Relative Frequency of Rotavirus Subgroups 1 and 2 in Venezuelan Children with Gastroenteritis as Assayed with Monoclonal Antibodies

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Monoclonal antibodies recently developed against the 42,000-dalton protein of two rotavirus strains were used in an enzyme-linked immunosorbent assay to determine the subgroup specificity of 252 specimens collected during a 45-month period from Venezuelan children with rotavirus gastroenteritis. Subgroup 2 rotavirus was shed by 85% of the children, whereas only 14% shed subgroup 1 rotavirus (one-half of them in a 3-month period). No differences were found in the occurrence of fever and vomiting between children shedding either rotavirus subgroup, but it appeared that the syndrome tended to last longer in children shedding subgroup 2 rotavirus. The monoclonal subgrouping enzyme-linked immunosorbent assay seemed to be more sensitive than an immune adherence hemagglutination assay, an enzyme-linked immunosorbent assay with polyclonal antibodies, or the electrophoretic analysis of RNA extracted from the virus. Overall, 99% of the specimens could be subgrouped by this assay.

Several studies from different parts of the world have shown that rotavirus is the single most important etiological agent of gastroenteritis in hospitalized children, and thus it appears likely that this agent is a major cause of infant mortality in undeveloped countries. The urgent need for rotavirus vaccines has prompted many attempts to characterize serologically the many strains identified in human stools and to study the interrelationships among them. Various tests such as complement fixation (9, 19), immune electron microscopy (16), enzyme immunoassays (18), and immune adherence hemagglutination assay (IAHA) (8) have been employed which identify antigenic differences among rotaviruses. It is now known that the differences detected by these assays reside in the protein coded by rotavirus gene 6 (7), a 42,000-dalton component of the inner capsid of the virus (13). This antigenic specificity permits the classification of rotaviruses into two distinct subgroups (8). Recently developed monoclonal antibodies against the 42,000-dalton protein from two rotavirus strains (5) have allowed the easy recognition of these subgroups in simple solid-phase assays. We describe in this paper the utilization of two such monoclonal antibodies in an enzyme-linked immunosorbent assay (ELISA) for the subgrouping of rotavirus present in stools of children with rotavirus gastroenteritis.

MATERIALS AND METHODS

As part of an etiological study of infantile gastroenteritis in two hospitals of Caracas, Venezuela, 698 stool samples were obtained during the period between January 1979 and September 1982 from children under 3 years of age. Samples of the stools were made up into 5% suspensions in phosphate-buffered saline and kept at −20°C until testing. The presence of rotavirus was recognized in 252 (36.1%) of them by employing a confirmatory ELISA which utilizes goat preimmune and hyperimmune rotavirus antisera as capture antibody in separate wells of microtiter plates (10). A hyperimmune guinea pig antirotavirus antisera was used as the second antibody, and alkaline phosphatase-labeled anti-guinea pig antibody was used as the conjugate.

For the subgrouping assay, the same capture hyperimmune antisera was used, whereas the second antibody was ascites fluid from mice inoculated with hybridomas which specifically recognized rotavirus subgroup 1 (SG1) (hybridoma 255/60, derived from mice immunized with rhesus Rh2 rotavirus) or subgroup 2 (SG2) (hybridoma 631/9, derived from mice immunized with human Wa rotavirus) (5). The ascitic fluid was obtained 2 weeks after peritoneal inoculation with 5 × 10⁶ hybridoma cells. The mice had been protected by intra-peritoneal injection of 1 ml of tetraethylentetramine (Aldrich Chemical Co., Inc., Milwaukee, Wis.) 2 weeks before the inoculation of the cells. Optimal antibody dilutions for subgrouping were determined by checkerboard titration. After incubation with the monoclonal antibodies, an anti-mouse immunoglobulin G conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was incubated in the wells. The test was completed with the addition of p-nitrophenylphosphate as substrate, and the optical density (OD) was determined at 420 nm. Negative and positive control specimens were assayed in each plate. Stool suspensions from gnotobiotic calves infected with DS-1 and D rotavirus strains were used as positive controls for SG1 and SG2, respectively (14). A stool specimen free of rotavirus was used as the negative control. All of the samples were analyzed in duplicate, and more than 70% of them were retested for confirmation.

