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Characterization of Three New Rhinovirus Serotypes.* (30304)

C. ALAN PHILLIPS, JOSEPH L. MELNICK AND CATHERINE A. GRIM
WHO International Reference Centre for Enteroviruses, Baylor University College of Medicine, Houston, Texas

Although the rhinoviruses have only recently been recognized as a subgroup of the picornaviruses(1,2), a large number of apparently distinct serotypes has been isolated in various laboratories(3-11). The rhinoviruses have been noted to be small RNA viruses of approximately 15-30 mμ in size, ether stable, acid labile, and to grow best when rolled at 33°C(2). Three new serotypes were isolated in Houston, Texas, during a recent study involving a student population(12)and have been submitted to the World Health Organization collaborative study group as candidate rhinoviruses. Their properties are described in this report.

Materials and methods. Tissue culture. Primary African green monkey kidney (GMK), rhesus monkey kidney (RMK), human diploid (WI-38), and human aorta (A-39) cells were employed. The growth and maintenance of GMK(13) and of WI-38(14) have been described. The aorta cell lines have recently been described from this laboratory(15). All cultures were prepared in disposable constricted tubes(16) and grown stationary at 37°C until inoculation, after which they were rolled at 33°C.

Antisera. Antisera for the 3 new serotypes were prepared in baboons (*Papio doguera*) according to the method recently described (17). Antigens used for preparation of these antisera were purified by terminal dilution passages in A-39 and WI-38 (Table I). Thirty-one additional types of rhinovirus antisera were supplied by Dr. Maurice Mufson of the WHO International Reference Centre for Respiratory Viruses, National Institute for Allergy and Infectious Diseases, Bethesda, Md., and by Dr. Dorothy Hamre of the University of Chicago. Enterovirus antisera and antiserum for echovirus 28 (rhinovirus 1) were obtained from the stocks of the WHO International Reference Centre for Enteroviruses. The rhinoviruses used in the reciprocal neutralization tests were kindly supplied by Drs. Mufson and Hamre.

Acid lability test. This test and all additional tests performed in characterizing the candidate rhinoviruses were performed in continuous cell cultures of human origin (WI-38 or A-39). The method followed for determining acid lability has been described(18).

Ether sensitivity. Viruses were mixed with 20% fresh diethyl ether and incubated for 18 hours at 4°C. Viruses mixed with Eagle's medium containing 0.15% $NaHCO_3$ were treated in a similar manner. After incubation, ether was removed by evaporation at 33°C for 30 minutes and infectivity titer was determined in roller tube cultures.

Nucleic acid determination. Indirect evidence for determining the type of nucleic acid present was obtained by noting the sensitivity

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TABLE I. Preparation of Antiserum for New Rhinovirus Prototypes.

Serotype	Strain	Passage history	Animal	Reciprocal of neutralization endpoint†	TCD_{50} in test
Baylor 1*	Tippett	$A39_1$ $WI\text{-}38_9$	Baboon	6400	100
" 2*	Crell	$A39_4$ $WI\text{-}38_{10}$	"	600	100
" 3*	Calvert	$A39_1$ $WI\text{-}38_8$	"	600	50

* All viruses used in production of antisera were purified by 3 terminal dilution passages.
† Third post bleedings.

TABLE II. Strain Characteristics of New Rhinovirus Serotypes.

Serotype	Strain	Date of onset of illness	Tissue culture in which virus was isolated	M or H strain†	Recovery after 6 mo of storage
Baylor 1	Tippett*	3/15/64	A39	H	+
" 2	Mabray	2/17/64	A39, WI-38	H	+
	Crell*	3/10/64	A39, WI-38	H	+
	Montgomery	3/14/64	WI-38	H	+
	Todd	3/23/64	A39	H	+
	Einspahr	3/30/64	A39	H	+
" 3	Kracke	10/ 8/63	A39	M	—
	Calvert*	10/20/63	A39	M	+
	Schneider	11/ 3/63	A39, WI-38	M	—

* Prototype strain.
† M = strains which grow both in tissue culture cells of human origin and monkey kidney.
H = strains which grow only in tissue culture cells of human origin.

of the candidate viruses to 5-iododeoxyuridine (IUDR). Ten-fold dilutions of viruses were carried out and inoculated into 2 to 4 tubes of WI-38 or A-39 containing 100 μg/ml of IUDR and into 2 to 4 tubes without IUDR. Each test included both poliovirus type 1 (a human RNA virus) and either vaccinia virus or herpes simplex virus (human DNA viruses). A test was considered satisfactory only if the DNA virus was completely or almost completely inhibited by IUDR while the RNA virus was unaffected.

