Antibody to Rhinovirus in Human Sera. I. Standardization of a Neutralization Test* (32724)

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There is at present only one serological method, virus neutralization, for measuring antibody to rhinoviruses. As usually performed, this test is quite insensitive (1,2). The desirability of a rapid, reproducible, and more sensitive test is apparent. In order to improve sensitivity, it has been suggested that smaller doses of virus be used in neutralization procedures (3). Using such a test, increased sensitivity has been reported in measuring antibody rises following experimental and in natural infections in humans (4,5). The present study was designed to evaluate the reproducibility of a neutralization test for rhinoviruses using low doses of viruses.

Materials and Methods. Sera. Serum samples were obtained from 20 adult males and divided into 2 aliquots. Each aliquot was assigned a code number and labeled with this number only. Ten pairs of aliquots with code numbers 1-1 through 1-20 were used in neutralization tests against rhinovirus types 1A (strain 2060), 13 (strain NIH 353), 14 (strain NIH 1059), 15 (strain NIH 1734), and 16 (strain NIH 11757) (6,7), and 10 duplicate pairs of aliquots with code numbers 2-1 through 2-20 were tested against rhinovirus types 2 (strain HGP), 17 (strain NIH 33342), and Oliver (6,8). Oliver is the designation given a strain isolated in a previous volunteer study (4); it is an acid-labile, ether-stable virus that produces a picornavirus-like cytopathic effect (CPE) in human diploid cells, but not in monkey kidney cells. In cross-neutralization tests with hyperimmunized guinea pig sera, it is not neutralized by antisera to the other rhinoviruses used in this study. These tests were all performed by the same technician who did not have access to the key to the serum code.

Neutralization tests. Neutralization tests were performed in human embryonic fibroblast tissue cultures (WI-38) maintained in equal parts of medium no. 199 and Eagle's medium no. 2 to which were added 2% heat-inactivated calf serum, 0.4% saturated solution sodium bicarbonate, and antibiotics. Virus stocks were derived from prototype strains which had been passaged 14–22 times (including triple terminal dilution) in human embryonic carcinoma (KB) and human embryonic fibroblast cultures. Equal volumes of virus stocks calculated to contain 3.2 to 16 fifty percent tissue culture infectious doses ($TCID_{50}$) per 0.1 ml were incubated at room temperature for 2 hours with serial 4-fold dilutions of heat-inactivated serum (56°C, 30 min). A 0.2 ml portion of this mixture was inoculated into each of 2 culture tubes, and 10-fold dilutions of the test dose of virus were inoculated into each of 10 culture tubes. Cultures were incubated at 34°C on a drum rotating at 12 rev/hour and were observed for CPE for 3 consecutive days beginning when 3.2 or more $TCID_{50}$ of test virus were estimated to be present by the Spearman-Karber method. Results were recorded by the following system: <5 = one to four foci of CPE observed, 0-1 = five or more foci of CPE to 25% of the cell sheet involved with CPE, 1+ = 25%, 2+ = 50%, 3+ = 75% and 4+ = 100%. Serum titers were estimated by the Karber method as the initial

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dilution of serum inhibiting CPE in 50% of cultures for each day of assessment considering cultures with <5 foci of CPE as negative and calculated again considering cultures with <5 foci as positive. After recording the corresponding titers to each day and each method of assessment for each serum number, the code was broken by one of the investigators, and the results on each aliquot of each pair compared.

Results. Neutralizing antibody titers. The results for each day of observation and each method of assessing neutralization tests utilizing the first series of 10 duplicate serum samples against rhinovirus types 1A, 13, 14, 15, and 16 are shown in Table I, and the results of testing the second series of 10 duplicable serum samples against rhinovirus types 2, 17, and Oliver, are shown in Table II. As can be seen, serum antibody titers ranged between $<1:2$ and 1:1024, and for a single serum specimen, titers varied over the 3-day period of observation by as much as 16-fold. Doses of virus in each test varied between 3.2 and 100 $TCID_{50}$.

Testing (Wilcoxon test) revealed that the two groups of sera were similar in regard to geometric mean antibody titers to the test viruses, average differences between antibody titers on duplicate aliquots, and standard deviations of differences. Therefore, the data from the two groups of sera (Table I and Table II) were combined for analysis.

