Rotavirus-Associated Traveler’s Diarrhea: Neutralizing Antibody in Asymptomatic Infections

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Received 12 October 1982/Accepted 20 April 1983

The sera of patients with traveler’s diarrhea were assayed for rotavirus-specific immunoglobulins in enzyme-linked immunosorbent and micro-neutralization assays. The antigenically related simian rotavirus SA-11 was used in both assays. Although similar titers of rotavirus-specific binding immunoglobulin G and A were observed in the sera of both symptomatic and asymptomatic subjects, clinical illness appeared to correlate with the failure to detect increased (greater than or equal to fourfold) titers of neutralizing antibodies in the post- compared with the pretravel sera.

Human rotavirus is recognized as a causative agent of acute diarrheal disease in children (2, 8), but its role in adult illness remains to be established. The highest incidence of symptomatic infection, characterized by virion- and viral-antigen-positive stools, occurs between 6 months and 1 year of age (8), and rotaviruses serologically related to the human virus (19, 20) cause diarrhea in the young of numerous species (19). In adults, rotavirus was identified as the cause of gastroenteritis in a relatively high (25%) proportion of staff members from a University Personnel Health Center (18), and it was associated with traveler’s diarrhea (TD) (1, 4, 5, 13), a syndrome caused by various agents (12). However, most adult infections are asymptomatic, and it is unclear whether virus-specific immunity plays a role in the development of clinical illness. The studies described in this report indicate that, in adults, subclinical (but not symptomatic) rotavirus infections are associated with the ability to develop increased titers of rotavirus-specific neutralizing antibody.

The sera studied in these series were selected from panels obtained from American travelers to Honduras (12) and Panamanian travelers to Mexico (11, 13) before and after travel. A total of 25 pairs were obtained from the American travelers (15 from symptomatic and 10 from asymptomatic subjects), and 9 pairs were obtained from Panamanian travelers (4 symptomatic and 5 asymptomatic). Clinical information was obtained as described elsewhere (11–13). In the Honduras series (12), questionnaires and interviews were administered at the beginning (pretravel) and at 6 weeks after arrival in Honduras (posttravel). TD was defined as the passing of one or more watery stools per day with cramps, vomiting, fever, or prostration. In the Panamanian series (11, 13), TD was defined as the passing of more than two watery stools in a 24-h period. Serum specimens were obtained before departure (pretravel) and within 10 days after returning home. Rotavirus-specific antibodies were assayed by the enzyme-linked immunosorbent assay (ELISA; binding antibodies) and by the neutralization assay using simian rotavirus (SA-11). The ELISA has been previously described (13). Briefly, we coated microtiter plates with SA-11 antigen (30 μg of protein per ml), using guinea pig anti-SA-11 serum in carbonate buffer as a precoat. The antigen-coated plates were exposed to serial dilutions of the patient sera for 12 h at 4°C, followed by enzyme-linked anti-immunoglobulin G (IgG) or IgA for 2 h at 37°C. At this time, the plates were exposed to p-nitrophenyl phosphate substrate in diethanolamine buffer (pH 9.6) for 30 min at room temperature, and the reaction was stopped by the addition of 3 M NaOH. The amount of yellow color produced by the reaction of the enzyme on the substrate was measured. A Beckman DBG spectrophotometer at 400 nm. Antibody titers were expressed as the reciprocal of the highest serum dilution that yielded an optical density at 400 nm of >2 standard deviations over the mean background. In the micro-neutralization assay, 10^2 PFU of SA-11 was mixed at a 1:1 ratio with twofold serial dilutions of the patient sera in minimal essential medium (MEM) containing 2.5 μg of trypsin (Difco Laboratories) per ml. After 30 min of incubation at 37°C, the survivors were assayed on confluent African green monkey kidney cells grown in 96-well microtiter plates (Falcon Plastics). Four wells were inoculated for
each virus-antibody mixture. After 2 h of incubation at 37°C to allow virus adsorption, the cells were overlaid with MEM containing 2.5 μg of trypsin per ml and incubated at 37°C for 4 days. At this time, complete cytopathic effect was observed in the control wells inoculated with a mixture of SA-11 and phosphate-buffered saline (pH 7.0) rather than serum. Antibody titers were expressed as the reciprocal of the highest serum dilution that gave rise to a 50% reduction in wells with detectable cytopathic effect. In both assays, statistical analyses were performed on the log transformation of these reciprocal values.