Estimation of size. Virus preparations were examined by Sandy McGregor with the electron microscope and size measurements made by using methods employed in this laboratory(19).

Neutralization tests. All sera were inactivated at 56°C for 30 minutes. Equal volumes of virus (approximately 100 TCD_{50}) and 4-fold dilutions of serum were mixed and incubated at 25°C for 2 hours. Then 0.2 ml of this mixture was inoculated into 2 to 4 tubes of WI-38 cells. Tubes were read every 2-3 days for one week. Simultaneous virus titrations were carried out in all instances. Neutralization with enterovirus antisera was done using combination pools described by Lim and Benyesh-Melnick(20).

Stabilization with inorganic salts. Rhinovirus candidates were heated at 50°C for one hour with and without molar $MgCl_2$(21) and molar $MgSO_4$(22) and infectivity titers determined.

Mouse pathogenicity. Newborn mice were inoculated intracerebrally and intraperitoneally with 2000 TCD_{50} of each rhinovirus. Mice were observed for signs of illness for at least 3 weeks before they were sacrificed.

Results. Isolation of the viruses. Rhinoviruses Baylor 1, 2 and 3 were isolated from naso-pharyngeal swabs of students in Houston, Texas between October 8, 1963 and March 30, 1964 (Table II). One strain of Baylor 1, 5 strains of Baylor 2, and 3 strains of Baylor 3 were found(12). Eight of the 9 strains were isolated initially in A-39 with 5 in A-39 *alone*. Four of the 9 were isolated in WI-38 with 1 strain in WI-38 *alone*. Once isolated, however, all strains grew readily in both A-39 and WI-38. Baylor 1 and Baylor 2 strains were isolated in the late winter and early spring of 1964 and grew only in tissue culture cells of human origin (H

TABLE III. Properties Which Characterize the Candidates as Rhinoviruses.

Virus	Size* (mμ)	Indirect evidence for nucleic acid type: Infectivity titer† With IUDR	No IUDR	Nucleic acid	Acid lability: Infectivity titer after exposure to pH 3.0	pH 7.0	Ether stability: Infectivity titer After ether exposure	Without ether
Baylor 1 (Tippett)	18-23	4.9	4.9	RNA	<1.8	4.3	5.5	4.8
" 2 (Crell)	18-23	5.0	5.0	"	<1.8	4.3	5.5	5.5
" 3 (Calvert)	18-23	5.0	4.5	"	<1.8	4.3	4.8	5.5

* Determined by electron microscopy.
† Infectivity titers expressed as $\log_{10}$ TCD_{50} per ml inoculum.

strains) while Baylor 3 strains were isolated in the fall of 1963 and could be passed in monkey kidney cells (M strains) after several passages in A-39 and WI-38. All strains of Baylor 1 and 2 could be recovered from initial naso-pharyngeal swabs after 6 months of storage in veal infusion broth at −30°C while only 1 or 3 strains of Baylor 3 were recovered after the 6 months storage period.

Properties of the viruses. Table III summarizes the essential properties of a rhinovirus. Baylor 1, 2, and 3 were found to be small viruses 18-23 mμ in diameter, uninhibited by treatment with IUDR and therefore probably RNA viruses, ether stable, and acid labile at pH 3.0. Baylor 2 and 3 were stabilized when heated at 50°C for one hour at pH 7 in the presence of M $MgCl_2$ or M $MgSO_4$, but Baylor 1 was only partially stabilized when heated in the presence of these salts (Table IV). When tested in the absence of Mg salts, Baylor 2 and 3 were more stable than Baylor 1 rhinovirus.

