Human Rotavirus and Its Antibody: Their Coexistence in Feces of Infants

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Rotavirus and its antibody were detected by paper disk solid-phase radioimmunoassay or electron microscopy in feces of infants and young children with acute diarrhea. The fecal specimens in which rotavirus was detectable often contained a high titer of antibodies, which were shown by radioimmunoassay to belong mainly to the immunoglobulin G class. Rotavirus was rarely detected in the specimens containing immunoglobulin A antibodies. By dissociation tests carried out by radioimmunoassay, it was shown that the rotavirus particles in some specimens had the same antibody-binding capacity as did cultured simian rotavirus (SA-11), but antibodies in feces usually had low avidity, probably resulting from enzymatic digestion.

Infantile enteritis is primarily a viral disease caused by human rotavirus and characterized by severe diarrhea in children under 2 years of age (3, 7). In a previous paper we reported that rotavirus isolated from feces of infants with acute enteritis was already associated with immunoglobulin G (IgG), IgM, and IgA antibodies (25). This finding aroused our interest in the possible presence of infectious rotavirus-antibody complexes in intestinal contents in the acute phase of infection. Coexistence of virions and antibodies in feces has been shown with poliovirus (8) and with hepatitis-associated antigen (14). The competition of low-avidity antibodies with high-avidity ones for binding to the antigenic determinants of the viral surface might contribute to the formation of such complexes (1, 10, 12).

It is clear that secretory IgA plays the most important role in the intestinal resistance of mammals, eliminating foreign pathogens from the intestine (2, 8, 15). Nevertheless, the role of IgG, sometimes confirmed to be the predominant immunoglobulin in colostrum or milk (2, 11, 16, 19, 22), is not so clear as that of secretory IgA, as shown in work on macromolecule uptake by the rat small intestine by Walker and his co-workers (23, 24).

The detection of rotavirus or its antibody in feces of some mammals has been done by immune electron microscopy (EM), fluorescent virus precipitin tests, neutralization tests, comple-

ment fixation tests, immunofluorescent tests, counter immunoelectrophoresis, radioimmunoassay (RIA), or enzyme immunoassay (3, 6, 7, 13, 17, 20, 21, 27-29). Immunoglobulin classes of the antibodies to human rotavirus in serum have been studied by 2-mercaptoethanol treatment (9). We now describe the detection and the titration of rotavirus and its antibody in feces of diarrheic infants and the classification of the antibody by EM or paper disk solid-phase RIA. The antibody classification was also applied to colostrum and serum samples.

MATERIALS AND METHODS

Collection of specimens. Fecal samples were collected from infants and young children with acute nonbacterial diarrhea, who were admitted to the hospital. The feces were made into a 20% (wt/vol) suspension in Hanks balanced salt solution and homogenized, and a 0.5 volume of fluorocarbon was added. After another homogenization, the mixtures were centrifuged for 30 min at $10,000 \times g$, and the supernatant fluids were collected to test in the following experiments. Colostrum and blood samples were collected from adults at random, and the former was made into a 50% (vol/vol) solution. The supernatant fluids after centrifugation were titrated for rotavirus antibodies by RIA.

EM. The supernatant fluids of fecal samples were placed in 0.002 M tris(hydroxymethyl)aminomethane buffer, pH 7.0, containing 45% (wt/vol) sucrose and centrifuged at $100,000 \times g$ for 75 min. The resultant pellets were suspended in 2 to 5 drops of tris(hydroxymethyl)aminomethane buffer. The specimens were negatively stained and observed by an electron microscope as previously reported (25).

Paper disk solid-phase RIA. Purified SA-11 (18), rabbit anti-SA-11 serum globulin, chicken IgG (26), and bovine serum albumin (BSA) were labeled with

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¹²⁵I, and RIA was done as described previously, with slight modifications (25). In comparison with previous results obtained when human fecal rotavirus was iodine labeled, the SA-11 labeling experiments resulted in a marked high peak of labeled virions after gel filtration on Sephadex G-200 and a much smaller secondary peak of labeled proteins.

