Attenuation of Rhinovirus Type 15: Relation of Illness to Plaque Size

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Received for publication 8 November 1973

Rhinovirus type 15 passed three times in WI-38 cells produced illness in only one of 35 volunteers, but infection resulted in formation of significant quantities of antibodies in the sera of 100%, and in nasal secretions of 89%, of infected volunteers. The inoculum contained large- (60%) and small- (40%) plaque variants. “Purified” large- and small-plaque inocula were prepared, and each was administered to 20 volunteers. There was a significant association of illness with the small-plaque inocula (P < 0.01) but the incidence of infection, quantitative virus shedding patterns, and mean serum and nasal secretory antibodies were not significantly different between the two groups. These findings suggest that plaque size may be an in vitro marker of attenuation of illness production by rhinoviruses.

Rhinoviruses are the most frequently isolated viruses from adults with common colds (9). Attempts to develop conventional parenteral vaccines as a means for control of these illnesses have not been successful, since such vaccines have been poorly antigenic or have failed to induce significant protection against infection (6, 10). Therefore, other methods for control of these infections are needed.

We recently reported attenuation of illness in humans with rhinovirus type 15 passed three times in tissue cultures (5). In the present studies, this work was extended to 35 additional volunteers, and serial determination of serum and nasal secretion antibody was performed. Attenuation of illness was confirmed, and development of antibody in both locations resulted from this infection. Studies of plaque size revealed that the third-passage inoculum contained predominantly large-plaque-producing variants. “Purified” large- and small-plaque inocula were then made and administered to volunteers. Illness developed more frequently in volunteers given the small-plaque variant, suggesting that in vitro markers of attenuation may be found which could lead to selection of attenuated rhinovirus variants for vaccine development.

MATERIALS AND METHODS

Studies were performed at the Ramsey Unit of the Texas Department of Corrections. Procedures for acquiring volunteers and performing studies in the institution are essentially the same as those previously reported (3, 8).

Clinical evaluation. Volunteers were examined once daily for 10 days by physicians who knew the date of inoculation but not the nature of the virus inoculum. Oral temperatures were recorded every 12 h. A designation of respiratory illness was based on daily evaluations and graded according to severity (1++, 2+, 3+, 4, 5); predominant site of involvement (rhinitis, pharyngitis, tracheobronchitis, or systemic illness), and occurrence of fever (>38 C).

Virus inocula. The challenge stock of rhinovirus type 15 WI was prepared from third-passage harvests in human embryonic lung fibroblast (WI-38) tissue cultures and safety tested as previously described (1). The 50% human infectious dose (HID₅₀) for this inoculum was previously reported to be 1.0-50% tissue culture infectious dose (TCID₅₀) (5); with further inoculations, this estimate is revised to 1.5 TCID₅₀. The small-plaque (WI₁₋₁SP) and large-plaque (WI₁₋₁LP) stocks were each made from eleventh-passage harvests in WI-38 cells. Each was purified by picking single plaques of the designated size four times serially by using the plaque techniques described below, except that the overlay additive consisted of 0.5% Noble agar. The additional passages were made in tube cultures with fluid media.

Inoculation of volunteers was by nasal drops, 0.25 ml per nostril of 1:200 dilution of type 15 WI pool and a 1:10 dilution of the WI₁₋₁LP and -SP pools. Quantities administrator were determined by simultaneous titration of the inoculum in WI-38 tube cultures.

Virus isolations and identifications. Nasal wash specimens were collected and tested in WI-38 cultures as previously described (1). The first and last virus isolates from each volunteer were identified by neu-
neutralization tests (1). Quantities of virus in nasal wash specimens were determined by tube dilution titrations in WI-38 cells (4).

Neutralization tests and immunoglobulins. Nasal secretions (NS) were collected and processed as previously described (2). They were then concentrated approximately 10-fold by dialysis against dry Sephadex G200 powder, a method shown to provide concentration without significant loss of immunoglobulin A (IgA) or neutralizing activity.