Neutralization tests. Reciprocal neutralization tests were performed using the 35 rhinovirus antisera listed in Table V and their corresponding viruses with the exception of rhinovirus 1734 which was not available but including rhinoviruses 11757 and Norman for which no antisera were available. No cross-reactions were detected between serotypes tested. In addition, rhinoviruses Baylor 1, 2, and 3 were tested against 62 enterovirus antisera (polioviruses 1, 2, and 3; group A cox-

TABLE IV. Stabilization of Rhinoviruses with Magnesium Salts.

Virus	Infectivity titer* room temperature in H_2O	Infectivity titer after heating for 1 hr at 50°C: With M $MgCl_2$	With M $MgSO_4$	Without Mg salts
Baylor 1	5.5	3.0	2.5	<1.8
" 2	5.5	5.2	5.4	2.0
" 3	4.8	4.3	3.8	3.0

* Infectivity titer expressed as $\log_{10}$ TCD_{50} per ml inoculum.

TABLE V. Rhinovirus Antisera Used in Reciprocal Neutralization Tests.

Antiserum	Animal in which sera made	Homologous titer	$TCID_{50}$ in test	Dilution used*
ECHO 28	Monkey	20,000	100	1:25
Feb	Goat	1280	32	1:10
Thompson	"	320	10	1:10
16/60	Calf	2560	32	1:10
HGP	"	640	100	1:10
B632	"	2560	32	1:10
1059	Goat	640	200	1:10
1734	Calf	640	320	1:40
33342	"	640	100	1:10
353	Goat	3200	200	1:10
363	"	640	32	1:4
1200	Calf	160	100	1:5
209	Guinea pig	320	32	1:4
1794	Goat	1280	32	1:10
56110	Guinea pig	5120	100	1:50
58750	" "	320	32	1:4
56822	" "	320	100	1:4
71560	" "	320	32	1:4
106F	Calf	1280	30	1:10
140F	"	1024	50	1:10
179E	"	640	30	1:10
164A	Guinea pig	1280	20	1:5
127-1	Calf	1280	30	1:10
113E	Guinea pig	20,480	30	1:100
137F	" "	3200	100	1:50
101-1	" "	5120	10	1:10
143-3	" "	640	30	1:4
151-1	" "	1600	20	1:6
342-H	" "	640	100	1:10
147-H	" "	1280	50	1:10
313G	" "	640	30	1:4
137-3	" "	800	100	1:10
Baylor 1	Baboon	600	100	1:20
" 2	"	3200	100	1:25
" 3	"	600	50	1:10

* All sera contained ≧20 antibody units against approximately 100 $TCID_{50}$.

sackieviruses 1-22 and 24; group B coxsackieviruses 1-6; echoviruses 1-7, 9, 11-27, 29-33) and against reovirus 1 antiserum. No neutralization was noted.

Mouse pathogenicity. Intracerebral and intraperitoneal inoculation of newborn Swiss mice failed to produce any detectable illness.

Discussion and summary. Three new rhinoviruses isolated from young adults have been shown to be serologically distinct from each other and from 33 previously described rhinoviruses, 62 enteroviruses, and reovirus type 1. These new viruses are small RNA viruses (18-23 mμ) that are ether stable and acid labile, thereby fulfilling the criteria necessary for classification as belonging to the rhinovirus subgroup of the picornaviruses(1,2). They are nonpathogenic for mice. Rhinoviruses Baylor 1 and 2 appear to be group "H" rhinoviruses since all strains of these serotypes grow only in tissue culture cells of human origin (A-39 and WI-38) while Baylor 3 seems to be a group "M" rhinovirus since strains of this serotype could be passed in tissue culture cells of monkey kidney. Several passages of Baylor 3 strains in A-39 and WI-38 were necessary, however, before successful passage in monkey kidney could be made. Eight of 9 strains were isolated in A-39 cells, which have proved to be very useful in working with the rhinoviruses. Most previously described rhinoviruses have been found to be stable when heated in the presence of M $MgCl_2$, a property described for the enteroviruses(21). Baylor 2 and 3 serotypes are stabilized by M $MgCl_2$ or $MgSO_4$ but Baylor 1 is only partially stabilized by these salts.

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