The ratio between the OD readings of each sample with the two monoclonal antibodies was determined. A ratio of 1.7 or higher between the readings observed with the SG1 monoclonal antibody over the SG2 monoclonal antibody was used as the criterion to classify a rotavirus as SG1. Speci-
mens with SG2/SG1 OD ratios of 2.0 or higher were classified as SG2. Specimens with ratios lower than 1.7 (SG1/SG2) or lower than 2.0 (SG2/SG1) were recorded as of undetermined subgroup. In cases in which a first assay did not allow the classification of a rotavirus into a given subgroup, the test was repeated after dilution of the samples (1/10) or treatment with Genetron 113 (Allied Chemical Corp., Morristown, N.J.) or both. Genetron treatment was performed by thoroughly mixing equal volumes of the 5% stool suspensions and Genetron. The aqueous phase containing the virus was separated by centrifugation for 10 min at 3,000 rpm in a clinical centrifuge.

Fifty-four of the samples were analyzed by an IAHA with sera from gnotobiotic calves infected with DS-1 (SG1) or Fh (SG2) rotavirus. The same sera were employed in a subgrouping ELISA on 61 of the specimens. These tests were performed as previously described (8).

RNA for electrophoretic analysis was obtained by phenol extraction of viruses partially purified by centrifugation through a 30% sucrose cushion (35,000 rpm in a Beckman SW40 rotor for 2 h) from 102 of the samples. Polyacrylamide gel electrophoresis in 7.5% or 10% gels was carried out by the method of Laemmli (11); the gels were stained with ethidium bromide and photographed under UV light.

RESULTS

Rotavirus from the stools of 252 children with gastroenteritis were studied in the subgrouping ELISA with monoclonal antibodies specific for the detection of rotavirus SG1 or SG2. The OD values obtained are depicted in Fig. 1. A total of 85% of the samples studied were classified as SG2 since they had OD ratios (reading with monoclon 631-9/reading with monoclon 255-60) higher than 2, whereas 14% were classified as SG1 (255-60/631-9 OD ratios higher than 1.7). With four exceptions, every one of the samples tested was recognized by either the SG1- or the SG2-specific monoclon. One of the four undetermined samples reacted equally well with both monoclonal antibodies and was later found to contain two rotaviruses of different electrophoretic mobilities. The other three samples did not react appreciably with either monoclonal antibody; however, their reactivity in the confirmatory ELISA used for initial diagnosis was low, and no RNA could be obtained from them for electrophoretic analysis.

Figure 2 shows the occurrence of the two rotavirus subgroups during the period studied. SG1 rotaviruses were detected at a lower frequency throughout the whole period and particularly during the first 9 months of 1982. Of the 34 SG1 rotaviruses identified, 18 were clustered in the period between December 1980 and February 1981. Of the 252 rotavirus-positive children studied, 89 were seen as outpatients, whereas 163 required hospital admission. Although a small difference in the rate of shedding of SG1 rotavirus was detected (11% of the outpatients versus 15% of the hospitalized children shed rotavirus SG1), this may be accounted for by the fact that a relatively larger number of the children examined after July 1981 were outpatients.

Information concerning the symptoms at the time of admission and the duration of illness (from the first vomiting or diarrheal episode until discharge) in 111 of the children who required hospitalization is shown in Table 1. No differences were observed in the relative frequency of diarrhea, vomiting, or fever in children who shed rotavirus SG1 as compared with those who shed rotavirus SG2. The duration of illness, however, seemed to be longer in children who shed SG2 rotavirus. A statistically significant difference (P < 0.05) was found in the percentage of children whose illnesses lasted fewer than 3 days between children who shed rotavirus SG1 and those who shed rotavirus SG2.

Table 2 shows a comparison of the efficiency of subgroup detection by monoclonal antibodies with those of the ELISA with polyclonal antibodies and those of the IAHA. Only 37 and 34% of the specimens could be subgrouped by the IAHA and the ELISA, respectively, with polyclonal antibodies as compared with 99% subgroup detection with monoclonal antibodies by ELISA. In every case in which the rotavirus
subgroup could be determined by either IAHA or ELISA with polyclonal antibodies, there was agreement with that determined with monoclonal antibodies.