Sucrose density gradient ultracentrifugation was performed as previously described (2). Tests for neutralizing antibody were performed in WI-38 cultures by described methods (1). The lowest dilution of serum tested was 1:2, and that of concentrated NS was 1:4. Quantities of IgA were determined in each concentrated nasal secretion specimen and in sucrose density gradient fractions by the single radial diffusion method against an IgA standard made from nasal secretions and containing predominantly 11S IgA.

Plaque techniques. Plaquing was performed in 25-cm² plastic flasks containing WI-38 cell monolayers with an overlay consisting of minimum essential medium (Eagle) supplemented with 1.0% Noble agar, 0.25 µg of diethylaminoethyl-dextran per ml, and 0.36 mmol of MgCl₂ per ml. Flasks were incubated at 33 C in 5% CO₂ for 5 or 9 days. Fluid neutral red (1:1,000) was overlaid, and plaques were examined 18 to 24 h later. Small plaques were 2 to 3 mm at 6 days and 6 to 10 mm at 10 days. Large plaques were 5 to 7 mm at 6 days and 12 to 17 mm at 10 days.

RESULTS

Responses of volunteers to nasal inoculations with rhinovirus type 15 WI₂. Rhinovirus type 15 WI₂ was administered to 35 volunteers with low or undetectable levels of serum antibody (≤ 1:4). The inoculum dose was 320 TCID₅₀, approximately 200 HID₅₀. Twenty-seven volunteers shed the inoculated virus type, and the mean number of isolates per man was 4.8. All of these volunteers, and four others who did not shed virus, developed fourfold or greater serum neutralizing antibody responses, making a total of 31 of 35 infected volunteers (89%). Of the four volunteers who resisted challenge, none had detectable neutralizing antibody in nasal secretions, and only one had serum antibody (1:4).

The illness results are shown in Table 1 and are compared with previous results with second-passage material (WI₂) (4). Although these studies were not performed simultaneously, the continued ability of the second-passage inoculum to produce illness has been demonstrated. Only one of 31 volunteers who received WI₂ developed illness, and the illness was accompanied by fever (38 C). His specimens were also tested in human embryonic kidney and rhesus monkey kidney cultures and, after neutralization with type 15 antiserum, in WI-38 cells. No other viruses were isolated.

The results of testing sera and nasal secretions for neutralizing antibody from the 31 volunteers that became infected are shown in Fig. 1. Significant serum antibody responses had occurred in 14 of 31 volunteers by 2 weeks, 30 of 31 by 3 weeks, and all 31 by the 4th week after inoculation. In addition, 25 of 29 volunteers who were tested developed significant nasal secretion antibody responses.

Sucrose density gradient ultracentrifugation studies were performed on four NS specimens obtained on day 28 after inoculation from four different volunteers. The major portion of neutralizing antibody sedimented at the 11S region and was associated with the presence of IgA.

Plaque size distribution of volunteer inocula. The WI₂ inoculum contained a higher proportion of large-plaque variants than did the WI₁ inoculum (Table 2).

Volunteer inocula containing predominantly small- and large-plaque variants were prepared from the WI₂ stock as described in Materials.

TABLE 1. Comparison of clinical responses of volunteers to nasal inoculations with two different stocks of rhinovirus type 15

<table>
<thead>
<tr>
<th>Virus inocula*</th>
<th>No. of volunteers infected</th>
<th>No. of volunteers ill</th>
<th>No. of volunteers with indicated illness response</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI₁</td>
<td>24</td>
<td>20</td>
<td>18 (75)⁹ 0 2 (8) 1 (3)</td>
</tr>
<tr>
<td>WI₂</td>
<td>31</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

* See Materials and Methods for details concerning each stock.
* P < 0.001 by chi square test.
* Numbers in parentheses are percentages.

