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Grouping and Occurrence of RI (Prototype RI-67) Viruses.* (22097)

M. R. HILLEMAN, J. H. WERNER, AND M. T. STEWART.

Department of Virus and Rickettsial Diseases, Walter Reed Army Institute of Research, Washington, D.C.

Viruses of the recently discovered RI family(1-4) which includes agents designated APC(5-8) or ARD(9) may cause undifferentiated acute respiratory disease (ARD)(10), non-*Streptococcal* exudative pharyngitis(11), primary atypical pneumonia unassociated with cold agglutinins(11), pharyngo-conjunctival fever(5-7), mesenteric lymphadenitis(12) or inapparent infections(2,4) in man. These viruses are heterogeneous antigenically(1,6,7,13) comprising a group of distinct serotypes(6,7) which are readily differentiable by the serum neutralization technic; however, they also elaborate a common group-specific "soluble" complement-fixing antigen which is readily separable from the virus(7,14,15) and which appears to be of little if any significance in immunity to RI disease(2,4). The seemingly ubiquitous distribution of RI agents not only in patients who are sick but also in latent or "masked" form in human adenoid or tonsil tissue(6,7, 16) poses the problem of determining which

* The majority of these observations were presented at 39th annual meeting of Am. Assn. of Immunologists, San Francisco, Apr. 11-15, 1955.

serotypes are the more important in causing respiratory illness and which are less frequently associated with human disease. The present paper summarizes the results of a study to determine the occurrence of the RI virus types in epidemics of acute respiratory illness among newly recruited soldiers in training. The antigenic grouping of the RI viruses based on tests with serum pairs from patients is also presented. Additionally, information concerning the serological response to homologous and heterologous virus serotypes in natural and experimental infections in man is summarized.

Materials and methods. Clinical. The patients with acute respiratory illness from whom blood samples and throat washings were obtained were U.S. Army personnel stationed at Fort Dix, N. J., Fort Leonard Wood, Mo. or Fort Ord, Calif.† during the winter of 1953, 1954 or 1955 when there was a high prevalence of infection with the RI viruses. Other articles(1-4,17) describe these epidemics. Sera were also available from volunteers who had been inoculated experimentally with the RI-67 strain of virus (18). *Laboratory. Virus isolation.* The methods employed for recovery of viruses from patients' throat washings, for propagating these agents, and for preparing complement-fixing (CF) antigens in HeLa(19) cell tissue cultures‡ were described earlier(1). *Antigenic typing of strains.* The recovered viruses were typed by the serum neutralization procedure(1) in HeLa cell cultures employing specific rabbit antisera according to the classification system§ of Rowe *et al.*(7). Seed virus stocks used to immunize rabbits or employed in the neutralization tests were prepared in HeLa cell tissue cultures. Neither antiviral antisera nor antisera prepared in rabbits against uninfected HeLa culture material had a demonstrable adverse effect upon the cells in the tissue cultures when tested at a dilution of 1:4. *Serology.* Serum neutralization and CF tests with the RI viruses and patients' sera were performed according to procedures outlined(1) for use with the RI-67 strain. Serum titers were expressed as the greatest initial dilution of patient's serum which caused complete or nearly complete suppression of viral growth (neutralization) or of fixation of complement. In the neutralization tests presented in Table I, one serum pair was tested with all 11 viruses on one day. The same seed virus stocks were employed throughout.

Results. Determination of viruses important in recruit disease and antigenic grouping of strains. The acute and convalescent sera from 20 soldiers with undifferentiated acute respiratory disease (ARD) or primary atypical pneumonia of RI etiology as proved by results of CF tests were examined for increase in neutralizing antibody against 11 RI virus strains which had been isolated from patients suffering with these illnesses and also against types 1, 2, 5 and 6 of the latent viruses recovered by Rowe, Huebner *et al.* from human adenoid or tonsil tissue. The findings, presented in interpretive form in Table I, express in terms of numerical designations the relative fold-increase in serum titer of each serum pair against each virus. In the tests, each convalescent serum showed a 32-fold or greater rise in titer for at least 1 virus of the group. It is seen in the table that by far the greatest increase in antibody was against the viruses which were recovered from soldiers who were ill with respiratory disease. By contrast, the serological response to the types 1, 2, 5 and 6 latent viruses was notably weak or absent. Thus, these particular latent agents did not appear to be of etiological significance in the illnesses among the military population in this study. Further, they were of little importance in the studies of others (9) who have examined military populations.