We performed two series of experiments to define the specificity of the neutralization assay with SA-11 virus. In the first series, we compared the neutralizing potentials of paired acute and convalescent sera from murine (EDIM), bovine (NCDV), porcine (OSU), human, and caprine rotavirus infections with those of hyperimmune bovine anti-human Wa rotavirus serum (obtained from R. Yolken, Johns Hopkins University) and guinea pig anti-SA-11 serum (homologous system) prepared by subcutaneous inoculation in complete Freund adjuvant as described previously (15). The human paired sera were obtained from neonates with rotavirus-induced diarrheal disease confirmed by (i) the presence of virus particles in stool specimens and (ii) seroconversion to rotavirus specificity as determined by complement fixation with bovine rotavirus (NCDV) (8). The paired animal sera were obtained at the time of experimental infection (acute) and at convalescence (13 to 21 days postinfection). The murine and porcine pairs were obtained from J. Sheridan, and the bovine and caprine pairs were the gift of R. Yolken, both at the Johns Hopkins School of Medicine. Antibody titers determined by ELISA, the specificity of which had been previously established (13, 21), served as a positive control.

The results are summarized in Table 1. The highest neutralizing potential for SA-11 was displayed by the homologous anti-SA-11 serum. All convalescent sera were also reactive. The acute sera were negative. The reaction was specific as evidenced by the findings that (i) the hyperimmune serum and convalescent sera were also positive for virus-specific antibodies by ELISA; (ii) acute sera were negative for neutralization of the virus and did not bind SA-11 in the ELISA; and (iii) SA-11 was not neutralized by antiserum to the unrelated viruses herpes simplex virus type 2 and influenza, whereas antisera to the various other rotaviruses neutralized it to a different extent. However, it should be pointed out that antiserum to various rotaviruses appeared to have different reactivities with these antisera.

In a second series of experiments designed to determine the specificity of the neutralization assay with SA-11 virus, posttravel sera from two TD patients (nos. 37 and 9) were (i) diluted 1/10, assayed for their ability to neutralize SA-11 before and after three cycles of adsorption with SA-11 or mock antigen (50 μg of protein per ml), and fixed with glutaraldehyde as described previously (15); and (ii) assayed after immunoglobulin fractionation by sucrose gradient centrifugation (13, 15). The data (Table 2) indicate that both sera had relatively high titers of neutralizing antibodies (200 and 500, respectively) before adsorption. Their neutralizing potential was adsorbed with SA-11 but not with mock antigen preparations, and it fractionated with the 7S (IgG) fraction.

Based on these findings, pairs of sera were
selected for rotavirus seropositivity from a panel previously shown to have a relatively high proportion of rotavirus-positive samples (13). The sera (34 pairs) were assayed for rotavirus-specific antibodies by ELISA and neutralization. Consistent with previous findings (13), increased titers (greater than or equal to fourfold) of rotavirus-specific binding IgG (assayed by ELISA) were observed in the post- as compared with the pretravel sera of 28 of 34 subjects (positive pairs). Twelve of these positive pairs also demonstrated increased (greater than or equal to fourfold) post- compared with pretravel titers of rotavirus-specific IgA. However, these increased IgG and IgA titers did not correlate with a history of diarrheal disease (Table 3).

On the other hand, increased titers of rotavirus-specific neutralizing antibodies in the post- as compared with the pretravel samples appeared to correlate with clinical status. Thus, based on the ELISA findings, 19 patients had diarrhea associated with rotavirus. Only one of these (5.3%) evidenced a greater than or equal to fourfold increase in neutralizing antibody titers in the post- as compared with the pretravel sera. The titers of the virus-specific neutralizing antibodies were virtually identical in the pretravel (geometric mean titer [GMT] = 342; 95% confidence interval 37; 3,110) and the posttravel (GMT = 427; 95% confidence interval 50; 3,651) sera of the other 18 patients (Table 3). This compares with a greater than or equal to fourfold increase in the titers of neutralizing antibodies in the post- (GMT = 898; 95% confidence interval 482; 2,108) as compared with the pretravel (GMT = 99; 95% confidence interval 26; 381) sera from 10 of 15 (66.7%) pairs obtained from asymptomatic individuals. The titers of the neutralizing antibodies in the pretravel sera from the symptomatic and asymptomatic patients were not significantly different (Table 3).

The reproducibility of the neutralization assay was determined by assaying six pairs of sera (two negative and four positive pairs) at two different times. Similar or twofold-different titers were observed in 8 of the 12 sera (67%). The titers of the other four sera showed a four- to sixfold difference in the two assays. However, it is significant that the differential response of the post- as compared with the pretravel serum specimen (greater than or equal to fourfold-higher titers) was reproducible in all six pairs. The reproducibility of the differential response of the post- as compared with the pretravel specimen assayed by ELISA was described previously (13).

