Immunogenicity of Rhinoviruses[1] (34536)

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Reports in the literature (1, 2) have indicated variable success in producing rhinovirus antisera of adequate potency for identification and antigenic comparison of rhinoviruses. The definition of 55 serotypes and 1 subtype (3), and the large number of candidate strains which are probably additional serotypes, have made it imperative that a method be devised for producing the potent antisera needed for identification of isolates collected in the course of epidemiologic studies (4). More importantly, potent antisera are needed for antigenic analysis of known serotypes. The present report describes the results of our attempts to define a minimum rhinovirus antigen dose for eliciting immune response in rabbits. In general, rhinovirus immunogens with a virus content of 10^7 plaque-forming units (pfu)/ml or greater elicited antibody response of high potency. Some of these data have been presented in a brief preliminary report (5).

Materials and Methods. Cell cultures. M-HeLa cell cultures were employed for propagation and plaque assay of rhinoviruses. Growth medium was Eagle's minimum essential medium (MEM) with 10% fetal calf serum. For virus propagation 2% agamma calf serum was used in MEM with sufficient $MgCl_2$ added to give a concentration of 30 m*M* Mg^{2+}. This medium, with 0.3% agarose (SeaKem brand) incorporated, was used as overlay for plaque assay, which was performed as described in a previous publication (6).

Virus. The HeLa-propagated prototype strains of rhinovirus serotypes were obtained from Dr. V. Hamparian.

Neutralization tests. All sera were heated at 56° for 30 min. Equal volumes of serial 2-fold dilutions (1:256–1:4096) and virus (approximately 100 TCD_{50}) were mixed. After 1 hr at room temperature, 0.2 ml of serum–virus mixture was inoculated into each of 4 HeLa cell tubes. Tubes were incubated at 33°, and examined for cytopathic effects (CPE) when virus control titration indicated the virus test dose to be between 32 and 100 TCD_{50}. Serum neutralization end points (*N* titers) were expressed as the reciprocal of serum dilution which completely neutralized virus in 50% of inoculated tubes as calculated by the method of Reed and Muench (7). If titers were below 256 or above 4096, sera were retested at appropriate dilutions.

Determination of neutralization rate constants (k values). Equal volumes of diluted serum and virus (20,000 pfu/ml) were mixed and incubated in a 37° water bath. A virus control was incubated under the same conditions. The serum–virus mixture and virus control were sampled at several time intervals, diluted 1:100 in cold diluent to stop neutralization, and 0.2 ml was plated on HeLa monolayers (60-mm plastic dishes, Falcon Plastics) to assay the unneutralized virus. The first-order reaction constant, *k*, was calculated (8).

Anticellular antibody titrations. A mixed cell agglutination test was performed in which equal volumes of a 1% suspension of HeLa cells and 1% suspension of human O red blood cells were used as antigen. Serial 2-fold dilutions of serum were prepared in 0.5-ml amounts, 0.5 ml of mixed cell suspension was added, and tests were incubated for 2 hr in a 37° water bath. Results were read by pattern after overnight refrigeration. Anti-HeLa cell antibody titers also were determined by indirect fluorescent antibody tests.

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HeLa cells were fixed to a slide, incubated with rabbit sera, washed, and stained with fluorescein-tagged antirabbit gamma globulin (Antibodies Incorporated, Davis, California). Anticellular antibody titers were expressed as the reciprocal of the highest dilution of serum which showed agglutination or specific fluorescence. Titers of anticellular antibody measured by the two methods agreed within one 2-fold dilution.

Production of immunogens. A stepwise procedure was followed to raise the virus titer to a maximum, in order to provide an inoculum with a high multiplicity of virus to produce the final immunogen. Fiala and Kenny (9) showed that presence of 30 m*M* $MgCl_2$ enhanced adsorption and increased virus release of rhinovirus 2 in HeLa cells. Enhancement of plaque-forming capacity of most rhinovirus types was reported by Kenny and Cooney (10). Serial transfer of rhinoviruses in the presence of 30 m*M* Mg^{2+} was performed to attempt to raise virus titers. Usually, passage level in which 4+ CPE was noted in 24 hr coincided with attainment of maximum virus titers.

