PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, **147** 541-545 (1974)

# Rhinoviruses: An Antigenic Study of the Prototype Virus Strains (38383)

JACK H. SCHIEBLE, VIRGINIA L. FOX, FLORENCE LESTER, AND EDWIN H. LENNETTE

*Viral and Rickettsial Disease Laboratory, California State Department of Health, Berkeley, California 94704*

Rhinoviruses are one of the more important etiologic agents of upper respiratory illnesses. One of the principal reasons why repeated infections with this virus group is so common is the existence of a large number of distinct antigenic types. In 1967 a numbering system (1) was established in which 55 distinct virus immunotypes and one subtype were described. Several years later this classification for rhinoviruses was enlarged so that presently there are 89 immunotypes and one subtype (2). There are additional candidate rhinoviruses which will undoubtedly increase the number of immunotypes to perhaps a hundred or more.

Subsequent to the establishment of this classification system for rhinoviruses several reports relating to antigenic variation (3, 4) and antigenic similarities among various rhinovirus immunotypes (5–7) have suggested varying degrees of relatedness among several different rhinovirus immunotypes. The purpose of this report is to describe our experience in performing reciprocal neutralization tests with all the currently classified rhinoviruses.

*Materials and Methods. Cell culture.* Cell cultures of human fetal diploid lung (HFDL) tissue were exclusively used in this study. The HFDL cell cultures were initiated and maintained in this laboratory by the usual procedures (8). Tube cultures of HFDL cells were seeded with approximately $10^5$ cells in Eagle's minimum essential medium (MEM) and 10% inactivated (56° 30 min) fetal bovine serum (FBS). For maintenance, a modified Eagle's MEM and 2% inactivated FBS was used. The modification consisted of substituting galactose for glucose in Eagle's MEM. All media contained 100 units of penicillin, 100 $\mu$g of streptomycin, and 10 $\mu$g amphotericin B per ml.

*Immune serum.* Rhinovirus (RV) immune sera were produced in guinea pigs immunized with concentrated and partially purified antigens grown in HFDL cells. Infectious tissue-culture fluids were centrifuged overnight at 30,000 rpm; the pelleted virus was resuspended in 1/10 the original volume using serum-free MEM. The resuspended antigens were treated with genetron and homogenized with an equal volume of complete Freund's adjuvant prior to injection.

Eight guinea pigs for each virus type were given two intramuscular injections of 1.0 ml of a virus–adjuvant mixture at an interval of 6 wk and then bled 2 wk after the second injection.

Except for rhinovirus type 74, the prototype virus strains were used for preparation of 91 rhinovirus immune sera. The Dirden virus strain, isolated in this laboratory and shown to be identical to the prototype strain (328A) of rhinovirus type 74, was used as antigen for preparing anti RV 74 serum.

*Immune serum pools.* Ninety-one RV immune sera were incorporated into pools using the intersecting pool scheme of Schmidt (9). Included were antisera to the 89 RV immunotypes, RV subtype 1B, and the Chase virus strain, a prime strain of rhinovirus type 22 (3).

In most cases immune sera with a minimum neutralization titer of 1:512 were selected for inclusion in the intersecting immune serum pools. Each serum pool consisted of equal volumes of either six or seven RV antisera. Sufficient Hanks' balanced salt solution was then added to each serum pool to make a final dilution of 1:30 for each of the sera in the pool. Thus, each serum pool contained approximately 20

[1] The studies on which this paper is based was supported by Grant AI-01475 from the National Institute of Allergy and Infectious Diseases, United States Public Health Service, Department of Health, Eduction, and Welfare, and supported by the Office of the Surgeon General, Department of the Army.

antibody units, or more, of each RV antiserum represented in the pool.

*Serum neutralization test.* Equal volumes of the inactivated (56° 30 min) diluted serum pools or individual antiserum, appropriately diluted, and virus were mixed and incubated at room temperature for 1–2 hr. Serum–virus mixtures were inoculated into tube cultures of HFDL cells in a volume of 0.2 ml. A concurrent virus titration accompanied each test. The neutralization test was read when the virus titration indicated that approximately 100 tissue culture infectious doses ($TCID_{50}$) were present in the test.

