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Characterization by Enzyme-Linked Immunosorbent Assay Using Subgroup- and Serotype-Specific Monoclonal Antibodies of Human Rotavirus Obtained from Diarrheic Patients in Bangladesh

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By enzyme-linked immunosorbent assay with group A-, subgroup-, and serotype-specific monoclonal antibodies (MAbs), we tested 414 stool specimens collected from pediatric and adult patients hospitalized with acute gastroenteritis between January and June 1988. Of 414 specimens tested, 124 (30%) were positive for group A rotavirus. The subgroup was determined in 110 specimens (88.7%); 16.1% were subgroup I, and 72.6% were subgroup II. Two specimens reacted with both subgroup I- and subgroup II-specific MAbs. Serotype determinations showed that serotype 1 (38.4%) was predominant over serotypes 2 (28.2%), 3 (2.5%), and 4 (23%). Three specimens reacted with more than one serotype-specific MAb. While the frequency of serotype 1 was highest in the two hospitals in Mymensingh, serotype 2 was most prevalent in one hospital in Dhaka. All human rotavirus strains with subgroup I and serotype 2 specificities showed a short electropherotype, and all but one strain with subgroup II and serotype 1, 3, or 4 specificities exhibited a long electropherotype.

Human rotavirus (HRV) has been recognized as the major etiological agent causing acute gastroenteritis in infants and young children worldwide (5, 6, 12). In developing countries, where malnutrition is common among infants and children, severe dehydration following rotavirus diarrhea leads to a high rate of mortality (2, 7, 12). Development of an effective rotavirus vaccine is therefore urgently needed to control rotavirus diarrhea (12, 31). The presence of at least six serotypes of HRV has hampered development of a broadly protective HRV vaccine (3, 10, 17, 27, 29, 32). Indeed, recent vaccine trial studies using rhesus rotavirus with serotype 3 specificity showed failure to protect against rotavirus illness due to heterotypic strains (14). Thus, extensive epidemiological studies on HRV serotypes in developing countries are required for development and evaluation of the vaccine.

The rotavirus virion contains 11 double-stranded RNA segments enclosed within a double-shelled capsid. The two capsid proteins, VP4 and VP7, are the major constituents of the outer capsid (12). Serotype specificity, which is defined by the virus neutralization test, is associated with VP7, which is encoded by RNA segment 8 or 9 (12). Another outer capsid protein, VP4, the product of RNA segment 4, also has independent neutralization specificity (9, 16, 19). In recent studies, neutralization epitopes common to serotypes 1, 3, and 4 or specific to serotype 2 have been identified on VP4 of HRV (21, 22). An inner capsid protein, VP6, encoded by RNA segment 6, has subgroup specificity (6, 12, 13), and two or three subgroups have been described (15, 25, 26).

In our laboratory, numerous monoclonal antibodies (MAbs) to VP2, VP4, VP6, or VP7 have been prepared and applied to an enzyme-linked immunosorbent assay (ELISA) for characterizing HRV in stools (20–24). The validity of ELISA subgrouping and ELISA serotyping with subgroup-specific and serotype-specific MAbs has been confirmed (24, 30). In this study, we characterized the HRV found in stools from 414 patients with gastroenteritis, including 98 adults in Bangladesh, by determining subgroup and serotype specificities with MAbs.

We collected 414 stool specimens of patients with acute gastroenteritis from three different hospitals between January and June 1988; 158 specimens were from Dhaka Medical College (DMC) hospital, 158 were from Mymensingh Medical College (MMC) hospital, and 98 were from SK hospital in Mymensingh, Bangladesh. The ages of the patients from the DMC and MMC hospitals were 1 month to 2 years, except for one patient, who was 5 years old. All patients from SK hospital were 21 to 46 years old. Most of the adult patients lived in rural areas, and they generally belonged to a low-income group. The symptoms and severity of the disease found in adults were almost the same as those found in infants. All of the patients suffered from watery diarrhea (8 to 15 times per day), vomiting, abdominal pain, and low fever. Approximately 10% stool suspensions prepared in phosphate-buffered saline were clarified by low-speed centrifugation, transported to the Department of Hygiene and Epidemiology, Sapporo Medical College, and kept at −20°C until tested. Anti-VP6 MAb YO-156, which recognizes the common antigen of rotavirus, was used to detect group A rotavirus. For the subgrouping assay, subgroup I-specific MAb S2-37 and subgroup II-specific MAb YO-5 directed to VP6 were used. For the serotyping assay, the following four anti-VP7 serotype-specific MAbs and one anti-VP4 commonly reactive neutralizing MAb were used: serotype 1-specific KU-6BG, serotype 2-specific S2-2G10, serotype 3-specific YO-1E2, serotype 4-specific ST-2G7, and commonly reactive YO-2C2. The properties of the above-listed MAbs have been previously described (20–24).

ELISA with MAbs was performed as described previously, with some modifications (24; S. Urasawa, T. Urasawa, K. Taniguchi, F. Wakasugi, N. Kobayashi, S. Chiba,

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N. Sakurada, S. Morita, O. Morita, M. Tokieda, T. Kawamoto, M. Minekawa, and M. Ohseto, J. Infect. Dis., in press). Briefly, polyvinyl microtiter plates were coated with a 1:10,000 dilution of ascitic fluid and incubated overnight at 4°C. After being washed, the wells were incubated with 1% bovine serum albumin overnight at 4°C and subsequently washed. A mixture of a 10% stool suspension (375 μl) and 10% skim milk (125 μl) was allowed to react in the wells overnight at 4°C. After being washed, 50 μl of rabbit anti-HRV serum (a 1:10,000 dilution) was added and incubated for 1 h at 37°C. After the washing, 50 μl of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Chemical Credential ICN ImmunoBiologicals, Israel) was added. The plates were incubated for 1 h at 37°C and washed. The reaction with the substrate, *o*-phenylenediamine, was allowed to develop for 30 min at room temperature and then stabilized by addition of 25 μl of 3 N sulfuric acid. The optical density was measured at 492 nm with a micro-ELISA reader (EAR400; SLT-Labinstruments, Salzburg, Austria). In the ELISA for serotype determination, a virus was assigned to a specific serotype only when the following two requirements were fulfilled simultaneously. (i) The optical density value for the reaction with the MAb corresponding to that serotype exceeded 0.2 per well, and (ii) the optical density value for the reaction corresponding to that serotype was greater than twice the value corresponding to any other serotype (Urasawa et al., in press). The criteria used to determine group and subgroup specificities were reported previously (24).

RNA electropherotyping was performed as follows. A 150- to 500-μl sample of a 10% stool suspension clarified after low-speed centrifugation was layered on 40% (wt/vol) sucrose and centrifuged at 100,000 × *g* for 3 h. The pellet was suspended in 300 μl of 10 mM Tris hydrochloride buffer (pH 8.0; partially purified virus). After the virus was disrupted with sodium dodecyl sulfate–2-mercaptoethanol–EDTA, genomic double-stranded RNA was extracted with phenol, precipitated with ethanol, and then analyzed by polyacrylamide gel electrophoresis on 10% slab gels. After electrophoresis at 40 mA for 6 h, the double-stranded RNA bands were visualized by the silver staining method.

For electron microscopy, 1 drop of the partially purified virus suspended in distilled water was placed on a 400-mesh carbon-collodion-coated grid for 1 min and excess fluid was withdrawn with the edge of a paper filter. Soon after staining with 1 drop of 2% uranyl acetate, the staining solution was withdrawn. The grid was examined in a Hitachi H500 electron microscope.

In the ELISA using MAb YO-156, 124 stool samples (30%) (46 from the DMC hospital, 54 from the MMC hospital, and 24 from the SK hospital) were positive for group A rotavirus. The 124 specimens were subjected to ELISA subgrouping with subgroup-specific MAbs, and 110 (88.7%) could be subgrouped. Twenty (16.1%) belonged to subgroup I, 90 (72.6%) belonged to subgroup II, 2 (1.6%) belonged to both subgroups, and the subgroup specificities of 12 specimens (9.7%) were not determined. There was some difference in subgroup distribution among the three different hospitals, although subgroup II strains were predominant in all three hospitals. Two specimens (no. 302 and 446) reacted with both subgroup I- and subgroup II-specific MAbs. By RNA analysis, specimen 446 seemed to contain both subgroup I and subgroup II strains since it showed a mixed RNA profile of so-called short (segment 11 migrates slowly) and long (segment 11 migrates fast) patterns. Specimen 302 failed to exhibit an RNA profile because too little viral RNA was present in the stool specimen; this was confirmed by electron microscopy. Of 124 specimens analyzed, 12 (9.7%) reacted with neither subgroup I-specific MAb S2-37 nor subgroup II-specific MAb YO-5, although they reacted very strongly with MAb YO-156. Of these 12 specimens, 5 also reacted moderately with YO-2C2, a commonly VP4-reactive MAb. These results suggest the existence of a third subgroup of group A human rotavirus, as reported recently (15, 25, 26). In this context, production and characterization of MAbs to one of the specimens whose subgroup specificity could not be determined would be useful for further characterization of the subgroup antigen of HRV.

TABLE 1. Characterization of HRV from stool specimens using subgroup- and serotype-specific MAbs in an ELISA

Subgroup(s)	No. of specimens with the following serotype(s):						No. of specimens whose serotypes were not determined
	1	2	3	4	2 and 4	1 and 4	
I		10					10
II	15	1	1	9	1	1	62
I and II					1		1
Not determined							12

The serotype specificities of the 124 specimens were tested by using four kinds of serotype-specific neutralizing MAbs in an ELISA (Urasawa et al., in press). Overall, 39 (31.5%) could be serotyped; 15 (38.5%) of these were assigned to serotype 1, 11 (28.2%) were serotype 2, 1 (2.6%) was serotype 3, and 9 (23.1%) were serotype 4 (Table 1). The distribution of the four serotypes was different among the three hospitals (Table 2). The frequency of serotype 1 was highest in the two hospitals in Mymensingh, but it was especially high in the SK hospital, where all of the patients were adults. In contrast, in the DMC hospital in Dhaka, serotype 2 was most frequent. Three specimens were recognized by two serotype-specific MAbs, two (no. 446 and 587) by serotype 2- and serotype 4-specific MAbs and one (no.

TABLE 2. Distribution of subgroups and serotypes in three different hospitals in Bangladesh as characterized by ELISA using subgroup- and serotype-specific MAbs

Hospital and subgroup(s)	No. of specimens with the following serotype(s):						No. of specimens whose serotypes were not determined
	1	2	3	4	1 and 4	2 and 4	
MMC							
I		2					4
II	6		1	3		1	32
Not determined							4
SK[a]							
I							1
II	8	1	0	1	1		10
Not determined							2
DMC							
I		8					5
II	1			5			20
I and II						1	1
Not determined							5

[a] All of the patients in this hospital were adults.

653) by serotype 1- and serotype 4-specific MAbs. Specimen 446 was found to be a mixture of two strains, as described above. However, there is a possibility that specimens 587 and 653 bear dual serotype specificities (serotypes 1 and 4 or 2 and 4) on VP7, since RNA profiles of these specimen showed a typical long RNA pattern and no extra RNA bands could be observed. Six specimen showed no reactivity with MAb YO-2C2, although they reacted with one of the four serotype-specific neutralizing MAbs. It has been found that MAb YO-2C2 can recognize VP4 in the outer capsid of most HRV strains, since in our previous examinations MAb YO-2C2 recognized all 15 representative, cultivable HRV strains except strain K8 (serotype 1, subgroup II) in an ELISA (22). The VP4 amino acid sequence of strain K8 was quite different (64 to 66% homology) from those of other virulent HRV strains (K. Taniguchi, K. Nishikawa, T. Urasawa, S. Urasawa, K. Midthun, A. Z. Kapikian, and M. Gorziglia, submitted for publication). Some of the six specimens with no reactivity with MAb YO-2C2 might have a unique VP4 gene, like strain K8.

The remaining 73 specimens did not react with any serotype-specific MAbs. Examination of some (27 specimens) of these unserotyped specimens by direct electron microscopy revealed the presence of insufficient numbers of double-shelled virus particles, in contrast to the serotype-determined specimens, in which more than 100 double-shelled virus particles per grid square were seen. Since the efficiency of serotyping directly depends on the number of double-shelled virus particles in the stool specimens, this result explains why many specimens failed to be serotyped. Double-shelled particles may be broken down or lost because of pH and enzyme (trypsin) influences in the gut or during processing of stool specimens (1). Under the existing circumstances in Bangladesh, considerable time elapsed between onset of diarrhea, hospitalization, and collection of stool specimens; this may have affected virus degradation. It should also be pointed out that stool specimens transported from Bangladesh by mail were kept at relatively high temperature for about 10 days. This might have caused degradation of the outer capsid and its constituent proteins. The concentration of the stool suspension was expected to increase the rate of serotyping (4). Indeed, when we used three concentrated stool specimens, all of the specimens could be serotyped. Of the 73 specimens of undetermined serotype (excluding 12 subgroup-undetermined specimens), 35 reacted with commonly reactive anti-VP4 MAb YO-2C2, which was included in ELISA serotyping for detection of double-shelled particles. It was found that the serotype-specific neutralization epitopes on VP7 form the conformational antigenic site(s) (20), whereas the cross-reactive epitope on VP4 recognized by YO-2C2 is linear, as described previously (22). These findings might also explain the low frequency of serotyping.

The RNA electropherotypes of 97 HRV specimens were examined by polyacrylamide gel electrophoresis. Of 39 specimens which could be serotyped, 8 (20.5%) showed a short electropherotype and 29 (74.4%) showed a long electropherotype. One serotyped specimen failed to show any RNA electropherotype. This may have been due to the low amount of RNA present in the specimen. Interestingly one specimen showing subgroup II and serotype 2 specificities exhibited a long RNA electropherotype. The strain might possess unusual genetic characteristic suggestive of reassortment between different HRV strains (18). In fact, it has been demonstrated under experimental conditions that interchange of genomic segments takes place with high efficiency in cells coinfected with two different rotaviruses (8, 28). The reason for and significance of the unusual genetic characteristics observed in this study remain to be elucidated further after propagation of the specimen in cell culture. We also performed RNA electropherotyping of 58 serotype-undetermined specimens, and 30 (51.7%) showed a long electropherotype and belonged to subgroup II while only 1 (1.7%) showed a short electropherotype and belonged to subgroup I. It has been reported by several workers that subgroup I rotaviruses showing a short electropherotype belong to serotype 2, and subgroup II rotaviruses showing a long electropherotype belong to serotype 1, 3, or 4 (11, 12; Urasawa et al., in press). In the present study, we also confirmed the correlation of the short RNA electropherotype with subgroup 1 and serotype 2.

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LITERATURE CITED

1. **Birch, C. J., R. L. Heath, and I. D. Gust.** 1988. Use of serotype specific monoclonal antibodies to study the epidemiology of rotavirus infection. J. Med. Virol. **24**:45–53.
2. **Black, R. E., M. H. Merson, A. S. M. Rahman, M. Yunus, A. R. M. A. Alim, I. Huq, R. H. Yolken, and G. T. Curlin.** 1980. A study of bacterial, viral, and parasitic agents associated with diarrhea in rural Bangladesh. J. Infect. Dis. **142**:660–644.
3. **Clark, H. F., Y. Hoshino, L. M. Bell, G. H. Groff, P. Bachman, and P. A. Offit.** 1987. Rotavirus isolate W161 representing a presumptive new human serotype. J. Clin. Microbiol. **25**:1757–1762.
4. **Coulson, B. S., L. E. Unicomb, G. A. Pitson, and R. F. Bishop.** 1987. Simple and specific enzyme immunoassay using monoclonal antibodies for serotyping human rotavirus. J. Clin. Microbiol. **25**:509–515.
5. **Cukor, G., and N. R. Blacklow.** 1984. Human viral gastroenteritis. Microbiol. Rev. **48**:157–179.
6. **Estes, M. K., E. I. Palmer, and J. F. Obijeski.** 1983. Rotaviruses. A review. Curr. Top. Microbiol. Immunol. **105**:123–184.
7. **Georges, M. C., C. Roure, R. V. Tauxe, D. M. Meunier, M. Merlin, J. Testa, C. Baya, J. Limbassa, and A. J. Georges.** 1987. Diarrheal morbidity and mortality in children in the Central African Republic. Am. J. Trop. Med. Hyg. **36**:598–602.
8. **Gombold, J. L., and R. F. Ramig.** 1986. Analysis of reassortment of genome segments in mice mixedly infected with rotaviruses SA11 and RRV. J. Virol. **57**:110–116.
9. **Hoshino, Y., M. Sereno, K. Midthun, J. Flores, A. Z. Kapikian, and R. M. Chanock.** 1985. Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. Proc. Natl. Acad. Sci. USA **82**:8701–8704.
10. **Hoshino, Y., R. G. Wyatt, H. B. Greenberg, J. Flores, and A. Z. Kapikian.** 1984. Serotype similarity and diversity of rotaviruses of mammalian and avian origin as studied by plaque reduction neutralization. J. Infect. Dis. **149**:694–702.
11. **Kalica, A. R., H. B. Greenberg, R. T. Espejo, J. Flores, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock.** 1981. Distinctive ribonucleic acid patterns of human rotavirus subgroups 1 and 2. Infect. Immun. **33**:958–961.
12. **Kapikian, A. Z., and R. M. Chanock.** 1985. Rotaviruses, p. 863–906. *In* B. N. Fields, D. N. Knipe, R. M. Chanock, J. L. Melnick, B. Roizman, and R. E. Shope (ed.), Virology. Raven Press, New York.
13. **Kapikian, A. Z., W. L. Cline, H. B. Greenberg, R. G. Wyatt, A. R. Kalica, C. E. Banks, H. D. James, Jr., J. Flores, and R. M. Chanock.** 1981. Antigenic characterization of human and animal rotaviruses by immune adherence hemagglutination assay (IAHA): evidence of distinctness of IAHA and neutralization antigen. Infect. Immun. **33**:415–425.
14. **Kapikian, A. Z., J. Flores, K. Midthun, Y. Hoshino, K. Y.**

Green, M. Gorziglia, K. Taniguchi, K. Nishikawa, R. M. Chanock, L. Potash, I. Perez-Schael, R. Dolin, C. Chrysty, M. Santosham, N. A. Halsey, M. L. Clements, S. D. Sears, R. E. Black, M. M. Levine, G. Losonsky, M. Rennels, L. Gottefors, G. Wadel, R. I. Glass, T. Vesikari, E. L. Anderson, R. B. Belshe, P. F. Wright, and S. Urasawa. 1988. Development of a rotavirus vaccine by a "Jennerian" and modified "Jennerian" approach. Vaccines (Cold Spring Harbor) **88:**151–159.

15. **Lambert, J. P., D. Marissens, P. Marbehant, and G. Zissis.** 1983. Prevalence of subgroup 1, 2, and 3 rotaviruses in Belgian children suffering from acute diarrhea (1978–1981). J. Med. Virol. **11:**31–38.
16. **Liu, M., P. A. Offit, and M. K. Estes.** 1988. Identification of the simian rotavirus SA11 genome segment 3 product. Virology **163:**26–32.
17. **Matsuno, S., A. Hasegawa, A. Mukoyama, and S. Inouye.** 1985. A candidate for a new serotype of human rotavirus. J. Virol. **54:**623–624.
18. **Midthun, K., J. Valdesuso, Y. Hoshino, J. Flores, A. Z. Kapikian, and R. M. Chanock.** 1987. Analysis of RNA-RNA hybridization assay of intertypic rotaviruses suggests that gene reassortment occurs in vivo. J. Clin. Microbiol. **25:**295–390.
19. **Offit, P. A., and G. Blavat.** 1986. Identification of the two rotavirus genes determining neutralization specificities. J. Virol. **57:**376–378.
20. **Taniguchi, K., Y. Hoshino, K. Nishikawa, K. Y. Green, W. L. Maloy, Y. Morita, S. Urasawa, A. Z. Kapikian, R. M. Chanock, and M. Gorziglia.** 1988. Cross-reactive and serotype-specific neutralization epitopes on VP7 of human rotavirus: nucleotide sequence analysis of antigenic mutants selected with monoclonal antibodies. J. Virol. **62:**1870–1874.
21. **Taniguchi, K., W. L. Maloy, K. Nishikawa, K. Y. Green, Y. Hoshino, S. Urasawa, A. Z. Kapikian, R. M. Chanock, and M. Gorziglia.** 1988. Identification of cross-reactive and serotype 2-specific neutralization epitopes on VP3 of human rotavirus. J. Virol. **62:**2421–2426.
22. **Taniguchi, K., Y. Morita, T. Urasawa, and S. Urasawa.** 1987. Cross-reactive neutralization epitopes on VP3 of human rotavirus: analysis with monoclonal antibodies and antigenic variants. J. Virol. **61:**1726–1730.
23. **Taniguchi, K., S. Urasawa, and T. Urasawa.** 1985. Preparation and characterization of neutralizing monoclonal antibodies with different reactivity patterns to human rotaviruses. J. Gen. Virol. **66:**1045–1053.
24. **Taniguchi, K., T. Urasawa, Y. Morita, and S. Urasawa.** 1987. Direct serotyping of human rotavirus in stools by an enzyme-linked immunosorbent assay using serotype 1-, 2-, 3- and 4-specific monoclonal antibodies directed to VP7. J. Infect. Dis. **155:**1159–1166.
25. **Taniguchi, K., T. Urasawa, S. Urasawa, and T. Yasuhara.** 1984. Production of subgroup-specific monoclonal antibodies against human rotaviruses and their application to enzyme-linked immunosorbent assay for subgroup determination. J. Med. Virol. **14:**115–125.
26. **Tufvesson, B.** 1983. Detection of human rotavirus strain different from types 1 and 2—a new subgroup? Epidemiology of subgroups in a Swedish and Ethiopian community. J. Med. Virol. **12:**111–117.
27. **Urasawa, S., T. Urasawa, and K. Taniguchi.** 1982. Three human rotavirus serotypes demonstrated by plaque neutralization of isolated strains. Infect. Immun. **38:**781–784.
28. **Urasawa, S., T. Urasawa, and K. Taniguchi.** 1986. Genetic reassortment between two human rotaviruses having different serotype and subgroup specificities. J. Gen. Virol. **67:**1551–1559.
29. **Urasawa, S., T. Urasawa, K. Taniguchi, and S. Chiba.** 1984. Serotype determination of human rotavirus isolates and antibody prevalence in pediatric population in Hokkaido, Japan. Arch. Virol. **81:**1–12.
30. **Urasawa, S., T. Urasawa, K. Taniguchi, Y. Morita, Y. Sakurada, Y. Saeki, Y. Morita, and S. Hasegawa.** 1988. Validity of enzyme-linked immunosorbent assay with serotype-specific monoclonal antibodies for serotyping human rotavirus in stool specimens. Microbiol. Immunol. **32:**699–708.
31. **Vesikari, T., E. Isolauri, E. Hondt, A. Delem, F. E. Andre, and G. Zissis.** 1984. Protection of infants against rotavirus diarrhoea by RIT4237 attenuated bovine rotavirus strain vaccine. Lancet **i:**977–981.
32. **Wyatt, R. G., H. D. James, A. L. Pittman, Y. Hoshino, H. B. Greenberg, A. R. Kalica, J. Flores, and A. Z. Kapikian.** 1983. Direct isolation in cell culture of human rotaviruses and their characterization into four serotypes. J. Clin. Microbiol. **18:** 310–317.