Days and methods of assessment. To assess the variability between tests on duplicate serum samples according to day of observation and method of assessment, titer differences in $\log_2$ units were calculated for each method of assessment and for each of the 3 days of observation. The difference was considered positive if the higher code numbered member of a pair had a higher titer than the lower numbered member, and negative if the reverse were true. As shown in Table III, the results suggest that there was greater variability between duplicate samples on the first than on the second or third days of observation, and this finding is confirmed by the standard deviations of the differences. Moreover, on the second and third day of observation, there was less variability between samples when cultures with 1–4 foci of CPE (<5) were considered negative than when these were considered positive. The standard deviation of results for the second day, <5 negative method, is the smallest of the six. In addition, considering cultures with <5 foci of CPE as negative has the added technical advantage of faster recording of results since 0–1+CPE is quickly and easily recognized whereas searching for a single small focus is time consuming. For these reasons, subsequent neutralization test results were calculated from observations of CPE on the second day of observation and cultures with <5 foci of CPE were considered negative.

Distribution of differences. As shown in Fig. 1, the frequency distribution of the differences between antibody titers of duplicate samples for all tests (<5 negative, second day reading) suggests a normal distribution. The frequency of 4-fold or greater differences between duplicate samples was 15.6%, of 8-fold or greater differences was 5.2%, and of 16-fold or greater differences was 2.6%.

Influence of rhinovirus serotype. Antibody titer differences, in $\log_2$ units, between duplicate serum samples were calculated for each of the 8 rhinovirus serotypes tested. Standard deviations of the differences and the geometric mean titers of the lower numbered member of each pair were also calculated and are shown

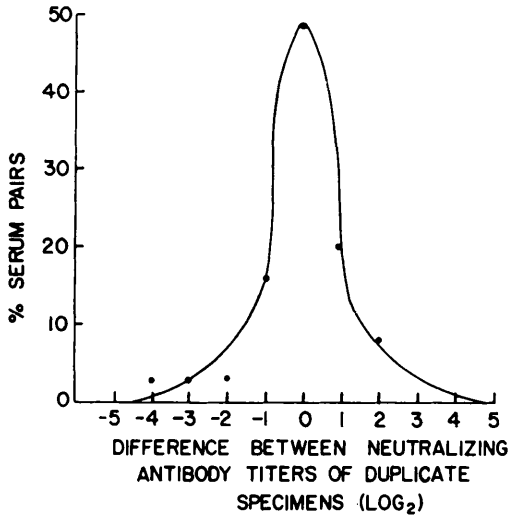

FIG. 1. Distribution of $\log_2$ differences between neutralizing antibody titers of 77 duplicate serum samples, Day 2 observation, cultures with <5 foci of CPE considered negative.

TABLE I. Rhinovirus Neutralizing Antibody Titers in Duplicate Serum Samples According to Day and Method of Assessment.

Virus type:	1A						13						14						15						16					
Day:	1		2		3		1		2		3		1		2		3		1		2		3		1		2		3	
Matching serum pairs	<5 Neg[a] 6[c]	<5 Pos[b] 10	<5 Neg 10	<5 Pos 10	<5 Neg 10	<5 Pos 10	<5 Neg 3.2	<5 Pos 6	<5 Neg 10	<5 Pos 10	<5 Neg 32	<5 Pos 32	<5 Neg 3.2	<5 Pos 10	<5 Neg 10	<5 Pos 10	<5 Neg 10	<5 Pos 10	5 Neg 3.2	5 Pos 6	5 Neg 6	5 Pos 6	5 Neg 10	5 Pos 10	5 Neg 3.2	5 Pos 3.2	5 Neg 3.2	5 Pos 3.2	5 Neg 3.2	5 Pos 3.2
1–1	NT[d]	NT	NT	NT	NT	NT	<2		<2		<2		16	16	4		4	4	<2		<2		<2		64		64		64	
1–17	NT	NT	NT	NT	NT	NT	<2		<2		<2		16	8	4		4	2	<2		<2		<2		256		256		256	
1–2	16		4		4		2		2		2		8	2	2		<2		16	8	4	2	2		64	64	64		64	
1–15	2		2		2		2		2		2		<2	<2	<2		<2		8	8	8	8	8		16	8	8		8	
1–3	4		4		4		8	4	4		4		8	8	8	8	8		4		4		2		64	32	32		32	
1–19	<2		<2		<2		8	8	16		16		32	8	8	4	4		4		4		4		512	128	32		32	
1–4	16		32		32		<2		<2		<2		32	32	16		16		<2		<2		<2		≥1024		≥1024		≥1024	
1–18	16		16		16		<2		<2		<2		64	32	32		32		4		4		4		≥1024		512		512	
1–5	64		32		32		4		4		4		4	2	2		2		4	2	<2		<2		8		8		8	
1–7	64		64		64		8		2		2		2	2	2		2		2	2	2		<2		16		16		16	
1–6	64		64		64		8	8	8		8		16	2	2	2	2		<2		<2		<2		2	2	<2		<2	
1–10	128		128		128		16	8	4		4		8	2	2	<2	<2		2		<2		<2		8	4	2		<2	
1–8	128		128		128		16	8	4		4		64	4	8	4	4		4		8		8		16		16		16	
1–9	64		64		64		2	2	2		2		32	8	8	4	4		16		16		16		16		16		16	
1–11	128		128		128		16		8	4	4		64	32	16		8		32		32		16		128		128		128	
1–13	16		16		16		8		8	8	4		16	16	16		16		64		32		32		128		128		128	
1–12	16		16		16		16	16	16	16	16		64		64		64		2		2		2		32		32		32	
1–14	16		16		16		8	4	8	4	4		64		64		64		4		2		2		4		2		2	
1–16	8		8		8		NT	NT	NT	NT	NT	NT	8	8	8		8		64	64	8	4	4		128	32	32		32	
1–20	8		2		2		NT	NT	NT	NT	NT	NT	32	16	16		8		16	8	8	8	4		32	32	32		32	

