Complement-Fixing Antibody Response to Rotavirus Infection

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Received for publication 9 September 1976

A human rotavirus complement-fixing (CF) antigen, prepared by purification of large volumes of fluid feces collected from children with winter diarrhea, was used to study the development and persistence of antibody in children with diarrhea and the prevalence of rotavirus antibody in Melbourne. In children with diarrhea, antibody rises were detectable within 4 to 6 weeks of the onset of illness, and the titers usually remained elevated for the next 1 to 2 years. CF antibody did not develop in two children with proven rotavirus infection aged less than 6 months, an age at which poor CF responses to other viruses have also been observed. A study of CF antibody levels in the general community showed that in Melbourne, most children have been infected with human rotavirus by the age of 3 years.

A new virus has recently been associated with acute nonbacterial gastroenteritis in infants and children (19). The virus has been designated as orbivirus (1), reovirus-like (8, 17), rotavirus (10), duovirus (6), and infantile gastroenteritis virus (21). Although it can definitely be placed in the family Reoviridae (14, 23), no formal name has yet been approved by the International Committee on Taxonomy of Viruses. In this communication it will be referred to as rotavirus.

Human rotavirus has been located in epithelial cells of the duodenal mucosa (1, 7, 26) and in duodenal aspirates (22) and stool extracts (2, 8) obtained during the symptomatic stage of the disease. Serological evidence of infection has been obtained by immune electron microscopy (EM) (17), indirect immunofluorescence (7, 9, 17, 22, 28), neutralization in cell culture (9), complement fixation (CF) (15, 17, 21, 27), and counter-immuno-osmophoresis (21). The most useful and generally available of these techniques is CF, using as antigen either virus-positive extracts of human stools (17) or concentrates of the antigenically related Nebraska calf diarrhea virus grown in cell culture (15). The homologous system using human stool extract has been shown to be more sensitive than the heterologous Nebraska calf diarrhea virus for detecting antibodies in humans (15).

Serological studies of diarrhea due to rotavirus have been hampered by difficulties in obtaining sufficient quantities of human virus and, in some countries, by the unavailability of Nebraska calf diarrhea virus. However, the development of a technique for collecting large quantities of feces from children with diarrhea (12) has allowed the preparation of human rotavirus CF antigen in sufficient quantity to permit a serological study of the development and persistence of CF antibody in children with severe enteritis and of the occurrence of CF antibody in all age groups in the community. The results are described below.

MATERIALS AND METHODS

Sera were collected from four groups of patients. Patient or parental consent was obtained when blood was required for other than routine tests. All sera were stored at −20°C before testing.

Group 1. Group 1 was comprised of 30 children, aged 8 months to 6 years, admitted to Fairfield Hospital, Melbourne, during an outbreak of "winter diarrhea" (June to August 1974). Fecal specimens were obtained from 19 of these children on the day of admission to the hospital, and rotavirus was detected by EM in 15. Sera were collected from all children by venipuncture within 1 week of onset of diarrhea ("acute" sera) and 2 to 20 weeks later ("convalescent" sera). Additional specimens were obtained from 19 of the children 12 to 24 months later ("follow-up" sera).

Group 2. Group 2 was comprised of 20 children, aged 10 days to 2.5 years, admitted to the gastroenteritis ward of the Royal Children's Hospital, Melbourne, during 1973 and 1974, in whom rotavirus...
infection had been diagnosed by EM of diarrheal feces. Acute and convalescent sera were collected as for group 1; follow-up sera were available from all but one child.

Group 3. Group 3 was a control group of eight children aged 1 month to 5 years, admitted to Fairfield Hospital, Melbourne, during June to August 1974 with illnesses other than diarrhea, namely, mumps (2), aseptic meningitis (2), pneumonia (2), measles, and mononucleosis due to cytomegalovirus. Acute and convalescent sera were obtained from all eight children.