RNA electrophoretic analysis was performed on 82 of the 102 samples from which attempts were made to extract RNA. Attention was directed only to the migration pattern of segments 10 and 11. RNAs with short migration patterns were referred to as 2S, and those with long patterns were referred to as 2L. These patterns correspond with SG1 and SG2, respectively, of human rotaviruses (8). An example of the two patterns is given in Fig. 3. The results obtained by the RNA electrophoretic analyses are also shown in Table 2. The efficiency of subgroup detection by this assay was 80%, and there was full agreement in each case with the subgroup determined by the subgrouping ELISA with monoclonal antibodies. One of the specimens was found to contain a mixture of two rotaviruses with both patterns of migration. As mentioned above, this specimen reacted with both subgrouping monoclones.

**DISCUSSION**

Antigenic diversity among rotaviruses was noted soon after their discovery. Through a variety of serological assays, including immune electron microscopy (19), complement fixation (9, 19), and ELISA (18), two types of human rotavirus were initially identified, and their relative importance was defined in epidemiological studies (18). Assumptions were then made that the serological differences defined by these methods were similar to those defined for other viruses by neutralization assays, and hence they were of immediate relevance to the development of strategies for vaccine production. The lack of cultivatable human rotavirus strains impeded serotyping by conventional neutralization assays and, thus, the proof of any connection between neutralization specificity and the antigenic specificity defined by immune electron microscopy, complement fixation, and ELISA. However, recent evidence indicates that these two properties are distinct and can be dissociated. Thus, Greenberg et al. (4, 5), Flores et al. (1), and Kapikian et al. (8) have obtained reassortant viruses derived from coinfecting cells with a bovine rotavirus strain and the human rotavirus Wa (which are antigenically distinct by subgrouping ELISA and the plaque neutralization assay); some of those reassortants conserved the bovine rotavirus serotype while they reacted similarly to Wa in subgrouping ELISAs. Conversely, some of the reassortants derived possessed the human rotavirus neutralization phenotype but reacted like the bovine virus in the subgrouping ELISA. Genotyping of a series of those reassortants led to the conclusion that the ELISA subgroup specificity is coded for by gene 6, whereas the serotype specificity as detected by neutralization assays is a property residing in a different gene (gene 9 for the Wa virus). More direct proof comes from the recent development of monoclonal antibodies against the protein encoded by rotavirus gene 6, which appears to be the most abundant component of the virion. Analysis of those hybridomas has clarified the fact that although this protein possesses antigenic domains common to human and animal rotaviruses of many different origins, it also has epitopes which confer antigenic specificities similar to those assayed by subgrouping ELISA and IAHA. Two such hybridomas (639/1 derived from human Wa rotavirus and 255/60 derived from the rhesus Rh-2 rotavirus) have been particularly useful in assaying this reactivity (5). In the present study, we used ascites fluid from mice inoculated with those two hybridomas to determine the relative frequency with which the subgroups are present in children with rotavirus gastroenteritis.

The ascites fluids were used as second antibodies in ELISAs and were more sensitive than the subgrouping IAHA and ELISA tests in which polyclonal antibodies were employed and which only subgrouped 37 and 34% of the

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**TABLE 1. Symptoms at admission and duration of illness in children hospitalized with rotavirus gastroenteritis**

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Patients infected with rotavirus:</th>
<th>SG1</th>
<th>SG2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Diarrhea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vomiting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration (days)</td>
<td>&lt;3°</td>
<td>6</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>3 to 7</td>
<td>10</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>&gt;7</td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>

* A significant difference (*P* < 0.05, Fisher's two-tailed exact test) was found between the number of children who shed rotavirus SG1 and SG2 and whose illness lasted fewer than 3 days.

**TABLE 2. Subgrouping of rotavirus by four different assays**

<table>
<thead>
<tr>
<th>Assay</th>
<th>No. tested in subgroup:</th>
<th>1</th>
<th>2</th>
<th>Undetermined</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA (monoclonal antibodies)</td>
<td></td>
<td>35</td>
<td>214</td>
<td>3</td>
</tr>
<tr>
<td>ELISA (polyclonal antibodies)</td>
<td></td>
<td>9</td>
<td>12</td>
<td>40</td>
</tr>
<tr>
<td>IAHA</td>
<td></td>
<td>8</td>
<td>12</td>
<td>34</td>
</tr>
<tr>
<td>RNA electrophoretic pattern</td>
<td></td>
<td>19</td>
<td>62</td>
<td>21</td>
</tr>
</tbody>
</table>
specimens, respectively. Furthermore, performing this test is simpler than performing the IAHA; the reagents are more uniform, and their availability is unlimited. In all, 248 of 252 samples assayed could be subgrouped, although in a few cases (about 21%) the assay had to be repeated when the ratios required for subgrouping were approached but not achieved in the initial test. Dilution of the sample or treatment with Genetron enhanced the differences in the ODs observed with the two monoclones.