[a] Reciprocal of titer calculated considering tissue culture tubes with <5 foci of CPE as virus negative.
[b] Reciprocal of titer calculated considering tissue culture tubes with <5 foci of CPE as virus positive.
[c] Virus dose, 50% tissue culture infectious dose.
[d] NT = Unsatisfactory test.
[e] No result in <5 Pos column indicates no tubes in either serum with <5 foci of CPE.

TABLE II. Rhinovirus Neutralizing Antibody Titers in Duplicate Serum Samples According to Day and Method of Assessment.

Virus type:	2						17						Oliver					
Day:	1		2		3		1		2		3		1		2		3	
Matching serum pairs	<5 Neg[a] 10[c]	<5 Pos[b,d] 10	<5 Neg 16	<5 Pos 16	<5 Neg 16	<5 Pos 16	<5 Neg 6	<5 Pos 6	<5 Neg 60	<5 Pos 60	<5 Neg 100	<5 Pos 100	<5 Neg 6	<5 Pos 6	<5 Neg 16	<5 Pos 16	<5 Neg	<5 Pos
2–1	128		64		64		32		8		4		64		64	32		
2–4	256		256		256		16		8		8		128		128	128		
2–2	8		8		8		16		4		4		<2		<2			
2–3	16		16		16		32		4		4		<2		<2			
2–5	2		<2		<2		128		64		64		32		16	8		
2–13	<2		<2		<2		256		32		32		64		64	64		
2–6	32		32		32		<2		<2		<2		<2	<2	<2			
2–8	32		32		32		<2		<2		<2		2	<2	<2			
2–7	8		8		8		2		<2		<2		32	32	8			
2–12	16		16		16		4		<2		<2		32	16	8			
2–9	4		4		4		<2		<2		<2		32		32	16		
2–18	4		2		2		<2		<2		<2		16		16	16		
2–10	512		512		512		16		4		4		<2		<2			
2–17	64		32		32		16		4		4		<2		<2			
2–11	16		16		16		64		32		32		2	<2	<2			
2–14	32		16		16		64		32		32		2	2	2			
2–15	16		16		16		32	32	4	4	4	2	64		64			
2–20	16		16		16		16	8	8	4	4	4	32		32			
2–16	16		16		16		64		16		16		128		128			
2–19	32		16		16		256		64		64		256		256			

[a] Reciprocal of titer calculated considering tissue culture tubes with <5 foci of CPE as virus negative.

[b] Reciprocal of titer calculated considering tissue culture tubes with <5 foci of CPE as virus positive.

[c] Virus dose, 50% tissue culture infectious dose.

[d] No result in <5 Pos column indicates no tubes in either serum with <5 foci of CPE.

TABLE III. Frequency of Differences between Duplicate Samples According to Day of Observation and Method of Assessing Rhinovirus Neutralization Tests.

