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Cross Relationships Among 37 Rhinoviruses Demonstrated by Virus Neutralization with Potent Monotypic Rabbit Antisera

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Antisera produced in rabbits to 37 rhinovirus (RV) types have been examined for antibody to the 36 corresponding heterologous types. Reciprocal neutralization was noted between RV types 2 and 49 and RV types 13 and 41. Five additional monotypic rhinoviral rabbit antisera neutralized one heterologous rhinovirus. Neutralizing antibody titers against heterologous RV serotypes were similar to those shown in the RV 1A, 1B and RV 9, 32 reciprocal cross-reactions, and would not be expected to result in false identification of serotypes. Comparison of neutralizing antibody titers and neutralization rate constants in serial serum specimens of immunized rabbits showed that antibody response to the immunizing RV type and the cross-reacting type followed a similar time pattern.

We have previously reported a relationship between rhinovirus (RV) types 9 and 32, as shown by reciprocal neutralization by antisera produced in rabbits in our laboratory. Except for the A and B subtypes of RV 1, this was the only reciprocal cross-reaction seen in rabbit antisera to 25 RV serotypes against the corresponding rhinoviruses, although several antisera neutralized one heterologous rhinovirus type. Examination of sera from all rabbits immunized with rhinoviruses involved in cross-reactions and extension of testing to include 37 RV serotypes have revealed two additional reciprocal relationships: RV 2 × RV 49, and RV 13 × RV 41. Five antisera neutralized one heterologous rhinovirus. The purpose of this report is to present data on further investigation of relationships between these rhinovirus types.

MATERIALS AND METHODS

Production of antiserum in rabbits. Antisera to 30 RV serotypes were produced after the protocol previously published (5), in which pairs of rabbits were injected intramuscularly with 2 ml of immunogen mixed with Freund incomplete adjuvant initially. After 21 days, rabbits were bled and a series of graduated doses of immunogen (0.1, 0.2, 0.3, and 0.4 ml) without adjuvant were given intravenously (IV) at 3-day intervals. Rabbits were bled 1 week after the completion of the IV series, and a final injection of 1 ml of immunogen was injected IV on that day. Rabbits were bled subsequently at 1-week intervals until death, usually at the third post-immunization bleeding. Since rabbits in this initial series infrequently showed further antibody response to the final dose of immunogen, this dose was eliminated and rabbits were bled out 1 week after the final graduated IV dose or 38 days after the initial injection of immunogen.

Neutralizing antibody determination. The procedure was previously employed for titrations of human serum (11). Flat-bottomed 96-cup microtiter plates were employed in the test (Microtest II; Falcon Plastics). Serial twofold dilutions of serum in 0.025-ml volumes were prepared in quadruplicate with microtiter loops. Twenty-five microliters of virus suspension (30–100 50% tissue culture doses [TCD_{50}]) were added to each cup. Serum-virus mixtures were allowed to stand for 1 h at room temperature, after which 0.05 ml of HeLa cell suspension from a spinner culture, approximately 50,000 cells, was added. All diluent and suspending medium in the system consisted of Eagle minimum essential medium with 5% fetal bovine serum containing 30 mM Mg^{2+} plus 100 units of penicillin and 100 μg of streptomycin per ml. Virus titrations, cell controls, and serum controls were included in each experiment. An overlay of sterile mineral oil was used to seal the cups, and plates were incubated in a CO_2 incubator at 35 C until cytopathic effects in the virus controls indicated the presence of 30 to 100 TCD_{50} of virus, usually on the third day of incubation. Monolayers were stained with crystal violet and examined. The 50% end point of neutralization was calculated by the method of Reed and Muench.

RESULTS

Reciprocal cross-reaction. Rabbit antirhinovirus antisera were first tested at a 1:20 dilution against 30 to 100 TCD_{50} of the 36 heterologous RV types. If any heterologous RV was neutralized, all available rabbit antisera to the same RV type were titrated for neutralization of the heterologous RV type. Neutralization of one heterologous rhinovirus was shown in 13 of 37 antisera tested, as shown in Table 1. In addition to RV 1, subtypes A and B, and RV 9 and 32, previously reported (6), four types showed reciprocal neutralization. These pairs are RV 2 × 49 and RV 13 × 41. The extent of the reciprocal cross-reactions is shown in Table 2 in which neutralizing antibody titers and neutralization rate constants (k values) determined on the same sample of rabbit antiserum are presented. RV 1A × 1B and RV 9 × 32 are presented for purposes of comparison, and it will be noted that the degree of relationship is similar among the four pairs shown.