† We are indebted to Lt. Col. T. O. Berge for supplying the 5 strains of virus recovered from patients at Fort Ord, and to Lt. Col. Berge, Lt. Col. H. E. Shuey, Col. L. G. Thomas, Dr. E. H. Lennette, and Capt. J. J. McCue for the serum specimens from this location.

‡ HeLa cell cultures and tissue culture fluids employed were obtained from Microbiological Associates, Inc., Bethesda, Md.

§ Prototype 1, 2, 3 (strain GB), 5 and 6 viruses were obtained from Dr. R. J. Huebner and type 7 virus, recently described by Berge *et al.*(20) was supplied by Lt. Col. T. O. Berge.

TABLE I. Grouping of RI Virus Strains Based on Increase in Neutralizing Antibody in Paired Sera from Patients with Respiratory Illness. Interpretive summary.

Sera from RI patient		Increase in neutralizing antibody against RI strains													
		Viruses recovered from soldiers ill with respiratory disease										Latent viruses			
		Group A–Type 4		Group B–Type 7					Group C–Type 3				T1†	T2	T5	T6
No.	Fort*	67	C-1	4-202	4-239	4-204	4-218	3-83	3-110	C-2	3-131	GB	AD-71	AD-6	AD-75	TD-99
4-385	Ord	5	5	2	2	2	2	2	2	0	0	0	0	0		
465	"	5	5	0	3	1	1	3	0	0	0	0	2	0		
3- 33	L. Wood	5	5	1	2	2	0	2	0	2	2	2	0	0		
63	"	5	5	0	0	0	0	0	0	3	0	0	0	0		
4-245	Dix	5	5	2	0	0	0	0	1	1	2	1	1	0	0	0
306	L. Wood	5	5	1	0	1	3	0	0	0	0	0	2	1	0	3
354	Ord	5	5	0	0	0	0	0	0	0	0	0	2	0		
4-305	L. Wood	1	1	5	5	3	1	1	0	0	0	1	0	0		
316	"	0	2	5	3	5	3	3	0	0	0	0	0	0		
472	Ord	1	0	1	5	0	3	5	0	0	1	0	0	0		
488	"	1	0	0	5	0	1	3	0	0	0	0	0	0	0	0
302	L. Wood	2	1	5	3	3	3	3	0	0	0	0	0	0		
202	Dix	0	0	5	3	3	1	1	0	0	0	0	0	0	0	0
239	"	0	0	4	5	3	3	3	2	0	2	0	0	0		
4-233	Dix	5	5	3	5	3	5	3	5	4	5	3	0	0	0	0
3- 57	L. Wood	3	3	1	5	1	2	3	2	0	2	2	0	0		
26	"	5	5	0	0	0	0	0	3	5	3	5	0	0		
4-237	Dix	0	0	4	4	2	4	4	4	5	4	5	0	0		
353	Ord	0	0	5	3	3	3	4	2	2	4	4	0	0		
3-134	L. Wood	0	0	1	5	1	0	3	1	2	0	2	0	0	0	0

A designation of 5 indicates the maximum increase in titer obtained with the given pair of sera. A 4 indicates a rise in titer half as great as the maximum; 3, ¼ as great; 2, ⅛ as much progressing consecutively to no rise which is represented by 0.

* Fort Ord, Calif.; Fort Leonard Wood, Mo.; Fort Dix, N. J.

† T = type.

TABLE II. Interpretive Summary of CF Test Results in Paired Sera from Patients with RI Infection. (The system for designation is explained in Table I.)

Sera from RI patient No.	Increase in complement-fixing antibody against RI strains											
	Group A–Type 4		Group B–Type 7				Group C–Type 3		T1*	T2	T5	T6
	67	C-1	4-202	4-239	4-204	4-218	3-110	GB	AD-71	AD-6	AD-75	TD-99
4-245	4	5	5	5	5	5	5	5	5	5	5	5
306	5	5	5	5	5	5	5	5	5	5	5	5
354	5	5			5	5			4	5		
4-488	4	4	4	5	5	5	4	4	4	4	5	5
202	5	5	5	5	5	5	5	5	4	4	4	4
239	3	3	4	4	5	4	5	5	2	2	0	2
4-233	5	5	5	5	5	5	5	5	5	4	5	4
3- 57	4	5	4	4	4	4	4	3	3	2	5	4
4-237	5	5	5	5	5	5	5	5	5	4	5	5
353	3	4	4	5	5	4	5	5	3	3	4	3
134	5	5	4	5	4	5	5	5	5	5	5	5

* T = type.