Day	Result	Total no. pairs	No. of serum pairs with indicated difference (log_2)								SD of differences
			−4	−3	−2	−1	0	1	2	3	
1	<5 neg	77	0	6	5	12	25	21	7	1	1.390
	<5 pos	77	0	6	4	11	29	22	5	0	1.266
2	<5 neg	77	2	2	2	12	38	15	6	0	1.182
	<5 pos	77	2	2	3	12	36	14	7	1	1.249
3	<5 neg	67	2	2	3	10	33	11	6	0	1.236
	<5 pos	67	2	2	3	11	31	12	6	0	1.246

in Table IV. Considerable variability in the standard deviations of the differences among tests with different rhinovirus types was observed. As shown in Table IV, tests with rhinovirus types 2 and 16 exhibited the largest standard deviations, and tests with rhinovirus types 14 and 15 exhibited the smallest standard deviations. In order to determine if the variability between titers of duplicate samples was related to virus dose, standard deviations for virus types were compared according to quantity of virus in the test. There was no significant correlation between virus dose and standard deviation of the differences (Spearman rank correlation test, $r = .304$, $p > .25$). However, it is apparent that the largest standard deviations were observed in tests with virus types to which the greatest amounts of antibody were present; moreover, in tests with virus types to which antibody levels were low, standard deviations were smallest. A significant positive correlation was observed between the geometric mean antibody titers and standard deviations (Spearman rank correlation test, $r = 0.738$, $p < .05$).

TABLE IV. Effect of Virus Type and Geometric Antibody Titer on Mean Changes Between Duplicate Samples and Standard Deviations of the Differences.[a]

Virus type	Geometric mean antibody titer (reciprocal)	Virus dose ($TCID_{50}$) in test	SD of differences
16	33.9	3.2	1.94
1A	23.2	10	1.37
2	15.9	16	1.60
Oliver	8.5	16	.95
14	6.9	10	.57
17	5.2	60	.79
13	3.7	10	.98
15	3.0	6	.71
All types			1.182

[a] Spearman rank correlation test: Virus dose and standard deviation, $r = .304$, $p = .25$; mean antibody titer and standard deviations, $r = .738$, $p < .05$.

Discussion. The lack of sensitivity of rhinovirus neutralization tests using conventional doses of virus has been amply demonstrated by epidemiologic studies (1, 2, 9). In these studies the frequency of neutralizing antibody responses to rhinovirus infection in individuals from whom rhinovirus has been isolated has been shown to vary between 37% and 90%. This lack of sensitivity is particularly apparent when antibody to so-called "H-strains" is tested (37–53% of infected individuals respond). Using small doses of virus in the neutralization procedure as in the present study, Cate *et al.* (4) reported that 84% of infected volunteers developed neutralizing antibody responses, and more recently, Mufson *et al.* (5) demonstrated increased frequency of detection of antibody response to infection with a rhinovirus when the dose of virus used in neutralization tests is reduced. Thus, it appears that by using small doses of virus in the test, the sensitivity of the neutralization test can be improved.

The present studies have demonstrated the reproducibility of rhinovirus neutralization tests employing small doses of virus by testing, in blind fashion, duplicate serum samples from 20 individuals against 8 rhinovirus serotypes. It was shown that the most reproducible results were obtained by reading the test at the second day on which 3.2

$TCID_{50}$ or more were present in the virus titration. More prolonged observation did not improve this reproducibility. Since the day of reading in question usually corresponded to the fourth day after inoculation of the tissue cultures, it is apparent that the test is rapid. In addition, the test can be made technically easier to interpret by classifying cultures with 1–4 foci of CPE as negative and only those with diffuse involvement of the cell sheet as positive.

It is apparent that the estimates of the neutralizing antibody titers by the proposed test procedure are variable to some extent. In the test as defined, virus dose did not appear to influence variability; however, it did appear that the variability was different for different types of virus. These latter differences in variability may be due to the level of antibody present as suggested by the finding of a positive correlation between the level of antibody and the standard deviation of the differences between duplicate pairs. The probable explanation for the lower standard deviations of the rhinovirus types with low mean antibody titers is restriction of the range of variability below the mean.

Rhinovirus neutralization tests must be interpreted carefully in view of the findings in the present study. Eightfold or greater rises in antibody titer between acute and convalescent phase sera would strongly indicate the presence of infection since it would occur by chance only about once in 40 tests; however, lesser rises could not be interpreted with the same degree of confidence.

Summary. The reproducibility of rhinovirus neutralization tests employing small doses of virus was tested by performing, in blind fashion, neutralization tests on duplicate serum samples from 10 individuals against 5 rhinovirus serotypes and from another 10 individuals against 3 different rhinovirus serotypes. The most reproducible results were obtained on the second day in which 3.2 $TCID_{50}$ or more were present in the simultaneous virus titration, and the test was made technically easier by classifying as positive only those cultures with diffuse involvement of the cell sheet with CPE. The neutralizing antibody differences between duplicate serum samples appeared to follow a normal distribution. The frequency of 4-fold or greater differences between duplicate samples was 15.6%; 8-fold or greater differences, 5.2%; and 6-fold differences, 2.6%. Variability between duplicate serum samples was found to be related to the level of antibody present.

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