TABLE 1. *Relationships among 37 rhinovirus serotypes as revealed by cross-neutralization with antirhinovirus rabbit sera*

Antirhinovirus rabbit sera	Neutralization of heterologous rhinovirus type
1A	1B
1B	1A
2	49
5	42
6	
8	
9	32
11	40
12	
13	41
14	
16	
17	42
18	
29	
30	
31	
32	9
33	
34	
35	
36	50
37	
38	
39	54
40	
41	13
42	
43	
45	
47	
49	2
50	
52	
53	
54	
55	

One-way cross-reactions. Neutralizing antibody titers and k values for homologous and heterologous RV types are shown in Table 3 for five antisera, anti-RV 5 and 17 versus 42, anti-RV 11 versus 40, anti-RV 36 versus 50, and anti-RV 39 versus 54, which neutralized one heterologous type. Again the relationships appear to be similar to those seen in the reciprocal cross-reactions. The neutralization of RV 54 by anti-RV 39 serum deserves special comment.

The standard challenge dose of RV 54 was completely neutralized by a dilution of 1:160 of each of the four available anti-RV 39 rabbit sera, and partially neutralized by dilutions of 1:320 and 1:640. However, RV 54 virus harvested from tubes showing partial neutralization was less susceptible to neutralization by anti-RV 39 serum than the parent virus (titer reduced from 160 to 20) but unchanged in neutralizability by anti-RV 54 serum. Presumably, growth in the presence of anti-RV 39 antibody had selected against the cross-reacting component of the virus population. Acornley et al. selected variants of RV 5 by passage in the presence of immune serum (2).

In most instances, a pair of rabbits injected with the same lot of immunogen showed little variation in homologous or heterologous antibody response. Response to one heterologous type of rhinovirus was elicited in all rabbits injected with seven immunogens, although variation in neutralizing antibody titer was seen. In two of the one-way crosses examined, anti-RV 11 versus RV 40 and anti-RV 36 versus RV 50, not all the rabbits injected showed the heterologous antibody. Of five rabbits immunized with RV 11, three had neutralizing antibody against RV 40, and two of four rabbits immunized with RV 36 showed neutralizing activity against RV 50. These results are shown in detail in Table 4. Extreme variation in heterologous response sometimes occurred in the same pair of rabbits responding uniformly to the homologous antigen, as exemplified by homologous and heterologous titers in anti-RV 11 rabbits numbers 347 and 348. Homologous and heterologous (RV 49) titers in three pairs of rabbits injected with RV 2 immunogen demonstrate variation in heterologous titer among three pairs of rabbits. Rabbits immunized according to the initial schedule were examined for heterologous antibody response. Results (Fig. 1), show that the heterologous response, with maximum

TABLE 2. *Reciprocal cross-neutralizations among rhinovirus (RV) serotypes*

Rhinovirus type	RV antiserum		Rhinovirus type	RV antiserum	
	1A	1B		9	32
1A	k:1,012[a] N:5,120[b]	k:8.6 N:40	9	k:575 N:2,882	k:5.8 N:40
1B	k:4.6 N:160	k:110 N:2,882	32	k:3.1 N:160	k:194 N:1,620
	RV antiserum			RV antiserum	
	2	49		13	41
2	k:200 N:2,048	k:9.3 N:20	13	k:52.4 N:730	k:6.3 N:80
49	k:11.2 N:40	k:171 N:5,760	41	k:5.3 N:320	k:552 N:4,096

[a] k value, neutralization rate constant calculated by the equation: $k = 2.3 \times (D/t) \times \log (Vo/Vt)$, where D = reciprocal of final serum dilution in virus-serum mixture, t = time (min), Vo = virus PFU at time 0, and Vt = virus PFU at time t.

[b] N titer, reciprocal of serum dilution which neutralizes 30 to 100 TCD_{50} of virus.

TABLE 3. *Rhinovirus (RV) antisera: neutralization of heterologous RV*

Antibody vs. RV type	Heterologous RV type neutralized	Homologous		Heterologous	
		N titer[a]	K value[b]	N titer	k value
Anti-RV 5	RV 42	730	208	160	2.0
Anti-RV 17	RV 42	2,882	900	240	2.4
Anti-RV 11	RV 40	4,096	380	160	18.5
Anti-RV 36	RV 50	5,760	130	160	4.6
Anti-RV 39	RV 54	8,192	ND[c]	160	ND

[a] N titer, reciprocal of serum dilution which neutralizes 30 to 100 TCD_{50} of virus.