Group 4. Group 4 was comprised of 1,019 patients ranging in age from 1 day to 90 years. None had diarrhea at the time of collection of sera. Sera from all children less than 6 years of age and from most children aged 6 to 10 years were obtained from patients admitted to the Royal Children's Hospital, Melbourne, between December 1973 and April 1976 for elective surgery or for noninfectious illness. There was no apparent socioeconomic bias in this group. The remaining sera from older children, adolescents, and adults had been submitted to the Virus Laboratory, Fairfield Hospital, during April 1974 to May 1976 for a variety of serological tests used in diagnosis of respiratory disease, neurological disorders, and skin rashes.

EM. Fecal specimens were examined by EM by the technique of Bishop et al. (2), with the exceptions that 4% ammonium molybdate at pH 7.0 was used for negative staining, and the preparations were examined with a Philips EM 301.

Preparations of CF antigens from human fecal specimens. Rotavirus antigen was prepared from 1,500 ml of fluid feces obtained from a 2-year-old male with acute enteritis. Feces were collected during the acute phase of diarrhea by nursing him on a specially designed frame covered with strong webbing (12). No bacterial or viral enteric pathogens were isolated by routine techniques. EM revealed large numbers of rotavirus particles. Feces were clarified by centrifugation at 10,000 × g for 15 min at 4°C. Approximately 12 ml of supernatant fluid was then layered onto a 2-ml cushion of 45% (wt/vol) sucrose in tris(hydroxymethyl)aminomethane buffer (0.002 M, pH 7.0) and centrifuged at 100,000 × g for 1.5 h in a Beckman ultracentrifuge using an SW41 rotor. The resulting pellets were resuspended in veronal-buffered saline to one-eighth the original volume, and the pH was adjusted to 7.2. Material acquired from several ultracentrifuge runs of the same original fecal specimen was pooled and stored in aliquots at −20°C. Chessboard titration of this antigen against convalescent serum from a child with proven rotavirus diarrhea showed that it contained 16 U of CF antigen, and that it was not anti-complementary.

Control antigen. Control antigen was collected and prepared by the procedure described above, using diarrheal stools from a 2-year-old male infected with Shigella sonnei. No rotavirus particles were detected by EM.

CF test. All sera were coded to exclude bias in interpretation of results. The CF test was a microtiter method based on that of Bradstreet and Taylor (4). Test sera were inactivated at 62°C for 15 min. Serial twofold dilutions (beginning with a dilution of 1:8) were held at 4°C for 16 to 18 h with 2 U of antigen and 3.5% hemolytic doses of complement. After further incubation for 45 min at 37°C, a 1.5% suspension of sensitized sheep erythrocytes was added. A fourfold or greater increase in CF antibody titer between acute and convalescent serum specimens was considered a significant response, diagnostic of rotavirus infection.

RESULTS

Specificity of the rotavirus CF antigen. Acute and convalescent sera from eight of the children with diarrhea in whom human rotavirus infection had been confirmed by EM (group 2), and from the eight children with other illnesses (group 3), were titrated by CF against both human rotavirus and control antigen. None of the sera from either group reacted (at a 1:8 dilution) with the control antigen. Using human rotavirus as antigen (Table 1), no changes in CF antibody levels occurred in paired sera from children in the control group, whereas fourfold or greater rises in CF antibody levels occurred during convalescence in all eight children with rotavirus diarrhea.

Antibody response in children with rotavirus diarrhea. Of 50 children with acute nonbacterial gastroenteritis studied (groups 1 and 2), 46 developed a significant rise in CF antibody titer in convalescent sera compared with that in acute sera. The antibody titers obtained on acute, convalescent, and follow-up sera from these 46 children are shown in Fig. 1. CF antibody was absent from sera collected within 1 week of the onset of diarrhea in all but two of these children (aged 13 and 9 months), in whom titers of 1:8 were present 1 and 6 days after the onset of illness. In most patients significant