Virus was serially transferred in HeLa cell cultures until 4+ CPE was observed in 24 hr. When this result was attained, 3 ml of undiluted virus suspension (about 10^7 pfu/ml to give a virus: cell multiplicity of at least 2:1) were inoculated per bottle into each of 10 HeLa cell bottles (120 cm^2 surface) and incubated at 33° for 12–15 hr. Maintenance medium was removed as soon as cells showed rounding, but before marked CPE was evident, in order to harvest intracellular virus. Cells were scraped off the glass surface with a rubber policeman, suspended in 1.5 ml of Hanks' solution/bottle, and pooled. The cell suspension was homogenized in a Virtis "45" homogenizer. The homogenate was centrifuged at 7000*g* for 20 min to remove cell debris, and the supernatant fluid was treated

TABLE I. Immunogenicity of Rhinovirus Types 1A and 2.

Titer of immunogen (pfu/ml)	Rabbit no.	Antibody response							
		21 days[a]		38 days		45 days		46–50 days	
		Titer[b]	*k* Value[c]	Titer	*k* Value	Titer	*k* Value	Titer	*k* Value
Rhinovirus 1A									
1.4×10^9	1	512	23	5760	763	4096	406	5760	920
	2	281	14.5	4096	128	2882	77	4096	ND[d]
1.4×10^8	3	281	7.2	5140	387	5140	352	5140	396
	4	Rabbit died							
1.4×10^7	5	<10	<0.5	2048	47	2882	55	4096	66
	6	20	0.6	804	ND	2882	74	5140	121
1.4×10^6	7	<10	<0.5	<16	<0.5	<16	<0.5	32	0.8
	8	<10	<0.5	<16	<0.5	<16	<0.5	32	0.8
Rhinovirus 2									
3×10^8	9	64	8.3	2048	296	1448	460	646	112
	10	64	4.6	1620	322	1448	99	646	69
3×10^7	11	ND	ND	<16	ND	<16	ND	<16	ND
	12	ND	ND	<16	ND	<16	ND	<16	ND

[a] Number of days after initial injection of immunogen.

[b] Reciprocal of dilution of serum which completely neutralized virus in 50% of inoculated tubes as calculated by the method of Reed and Muench.

[c] Neutralization rate constant calculated by the equation: $k = 2.3 \times (D/t) \times \log (V_0/V_t)$ where $D =$ reciprocal of final serum dilution in virus-serum mixture, $t =$ time (min), $V_0 =$ virus pfu at time 0, $V_t =$ virus pfu at time t (8).

[d] ND = not determined.

TABLE II. Production of Rhinovirus Immunogens.

Sample protocol: rhinovirus 38

1. Virus: rhinovirus 38 (Strain CH 79)
   Passage history: HEK_1 Wi_8 $HeLa_7$
   Virus titer:[a] 2×10^4 pfu[b]/ml (30 m*M* Mg^{2+} in overlay).
2. Seven further transfers in HeLa cells with 30 m*M* Mg^{2+} in fluid medium, transferred when 4+ CPE was evident
   Virus titer: 4.5×10^6 pfu/ml.
3. Preparation of inoculum for final immunogen: 40 ml of infected cells and fluid were homogenized, cell debris was removed by centrifugation. Approximately 3 ml of supernatant were used as inoculum per 120-cm^2 bottle of HeLa cells.
   Virus titer of inoculum: 3.15×10^7 pfu/ml.
4. Final immunogen: infected cells from 10 HeLa bottles (120 cm^2) harvested before virus release (12–15 hr after inoculation). Approximately 10× concentration of virus effected by harvesting cells in 1.5 ml of Hanks' BSS/bottle. Cell suspension was homogenized and treated with fluorocarbon.
   Virus titer: 1.1×10^8 pfu/ml.

[a] HeLa cell monolayers used for plaque assay.
[b] pfu = plaque-forming units.

with fluorocarbon (Genesolv-D Allied Chemical Company). The fluorocarbon-treated virus suspension constituted the final immunogen, and was stored at —70°.