Table I also showed that the viruses recovered from patients with respiratory disease were antigenically heterogeneous. Nevertheless, the strains could be divided into 3 separate groups, designated A, B or C in Table I, based on the reaction with the paired sera from the patients. The first 7 pairs of sera listed showed a sharp rise in titer (designated 5) against the group A strains but little or none against the B or C agents. Conversely, the next 7 pairs of sera listed in Table I usually presented a marked increase in antibody against the B but not against the A or C viruses. The last 6 pairs of sera gave strong rises against the C viruses, and also against either the A or B strains or both. Subsequent typing of these agents with monotypic rabbit antisera demonstrated all of the A strains to belong to Rowe *et al.'s* type 4 while all of the B and C strains belonged to types 7 and 3, respectively. Thus, there was complete agreement as regards grouping of the strains either when the viruses were typed with paired sera from cases or with monotypic rabbit antisera. It is to be noted additionally in Table I that there was greater heterogeneity within the B and C groups than among the A strains. Thus, the 2 A viruses reacted to the same extent with all of the first 7 pairs of sera while, by contrast, the B and C strains varied markedly in their reaction to the second and third groups of sera, respectively. This indicated that there was minor antigenic heterogeneity within these types which could be detected with human sera but which was not revealed in the tests with rabbit antisera.

Complement-fixation findings. The results of CF tests performed with a portion of the paired human sera shown in Table I and representative viruses are presented in Table II. The CF findings were strikingly group-specific in contrast to the highly type-specific reactions obtained in the neutralization test. Serum pair 4-239 was the notable exception in that it showed a weak reaction with the type 1, 2, 5 and 6 latent virus antigens even though the reaction with types 4, 7 and 3 was strong. This finding suggested that there may be a small degree of specificity demon-

TABLE III. Distribution of RI Virus Types among Strains Recovered from Military Patients.

Installation	Time of collection of specimens	No. of strains of type: 3	4	7	Total No. of strains
Fort Dix, N. J.	Jan. 11-13, '54			11	11
	" 31, '55	2	5	2	9
Leonard Wood, Mo.	" 4-9, '53	2	1	2	5
Ord, Calif.	Winter, '54	1	2	2	5*
	Total	5	8	17	30

* Viruses recovered by Lt. Col. T. Berge, Fort Baker, Calif.

TABLE IV. Distribution Neutralizing Antibody Increases for RI Viruses among 25 Patients with RI Infection.

Fold-increase in neutralizing antibody titer	No. of persons showing antibody increase against RI strains		
	Type 3 (3-110)	Type 4 (RI-67)	Type 7 (4-202)
0	15	4	7
2	2	2	3
4	1	3	3
8	2	6	4
16	2	0	
32		9	3
64	1		
128	2	1	4
256			1
No. positive (4-fold or > increase)	8	19	15

strable among the "soluble" antigens of these viruses when tested with sera from certain patients.

Distribution of virus types among the patients. Thirty RI virus strains recovered from patients with respiratory disease at Fort Dix, Fort Leonard Wood or Fort Ord were typed and the distribution of the various serotypes in these military camps is shown in Table III. It is seen that types 3, 4 and 7 only were represented and that all 3 types were present in all 3 camps in spite of their wide geographic separation. The 1954 outbreak at Fort Dix was of special interest in that it was largely if not entirely due to type 7 virus alone(17). To obtain further information about the occurrence of these agents, the paired sera from 25 patients with RI infection selected from the same populations in the same epidemics from which the viruses in Table III were recovered were tested for increase in neutralizing antibody against type 3, 4 and 7 viruses. Table IV, which summarizes the increases in antibody against the strains, showed that 8 patients developed a significant rise in titer against type 3 virus, 19 against type 4 and 15 against type 7. Although one cannot always be confident of the infecting type when the serological response alone is measured (see Table V,) the data are sufficiently clear to permit the conclusion that types 4 and 7 virus were the most common in these particular military outbreaks.

Homotypic and heterotypic response to infection. The findings in tests to determine the type-specificity of the serological response to natural infection among patients in whom the infecting type was definitively established by recovery of virus are summarized in Table V. The increases in neutralizing antibody were predominantly homotypic although the heterologous response to type 3 or 4 virus in the last three cases in the table was as large or larger than that against the type 7 strains which were recovered from the throat washings. Patient 4-246, in whom the heterologous response was 16 times greater than the homologous, provided the most outstanding example of this experience. The serologic findings and the identity of the isolate were determined on several occasions. The possibility cannot be excluded, however, that the patient may have been infected with both type 3 and 7 virus within the time period for collection of the serum specimens. Similar tests to determine the heterotypic response in patients infected experimentally with the RI-67 strain of type 4 virus by several routes are summarized in Table VI. It is noteworthy that the neutralizing antibody response in these individuals was entirely type-specific and there was no rise to either heterologous type. It is of further interest that the artificial infection gave no heterotypic booster effect even in the patients who had antibody against the heterologous virus prior to inoculation with the RI-67 agent.