| Table 1. Rotavirus CF antibody titers in children |
| --- | --- | --- |
| Group | Patient | Rotavirus CF titer |
| --- | --- | --- | --- |
| 2 (rotavirus diarrhea) | M.A. | <8 | 64 |
| | R.A. | <8 | 32 |
| | O.B. | <8 | 32 |
| | J.B. | <8 | 32 |
| | F.C. | <8 | 64 |
| | J.C. | <8 | 32 |
| | B.C. | <8 | 32 |
| | J.D. | <8 | 64 |
| 3 (other illness) | T.S. | 32 | 32 |
| | S.P. | 64 | 64 |
| | C.P. | 16 | 16 |
| | V.A. | 32 | 32 |
| | S.D. | <8 | <8 |
| | N.O. | 128 | 128 |
| | L.S. | 8 | 8 |
| | D.S. | <8 | <8 |
rises in antibody titers were present 4 to 6 weeks from the onset of disease. In 33 of 35 patients, these titers did not alter significantly within the next 1 to 2 years. In one patient antibody fell to undetectable levels 14 months after infection, whereas a second child showed a 16-fold increase in antibody between sera collected 4 weeks and 14 months after the onset of illness. Four of the 50 children with acute nonbacterial gastroenteritis failed to develop a significant antibody response to rotavirus (Table 2). It seems probable that in one of these children (G.J.) illness was not due to rotavirus infection, since no virus particles were detected in her diarrheal stools. The other three children all showed rotavirus particles in acute-phase stools. Patient A.R. developed diarrhea 5 days after birth. CF antibody was detected in acute and convalescent sera and declined to undetectable levels during the following 12 months. Patient S.A. was a 4-month-old male in whom no CF antibody was detected even in sera obtained 3 weeks after onset of diarrhea. Patient D.C. was of particular interest, as rotavirus was visualized in his stools on two occasions, during a diarrheal illness at 7 months of age and again during a second attack of diarrhea at 14 months of age. CF antibody was detected in his serum during the acute phase of the second illness. There was no evidence of an anamnestic response in convalescent sera, and CF antibody levels declined fourfold during the next 12 months.

Detection of infection by CF and EM. Nineteen of the 30 children in group 1 and all 20 in group 2 were tested for evidence of rotavirus infection, by both serology and EM. The results are shown in Table 3. Of the 19 children in group 1, evidence of infection was obtained in 18 by serology and in 15 by EM. One child (G.J.) showed no evidence of infection by either technique. The failure of EM to detect virus in three children was not due to their late admission to the hospital, since fecal specimens were obtained 2, 3, and 4 days after onset of symptoms. Group 2, by definition, included only children with rotavirus in stools. Seroconversion occurred in 17 of the 20 children. Details of the three children who failed to develop a serological response (A.R., S.A., and D.C.) are described above (Table 2).

Community incidence of rotavirus antibody. The percentage of patients in group 4 in whom titers of $\geq 1:8$ were detected is shown in Fig. 2 to be related to age. During the first 2 months of life, 41% of infants showed detectable

![Fig. 1. CF antibody response in children with rotavirus diarrhea.](http://jcm.asm.org/)
levels of serum antibody. There was an abrupt decline in prevalence of antibody in children aged 3 months to 2 years, but by 3 years of age, 64% of the children showed evidence of past rotavirus infection. After the age of 5, the proportion of each group with detectable antibody remained relatively constant, with a minimum of 64% positive (6 to 10 years) and a maximum of 85% positive (36 to 40 years).

**DISCUSSION**

The CF test described by Kapikian et al. (17) to detect antibody to human rotavirus has not been widely used, partly because large quantities of diarrheal feces are difficult to collect from children and partly because many stool filtrates have been found to be anti-complementary. These problems can be overcome by nursing children on a specially designed webbing frame that allows collection of fluid feces and preparing rotavirus antigen by centrifugation of clarified feces through a sucrose cushion. This human rotavirus antigen is more sensitive in detecting seroconversions, and in titration of sera, than is CF antigen prepared from the serologically related Nebraska calf diarrhea virus grown in tissue culture (15).