Fig. 1. Geometric mean of serum and nasal secretion antibody responses to infection with rhinovirus type 15, WI₂. All specimens included in calculations.
Responses of volunteers to nasal inoculations with WI₁₁-SP and WI₁₁-LP pools. Twenty antibody-free volunteers were inoculated intranasally with the WI₁₁-SP and WI₁₁-LP pools (Table 3). In each case, titration revealed that the dose administered was large and was somewhat larger for the LP than the SP pool. Although infection rates were similar for the two pools, illness rates were significantly different. However, among ill volunteers, illness caused by the two inocula was indistinguishable in terms of severity, incidence of fever, and duration.

All volunteers in the WI₁₁-LP group began to shed virus on the first or second day after inoculation, whereas five volunteers in the WI₁₁-SP group had onsets on the third day or later ($P < 0.10$, $> 0.05$). Quantitative virus shedding patterns for the two inoculum groups are shown in Fig. 2. Although the WI₁₁-LP group consistently shed more detectable virus, these differences also are not significant ($P > 0.05$, $t$ test). Comparison of mean peak quantities detected (2.77 versus 2.30 log₁₀ TCID₅₀) and median duration of shedding (7.5 versus 7.5 days) also failed to reveal significant differences between the two groups ($P > 0.10$, $t$ test).

Antibody responses of the two groups were similar. The frequency of development of significant serum and nasal secretion antibody responses was nearly identical for both groups (Table 4). In addition, mean titers of antibody in the two sites were similar.

Distribution of plaque size variants in specimens obtained from volunteers inoculated with WI₁₁-SP or WI₁₁-LP. Testing of nasal wash specimens from volunteers given WI₁₁-SP pool revealed almost entirely small-plaque variants (Table 5) in a distribution frequency similar to that of the inoculum given those volunteers (Table 2). In contrast, in nasal wash specimens obtained from volunteers given the WI₁₁-LP pool, both large- and small-plaque variants were detected, but, whereas large plaques predominated in the inoculum (Table 2), small plaques predominated in nasal wash specimens.

**DISCUSSION**

The present studies have confirmed previous findings suggesting attenuation of a rhinovirus
TABLE 4. Comparative neutralizing antibody responses to nasal inoculations with WI1,,-SP and WI1,,-LP

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Serum</th>
<th>Nasal secretions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of volunteers demonstrating fourfold rise</td>
<td>Mean titer* 4 weeks after inoculation</td>
</tr>
<tr>
<td>WI1,,-LP</td>
<td>17</td>
<td>4.05</td>
</tr>
<tr>
<td>WI1,,-SP</td>
<td>18</td>
<td>4.50</td>
</tr>
</tbody>
</table>

*Log2.
* P > 0.10.

TABLE 5. Distribution of plaque size variants in specimens obtained from volunteers inoculated with WI1,,-SP or WI1,,-LP

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>No. of specimens tested</th>
<th>No. of plaques examined</th>
<th>No. small</th>
<th>No. large</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI1,,-SP</td>
<td>52</td>
<td>122</td>
<td>119 (98)*</td>
<td>3 (2)</td>
</tr>
<tr>
<td>WI1,,-LP</td>
<td>81</td>
<td>117</td>
<td>83 (70)</td>
<td>34 (30)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses are percentages.

strains after three passages in human embryonic lung fibroblast tissue cultures. In a previous report, none of eight volunteers who were infected with type 15 WI1 became ill. If these results are combined with those of the present report, illness has been observed in only one of 39 (2.6%) of volunteers who became infected with this inoculum. This finding is in striking contrast to the 75% illness rate observed with the type 15 WI1 inoculum. The 75% rate is similar to that which we have observed with types 13, 15, 17, and that reported in naturally occurring infections (9).

In addition to attenuation of illness production, it has been shown that type 15 WI1 inoculum was 50-fold less infectious for man (5). The alternative possibility, that the WI1 inoculum is more infectious for tissue cultures, seems unlikely since the WI1 and WI3 stocks exhibited similar titers in WI-3B culture (Table 2).