Immunization of rabbits. A schedule of injections, successfully used by Kenny (11) for preparing antisera in rabbits to various *Mycoplasma* species, was adopted. Two rabbits were injected with each immunogen. A preimmune blood sample was drawn 2-3 days prior to the first injection. On day 0, 2 ml of rhinovirus suspension were emulsified with 2 ml of Freund's incomplete adjuvant (Difco), and 1 ml of emulsion was injected into each of 4 intramuscular (im) sites. On day 21, rabbits were bled, and injected intravenously (iv) with 0.1 ml of immunogen. Injections of 0.2 ml, 0.3 ml and 0.4 ml, iv, were given at 3–4-day intervals through day 31. On day 38, rabbits were bled and a final iv injection of 1 ml was administered. Rabbits were bled on day 45 and several days thereafter (11).

Results. Estimation of minimal immunogenic dose. Pilot experiments with rhinoviruses 1A and 2 were conducted to determine the minimal immunogenic dosage in terms of pfu/ml. Four serial 10-fold dilutions of the rhinovirus 1A immunogen (highest concentration injected 1.4×10^9 pfu/ml) were inoculated into 2 rabbits each per virus dosage. Dosage levels of rhinovirus 2 were 3×10^8 pfu/ml and 3×10^7 pfu/ml. The minimum dose of rhinovirus 1A to elicit potent antibody was 1.4×10^7 pfu/ml (Table I). The antibody response as measured by tube neutralization test was of the same order of magnitude with 1.4×10^9 pfu/ml as with 1.4×10^7. A response to rhinovirus 2 required 10^8 pfu/ml. Accordingly, the minimal rhinovirus dosage for immunizing rabbits by this immunizing schedule appeared to be about 10^7 to 10^8 pfu/ml of immunogen. We therefore determined whether high-titered rhinovirus preparations could be made from a number of different serotypes using the procedure developed for rhinoviruses 1A and 2.

Production of rhinovirus immunogens. A sample protocol for production of rhinovirus 38 immunogen is shown in Table II. The only variation in this protocol with different rhinovirus types was in the number of transfers (step 2) needed to achieve total CPE in 24 hr. The titers of 24 of the immunogens thus produced are shown in Table III, ranked by increasing titer. Eighteen immunogens had titers of 1.0×10^7 pfu/ml, or greater. Usually, the virus content of the immunogen was increased 2- to 10-fold by harvesting intracellular virus. Removal of medium prior to harvest had the additional advantage of eliminating bovine serum from the immunogen.

Antibody response in rabbits. The neutralizing antibody response shown by rabbits injected with rhinovirus 38 immunogen, depicted in Fig. 1, was typical. Twenty of 30 rabbits showed peak neutralizing antibody titer on day 38 of the immunization schedule, 1 week after the "iv series." The final 1 ml, iv, injection of antigen on day 38 usually failed to elicit further antibody response. Fifteen of 20 rabbits with peak antibody titers at 38 days showed a slight (less than 4-fold) decrease in titer 1 week (day 45) after the final iv injection. Identical titers were shown in 5 rabbits at 38 and 45 days. Five rabbits showed a peak antibody titer at day 45. In 3

TABLE III. Relationship of Titer of Rhinovirus Immunogens to Antibody Response in Rabbits.

Rhinovirus type[a]	Time of harvest (hr post-inoculation)	Virus cell associated (%)[b]	Titer of immunogen (pfu/ml)	Antibody response[c] in rabbits	
				Rabbit 1	Rabbit 2
48	12	ND[d]	2.1×10^4	<16	<16
34	12	63	2.3×10^5	5760	Died
35	12	10	6.0×10^5	2576	1024
47	13	82	1.9×10^6	1024	1448
42	14	ND	2.5×10^6	2048	1024
54	12	11	2.6×10^6	730	256
31	12	83	1.0×10^7	1024	Died
39	13	ND	1.8×10^7	804	1620
32	14	52	2.0×10^7	1620	Died
37	13	ND	2.3×10^7	2576	4096
29	15	68	2.8×10^7	1024	1620
36	12	ND	3.0×10^7	5760	5760
33	12	49	3.0×10^7	2882	Died
11	15	74	4.5×10^7	2882	2882
40	13	ND	4.5×10^7	2576	1448
1B	13	94	6.0×10^7	1448	Died
38	12	88	1.1×10^8	4096	11,500
41	12	90	1.5×10^8	10,000	4096
14	15	74	1.6×10^8	4096	Died
30	14	75	1.6×10^8	730	Died
49	14	87	1.7×10^8	5760	5140
51	13	99	4.2×10^8	23,000	23,000
9	14	98	8.2×10^8	2882	Died
52	14	78	1.0×10^9	11,500	11,500

[a] Ranked according to virus titer.