Discussion. It is evident that the RI viruses recovered from patients with respiratory illness exhibited marked antigenic heterogeneity when tested by the neutralization method with paired sera from cases and that these strains were clearly differentiated into three serological groups. These groups, which correspond to types 3, 4 and 7 as determined by tests with monotypic rabbit antisera(7), showed further minor antigenic heterogeneity within types 3 and 7, the significance of which remains to be determined.

All the viruses recovered from patients belonged to type 3, 4 or 7 and the antibody response in the persons with respiratory disease was against these 3 types primarily. There was little or no antibody produced against the type 1, 2, 5 or 6 latent viruses(7,16) recov-

TABLE V. Homotypic and Heterotypic Neutralizing Antibody Response in Patients with RI Infection from Whom Virus Was Recovered.

Case No.	Virus isolate type	Serum specimen	Antibody titer: RI group CF	Antibody titer: Neutralizing Type 3	Antibody titer: Neutralizing Type 4	Antibody titer: Neutralizing Type 7
55- 11	3	A*	0†	0†	0	0
		C	80	512	0	8
55- 33	3	A	0	0	2	0
		C	5	128	2	0
55- 9	4	A	0	0	0	0
		C	160	8	32	0
55- 8	4	A	0	2	0	0
		C	20	8	32	2
4-229	7	A	0	0	0	0
		C	80	0	8	512
4-239	7	A	0	ND‡	0	2
		C	20		0	512
4-202	7	A	0	ND	0	0
		C	160		0	32
4-207	7	A	0	ND	0	0
		C	160		8	32
55- 6	7	A	0	0	0	0
		C	20	0	8	32
4-204	7	A	5	ND	0	0
		C	80		2	8
4-215	7	A	0	ND	0	0
		C	40		2	8
4-201	7	A	0	0	2	0
		C	20	0	2	8
4-218	7	A	0	ND	0	0
		C	80		8	8
4-233	7	A	0	ND	0	0
		C	80		128	32
4-246	7	A	0	0	0	0
		C	10	128	0	8

* A = acute, and C = convalescent specimen collected 3 or 4 wk later.
† 0 titer in CF test = less than 1:5 and in neutralization test = less than 1:2, the lowest serum dilutions tested.
‡ ND = Tests not done.

ered from human adenoid or tonsil tissue in tissue culture and these latter agents did not appear to be of etiological significance in any of the cases studied. All 3 serotypes 3, 4 or 7 were present in troops in each of the 3 camps and it is to be noted that the personnel in these installations were recruited from the eastern (Fort Dix), central (Fort Leonard Wood) and western (Fort Ord) regions of the United States. Types 4 and 7 virus appeared most often among the soldiers while type 3 was less prevalent. One may speculate that the less frequent occurrence of type 3 might have been related to immunity resulting from prior infection with this agent which is known to cause epidemics in children(5,8).

Patients infected naturally with type 3, 4 or 7 virus usually exhibited antibody increase of low order against at least one other type even though antibody against the heterotype was absent from the acute phase specimen. By contrast, volunteers given type 4 virus (RI-67) by the respiratory route or parenterally gave a homotypic response only even in persons who had antibody initially against another type. The reason for this difference was not evident although it may be related to the fact that the infection in the volunteers was inapparent or associated with minor illness(18).

The present findings are of importance in the laboratory diagnosis of RI infections and in any consideration of immunologic control of the disease. For routine laboratory diag-

TABLE VI. Homotypic and Heterotypic Neutralizing Antibody Response in Volunteers* Given RI-67 Virus.

