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Antigenic Groupings of 90 Rhinovirus Serotypes

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We have completed production in rabbits of potent antisera to the 90 classified rhinovirus serotypes by using methods previously described (M. K. Cooney and G. E. Kenny, Proc. Soc. Exp. Biol. Med. 133:645–650, 1970). Systematic testing by neutralization tests has revealed significant numbers of cross-relationships among rhinovirus types, some of which have already been reported. Herein, our observations are compared with cross-reactions reported in National Institutes of Health reference guinea pig antisera. Also, original rhinovirus isolates, representing serotypes known to be antigenically related to other rhinoviruses, were tested against rabbit antisera to the related serotypes. These tests revealed extensive antigenic variation among isolates identified as rhinovirus 12:78 or 36:58, which are reciprocally related pairs, 41, reciprocally related to 13, and 67, which is related to both 9 and 32. If the rhinovirus serotypes were grouped according to antigenic relationships, 50 types could be included in 16 groups.

When the numbering scheme for rhinoviruses 1 through 55 was published, only type 1 was assigned subtypes A and B; all others were assumed to be distinct serotypes, and no subtypes were identified in types 56 through 89 (2, 3). We and others have shown that there are relationships between many rhinovirus types as demonstrated by one-way and reciprocal cross-reactions shown by neutralization. Some of these relationships involve three different serotypes. Rhinovirus typing is a very cumbersome procedure, not only because of the large number of serotypes (at least 115 exist), but also because many serotypes circulate concurrently in the community, which means that each isolate must be tested against all antisera. Thus, even when combinatorial pools (13) are used for typing, antigenic variation in field strains presents a problem in typing. For example, a variant of 12:78 could be neutralized by the two pools containing anti-12 and the one pool containing 78, producing a pattern which does not identify any specific type.

We have examined the cross-relationships among rhinoviruses as revealed by neutralizing antibody in our rabbit antisera and similar reactions reported in reference guinea pig sera. We suggest a scheme for grouping antigenically related types which would include all serotypes related to any member of the group. This would reduce the number of serotypes and could form a basis for constructing antiserum pools to include related types in one pool, thus facilitating typing by reenforcing the neutralization reaction and eliminating problems in typing field isolates which are intermediate between two related types.

MATERIALS AND METHODS

Methods for preparation of rhinovirus immunogens (5), immunization of rabbits (6, 7), and antibody titrations in microtiter plates have been described in previous publications. Slight modifications of some procedures were introduced.

Preparation of rhinovirus immunogens. Seed virus for each prototype rhinovirus serotype was obtained from V. V. Hamparian, Ohio State University. Seed virus stocks were plaque purified in M-HeLa plates (12), and the serotype was confirmed with reference antiserum (9). HeLa cell monolayers in roller bottles were infected at a multiplicity of infection of 10. Infected cells were harvested in a reduced volume of cell culture medium. Cells were homogenized to effect virus release, and the homogenate was centrifuged to remove cell debris. The supernatant, after extraction with fluorocarbon to remove nonviral antigens, constituted the immunogen and contained at least 10^7 plaque-forming units per ml.

Immunization schedule for rabbits. The serotype of the immunogen was again confirmed before a pair of rabbits was injected with each preparation. A preimmunization blood sample was collected, and each rabbit received an intramuscular injection of 2 ml of immunogen mixed with 2 ml of Freund incomplete adjuvant (1 ml at each of four sites).

After 21 days, 0.1 ml of immunogen without adjuvant was injected intravenously, followed at 3-day intervals by intravenous injections of 0.2, 0.3, and 0.4 ml. Rabbits were bled out by cardiac puncture 7 days after the final intravenous injection.

Neutralizing antibody titrations. Appropriate twofold serum dilutions (1:10 to 1:160 for pre-immunization sera and 1:10 to 1:5,120 for post-immunization sera) were prepared in quadruplicate in flat-bottomed,

TABLE 1. Heterotypic rhinovirus antibody seen in rabbits immunized with prototype rhinovirus strains 1A, 1B, and 2 through 89

Antiserum to rhinovirus:	Neutralizes heterotypic rhinovirus(es):	Antiserum to rhinovirus:	Neutralizes heterotypic rhinovirus(es):
1A	1B	29	44
1B	1A	32	9, 67
2	49	36	50, 58, 89
3	14	39	54
5	42	41	13
6	14	44	29
9	32, 67	49	2
11	40, 74	58	36
12	78	60	38
13	41	66	77
14	3	74	15
15	74	76	11
17	42, 70	78	12
22	61		