An association between the RNA electrophoretic pattern of human rotaviruses and their subgroup specificity was observed by Kalica et al. (6) and recently confirmed by Thouless et al. (15). Such an association is also confirmed in this study, in which 81 of 82 specimens whose RNAs could be analyzed showed migration patterns characteristically associated with the two subgroups. However, the subgrouping ELISA with monoclonal antibodies was more sensitive than the electrophoretic analysis in that only 80% of the samples yielded enough RNA for analysis.

Whereas gene 6 codes for the subgroup specificity, it is interesting that the migration of genes 10 and 11 is characteristically different between rotaviruses belonging to SG1 or SG2. The existence of rotavirus gene linkages may account for this phenomenon. Although such linkages have not been demonstrated, they are suggested by the fact that many human rotaviruses belong to at least two genetic "families" corresponding to the different subgroups (2). High degrees of genomic homology are found within members of each family but not with members of the other family. Analysis of reassortants obtained between DS-1 (our SG1 prototype) and the UK bovine rotavirus (3) points out the correspondence between segment 10 of DS-1 and segment 11 of the bovine rotavirus, and by analogy with segment 11 of the Wa virus, both viruses (UK and Wa) have a long migration pattern (2L) such that in reality only one gene (gene 10) of DS-1 migrates slower than the corresponding gene in 2L viruses.

The high specificity and sensitivity of the subgrouping ELISA used in this work, imparted by the use of well-characterized uniform monoclonal antibodies, has allowed us to investigate unequivocally the relative prevalence of the two different rotavirus subgroups in a larger number of specimens. Throughout the 45 months of the study, there was a marked preponderance of rotavirus SG2, which represented overall 85% of all the specimens studied. Only during the 3-month period of December 1980 to February 1981 was there a noticeable increase in the occurrence of rotavirus SG1, which reached 28% of the total number of rotaviruses detected during that period. No other marked seasonal differences were observed either in the frequency of either subgroup or in the overall occurrence of rotavirus, which was 36.1%. Although the children from whom the samples were obtained were not closely observed clinically, some information was obtained from 111 of them. There were no differences in the occurrence of diarrhea, vomiting, or fever between hospitalized children shedding either rotavirus SG1 or SG2. However, there was a tendency in those shedding SG2 rotavirus to have a more prolonged illness (26% of those shedding SG2 virus had a syndrome longer than 7 days as compared with 11% of the children shedding rotavirus SG1) that might indicate a higher virulence of this subgroup. Similarly, 33% of the children who shed SG1 rotavirus had an illness of less than 3 days, whereas in only 11% of those who shed rotavirus SG2 did the syndrome last fewer than 3 days ($P < 0.05$).

No information is available on the serotypes of the specimens examined in this study since neutralization assays would be required, and the specimens have not been adapted to culture. Although it is likely that the SG1 rotaviruses belong to the second serotype of Thouless et al. (15), no inferences can be made about the SG2 specimens since they may represent viruses of two (15) or even three (17) different serotypes. Although an ELISA has recently been developed for serotyping rotaviruses (15), bypassing the need for cultivation, the test uses polyclonal antibodies with various combinations of blocking antigens, and hence the results obtained with it should be interpreted cautiously. Serotyping solid-phase assays are highly desirable and will require the development of monoclonal antibodies such as the ones employed here for subgrouping but with the capability of detecting the antigenic specificities of the protein(s) responsible for the induction of neutralizing antibodies.

Lambert et al. (12) have recently described the identification of a third subgroup of human rotavirus with polyclonal antibodies in a complement fixation assay. Although it is known that their SG1 corresponds to our DS-1 strain (18), it is possible that their SG2 and SG3 strains may be recognized as a single subgroup with the monoclonal antibodies used in the present work which were able to recognize every one of the specimens tested. Specific reactivities of rotaviruses as assayed with polyclonal antibodies may not necessarily reflect only antigenic differences in the 42,000-dalton protein, the subgrouping component of rotavirus.

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LITERATURE CITED