Patient No.	Age and sex	Virus inoc. route	Serum specimen†	Antibody titer: CF‡ RI group	Neutralizing‡ Type 3	Neutralizing‡ Type 4	Neutralizing‡ Type 7
1	71 ♂	Nebulizer	Pre	0	2	0	8
			Post	0	2	32	8
5	29 ♂	Nebulizer & intraderm.	Pre	0	8	0	0
			Post	160	8	128	0
6	53 ♂	*Idem*	Pre	0	2	8	0
			Post	5	2	128	0
7	54 ♂	"	Pre	0	0	8	0
			Post	40	0	512	0
8	43 ♀	"	Pre	0	0	0	0
			Post	160	0	512	0
9	63 ♂	Intranasal & intraderm.	Pre	0	0	2	0
			Post	10	0	32	0
10	21 ♂	Intramusc.	Pre	0	0	0	2
			Post	80	0	512	2
11	55 ♀	Intramusc. & intraderm.	Pre	0	2	0	0
			Post	40	2	512	0
12	43 ♀	Intramusc.	Pre	0	2	2	0
			Post	20	2	128	0
13	53 ♂	Intramusc. & in'tumor	Pre	0	2	0	0
			Post	0	2	128	2

* Details of the volunteer experiments will be reported elsewhere(18).

† Pre-inoculation specimen collected immediately prior to administration of virus and post-inoculation sample taken 8 to 45 days later.

‡ 0 titer = less than 1:2 in the neutralization test; less than 1:2 in the pre-inoculation CF samples and less than 1:5 in the post-inoculation specimen.

nosis of RI infections, the CF test is the method of choice since a single antigen suffices to detect antibody increase in all cases. Where additional information of the serotype is important, the specific neutralization test with types 3, 4 and 7 virus should be employed or the virus itself should be recovered and typed. As regards immunization of newly recruited soldiers, one would certainly wish to induce resistance against types 3, 4 and 7 viruses.

Summary. Viruses of the RI family which caused acute respiratory illness among soldiers at Fort Dix, N. J., Fort Leonard Wood, Mo., and Fort Ord, Calif. during the last few years were separated into 3 groups based on neutralization tests with paired sera from cases. These 3 groups of agents corresponded to serotypes 3, 4 and 7 of Rowe *et al.* determined by tests with monotypic rabbit antisera. Latent virus types 1, 2, 5 and 6 which were recovered originally from tonsil or adenoid tissues by others did not appear to be of etiologic importance in the military illnesses. The CF test results, in contrast to the neutralization findings, were markedly group-specific. The importance of strain heterogeneity in relation to diagnosis and immunity is discussed.

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Multiplication of Virulent Poliovirus in Capuchin Monkey Kidney Cultures Without Microscopically Observed Cytopathogenicity.* (22098)

ALBERT S. KAPLAN† AND JOSEPH L. MELNICK.
Section of Preventive Medicine, Yale University School of Medicine, New Haven.

Previous work from this laboratory demonstrated that the South American ringtail monkey (*Cebus capucina*) has a limited susceptibility to poliovirus *in vivo*(1). Recent experiments showed that the Type 2 Y-SK strain, highly cytopathic for rhesus kidney cultures, was without demonstrable effect in capuchin cultures(2). The present report extends the earlier findings in demonstrating that Type 1 human strains can multiply in epithelial cultures of capuchin kidney, even though no obvious cytopathic changes could be observed. In the course of these experiments, material became available to determine whether capuchin cultures, which supported virus growth with no apparent cell destruction, could act as a selective medium for the propagation of non-neurotropic variants of poliovirus‡(3-8).

Materials and methods. Four Type 1, four Type 2 and three Type 3 strains were employed, both as stool suspensions of patients ill with poliomyelitis and as the first passage material from infected rhesus kidney cultures (in which they were all markedly cytopathogenic). The procedures in use in this laboratory for growing cultures, for isolation and titration of viruses have been reviewed recently(9). In order to test for survival of virus under the conditions of the experiments, control tubes were inoculated and incubated together with the capuchin cultures. The control tubes contained medium alone (without cells) or rhesus cells that had been killed by freezing. Normal controls of uninoculated capuchin cultures were also included. For each test 0.1 ml of fluid was inoculated into a series of 8 to 10 monolayer cultures of capuchin kidney cells and 0.9 ml of lactalbumin hydrolysate medium(9) was added. The cultures were allowed to incubate for various times; at the end of each incubation period, the fluids were harvested, and fresh medium was added if the incubation was to be continued. Titrations were carried out in rhesus kidney cultures, using 8 tubes per dilution, since the capuchin cultures showed no apparent cytopathic alterations. The plaque technic was carried out according to the method of Dulbecco and Vogt(10). Tests for neurotropic properties of the Type 1 strains were carried out by inoculating the first or second passage capuchin culture fluid into

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† Present address: Department of Bacteriology, University of Illinois, Urbana.

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