Immunoglobulin classes of antiviral antibodies in feces were identified by inhibiting the reaction between antibodies present in the fecal specimens and labeled SA-11 virus by goat sera; anti-human γ -chain (lot 8257A002AC, Hyland), anti- μ -chain (lot 8211A001AA), or anti- α -chain (lot 8212A004AA) disks coated with fecal specimens were reacted with the antisera at 4°C overnight. After the reactions of anti-H-chain sera were completed, the antisera were discarded, and the disks were washed with buffer twice, since anti-rotavirus antibodies were shown to be present in these antisera. As a control, the specimen disks were treated with "normal" rabbit serum (containing very little or no rotavirus antibodies) instead of anti-H-chain sera. Total inhibition was measured by adding inhibition by antiserum to each class. Inhibition percentage was calculated as (inhibition by antiserum to each class/total inhibition by the antisera) $\times$ 100.

To measure the avidity of fecal antibodies and the capacity of fecal rotavirus to bind antibodies, dissociation tests were done as follows. Disks coated with fecal specimens were reacted with labeled SA-11 virus or labeled anti-SA-11 serum globulin for 3 days at 4°C, followed by washing with 2 ml of ice-cold 0.05 M phosphate buffer, pH 7.5, twice. The remaining radioactivity was considered to represent 100% binding of labeled SA-11 virus or labeled anti-SA-11 antibodies to rotavirus antibodies or rotavirus on the disks, respectively. The disks were then immersed in 2 ml of phosphate buffer at 24°C without shaking, and after the appropriate incubation time the buffer was removed and the radioactivity remaining on the disks was counted. The counting was done at 5, 10, 20, 40, and 80 min after the first addition of buffer at 24°C. Control disks were prepared by coating with rabbit anti-SA-11 serum, cultured SA-11 virus, and BSA.

RESULTS

Titration of rotavirus in feces. A total of 31 fecal specimens were tested by EM. In 16 of 31 (52%) specimens, rotavirus particles were identified (Table 1). A total of 23 fecal specimens were diluted to 1:10 and 1:100, and specific binding of labeled anti-SA-11 to the specimen disks was examined by RIA. All of the specimens gave the highest specific binding with the undiluted original solutions, which were 20% (wt/vol) suspensions of the original fecal samples. The highest titers ranged from 0.3 to 6.0% specific binding.

When considering the widespread incidence of rotavirus and the high sensitivity of RIA, we became aware of the difficulty of obtaining human feces that we could use as a negative control in RIA. For this reason we established a criterion for judging whether specimens were negative or

TABLE 1. *Detection of rotavirus and its antibody in feces by EM and RIA^a*

Specimen	Virus detection by RIA	Antibody detection by RIA
Group A ^b		
0270	ND	—
0271	—	±
0276	+	+
0293	+	+
0294	+	+
0303	ND	+
0308	+	+
0312	ND	+
0315	+	+
0318	ND	—
0393	+	+
0399	+	±
0418	+	+
0419	+	+
0421	—	+
0424	±	+
Group B ^c		
0265	—	—
0269	±	—
0272	—	+
0279	ND	+
0288	ND	+
0289	±	+
0290	±	—
0291	—	+
0296	—	+
0297	+	+
0299	—	+
0405	—	+
0411	—	±
0422	ND	—
0423	ND	—

^a ND, Not determined; —, negative for antibody or virus; ±, equivocal for antibody or virus; +, positive for antibody or virus.

^b Rotavirus detectable by EM.

^c Rotavirus not detected by EM.

positive for virus, for which no negative specimens were required. First, labeled chicken IgG and labeled BSA were reacted with disks coated with 20 different fecal specimens. The specific binding of the labeled proteins ranged from -0.4 to 0% and -0.3 to 0.3% at a 1:1 dilution of feces, respectively, showing that no binding of chicken IgG and BSA to the specimens occurred; i.e., the fecal specimens were considered not to bind such protein molecules nonspecifically. Second, it was assumed that dilution of the antigen or antibody would result in decreased binding in the antigen-antibody reaction as the antigen-antibody ratio became inadequate to react. In the present experiment, a >1% difference in binding levels over a 1:100 dilution range was supposed to be significant, since such a difference over a 1:100 dilution range as resulted from nonspecific reactions

was shown to be $\leq 0.7\%$ in the first step described above. According to this criterion, 9 of 23 (39%) samples were negative for virus; their binding ranged from 0.3 to 1.1% (average, 0.7%) at a 1:1 dilution. If the specimens that had $\geq 1.5\%$ binding were considered as positive, 10 of 23 (44%) samples were positive; the maximum specific binding ranged from 1.5 to 6.0% (average, 2.4%). Others were considered to be equivocal; i.e., 4 of 23 (17%) samples had binding ranging from 1.0 to 1.2% (average, 1.2%). This criterion appeared to be reasonable, since the results of this RIA correlated well with those of EM (70% correlation; Table 1).

