at Fort Ord, California. These new viruses were shown to be small RNA viruses which are inactivated at low pH and do not contain lipids as an essential structural component. Thus, they can be classified as human rhinoviruses. Reciprocal neutralization tests show them to be serologically distinct from the presently recognized 55 prototype rhinoviruses and on the basis of one-way neutralization tests they also appear to be distinct from certain other previously described candidate rhinoviruses.

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Study of the Effects of Chemotherapeutic Agents on the "Early" and "Late" Responses to Erythropoietin* (32683)

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The response of fasted rats to erythropoietin (EP) containing plasma, measured 24 hours after injection, the so called "early" response (1), is markedly dependant on the duration of fasting. It is maximum when EP is given at the start of fasting and drops sharply to a minimum by three days. On the other hand, the response measured 48 hours after EP the so called "late" response, is not markedly affected by the state of the recipient at the moment of injection (1). The "early" response to EP has been interpreted as reflecting the action of the hormone on erythroid precursors present at the moment of injection, and the "late" response as reflecting differentiation of erythropoietin sensitive stem cells (1).

In order to further characterize the "early" and "late" responses the effects of the following chemotherapeutic agents were studied: (a) Dactinomycin[1] known to suppress the differentiating effect of EP on stem cells in doses which do not affect other blood cells (2, 3). (b) Vinblastine[2] and methotrexate[3] (4-amino-10-methylfolic acid) drugs classified (4) as affecting cells in cycle. Vinblastine by blocking cells in mitosis (5), methotrexate by producing a thymidineless state and blocking DNA synthesis (6). A study of the effects of these drugs should given information as to whether cells involved in the "early" response must go through cycle for the response to be expressed.

Method. A × C male rats 160–180 gm were used. For the study of the early response, rats were injected intravenously at the start of fasting with 1 ml of anemic rat plasma containing 10 μ EP and a ^{59}Fe distribution assay (1) carried out 24 hours later. For the study of the "late" effect 10 μ EP were injected 2 days after the start of fasting and a ^{59}Fe distribution assay carried out 48 hours later (1). The drugs used were injected in the doses and schedules indicated in the results. Treatment with methotrexate was given in a "pulsed" form, by injecting 3 mg of leucovorin, (folinic acid), a drug factor known to reverse methotrexate effects (7), 4 hours after methotrexate injection.

Results. Methotrexate, 0.5 mg/kg, completely suppresses the early response to EP when given 4 hours after EP and reduces it to 50% and 30%, respectively, when given 4 hours before and together with EP, (Fig. 1). Only a slight effect on the "late" response is obtained when the methotrexate is given together with EP; the greatest effect is seen when it is given 4 hours before or after. The dose of methotrexate used is such that it does not affect the iron uptake of the control rats for the "early" and "late" responses.

When methotrexate, given together with EP, was not followed after 4 hours by folinic acid, the "late response observed (47 ±

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[1] Merck Research Laboratory, Rahway, New Jersey.

[2] Lederle Laboratories.

[3] Eli Lilly and Co. Indianapolis, Indiana.