*Absorption of sera with human liver powder.* Human liver powder used for absorption of certain anti-RV guinea pig sera was prepared according to the method described by Conant and Hamparian (10). For absorption, the sera were inactivated at 56° for 30 min and diluted 1:4 in Hanks' balanced salt solution. The diluted sera were mixed with a volume of washed packed liver powder equal to the volume of undiluted serum. The mixture was incubated overnight at 4° and shaken for 1 hr at room temperature after which it was clarified by low-speed centrifugation. The absorbed sera were subjected to two additional absorptions with fresh liver powder. After the third absorption the sera were further clarified at 30,000 rpm for 30 min and stored at 4° until tested.

*Results. Reciprocal neutralization tests.* Reciprocal neutralization tests, utilizing ninety-one RV immunotypes and the corresponding immune sera were greatly facilitated by use of the intersecting serum pool scheme (9). Homologous neutralization was observed for each of the RV immunotypes. In addition, several cases of heterologous neutralization were also noted (Table I). Reciprocal neutralization was observed between RV types 29 and 44, and a one-way cross-neutralization reaction occurred between the remaining virus–antiserum combinations.

The observed heterologous reactions were made using serum pools. Because we have on rare occasions noted nonspecific neutralization using serum pools, reciprocal neutralization tests were repeated employing individual immune sera for those RV immunotypes indicated in Table I.

*Reciprocal neutralization between RV types 29 and 44.* Conant and Hamparian (10, 11) reported that the heterologous reactions which they observed were due to "cyto-toxic" antisera and that they were able to remove the cyto-toxic factors by absorption with human liver powder. Accordingly, individual guinea pig anti-RV 29 (Se 2051-1) and anti-RV 44 (Se 1798-6) sera were absorbed three successive times and compared with the same unabsorbed antisera in reciprocal neutralization tests with RV types 29 and 44. The results are presented in Table II. Using the individual, unabsorbed sera there was significant cross neutralization with both anti-RV 29 and 44 guinea pig sera. With this particular anti-RV 29 guinea pig serum no distinction between RV virus types 29 and 44 was possible. The heterologous titer of anti RV 44 serum was eight fold lower than its homologous titer. After absorption of anti-RV 29 and 44 sera with human liver powder no significant difference in either the homologous or heterologous titers was observed.

TABLE I. Heterologous Neutralization of Various Rhinoviruses.[a]

| Rhinovirus type | Neutralized by anti-RV serum |
|---|---|
| 1A | 1B |
| 9 | 32 |
| 22 | Chase virus[b] |
| 29 | 44 |
| 36 | 58 |
| 44 | 29 |
| 49[c] | 2 |

[a]Results of reciprocal neutralization tests of 91 rhinoviruses using pooled sera.

[b]A "prime" strain of RV 22.

[c]Neutralization of RV49 could not be demonstrated in tests employing individual anti RV2 serum.

*One-way serologic cross reactions.* Results of reciprocal neutralization tests using intersecting serum pools indicated that RV 9 was neutralized by anti-RV 32 guinea pig serum and that RV 36 was neutralized by anti-RV 58 guinea pig serum. Reciprocal neutralization tests between these two virus pairs were repeated using individual antisera. For purposes of comparison, anti-RV 32 and 58 sera were also absorbed with human liver powder and tested simultaneously with the unabsorbed sera. The results are shown in Table III. The one-way cross reactions between RV 9 and 32, and RV 36 and 58 observed with serum pools were reproducible using individual antisera.

TABLE II. Effect of Absorption with Human Liver Powder on Reciprocal Neutralization Between RV Types 29 and 44.

| | RV antisera | | | |
|---|---|---|---|---|
| | 29 | | 44 | |
| Rhinovirus type | Unabsorbed | Absorbed | Unabsorbed | Absorbed |
| 29 | 256/100[a] | 128/100 | 64/100 | 32/100 |
| 44 | 256/63 | 256/63 | 512/63 | 512/63 |

[a]Reciprocal of serum dilution/$TCID_{50}$

The heterologous neutralization of RV 9 by anti-RV 32 serum was not significantly affected by human liver powder absorption. There was, however, a 4-fold reduction in the heterologous titer of anti-RV 58 guinea pig serum after absorption.

Heterologous neutralization between RV types 1A and 1B has been consistently observed by many investigators (5, 7, 11–13) and the data in Table IV are additional confirmation of this relationship. The one-way neutralization of RV 49 by anti-RV 2 guinea pig serum obtained with pooled sera, however, could not be demonstrated in repeated tests when the individual RV 2 antiserum was used (Table IV).