96-cup microtiter plates (Microtest II; Falcon Plastics). A 25-μl amount of virus suspension (30 to 300 50% tissue culture doses) was 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 media in the system consisted of Eagle minimum essential medium with 5% fetal bovine serum containing 30 mM Mg^{2+} plus 100 U of penicillin per ml and 100 μg of streptomycin per ml. Virus titrations, cell controls, and serum controls were included in each experiment. Plates were covered with sterile Lucite covers and incubated in a CO_2 incubator at 33°C until cytopathic effects in virus controls indicated the presence of approximately 100 50% tissue culture doses of virus, usually on day 3 of incubation. Monolayers were stained with crystal violet and examined. The 50% endpoint of neutralization was calculated by the method of Reed and Muench (14). Neutralizing antibody titers in guinea pig sera are those reported in the *NIAID Catalog of Research Reagents* (9).

Determination of *k* values. In rabbit sera that neutralized heterotypic rhinovirus, neutralization rate constants (k) were determined to assess the quality of the homologous antibody and to verify the specificity of heterologous response. Equal volumes of antisera (diluted 1:500 or 1:1,000 for homologous virus and 1:20 for heterologous virus) and virus (10^5 plaque-forming units per ml) were mixed and incubated in a water bath at 37°C. Samples of the homologous system were removed at 2, 5, 10, and 15 min, diluted 1:100 in cold diluent, and held in an ice bath until plated. The heterologous system was sampled at 10, 15, 20, and 30 min. The virus control was sampled at 0, 15, and 30 min. Plaque assays were performed on all samples in HeLa plates with 30 mM Mg^{2+} in the medium as previously described (12). Neutralization rate constants were calculated by the equation: $k = 2.3 \times (D/t) \times \log (V_0/V_t)$, where D is the reciprocal of final dilution of serum in serum-virus mixture, t is the time (minutes), V_0 is the plaque-forming units at time 0, and V_t is the plaque-forming units at time t (1).

RESULTS

Titration of the antisera from individual rabbits against each homotypic rhinovirus revealed 50% endpoint neutralization titers ranging from 1,000 to 5,000 in most cases. If homotypic antibody titers were lower than 512, a second pair of rabbits was immunized in the hope of obtaining more potent antisera. Since we intended to use the antisera in pools for typing isolates, it was important to have a high titer and also to assure specificity. A 1:20 dilution of each rabbit antiserum was tested against 30 to 300 doses of the 89 heterotypic rhinoviruses (1A and 1B are considered as two serotypes, for a total of 90). Sera that neutralized any heterotypic rhinoviruses at 1:20 were titrated for serum neutralization endpoints against those viruses. Many of the cross-reactions have been published previously (6, 8), and Table 1 includes these to give a complete report on the 90 antisera. Antibody titers to heterotypic rhinoviruses usually were low; the median titer was 40, and the mean titer was 88.5. A total of 27 rhinovirus immunogens elicited antibody to at least one of the 89 heterotypic rhinoviruses. Four antisera, 9, 11, 17, and 32, neutralized two heterotypic rhinoviruses, and antirhinovirus 36 neutralized three heterotypic rhinoviruses. Five pairs of rhinovirus serotypes (1A:1B, 2:49, 12:78, 13:41, and 29:44) showed reciprocal relationships, with no evidence of relatedness to any other serotype. Four pairs of serotypes showed reciprocal crosses in which one or both members of the pair showed a relationship to other serotypes.

In the reciprocal relationship between serotypes 3 and 14, 14 was neutralized by anti-6. Both anti-9 and anti-32 neutralized 67. In the 15:74 cross, 74 was neutralized by anti-11, which also neutralized 40, and anti-76 neutralized 11. In addition to showing a reciprocal relationship with 58, anti-36 neutralized 50 and 89. Four antisera neutralized only one heterologous serotype: 22 versus 61, 39 versus 54, 60 versus 38, and 66 versus 77. Finally, relationships among four serotypes are suggested by anti-5 neutralizing 42 and anti-17 neutralizing both 42 and 70. Thus, 37 of the 90 serotypes showed antigenic relationships to one or more heterologous serotypes.

Guinea pigs were chosen by the Research Resources Branch of the National Institute of Allergy and Infectious Diseases for production of reference antirhinovirus sera because of the reportedly more specific response in these animals (3), but some heterotypic responses were reported in the Institute's research reagents catalog (9). In Table 2, guinea pig reference antisera are tabulated for comparison with rabbit antisera. Among the guinea pig antisera, 10 showed heterotypic response to one rhinovirus type, 2

TABLE 2. Heterotypic rhinovirus antibody reported[a] in antirhinovirus guinea pig reference sera

Antiserum to rhinovirus:	Neutralizes heterotypic rhinovirus(es):	Antiserum to rhinovirus:	Neutralizes heterotypic rhinovirus(es):
1B.....	1A[b]	30.....	29, 43, 70
3.....	79	32.....	9[b]
9.....	32, 67[b]	44.....	29[b]
15.....	74[b]	50.....	18
19.....	42	55.....	48
21.....	43	57.....	56
		59.....	63, 85

[a] Data from reference 9.
[b] Cross-reactions also observed with rabbit antiserum; see Table 1.

sera showed antibody to two types, and 1, antirhinovirus 30, neutralized three heterotypic viruses. The antigenic cross-relationships involved 26 rhinovirus types, 13 of which were not involved in cross-reactions when tested with the rabbit antisera.

Although antibody to rhinovirus 1B was not seen in antirhinovirus 1A guinea pig serum, the titer against rhinovirus 1A, 1,280 in anti-1B serum, was almost identical to the homologous titer, 1,600. In other sera, the heterotypic titers ranged from 20 to 160, with a median titer of 40 and a mean of 63. Several cross-relationships were seen in both the guinea pig reference antisera (Table 2) and the rabbit antisera (Table 1): 1A:1B, 9:32:67, 15:74, and 29:44.

The final dilution of rabbit antiserum in our pools used for rhinovirus typing is usually 1:60 or higher to provide 20 U of homologous antibody for each serum. Since heterotypic antibody titers are usually less than 1:60, field strains are neutralized only by rabbit serum in the typing pool(s) containing homotypic antibody. The combinatorial pools are designed so that each of the 90 serotypes will be neutralized by only one, or no more than two, pools (13). However, we have noted confusing results in typing isolates of rhinovirus 36 or 58, and 12 or 78. Rabbit antisera were titrated for neutralizing activity against each available isolate identified as a strain of the cross-related (heterologous) rhinovirus type. The results shown in Table 3 illustrate antigenic variation in 12:78 and 36:58 isolates. Many of the 36:58 or 12:78 isolates were neutralized equally (unfortunately, the 36:58 isolates equally poorly) by the pools containing the antisera involved. Antigenic variation was also seen in isolates typed as 41 and 67. Of 11 type 41 isolates, 3 were not neutralized, 2 were partially neutralized, and 6 were completely neutralized by a 1:20 dilution of anti-13. Anti-9 and anti-32 sera were titrated against each of five isolates of type 67. Three of these strains were not neutralized by either anti-

TABLE 3. Evidence of antigenic variation in rhinovirus isolates[a] neutralized by anti-12, anti-78, anti-36, or anti-58 rabbit typing antisera

No. of isolates	Reciprocal of neutralizing antibody titer in typing antisera[b]			
	Anti-12	Anti-78	Anti-36	Anti-58
1	80	640		
3	160	80–160		
5	320	160–320		
2	640	80–160		
3	640	40		
Rhinovirus 12[c]	1,280	10		
Rhinovirus 78[c]	40	640		
1			80	<20
1			20	40
1			20	20
1			20	80
Rhinovirus 36[c]			1280	40
Rhinovirus 58[c]			80	640

[a] Rhinovirus isolates from subjects in the Virus Watch program, 1965–1970.
[b] Antisera raised in rabbits against prototype rhinovirus strains for use in typing isolates.
[c] Prototype rhinovirus strains used as antigen in the production of rabbit antisera.

9 or -32 at a 1:20 dilution, one was neutralized by anti-32 only, and one was neutralized by both diluted 1:40. Depending on the distribution of antisera in the typing pools, the isolate might be neutralized by three or four pools, thus necessitating testing the isolate against as many as 40 individual antisera to identify it.

Measurements of neutralization rate constants (k values) provide a measure of affinity and avidity, or "quality," of the antibody. The k value determination also precludes the false appearance of neutralization of virus due to cytotoxic effects of the antiserum (4). Therefore, the k value confirms the evidence for antigenic relatedness demonstrated by cross-neutralization. Table 4 gives neutralization titers and k values for antirhinovirus rabbit sera, indicating antigenic relationships among rhinoviruses not previously reported by us. Reciprocal neutralization was confirmed by k values for 12:78, 15:74, and 29:44. The anti-29 serum had a relatively low neutralizing antibody titer and a surprisingly low homologous k value. We subsequently found that this was reflected in a lack of success in typing field isolates of rhinovirus 29. When a rhinovirus isolate, classified as "untypable," was used as an immunogen in rabbits, the resulting antiserum showed a neutralizing antibody titer against itself, and against the prototype strain of 29, of 1:2,560, with a k value of 600. Anti-36 antiserum showed a much higher k value against 89, which is not reciprocally related, than against 58, which is reciprocally related.

With the information regarding antigenic relationships among the 90 rhinovirus serotypes (1A, 1B, and 2 through 89) demonstrated in our rabbit antisera and the guinea pig sera available from the Research Resources Branch, 50 of the 90 serotypes can be allocated to 16 groups (Table 5). Of these groups, 10 involve only two serotypes, and 5 groups involve three to five serotypes. Group IX involves 10 serotypes and includes one pair, 29:44, which shows a reciprocal cross. There seem to be several linkage points in this group, i.e., 42 is neutralized by anti-5, -17, and -19; 43 is neutralized by anti-21 and anti-30. Anti-30 also neutralizes 29 and 70, thus linking with 44 and 17 (Tables 1 and 2).

DISCUSSION

It has been demonstrated that rabbits immunized with a sufficiently high antigenic dose of rhinovirus immunogen respond uniformly with almost identical antibody titers, whereas a minimal antigenic dose results in extreme variation in response, from no antibody to a reasonably high titer in a group of rabbits injected with the same immunogen (5). When rabbits are injected with a rhinovirus antigen which is related to another type, the homologous antigen is adjusted to contain the optimum immunogenic dose, whereas the heterologous antigen is suboptimal. This probably accounts for the fact that the antibody titers to a heterologous related type may vary from less than 20 in one rabbit to 40 or 80 in others, whereas the homologous titers are essentially the same in all the rabbits injected with the same lot. This would also explain the differences reported from different laboratories and the differences seen in various animals used for production of immune serum. Despite these differences, there is surprising agreement about which rhinovirus types are related. Schieble et al. (16) examined antigenic relationships among prototype rhinovirus strains by cross-testing with guinea pig antirhinovirus sera. Using pools of antisera, with individual sera diluted approximately 1:30, these investigators found only a few heterotypic responses (1B:1A, 32:9, and 44:29), all of which were reported in the research reagents catalog (9) (Table 2). In addition, a relationship between rhinovirus types 2 and 49 was indicated which has previously been reported (7, 11, 15), as well as a relationship between types 36 and 58 in rabbit sera, included in the present report.

The use of different animals, rabbits and guinea pigs, is the most obvious source of differences in results. Other differences must be considered and probably have a significant influence on results. Immunogens used for rabbits were produced in HeLa cells in such a way as to produce a maximum yield of virus. These preparations,

TABLE 4. Neutralization rate constants for reciprocally cross-reacting rhinoviruses (rabbit antisera)

Anti:	N (*k*)[a]								
	Versus 12	Versus 78	Versus 15	Versus 74	Versus 29	Versus 44	Versus 36	Versus 58	Versus 89
12	1,448 (176)	40 (1.6)							
78	20 (4.0)	1,448 (197)							
15			4,096 (500)	20 (9.0)					
74			160 (2.5)	16,132 (650)					
29					512 (27)	80 (5)			
44					20 (5.7)	1,280 (300)			
36							5,760 (230)	20 (1.2)	80 (8.0)

[a] N, Neutralizing antibody titer. Reciprocal of serum dilution that neutralizes 30 to 300 50% tissue culture doses of virus. *k*, Neutralization rate constant.

TABLE 5. Groups of related rhinoviruses as revealed by heterotypic antibody in both rabbit and guinea pig antirhinovirus sera

Group	Prototype of group	Rhinovirus types included[a]
I	1A	**1B**
II	2	**49**
III	3	(79), 6, **14**
IV	9	**32, 67**
V	11	15, 40, 74, 76
VI	12	**78**
VII	13	**41**
VIII	22	61
IX	29	5, 17, (19, 21, 30), 42, (43), **44,** 70
X	36	50, (18), **58,** 89
XI	38	60
XII	39	54
XIII	(48)	(55)
XIV	(56)	(57)
XV	(59)	(63, 85)
XVI	66	77

[a] Boldface indicates a reciprocal cross-reaction with the group prototype. Parentheses indicate heterotypic antibody seen only in reference guinea pig antirhinovirus sera.

which undoubtedly contained varying amounts of subviral particles that may have been antigenic, thus contributed to the presence of heterotypic antibody in the rabbit sera. On the other hand, rhinovirus immunogens used in guinea pigs were produced in WI-38 cells and concentrated by ultracentrifugation, resulting in a preparation that probably contained proportionately more complete virus. Another source of variation was in assay of antibody. We used HeLa cells in a microtiter system, whereas the reference antisera were tested in tube cultures of WI-38 cells. Some rhinovirus strains have to be passaged several times in HeLa cells before high titers can be attained, indicating that a virus population is selected which replicates well in HeLa cells. Other serotypes replicate well in HeLa cells on first inoculation. Antigenic variation in high-passage virus preparations probably results in only minor variations in titer in a homologous system, but it could account for detection of a cross-reacting antibody. We have tested some of the reference guinea pig sera and found a heterotypic titer of at least 1:20 in anti-12 versus 78, anti-66 versus 77, and anti-17 versus 70. None of these reactions was reported.

The guinea pig reference antisera are designed to neutralize only homotypic virus when tested at a level of 20 antibody units against prototype strains of rhinovirus, and they meet this stipulation. However, it is antigenic variation among strains isolated from field studies which presents a problem in the use of antirhinovirus serum pools. The rhinovirus 22 "prime" strain of Schieble et al. (17), for example, is a duplicate of the response in guinea pigs to 1A and 1B, in which 1B antigen elicits essentially equal response to 1A and 1B, whereas anti-1A serum does not neutralize 1B. Previous practice would dictate that the prime strain should be used as antigen for production of typing serum. Stott and Walker (18) investigated a number of rhinovirus isolates typed as 51 and found a range of variation, from a minor cross-reaction to identity with the prototype. Similar variation was seen in our laboratories (Table 3), particularly in strains of 36:58 and 78:12. Strains of 41 (related to 13) and 67 (related to 9 and 32) showed variation in cross-reacting antigen but were type identified by antisera to prototype virus included in the typing pools. Some strains of rhinovirus 12 or 78 were neutralized by a high dilution of antirhinovirus 12 or 78. Several strains were neutralized by both antisera, but at much lower dilutions than either prototype virus. These strains are obviously different from rhinovirus 12 or 78 and probably represent a subtype of the rhinovirus 12:78 "group." Antisera to selected isolates are being prepared to further explore the relationship. Mogabgab et al. (15) studied heterotypic antibody response among related rhinoviruses 1A:1B, 2:49, 23:30, 29:44:62, and 13:41:82. These investigators found heterotypic antibody response to be higher in humans naturally infected than in type-specific animal antisera. Temporal antigenic drift, similar to that seen by Stott and Walker in rhinovirus 51, was reported. Since some rhinovirus types replicate less readily in HeLa cell cultures than others, the optimal immunizing dose of antigen is difficult to attain with all serotypes. One method of assuring identification of variants of known types is to construct typing pools based on groups of related viruses, thus enhancing antibody to each related antigen. Heterotypic stimulation with related rhinoviruses usually enhances antibody titers to each antigen employed. Both of these methods could be expected to greatly facilitate rhinovirus typing.

As documented in this report, extensive antigenic relationships among rhinoviruses can be demonstrated in rabbits and guinea pigs immunized with a single, supposedly unique, rhinovirus serotype. Only 40 antisera did not neutralize at least one heterotypic virus. Additional relationships can be shown by the use of heterotypic stimulation, and others have been revealed by studies on human serum (10, 15). It is possible that previously existing antibody in the infected person may cause antigenic changes in virus. The recently discovered existence of dual rhinovirus infection (6) suggests recombination as another mechanism whereby rhinoviruses might exchange genetic material. Study of antigenic

variation in field strains is greatly facilitated by examining those isolates which are related to two or more rhinovirus serotypes.

Existing "monospecific" antisera are generally useful for typing rhinoviruses according to the present classification and will also be useful in exploring possibilities for consolidation and simplification of the system. It is apparent that any hope for control of rhinovirus infections depends on the ability to isolate and identify these agents. Consolidation of the classification system, even to the extent suggested here, should make identification more feasible.

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