Infections with type 15 WI1 resulted in neutralizing antibody responses in sera of 100% and in nasal secretions of 89% of infected volunteers. These frequencies and the range of resultant antibody titers are comparable to those appearing after type 15 WI3 infections. Since infection with type 15 WI1 has been shown to protect against homotypic rechallenge, it seems likely that infection with type 15 WI1 would also be protective against homotypic rechallenge (1). Thus, it seemed fruitful to search for in vitro markers which might correlate with attenuation. If such markers were found, they might be used to develop attenuated stocks of other rhinovirus types.

In the present studies, it was shown that plaque size distribution of the WI1 pool was somewhat different from that of the WI3 pool in that there was a larger proportion of large plaques. To test the possibility that the large-plaque variant might be associated with attenuation of illness production, purified small- and large-plaque pools were made from this inoculum. Testing indicated that each volunteer inoculum contained 98 and 93%, respectively, of the indicated plaque size variants. The 2 and 7% apparent contamination with the opposite genotype was at least partially due to phenotypic variation or possibly other factors, since a few of these plaques were examined and small plaques appearing in the WI1,,-LP pool revealed large plaques on passage, and large plaques which appeared in the WI1,,-SP pool revealed small plaques on passage. However, since the volunteer inoculum was large (15,000 TCID50), a very small proportion of small-plaque variants in the large-plaque pool could account for the observed illness, and sufficient plaques have not been examined in order to be certain that contamination with the opposite genotype did not occur. In this regard, the semisolid agar (0.5%) used for preparation of the inoculum may have allowed for some contamination. However, preliminary experiments and reports in the literature indicated that more consistent results were obtained by using semisolid solidifying agents (7). As indicated in Materials and Methods, in later experiments we found results with 1.0% agar to be equally consistent.

Studies in volunteers with these inocula demonstrated a significant association of illness with the small-plaque pool. In fact, the WI1,,-SP pool reproduced an illness rate and severity indistinguishable from the WI1 inoculum. Conversely, attenuation of illness production was associated with the WI1,,-LP pool. The 25% illness rate observed with the WI1,,-LP pool is of interest, although not significantly different from the illness rate for the WI1 pool (P > 0.10). Several possible explanations for higher illness rate are apparent. First, the dose of WI1,,-LP pool administered to volunteers was 15,000 TCID50, whereas that for the WI1 pool was 320 TCID50. A dose-dependent difference in host response might account for more illness. Secondly, as
discussed previously, it may be that the volunteer inoculum was not 100% purified. This is suggested by the predominance of small-plaque variants detected in nasal wash specimens from volunteers infected with the WI11-LP inoculum, although this finding may be partially explained by phenotypic variation. Moreover, calculations using distribution of plaque size for the WI3 and WI11-LP pools and doses administered indicate that volunteers given the WI11-LP pool received about eight times as much small-plaque virus as did those given the WI3 pool. Thus, the opportunity for infection with illness-producing virus was greater. If this is the correct explanation, it would imply a growth advantage for the small-plaque variant in man and the large-plaque variant in tissue cultures, a finding supported by the uniformly greater recovery of SP as compared to LP from volunteers. A third possibility, also compatible with the plaque distribution of virus in nasal wash specimens, is that the large-plaque variant may be genetically unstable so that reversion to small-plaque occurs with human passage. Finally, it may be that plaque size variation and attenuation are not interdependent properties.

In any event, the findings presented suggest that plaque size may be an in vitro marker of attenuation of illness production by rhinoviruses. Thus, it might be possible to select large-plaque variants of other rhinovirus types which would be potential live virus vaccine candidates.

ACKNOWLEDGMENTS

This work was supported by a Public Health Service contract (PH-43-68-963) from the National Institute of Allergy and Infectious Diseases and by a general research support grant.

We wish to thank Terrel D. Hutto, Warden, and Clive Aldridge and Hudie Cottrell, Medical Officers, Ramsey Unit, Texas Department of Corrections, for their help in the volunteer program.

LITERATURE CITED