Reciprocal neutralization between RV types 13 and 41 was observed by Cooney *et al.* (7). Our screening tests of these two viruses, employing serum pools, did not confirm this relationship. Additional tests with individual anti-RV 13 and 41 guinea pig sera (Table IV) also failed to establish a serologic relationship between these viruses.

TABLE III. Effect of Absorption with Human Liver Powder on Heterologous Neutralizing Activity of Certain RV Antisera.

| Rhinovirus type[a] | Anti RV sera | | |
|---|---|---|---|
| | 9 (SE 1392-8) | 32 (SE 2021-1) | |
| | Unabsorbed | Unabsorbed | Absorbed |
| 9 | 256[b] | 32 | 16 |
| 32 | <16 | 1024 | 1024 |
| | Anti RV sera | | |
| | 36 (SE 1681-6) | 58 (SE 1805-6) | |
| | Unabsorbed | Unabsorbed | Absorbed |
| 36 | 2048 | 16 | 4 |
| 58 | <16 | 1024 | 512 |

[a]$TCID_{50}$ used in these tests ranged between 32 and 320.
[b]Serum titer expressed as reciprocal of serum dilution.

*Discussion*. Ninety-one rhinoviruses were examined in reciprocal serum neutralization tests employing pooled antisera. Each of 91 RV types was neutralized by serum pools containing their respective homologous immune serum. There was a single instance in which we observed reciprocal neutralization between two RV immunotypes. The reciprocal relationship between RV types 29 and 44 has not previously been reported. Conant and Hamparian (11) noted a one-way cross relationship between these viruses but concluded it was nonspecific since the heterologous reaction could be removed by absorption with human liver powder. We could not confirm this finding. Our data suggest that the relationship is not one-way but reciprocal especially since three successive absorptions of both anti-RV 29 and 44 guinea pig sera did not significantly alter the heterologous neutralizing activity of these sera. The serologic relationship between these viruses is reminiscent of the prime strain variation reported for RV type 22 (3). It is unfortunate that RV type 44 was not included in the study reported by Cooney *et al.* (7).

The serologic relationship between rhinovirus types 1A and 1B is one about which there is general agreement. However, even with this combination Monto (12) has clearly shown that the low-order cross reaction between RV types 1A and 1B may be either reciprocal or one-way depending upon whether the 2060 or JH virus strain of RV type 1A is used. This would suggest that minor intratypic variations may possibly be one reason for the divergent findings observed by various investigators.

Our findings also suggest a low-order one-way cross reaction in which RV 9 is neutralized by anti-RV 32 serum. The heterologous neutral-

TABLE IV. Reciprocal Rhinovirus Neutralization Tests.

| Rhinovirus type | Reacting RV antiserum | Serum titer | |
|---|---|---|---|
| | | Homologous | Heterologous |
| 1A | 1B | 512/100[a] | 64/100 |
| 1B | 1A | 512/320 | <16/320 |
| 2 | 49 | 2048/320 | <16/320 |
| 49 | 2 | 2048/63 | <16/63 |
| 13 | 41 | 128/32 | <8/32 |
| 41 | 13 | 512/32 | <8/32 |

[a]Reciprocal of serum dilution/$TCID_{50}$.

izing activity of this serum was reduced from 1:32 to 1:16 by three absorptions with human liver powder. Conant *et al.* (11) also observed a similar one-way relationship between these viruses when unabsorbed anti-RV 32 serum was used. However, these investigators indicated that five absorptions were required to remove the heterologous reactivity. Since we absorbed anti-RV 32 serum three times, it is possible that additional absorptions would have removed the heterologous reactivity we noted. A low-order reciprocal relationship was first reported between these same virus tpes by Cooney *et al.* (7), who did not, however, test for the heterologous reaction with absorbed sera.

The heterologous neutralization of RV 36 by anti-RV 58 serum was minimal with unabsorbed serum and was significantly reduced when absorbed serum was used. This was one instance in which we were able to observe a significant reduction in heterologous activity with absorbed serum.