Titration of antibodies in feces, colostrum, and sera. A total of 31 fecal specimens were serially diluted, and rotavirus antibodies in them were titrated by RIA. Specific binding at 1:1 dilutions ranged from 0 to 9.7%, and with some specimens maximal binding occurred with a 1:10 dilution of extract. The same criterion for positivity as in virus titration was also applied to antibody titration. On this basis, 7 of 31 (23%) specimens were negative for antibodies, having binding levels of 0.1 to 1.8% (average, 0.8%). As $\geq 2.0\%$ binding was considered to be significant, 21 of 31 (68%) specimens were shown to be positive; their binding ranged from 2.0 to 9.7% (average, 4.8%). Equivocal results were obtained with 3 of 31 (10%) samples, giving 1.5 to 1.9% binding (average, 1.7%). The results are shown in Table 1. The maximal specific binding of five colostrum and of six sera ranged from 5.9 to 11.0% and from 3.2 to 9.2%, respectively.

Immunoglobulin classes of antibodies. A total of 13 fecal specimens that had high antibody titers were subjected to inhibition tests as described above to determine the immunoglobulin classes of fecal antibodies (Table 2). It is notable that IgA antibodies were present in all of five specimens (23 to 41% inhibition) in which rotavirus was undetectable by EM and usually also by RIA. Three other specimens contained IgA antibodies (17 to 36% inhibition); in two of these virus was detectable only by EM, and in one it was detectable by EM and RIA. All five specimens negative for IgA antibodies were positive for rotavirus by EM and usually also by RIA.

Antibody class identification was also done in colostrum and sera (Table 3). The most outstanding finding was that serum samples contained a small amount of IgG antibodies (0 to 18% inhibition) as compared with fecal and colostrum samples (0 to 100% inhibition).

Capacity of virus to bind antibodies and antibody avidity. Dissociation tests were done as described above. As shown in Fig. 1a, the rotavirus antigens in the three specimens having

TABLE 2. *Classification of antibodies in feces by inhibition tests^a*

Specimen	Inhibition (%) ^b		
	Anti- γ -chain	Anti- μ -chain	Anti- α -chain
Group A ^c			
0276	47	36	18
0294	100	0	0
0308	100	0	0
0393	83	17	0
0419	76	24	0
Group B ^d			
0312	67	33	0
0421	30	34	36
0424	42	41	17
Group C ^e			
0272	35	29	36
0279	7	52	41
0288	16	55	29
0291	32	45	23
0405	34	34	32

^a The fecal specimen disks were treated with anti-H-chain antisera before addition of labeled SA-11 virus.

^b (Inhibition by antiserum to each class/total inhibition by the antisera) $\times 100$.

^c Rotavirus detectable by EM and RIA.

^d Rotavirus detectable by EM. 0312 was not determined by RIA.

^e Rotavirus not detected. 0279 and 0288 were not determined by RIA.

TABLE 3. *Classification of antibodies in colostrum and sera by inhibition tests^a*

Specimen	Inhibition (%)		
	Anti- γ -chain	Anti- μ -chain	Anti- α -chain
Colostrum			
1	19	32	49
2	20	36	44
3	34	48	17
4	34	39	27
5	0	61	39
Serum			
1	0	56	44
2	0	49	51
3	0	44	56
4	0	78	22
5	0	77	23
6	18	42	40

^a See footnotes to Table 2.

the highest virus titer behaved very much like cultured SA-11 virus, judging by capacity to bind antiviral antibodies, since dissociation rates of the specimens and SA-11 virus were similar, i.e., 3.4 ± 0.7 and $3.4\%/min$, respectively (the rates were determined within the first 10 min, since

FIG. 1. Antibody-binding capacity of fecal rotavirus (a) and avidity of fecal antibody (b) shown by dissociation tests. Specimen disks were reacted with labeled anti-SA-11 serum globulin or labeled SA-11 virus followed by successive washes to measure their dissociation at 24°C. One hundred percent binding was obtained by washing with ice-cold buffer. Symbols: ●, highest-virus-titer specimens or highest-antibody-titer specimens; ▽, lowest ones; ○, cultured SA-11 virus or rabbit anti-SA-11 serum; ×, BSA.

their dissociation became parallel after that). The sample having the lowest virus titer showed a rate of 5.2%/min, and the BSA control showed a rate of 6.2%/min.

In contrast, antibodies in feces showed high dissociation rates as compared with the anti-SA-11 control (Fig. 1b). The rates within 20 min of dipping time (parallel dissociation after that time) were 1.1, 2.2 ± 0.1 , 2.6, and 2.6%/min with anti-SA-11 serum, the highest-antibody-titer specimens, the lowest one, and the BSA control, respectively.

DISCUSSION

The results of the present study suggest that rotavirus may be capable of infecting infants or young children who have rotavirus antibodies of the IgG class in their intestinal contents. As a result of the degradation of antibodies by intestinal enzymes, feces may contain antibody fragments that are capable of only weak virus neutralization or antigen binding (5, 8); considering these reports and our results, we would conclude that a large amount of antibodies of low avidity may be contained in the intestinal cavity, without affording protection against infection.

Dissociation tests suggested that some fecal specimens contained intact virions as antigens that were indistinguishable from cultured rotavirus on the basis of resistance to enzymes. However, a large proportion of antibodies in feces appeared to be damaged or degraded by enzymes, resulting in low avidity. Antibodies bound to the virus surface might remain more intact and resistant to enzyme digestion (1). The free low-avidity antibodies were readily dissociated from rotavirus on disks during washing in dissociation tests. This finding may be important in the interpretation of standard serological tests that involve washing, as one may not detect specific antibodies having low avidity.

By the RIA method for detecting antigenic determinants, five of the six specimens (83%) that contained IgA antibodies gave a negative result for virus antigens, although by EM five of the eight specimens (63%) positive for IgA were negative for virus. On the other hand, all five specimens free of IgA antibodies contained rotavirus, which was detected by EM and usually also by RIA. These results, together with the known resistance of secretory IgA to enzymatic digestion, suggest that secretory IgA of stable avidity in the intestine may bind to the virus surface firmly, covering the virions and assisting in their elimination. IgA is likely to play the main role in the intestinal resistance to rotavirus infection, as in infections with other viruses (2, 8, 15, 19).

An unexpected finding involved the IgG antibody levels. As the amount of IgG antibodies increased in feces, the frequency of detection of rotavirus also increased in the specimens (Table 2). The presence of infectious virus-antibody complexes would be predicted in such specimens, and actually we have detected antibody-covered virions in feces (25).

The two methods applied in this study have different characteristics for the detection of rotavirus in feces. By EM, only intact or partially broken virions are detectable. Since antigenic determinants are detectable by RIA, virions covered completely with antibodies would not be detected. This is probably the reason why RIA gave negative results for two specimens positive by EM. Although the virus detection rate in specimens was 52 and 44% by EM and RIA, respectively, it may be misleading to compare the sensitivity of methods having such different characters.

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LITERATURE CITED

- Blank, S. E., G. A. Leslie, and L. W. Clem. 1972. Antibody affinity and valence in viral neutralization. *J. Immunol.* **108**:665-673.
- Bohl, E. H., and L. J. Saif. 1975. Passive immunity in transmissible gastroenteritis of swine: immunoglobulin characteristics of antibodies in milk after inoculating virus by different routes. *Infect. Immun.* **11**:23-32.
- Davidson, G. P., R. F. Bishop, R. R. W. Townley, I. H. Holmes, and B. J. Ruck. 1975. Importance of a new virus in acute sporadic enteritis in children. *Lancet* **ii**:242-245.
- Flewett, T. H., A. S. Bryden, H. Davies, G. N. Woode, J. C. Bridger, and J. M. Derrick. 1974. Relation between viruses from acute gastroenteritis of children and newborn calves. *Lancet* **ii**:61-63.
- Haneberg, B., and C. Endresen. 1976. Fragments of immunoglobulins in human feces. *Acta Pathol. Microbiol. Scand. Sect. C* **84**:31-36.
- Kalica, A. R., R. H. Purcell, M. M. Sereno, R. G. Wyatt, H. W. Kim, R. M. Chanock, and A. Z. Kapikian. 1977. A microtiter solid phase radioimmunoassay for detection of the human reovirus-like agent in stools. *J. Immunol.* **118**:1275-1279.
- Kapikian, A. Z., H. W. