Rhinovirus Infection in Nursery and Kindergarten Children. New Rhinovirus Serotype 54. (31868)

C. C. MASCOLI, M. B. LEAGUS, M. R. HILLEMAN, R. E. WEIBEL AND J. STOKES, JR.
Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pa., and Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia

Reports from these laboratories(1-3) described the development and field evaluation among children in families and classrooms during 1964-65 of a new heptavalent killed respiratory virus vaccine containing respiratory syncytial, parainfluenza 1, 2 and 3, influenza A and B viruses and *Mycoplasma pneumoniae*. The field study was carried out in the Havertown-Springfield suburb of Philadelphia. Respiratory secretions collected from children with respiratory illness were tested for agents homologous to those of the vaccine and the findings were recorded(2,3). Since that time, the retained throat samples were tested further for presence of rhinoviruses. Twenty-six rhinovirus strains were isolated, 17 of which belonged to the group of 53 serotypes designated previously(4,5). Nine of the isolates were different and were classed in a new serotype which is designated rhinovirus type 54. The present report describes the isolation of the viruses and presents the serologic and epidemiologic findings in the study.

Materials and methods. Field evaluation. Details of the conduct of the study to evaluate the heptavalent respiratory vaccine, the collection of the respiratory samples, and the isolation and identification of agents corresponding to those in the vaccine have been described elsewhere(2,3). Altogether, 35 nursery and kindergarten classrooms in 14 private and parochial schools in the Havertown-Springfield area were represented. *Rhinovirus isolation.* The frozen pharyngeal specimens which had been collected in veal broth and stored at —70°C up to 6 months in flame-sealed ampoules in a dry ice refrigerator were thawed, treated with 150 units per ml of penicillin, 100 μg per ml of streptomycin, and 30 μg per ml of Fungizone and planted into 4 to 6 roller tube cultures of WI-26 or WI-38 strain of diploid human embryonic fibroblasts. The cultures were prepared and maintained in the manner described previously(4). The cultures were observed microscopically for cytopathic change for 14 days at which time a subpassage was carried out in the same manner in WI-26 or WI-38 cells. Further propagation of the isolates was in the human diploid cells. *Identification and typing.* All suspect rhinoviruses were tested for acid lability, RNA type, chloroform sensitivity, and H and M type by established procedure(6-8). All strains showing appropriate rhinovirus properties were then typed in serum neutralization tests by procedures published earlier(4,6) employing guinea pig antisera against prototype strains prepared here. Initial typing was by the "intersecting serum scheme" neutralization technique utilizing serum pools according to Schmidt *et al*(9) and adapted to rhinovirus study(4). The identity of each isolate was confirmed in tests with the corresponding monovalent antiserum. *Tests of rhinovirus 54 serotypes.* The nine type 54 rhinovirus isolates which were not typable with the 53 prototype antisera were subject to further study. Antisera against each strain were prepared in guinea pigs by methods described earlier(6) employing as antigen virus which was propagated in human diploid cells after 3 limiting dilution passages. Cross-serum neutralization tests were performed as described in the text.

Results. Agents recovered in the studies. The children in the classrooms ranged in age from 3 to 5 years and in families from 5 months to 6 years. All children in the classroom study group were seen twice weekly by visiting nurses and the children in the family groups were seen when occurrence of respiratory illness was reported by the parents. Throat specimens were collected from all children with respiratory illness. Table I shows the numbers of specimens which were

available for test and the numbers and kinds of viruses which were isolated. The isolations of agents other than rhinoviruses are recorded elsewhere(2,3) and are reproduced here for purpose of completeness. Whether the child was vaccinated was of no consequence for present discussion. Identical agents recovered in sequential throat specimens were counted only once.

Altogether, 126 agents were isolated from 831 respiratory samples taken from 516 children. Rhinoviruses were found only in specimens taken from children in classrooms. These were recovered from children in 15

TABLE I. Summary. Agents Recovered During 1965 During Heptavalent Respiratory Vaccine Studies.

Agent recovered	Classroom			Family		
	Vaccinated (199)* No. isolates (357 specimens)	Control (208) No. isolates (384 specimens)	Total (407) No. isolates (741 specimens)	Vaccinated (59) No. isolates (59 specimens)	Control (50) No. isolates (31 specimens)	Total (109) No. isolates (90 specimens)
Rhinovirus 16	2	2	4	—	—	—
" 25	2	1	3	—	—	—
" 38	2	5	7	—	—	—
" 42	1	0	1	—	—	—
" 48	1	0	1	—	—	—
" 50	1	0	1	—	—	—
" 54	5	4	9	—	—	—
RS	14	12	26	4	2	6
Adenovirus	2	8	10	0	1	1
RS and adenovirus†	1	2	3	2	1	3
Parainf 1	—	—	0	—	—	—
" 2	2	1	3	—	—	—
" 3	0	1	1	—	—	—
Mycoplasma pneumoniae	1	7	8	0	1	1
Influenza A	0	1	1	—	—	—
" B	8	12	20	3	1	4
Mumps	1	2	3	1	1	2
Herpes simplex	2	0	2	—	—	—
Total			106			20

* Numbers of children in the groups.
† Isolation of 2 viruses.

TABLE II. Origin of 26 Rhinovirus Strains Recovered During January-April, 1965 in the Heptavalent Respiratory Vaccine Study.

Case No.	Severity of illness	Date of specimen	Rhinovirus serotype recovered							School No. and classroom letter
50-371A	Moderate	1/28							54	7S
50-454A	Mild	"							54	14Y
50-393	"	2/2		25						7T
50-91	"	2/8			38					12F
50-79A	"	2/11			38					12F
50-364	"	2/15		25						7S
50-467	"	2/16							54	14Z
50-590	"	2/18	16							13I
50-427	"	2/22							54	2W
50-233	Moderate	2/25							54	11N
50-370	Mild	"				42				7S
50-436	"	"	16							8X
50-579	"	"	16							13I
50-379A	"	"		25						7S
50-528A	"	"						50		13I
50-594	"	3/2	16							13J
50-607A	"	3/5			38					11P
50-402	"	3/8							54	9U
50-525	"	3/30							54	6D
50-561	"	"			38					6G
50-243	"	4/1			38					11O
50-522	"	4/5							54	6C
50-558	"	"			38					6G
50-564	"	"			38					6G
50-517	"	4/8							54	6C
50-528B	"	"					48			13I
Total No. of strains			4	3	7	1	1	1	9	

of the 35 classrooms of 9 of the 14 schools represented in the study. Twenty-six rhinoviruses representing 7 serotypes were isolated from 741 specimens collected from children in the classrooms, a rate of only 3.5%. The overall recovery rate for all agents from all groups was 15%.

Origin of the rhinoviruses. Pertinent clinical and epidemiologic information relating to the 26 rhinovirus isolates is shown in Table II. The illnesses in all but 2 of the patients were mild consisting generally of rhinorrhea, pharyngitis and temperature no greater than 100°F (0). Two children exhibited moderately severe illness with rhinorrhea, pharyngitis and maximum temperatures of 100.3 and 101°F (0). Sampling of cases was made during 19 weeks from January 3 to May 14, 1965. Rhinovirus infections occurred sporadically during the entire period. Types 54 and 38 were most frequently isolated. There was some tendency for multiple occurrence of a particular rhinovirus type among school contacts. Thus three of four rhinovirus type 16 strains came from children in school 13; all 3 type 25 strains from school 7; two type 38 strains from school 11; two from school 12; three from school 6; 2 rhinovirus 54 strains from school 14 and three from school 6.

Establishment of rhinovirus serotype 54. The 9 rhinovirus isolates which failed to be neutralized by "intersecting scheme" antiserum pool representing prototypes 1 through 53 were examined further as shown in Table III. All 9 isolates showed antigenic homogeneity in cross-neutralization tests with the viruses and their antisera and were designated serotype 54. None of the type 54 isolates reacted with antisera against any of the other serotypes represented in the classrooms and antisera against the latter types did not neutralize any of the type 54 strains. Strains 50-522 and 50-525 were tested with individual guinea pig antisera representing serotypes 1 through 53 and neither was neutralized. These data established definitively that a different rhinovirus had been isolated. The type 54 rhinoviruses were shown to be of H group based on their growth in human

TABLE III. Establishment of Rhinovirus Serotype 54.

Rhinovirus guinea pig antiserum		Reciprocal of neutralizing antibody titer obtained in tests with serotypes														
		54										25	38	42	48	50
Serotype	Strain	50-233	50-371	50-402A	50-402B	50-427	50-454	50-467	50-517	50-522	50-525	5146	6660	6692	1983	477
54	50-233	160	160	80	160	80	80	80	80	80	80	0	0	0	0	0
"	50-371	160	160	80	160	80	80	160	80	160	80	0	0	0	0	0
"	50-402A	160	80	80	160	160	80	80	80	80	80	0	0	0	0	0
"	50-402B	160	80	80	160	80	80	160	160	80	80	0	0	0	0	0
"	50-427	160	160	160	160	160	80	160	160	160	160	0	0	0	0	0
"	50-454	160	160	80	160	160	80	160	160	160	160	0	0	0	0	0
"	50-467	160	160	160	160	160	160	160	80	160	160	0	0	0	0	0
"	50-517	160	160	160	160	160	80	160	160	160	80	0	0	0	0	0
"	50-522	160	160	160	160	160	80	160	160	160	160	0	0	0	0	0
"	50-525	160	160	160	160	160	160	160	160	160	160	0	0	0	0	0
25	5146	0*	0	0	0	0	0	0	0	0	0	160	0	0	0	0
38	6660	0	0	0	0	0	0	0	0	0	0	0	160	0	0	0
42	6692	0	0	0	0	0	0	0	0	0	0	0	0	160	0	0
48	1983	0	0	0	0	0	0	0	0	0	0	0	0	0	160	0
50	477	0	0	0	0	0	0	0	0	0	0	0	0	0	0	160
All other serotypes	1-53	—†	—	—	—	—	—	—	—	0	0	—	—	—	—	—

* 0 = <1:10.
† — = Not done.

but not in monkey renal cell cultures.

Discussion. The findings present continuing evidence for the role of rhinoviruses in acute respiratory illnesses among children, in this instance, among children 3 to 5 years of age who attended nursery or kindergarten classrooms. The proportion of cases of total respiratory illnesses caused by rhinoviruses cannot be determined at this time since there is no basis for assessing the efficiency of the virus isolation procedure for diagnostic purpose. The 3.5% rhinovirus recovery rate is consistent with the 2-6% range ordinarily found in young children and expectedly less than the average 14 or 15% recovery rate found in adults, adolescents and older children with acute respiratory infections(5,10-12).

The illnesses in the children were clinically classed as common cold. Only 2 of the 26 were febrile. The clinical observations were not sufficiently extensive to permit detection of possible lower respiratory tract involvement noted earlier(5,13).

The present findings are consistent with the previous demonstration of simultaneous occurrence in the human population of multiple serotypes of rhinovirus. Types 16 and 42 rhinovirus had been isolated previously from both adults and children, types 25 and 38 from children only, and type 50 from an adult only(4,6). The finding of only one new serotype among 26 new rhinovirus isolates was somewhat less than expected in view of the previous demonstration of 46 serotypes among 93 isolates(4,6). There appeared to be a clustering of particular serotypes among contacts in particular schools suggesting that there was considerable spread of infection, once a virus was introduced.

Our laboratory group previously designated the criteria by which rhinoviruses might be separated from other agents(6,8) and established a system of serologic classification of which 53 numbered serotypes(4-6) were designated among more than 100 rhinovirus strains which were studied. Serotyping was by the neutralization method using guinea pig antisera prepared against prototype strains. The utility of the system for rapid and practicable identification and serotyping of new rhinovirus isolates was amply proved in the present study. The existence of a new serotype 54 was established and this was added to the present classification system. Strain 50-525 is tentatively designated as the prototype but it is possible that earlier isolations of this same virus might have been recorded(14-18) since we have not had the opportunity to examine these recently reported strains.

Summary. Twenty-six rhinoviruses were recovered from 741 throat specimens (3.5%) taken during acute respiratory illness in the winter and early spring of 1965 from 407 children 3 to 5 years of age who were attending nursery and kindergarten classrooms. No rhinoviruses were isolated from 90 specimens taken from 109 children aged 5 months to 6 years in families. Rhinovirus serotypes 16, 25, 38, 42, 48 and 50 were represented in addition to a new H group serotype which was designated type 54. Types 16, 38 and 54 were most frequently encountered. There appeared to be a clustering of particular serotypes among the contacts in particular schools indicating spread of infection once a virus was introduced. All cases were clinically common cold and only two had mild fever.

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1. Woodhour, A. F., Sweet, B. H., Tytell, A. A., Potash, L., Stokes, J., Jr., Weibel, R. E., Metzgar, D. P., Hilleman, M. R., Am. Rev. Resp. Dis., 1966, v94, 350.