[b] Virus cell associated (%) = (total pfu of intracellular virus/total pfu of extracellular virus + total intracellular virus).

[c] 50% neutralization end point.

[d] ND = not determined.

of these the difference in titer was less than 4-fold, but in 2 rabbits, each of which received a different antigen, titers in the 38-day bleeding were less than 16. On day 45, one of these rabbits had a titer of 4096, and the other a titer of 11,500. The geometric mean titer of the antibody response was directly related to the titer of the immunogen (Table IV).

Anticellular antibody. Antibody was detected by the mixed cell agglutinatiion test in 13 of 50 rabbits prior to immunization. Sera from the remaining 37 rabbits had anticellular antibody titers of <1:10 in the preimmunzation serum and 24 of these rose to 1:20 or above during immunization (Fig. 2). The maximum anticellular antibody titer found in 7 of the 50 rabbits, was 1:160.

TABLE IV. Antibody Response in Rabbits to Antigens of 25 Rhinovirus Serotypes.

Range of immunogen titer (pfu[a]/ml)	No. of rabbits in group	Range of antibody response[b]	Geometric mean of antibody response
2.3×10^5 to 2.6×10^6	9	256–5760	1200
1.0×10^7 to 6.0×10^7	16	804–5760	2100
1.1×10^8 to 8.2×10^8	13	730–23,000	4860
1.0×10^9 to 1.4×10^9	4	4096–11,500	7400

[a] pfu = plaque-forming units.

[b] Expressed as reciprocal of serum dilution which completely neutralized virus in 50% of tubes inoculated.

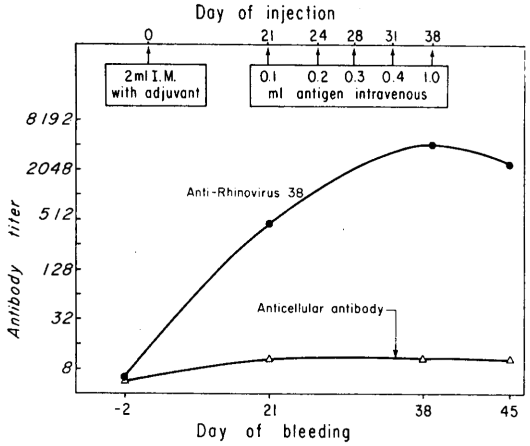

FIG. 1. Rabbit immunization and bleeding schedule: rhinovirus 38 immunogen. On day 0, 2 ml of antigen emulsified with an equal volume of Freund's incomplete adjuvant was injected intramuscularly. Antiviral antibody titer is expressed as the reciprocal of the 50% end point of neutralizing antibody titer as calculated by the method of Reed and Muench. Anticellular antibody titer is expressed as reciprocal of the highest serum dilution which agglutinated a mixture of human O red blood cells and HeLa cells.

These results indicate that the fluorocarbon purification step appeared to be quite successful in reducing cellular antigen content and consequent anticellular antibody production. The anticellular antibody could be removed effectively by adsorption with HeLa cell suspension or with human group O erythrocytes. Adsorbed sera were negative for anticellular antibody when tested by fluorescent antibody or agglutination methods, but specific antirhinovirus titers were not reduced.

Discussion. The data obtained on the immunogenicity of rhinoviruses 1A and 2 (Table I), indicated that a titer of 10^7 or 10^8 pfu/ml was sufficient to stimulate antibody response in rabbits. The protocol shown in Table II, with rhinovirus type 38 as an example, was used for preparation of all immunogens and usually resulted in an immunogen with a titer of at least 10^7 pfu/ml. When rabbits were immunized according to schedule shown in Fig. 1, variation in rabbit antibody response with respect to content of infectious particles injected was noted with several rhinovirus types (Table III). A remarkable uniformity of response was observed in pairs of rabbits injected with the same antigen, as shown in Table III. This would suggest that quantity of immunogens, not necessarily as measured by plaque assay, is a major factor in determining antibody response. The reason some immunogens with low titers (e.g., rhinovirus 34) elicited high antibody levels may be a variation in particle:infectivity ratio, either because of an insensitive assay or presence of noninfectious but immunogenic particles. Virus aggregation may also play a role by resulting in spuriously low plaque titers. However, data presented in Table IV show that injection of higher concentrations of virus generally results in higher antibody titers. As shown in Table I, *N* titer does not necessarily show a quantitative relationship to the rate of neutralization by the serum (*k* value). We can look upon *k* values as a measure of both potency and avidity. The procedure for determination of this constant requires a very large dilution, usually about 1:5000, when plated for plaque assay of the serum–virus mixture, which is a test for stability of the antigen–antibody union. Although the *N* titers of antibody in the postimmunization

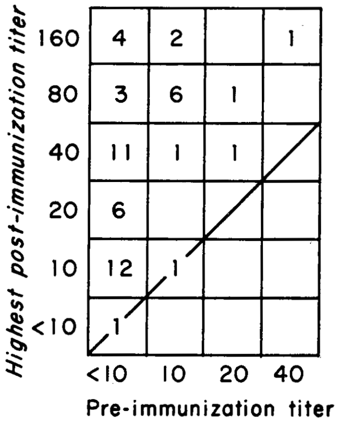

FIG. 2. Anticellular antibody response in rabbits immunized with rhinoviruses propagated in HeLa cells. Antibody titer expressed as the reciprocal of the highest dilution of serum which agglutinated a mixed suspension of HeLa cells and human O red blood cells. Preimmunization titers are correlated with maximum titer observed in any of the series of postimmunization bloods.

bleedings after a rhinovirus 1A dose of 1.4 × 10^7 pfu/ml were similar to those elicited by 1.4 × 10^9 pfu/ml, the *k* values of one rabbit's sera with the larger dose were consistently much higher and perhaps reflect increased avidity. Highly avid sera would be desirable for examining heterologous rhinovirus types for cross-reactions. We were particularly concerned about levels of anti-HeLa cell antibody in these sera since we plan to pool them in various combinations for typing rhinovirus isolates and would expect to have high serum concentration in the pools. We also plan to use low dilutions of the sera to look for cross-reactions (12) among rhinovirus serotypes. Habel *et al.* (13) showed that anticellular antibody could interfere with virus attachment to cells and simulate neutralizing effect. Conant and Hamparian (14) reported that a number of bovine, goat, and guinea pig antirhinovirus sera inhibited heterologous rhinovirus serotypes, and that this heterologous reactivity could be removed by absorption with human liver powder though such absorption did not affect homologous antibody titer. These authors noted the apparent lack of correlation between cytotoxicity of antisera and nonspecific inhibition of CPE of heterologous serotypes, and question the relationship of nonspecific inhibition and anticellular antibody. Garabedian and Syverton (15) used an indirect hemagglutination test to detect anti-HeLa cell antibody in immunized rabbits. We found that direct agglutination of mixed HeLa and human O cells could be demonstrated. We used cell agglutination as an independent measure of anticellular antibody levels (Fig. 1) since demonstration of cytotoxic effect is not satisfactorily reproducible. Although we demonstrated agglutinins in the rabbit sera, which increased in titer during the course of injections, 1:20 serum dilutions have not revealed any nonspecific inhibition of heterologous rhinovirus types.

Summary. Uniformly successful production of rabbit rhinovirus antisera depended on production of immunogens with virus concentrations of at least 10^7 pfu/ml. Response in pairs of rabbits immunized with the same antigen appeared to be remarkably uniform, and maximum antibody titers were attained at 38 to 45 days after beginning a relatively simple immunization schedule (1 im + 4 iv injections). Anticellular antibody levels were usually low in comparison to rhinovirus antibody titers and such antibody could be removed easily by adsorption with suspensions of whole HeLa cells without reducing the level of antiviral antibody.

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