[b] k value, neutralization rate constant calculated by the equation: $k = 2.3 \times (D/t) \times \log (Vo/Vt)$, where D = reciprocal of final serum dilution in virus-serum mixture, t = time (min), Vo = virus PFU at time 0, Vt = virus PFU at time t.

[c] ND, not done.

titer attained at the 38-day bleeding, is similar to that previously reported (5) for homologous response. Although the homologous titers usually remained at the maximum level at the 45-day bleeding, there was often a drop in titer in subsequent bleedings and occasionally a drop at the 45-day bleeding. Heterologous antibody was not present in the anti-RV 2 serum at 21 days; titers rose to high levels by 38 days and remained high at 45 days, whereas the neutralizing activity versus RV 2 in the anti-RV 49 serum appeared earlier (on the 21st day), rose to a peak on day 38, and was lower on day 45. Although heterotypic antibody continued to decay during the period of observation, detectable levels were still evident 21 days after the last injection of immunogen.

DISCUSSION

Several of the cross-reactions reported here have been reported and investigated by others. Fenters et al. (8) reported neutralization of two strains of RV 2 by anti-RV 49 bovine serum. These authors' data also suggested a relationship between RV 5 and RV 17, and although they did not test these sera against RV 42, our results also suggest a relationship between RV 5 and RV 17, since both of these antisera neutralize the same heterologous virus, RV 42. Conant and Hamparian investigated neutralization of RV 32

TABLE 4. *Examples of variation in antibody response in pairs of rabbits injected with the same lot of rhinovirus immunogen*

Rabbit no.	Immunogen RV 2 N[a] titers vs.		Rabbit no.	Immunogen RV 11 N titers vs.		Rabbit no.	Immunogen RV 36 N titer vs.	
	RV 2	RV 49		RV 11	RV 40		RV 36	RV 50
326	646	320	347	2,000	320	408	5,760	20
327[b]	2,048	20	348	1,448	<20	409	5,760	160
791	360	40	803	1,448	<20	1,025	1,448	<20
793	360	160	815	4,096	20	1,048	1,448	<20
1,028	1,000	640	1,033	8,192	480			
1,029	1,000	640	1,030	died	died			

[a] Reciprocal of highest serum dilution which neutralized 30 to 100 TCD_{50} of virus.
[b] Bracket indicates pair of rabbits receiving same lot of immunogen.

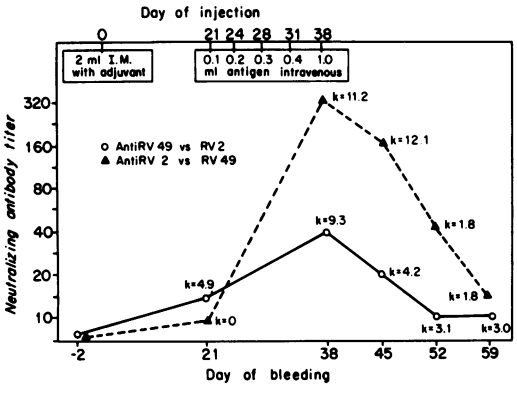

FIG. 1. *Heterologous antibody response in rabbits injected with cross-reacting rhinovirus types 2 (▲-▲) and 49 (O-O). k is neutralization rate constant for serum sample with indicated neutralizing antibody titer.*

by anti-RV 9 sera, and also reciprocal neutralization of RV 13 and RV 41 (3, 4). Since the neutralizing effect in all three instances could be removed by exhaustive absorption with human liver powder, these authors concluded that the apparent neutralizing activity was a nonspecific reaction due to cytotoxic antibodies. It is difficult to explain why an absorption of antiserum with a nonspecific absorbent should remove specific cross-reactions. In each of the cases here and in the previous examples, 1A, 1B, and 9 and 32 (6), serological cross-reactivity is seen only with specific viruses, and as many as four sub-specificities were observed among the rhinoviruses studied in the present report. Clearly, extensive absorption of antiserum with human liver powder must have removed components which cause specific heterotypic neutralization of a variety of rhinoviruses.