Presently available evidence indicates that clinical illness is age dependent and detectable in the young (2, 8), but generally not in the adults of all species. This age-dependence has been attributed to the loss of cell surface receptors for

TABLE 2. Specificity of neutralizing antibodies in sera of TD patients

<table>
<thead>
<tr>
<th>Serum</th>
<th>Antibody titer</th>
</tr>
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<tbody>
<tr>
<td>Serum 37</td>
<td>200</td>
</tr>
<tr>
<td>IgG fraction</td>
<td>200</td>
</tr>
<tr>
<td>Adsorbed with SA-11</td>
<td>0</td>
</tr>
<tr>
<td>Adsorbed with mock antigen</td>
<td>200</td>
</tr>
<tr>
<td>Serum 9</td>
<td>500</td>
</tr>
<tr>
<td>IgG fraction</td>
<td>700</td>
</tr>
<tr>
<td>Adsorbed with SA-11</td>
<td>0</td>
</tr>
<tr>
<td>Adsorbed with mock antigen</td>
<td>600</td>
</tr>
</tbody>
</table>

* Expressed as the reciprocal of the highest serum dilution that gave rise to a 50% reduction in wells with detectable viral cytopathic effect.

Infection (9). However, since infection, albeit asymptomatic, occurs with equal frequency in all age groups (14), it seems reasonable to assume that virus-specific immunity is at least partially involved. Indeed, in the murine model, reinfection has been associated with an immediate (2 to 5 days postinfection) suppression of virus-specific cell-mediated immunity (14), and in adult human volunteers, the absence of antibodies to the DS-1 rotavirus serotype was shown to correlate with diarrheal illness after the administration of a Wa-like rotavirus (A. Z. Kapikian, personal communication). Nevertheless, the role of virus-specific immunity in adult infections remains to be established.

The studies described in this report were designed to determine the relative titers of neutralizing antibodies in the post- as compared with the pretravel human sera of adult subjects with rotavirus-associated TD, as they relate to clinical illness. The experimental design was based on the following considerations. Increased (greater than or equal to fourfold) titers of virus-specific binding IgG (determined by ELISA) in the post- as compared with the pretravel samples (positive pairs) were considered indicative of rotavirus infection (13, 14), and a relatively high proportion of such positive pairs (28 of 34) were specifically selected for this study. Therefore, the data are not indicative of the rate of rotavirus infection in this population group. Also, they do not exclude the existence of dual infections with other pathogens, including enterotoxigenic Escherichia coli, or Norwalk agent or both, or the possible role of such agents in disease. However, it should be pointed out that enterotoxigenic E. coli was isolated from
only 2 of the 17 symptomatic and 1 of the 10 asymptomatic patients selected for study in these series. It should also be pointed out that selection may be responsible for the previously undetected (13) overlap in the confidence intervals about the GMT for virus-specific IgA in the asymptomatic sera (Table 3).

The data obtained by ELISA were consistent with previous findings in such population groups (13). The increased sensitivity of ELISA as compared with complement fixation (CF) was previously documented (5, 6, 13). Thus, a direct comparison of the CF and ELISA assays by Sheridan et al. (13) confirmed the greater sensitivity of the ELISA, both in terms of antibody titers and absolute seropositivity. Ghose et al. (5) found that virtually 100% of adult sera had relatively high rotavirus-specific antibody titers when studied by ELISA. A comparison of ELISA and CF titers at different ages suggested that for some reason, possibly reflecting the quality of the immunoglobulin class, the relative sensitivity of the ELISA increased with age, so that virtually all adult sera were strongly positive by ELISA, although manifesting minimal, if any, reactivity in the CF assay. A prevalence rate of only 45% rotavirus seropositivity and relatively low titers (8 to 32) were independently reported in a similar population group, using the CF assay (6). The differences between the prevalence of rotavirus-specific seropositivity in TD groups studied by CF (11), as compared with ELISA (13), probably reflect a situation similar to that described by Ghose et al. (5), in which the ELISA is much more efficient than the CF in detecting rises in antibody titers that may result from symptomatic or asymptomatic reinfection.

As suggested by Ghose and co-workers (5), this increased efficiency of the ELISA may be due to the quality of the immunoglobulin class reactive in the two assays.

The micro-neutralization used in these studies is simple and reproducible and yields results virtually identical to those obtained with the plaque reduction assay in terms of specificity and antibody titers (20; unpublished data).