The data from several investigations on antigenic similarities among rhinoviruses (5–7, 11, 12) have been for the most part conflicting and confusing. Cooney *et al.* (6, 7) reported a reciprocal relationship between RV types 2 and 49 and between RV type 13 and 41. Conant *et al.* (12) also observed a similar relationship between RV types 13 and 41 with unabsorbed sera but not when absorbed sera were used. In the present study, using individual antisera, we could find no evidence of a serologic relationship between either RV 2 and 49 or RV 13 and 41. Although in our hands RV 49 was neutralized by serum pools containing anti-RV 2 serum, we could not demonstrate neutralization of RV 49 using individual anti-RV 2 serum, despite the fact that it was the same anti-RV 2 guinea pig serum that was incorporated into the serum pools. We, therefore, do not consider the neutralization of RV 49 by serum pools containing anti-RV 2 serum to be specific. The data of Cooney *et al.* further indicate a one-way relationship between RV types 5 and 42, 11 and 40, 17 and 42, 36 and 50, and between types 39 and 54. The present study and that of Conant (11), on the other hand, clearly indicate that these viruses are unrelated and distinct virus immunotypes.

We are unable at the present time to explain fully the divergence of our findings from those of previous investigators. Further studies employing gel diffusion or radioimmuno-assay techniques with carefully prepared reagents may provide additional insight. In this respect, it is of the utmost importance for studies of this nature that antigens used for production of immune sera be purified by the best available procedures to reduce to a minimum the stimulation of antibodies to extraneous proteins. Attention should also be paid to the selection of immunization schedules which produce a highly specific antigenic response to the antigen. A long series of repeated injections serves not only to heighten the response to what is essentially a distinct antigen but more importantly to broaden the response to a minor antigenic component shared with another virus type.

*Summary.* Ninety rhinovirus types and a prime strain of RV 22 were compared in reciprocal serum neutralization tests. Neutralization was observed for each of the 91 homologous virus–serum mixtures. A single instance of reciprocal neutralization between two rhinovirus serotypes was noted. This reciprocal relationship, between RV types 29 and 44 was not altered by absorption with human liver powder. It is suggested that the relationship between RV types 29 and 44 may be a prime strain relationship similar to that described for RV type 22.

Other antigenic similarities noted in this study were one-way crosses between RV types 1A and 1B, 9 and 32, and between types 36 and 58. The heterologous neutralizing activity of anti-RV 58 guinea pig serum was significantly reduced by absorption with human liver powder. Absorption did not materially alter the cross reactivity observed with the remaining antisera.

1. Kapikian, A. Z., Conant, R. M., Hamparian, V. V., Chanock, R. M., Dick, E. C., Fenters, J. D., Gwaltney, J. M., Hamre, D., Holper, J. C., Jordan, W. S., Lennette, E. H., Melnick, J. L., Mogabgab, W. J., Mufson, M. A.,

Phillips, C. A., Schieble, J. H., and Tyrrell, D. A. J., Nature (London) **213**, 761 (1967).

2. A collaborative report, Virology **43**, 524 (1971).

3. Schieble, J. H., Lennette, E. H., and Fox, V. L., Proc. Soc. Exp. Biol. Med. **133**, 329 (1970).

4. Stott, E. J., and Walker, M., Nature (London) **224,** 1311 (1969).

5. Fenter, J. D., Gillum, S. S., Holper, J. C., and Marquis, G. C., Amer. J. Epidemiol. **84**, 10 (1966).

6. Cooney, M. K., and Kenny, G. E., J. Immunol. **105**, 537 (1970).

7. Cooney, M. K. , Kenny, G. E., Tam, R., and Fox, J. P., Infec. and Immun. **7,** 335 (1973).

8. Hayflick, L., and Moorhead, P. S., Exp. Cell Res. **25**, 585 (1961).

9. Schmidt, N. J., Guenther, R. W., and Lennette, E. H., J. Immun. **87**, 623 (1961).

10. Conant, R. M., and Hamparian, V. V., J. Immun. **100**, 107 (1968).

11. Conant, R. M., and Hamparian, V. V., J. Immun. **100**, 114 (1968).

12. Monto, R. S., and Johnson, K. M., Proc. Soc. Exp. Biol. Med. **121**, 615 (1966).

13. Taylor-Robinson, D., and Tyrrell, D.A.J., Lancet **1**, 452 (1962).

---

Received June 3, 1974. P.S.E.B.M. 1974, Vol. 147.