However, when we investigated a reciprocal cross-reaction between RV 9 and RV 32, we could show that although heterologous neutralizing antibody titers were low, a k value of 3 to 8 could be calculated, whereas values to other heterologous rhinoviruses were 0.6. In the procedure for determining k values, virus and antiserum are incubated and the mixture is sampled at various time intervals for virus concentration. If neutralization is demonstrated, the reaction obviously must have occurred in the reaction mixture and proceeds at some constant rate as shown in Fig. 2. Furthermore, antiserum is diluted at least 1:2,000, and usually more, before plating on cell monolayers. Therefore, it is highly unlikely that anticellular antibody plays any part in neutralization of virus. There are many indications that minor relationships demonstrated might have arisen through antigenic variation that eventually resulted in major differences from "parent" serotypes.

Antigenic variation in RV 22 (14) was shown by Schieble et al. in description of a "prime" strain. Strains of RV 51 isolated in different years were shown to have antigenic variation (16). Several reports in the literature suggest that human beings show heterotypic antibody response after natural infection with rhinovirus (7, 9, 10).

We have noted changes in biological characteristics of rhinoviruses during rapid passage in HeLa cells in the presence of 30 mM Mg^{2+}. With most of the rhinovirus types we have used to prepare immunogens, we have been able to select a population of virions with a titer of 2 to 4 $\log_{10}$ higher titer than the original seed virus. In most instances, the plaque characteristics have changed in that plaques are clearer and larger, probably a reflection of more complete release of virus. It is possible that along with these changes, there is a

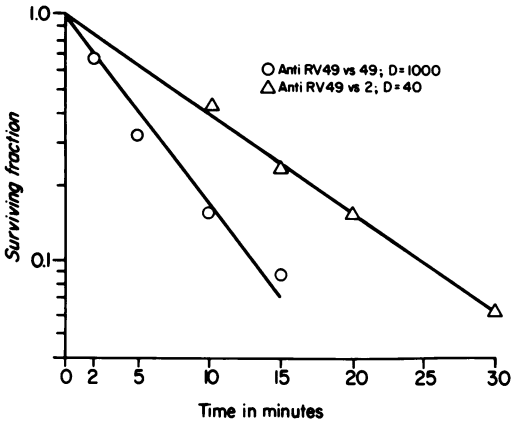

FIG. 2. *Comparison of rate of neutralization of homologous (RV 49) and heterologous (RV 2) cross-reacting rhinoviruses by rabbit anti-RV 49 serum. $k = 2.3 \times [D$ (final dilution of serum)$/t$ (time in minutes)$] \times \log V_0/V_t$. k value of anti-RV 49 serum vs. RV 2 ($D = 40$) is 3.68. k value anti-RV 49 vs. RV 49 ($D = 1{,}000$) is 177.*

shift in surface antigens on the virion surface which uncovers an antigen which is the major surface antigen of another rhinovirus type. It should be emphasized that the cross-reactions reported here are usually minor, as shown in Tables 2 and 3, and there is variation in antibody response of rabbits to the same immunogen, as illustrated in Table 4. This variation in response is similar to that shown when a suboptimal antigenic stimulus is given to rabbits. When five rabbits were immunized with a pentavalent rhinovirus antigen, the antibody response among rabbits varied greatly (unpublished data), whereas antibody response in pairs of rabbits injected with at least 10^7 plaque-forming units (PFU) per ml was much more uniform. The variation in response, together with the generally low level of cross-reactions, undoubtedly accounts for variations in results in different laboratories. On the other hand, the strongest evidence for the validity of the cross-reactions reported is the suggestion of relatedness between the same pairs of rhinoviruses shown in different laboratories after immunization of different animal species, although in some instances, a one-way cross was indicated in one laboratory and a reciprocal cross was detected in another (3, 7).

The fact that four reciprocal and five one-way cross-reactions were observed in the 37 rhinoviruses studied suggests that a substantially greater number of cross-reactions will be observed among the 90 (including rhinovirus 1B) presently classified viruses (1). There are undoubtedly candidate rhinoviruses which will increase this number (13). A simple comparison of the areas (37^2 versus 90^2) of the matrices of antisera and viruses suggests that perhaps six times as many crosses might be observed in the larger matrix than we have observed in our present small matrix of 37 by 37. The possible significance of these cross-reactions in immunity is presently unknown, but the knowledge of rhinoviruses involved in serological cross-reactions should permit further investigation of possible cross-reactions in human sera (7). Production of antisera with maximum cross-reacting antibody titers might also be applied to the presently cumbersome (12) methods for serotyping RV strains.

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