2. Weibel, R. E., Stokes, J., Jr., Leagus, M. B., Mascoli, C. C., Hilleman, M. R., ibid., 1966, v94, 362.

3. Leagus, M. B., Weibel, R. E., Mascoli, C. C., Stokes, J., Jr., Hilleman, M. R.,, ibid., in press.
4. Hamparian, V. V., Leagus, M. B., Hilleman, M. R., Proc. Soc. Exp. Biol. and Med., 1964, v116, 976.
5. Hilleman, M. R., Ergebnisse der Mikrobiol. Immunitatsforsch. und Exp. Ther., in press.
6. Ketler, A., Hamparian, V. V., Hilleman, M. R., Proc. Soc. Exp. Biol. and Med., 1962, v110, 821.
7. Hamparian, V. V., Ketler, A., Hilleman, M. R., ibid., 1961, v108, 444.
8. Hamparian, V. V., Hilleman, M. R., Ketler, A., ibid., 1963, v112, 1040.
9. Schmidt, N. J., Guenther, R. W., Lennette, E. H., J. Immunol., 1961, v87, 623.
10. Hilleman, M. R., Hamparian, V. V., Ketler, A., Reilly, C. M., McClelland, L., Cornfeld, D., Stokes, J., Jr., J.A.M.A., 1962, 180, 445.
11. Gwaltney, J. M., Jr., Jordan, W. S., Jr., Bact. Rev., 1964, v28, 409.
12. Chanock, R. M., Mufson, M. A., Johnson, K. M., Progr. Med. Virol., 1965, v7, 208.
13. Reilly, C. M., Hoch, S. M., Stokes, J., Jr., McClelland, L., Hamparian, V. V., Ketler, A., Hilleman, M. R., Ann. Int. Med., 1962, v57, 515.
14. Webb, P. A., Johnson, K. M., Mufson, M. A., Proc. Soc. Exp. Biol. and Med., 1964, v116, 845.
15. Phillips, C. A., Melnick, J. L., Grim, C. A., ibid., 1965, v119, 798.
16. Mufson, M. A., Kawana, R., James, H. D., Jr., Gauld, L. W., Bloom, H. H., Chanock, R. M., Am. J. Epidem., 1965, v81, 32.
17. Hamre, D., Connelly, A. P., Jr., Procknow, J. J., J. Lab. Clin. Med., 1964, v64, 450.
18. Gwaltney, J. M., Jr., Jordan, W. S., Jr., Am. Rev. Resp. Dis., 1966, v93, 362.

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Comparison of Buoyant Density of Two Mutants of ECHO 6 by Density Gradient Ultracentrifugation.* (31869)

ARLENE R. COLLINS, ALMEN L. BARRON AND SIDNEY SHULMAN†
Department of Bacteriology and Immunology, School of Medicine, State University of New York at Buffalo, Buffalo

Two plaque mutants of ECHO 6 virus, designated m^+(large) and m(minute) have been studied in primary rhesus monkey kidney cell culture (PRMK)(1,2). The characteristics which serve to differentiate these two mutants are as follows: The m^+ mutant predominated at low passage levels in PRMK, was weakly cytopathic, and yielded low infectivity titers. In contrast, the m mutant predominated at high passage levels, was more destructive to cells, and yielded high infectivity titers. It was further shown by Suto *et al*(3) that the m mutant had a selective growth advantage over the m^+ mutant in PRMK. The m^+ mutant was weakly neutralized by ECHO 6 antiserum, while the m mutant was well neutralized by the same antiserum. Plaques produced by m^+ in PRMK were large (3-7 mm), in contrast to m plaques which were much smaller (less than 1 mm) in size. In addition, plaque formation with m was inhibited by sulfated polysaccharides present in agar. This inhibition could be readily reversed by the addition of protamine to the agar medium(2).

For further characterization of the 2 mutants, the buoyant density in a cesium chloride density gradient was compared.

Materials and methods. Cell cultures. Primary rhesus monkey kidney cell cultures (PRMK) were grown in a medium containing 0.5% lactalbumin hydrolysate and 2.0% calf serum in a base of Hanks' balanced salt solution.

A continuous line of cells, designated BGM developed in this laboratory from African green monkey kidney was also used. BGM cells were grown in Eagle's minimum essential medium in a base of Earle's saline with 10% calf serum added.

Virus. The m and m^+ mutants studied in this investigation were obtained from the Charles strain of ECHO 6 virus. After 3

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