Rotavirus show a high degree of genetic polymorphism (10) and are serologically distinguishable. However, there is a significant degree of cross-reactivity (7, 18, 19). More recently, three distinct serotypes of human rotavirus were described, one of which includes both human and simian rotavirus (16, 19). Accordingly, it seemed reasonable to perform both serological assays (ELISA and neutralization) with SA-11 virus. Feasibility studies performed to verify the soundness of this choice indicated that the neutralizing potential of the human sera is adsorbed by SA-11 but not by mock antigen preparations, and it resides in the IgG fraction (Table 2). Preimmune or acute sera were negative (Table 1).

Consistent with previous findings (16, 17, 19), we found that antisera to various rotavirus strains reacted with SA-11 in both the ELISA and neutralization assays, although their anti-SA-11 titers differed in the two assays. Thus, the titers of neutralizing antibodies in the porcine and caprine postinfection sera were higher than those of the human sera, whereas in ELISA the titer of human serum no. 8 was higher than that of the porcine serum. Similarly, the convalescent murine serum had a low titer (titer = 384) of neutralizing anti-SA-11 antibodies, whereas the

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**TABLE 3. Rotavirus antibodies in travelers’ sera**

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>Serum</th>
<th>Antibody titer (GMT) in paired sera</th>
<th>Neutralization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG</td>
<td>IgA&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>Pre</td>
<td>1,290 (46; 5,417)</td>
<td>13 (10; 19)</td>
</tr>
<tr>
<td>(n = 17)</td>
<td>Post</td>
<td>6,287 (5,714; 130,136)</td>
<td>215 (26; 8,134)</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>Pre</td>
<td>813 (143; 4,600)</td>
<td>20 (3; 140)</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>Post</td>
<td>6,020 (4,864; 41,898)</td>
<td>82 (4; 1,465)</td>
</tr>
</tbody>
</table>

<sup>a</sup> We tested 19 symptomatic and 15 asymptomatic individuals. Data are only shown for those displaying increased (greater than or equal to fourfold) titers of virus-specific IgG in the post- (4 weeks) as compared with pretravel serum specimens (positive pairs). Eight patients did not show such increased titers posttravel (negative pairs). In these pairs, the titers of neutralizing antibodies in the pre- and posttravel specimens were identical. The values in parentheses represent the 95% confidence interval.

<sup>b</sup> Twenty-eight pairs of sera assayed with anti-IgA included eight negative and twenty positive pairs. Twelve of the twenty positive pairs were from patients with clinical illness. Eight were from asymptomatic subjects.

<sup>c</sup> The pretravel and posttravel titers in the 17 symptomatic pairs were not significantly different (P > 0.2 by Student’s t test).

<sup>d</sup> The pretravel titers of symptomatic and asymptomatic patients were not significantly different (P > 0.1 by Student’s t test).

<sup>e</sup> The pretravel and posttravel titers in the 10 asymptomatic pairs were significantly different (P < 0.01 by Student’s t test). The titers of neutralizing antibodies in the pre- and posttravel samples from five other positive pairs were not significantly different.
títer of binding antibodies to SA-11 was equal (titer = 10,000) to that of the porcine serum. The two human postinfection sera (nos. 5 and 8) had significantly different titers of binding antibodies to SA-11 (titers = 128 and 32,768), whereas their SA-11 neutralizing antibody titers were similar (titers = 512 and 986 for nos. 5 and 8, respectively). These differences were observed for both hyperimmune and convalescent sera and probably reflect the involvement of different viral antigens in ELISA as compared with virus neutralization (7).

The feasibility studies confirm the validity of using SA-11 in serological assays of TD patients. In this context, it is particularly significant that virus-specific neutralizing antibodies appear to be associated with subclinical infection in a relatively high (66.7%) proportion of patients. Thus, among 18 patients with serologically confirmed rotavirus-associated diarrheal disease (increased titers of virus-specific binding IgG or IgA or both in the post- as compared with the pretravel serum samples), only one had titers of neutralizing antibodies that were greater than or equal to fourfold higher in the post- compared with the pretravel specimen. This compares with 10 of 15 (66.7%) patients with asymptomatic infections. The exact interpretation of these findings is not immediately apparent. Possibly, the immune recognition of viral proteins involved in neutralization results in increased titers of antibodies capable of preventing further viral replication, thereby providing protection from clinical illness. An alternative but not mutually exclusive interpretation is that neutralizing 7S immunoglobulin represents an IgG subclass that has protective potential and is different from that detected by ELISA. Indeed, in the murine model, protection appears to be mediated by virus-specific IgG (14). However, its subclass remains to be established.

These studies were supported in part by WHO grant C6/181/85.

We thank R. Fishelevitch for excellent technical assistance and J. F. Sheridan for help with the statistical analyses of the data.

LITERATURE CITED