**Table 3. Comparison of CF and EM in detection of rotavirus infection in children**

<table>
<thead>
<tr>
<th>Patients</th>
<th>CF Positive</th>
<th>CF Negative</th>
<th>EM Positive</th>
<th>EM Negative</th>
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</thead>
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<tr>
<td>Group 1</td>
<td>18</td>
<td>1</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>(tested</td>
<td></td>
<td></td>
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<td>19/30)</td>
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<td></td>
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</tr>
<tr>
<td>Group 2</td>
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<td>17</td>
<td>0</td>
</tr>
<tr>
<td>(tested</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>20/20)</td>
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It is already established that seroconversion occurs during rotavirus infection in children (15, 17, 18, 27, 28). Our study confirms this. The pattern of acquisition of antibody is similar to that seen as a primary response in other viral infections (20), with high titers of antibody present after 14 days from onset of symptoms. CF antibody to rotavirus was persistent, with no significant fall in titer in the majority of patients 1 to 2 years after primary infection.

In agreement with Kapikian et al. (16), a comparison of the sensitivity of CF and EM showed that, ideally, a combination of the two techniques is required in diagnosis of rotavirus infections. In children aged 6 months or more, serology was a slightly more reliable means of detecting infection than EM. This is not surprising, since EM is only likely to be positive if stools contain more than 10⁵ virus particles per ml. However, seroconversion did not always occur after infection diagnosed by EM.

Two of the children who failed to show a serological response were less than 6 months old, an age at which poor CF antibody responses to other viruses have been observed (11, 25). Indirect immunofluorescence may be more useful than CF in detecting rotavirus antibody in such young children (28). The third child who failed to develop a serological response (D.C.), aged 14 months, was of special interest as he had previously been admitted with rotavirus diarrhea (proven by EM) at the age of 7 months. Despite a normal antibody response to the first infection, he developed a second attack of rotavirus diarrhea, again proven by EM, 7 months later. This child may have been a chronic carrier of the virus or may have suffered a second infection, perhaps due to a different strain of the virus. It is also possible that he had a subtle immunological defect,

**Fig. 2. Incidence of rotavirus CF antibody in different age groups in Melbourne, Australia. (Number on column = total number of sera tested.)**
since his serum antibody level did not rise during the course of the second attack, and sera taken 12 months later showed an unusual four-fold decrease in titer.

Survey of community levels of rotavirus CF antibody shows that most children in Melbourne, Australia, as in Washington, D.C. (15), Boston (3), and Toronto (21), have experienced infection by the age of 3. A high percentage of adult sera showed detectable antibody. This could be explained by persistence of virus or by repeated asymptomatic reinfection. The increased prevalence of antibody in adults of child-rearing age suggests the latter may be the case. Although occasional symptomatic infection has been described in older children and adults (13, 29), rotaviruses are thought to be a rare cause of diarrhea after 6 years of age (13, 29).

The serological response to rotavirus infection, and incidence of antibody in adolescence and in adults, closely resembles the response to other viruses infecting mucous surfaces of the body, such as respiratory syncytial virus (11) and parainfluenza virus (24). Secretory antibody present at the initial site of infection is more effective in conferring resistance to reinfection by these and other viruses. It is now necessary to establish the importance of secretory antibody response to the mucus membrane in prevention of disease or infection due to rotavirus.

ACKNOWLEDGMENTS

This study was assisted by a generous grant from the Aboriginal Health Branch of the Australian Department of Health. Further support came from the National Health and Medical Research Council of Australia (G.L.B., R.F.B.) and from the Royal Children's Hospital Research Foundation (G.P.D.).

We thank M. Homola for the electron microscope studies, S. McNaught for help with collection and coding of sera, Robert Warren and Greg Cooper for technical assistance, and Judy Westwood and Anne Peace for help in preparation of the manuscript.

LITERATURE CITED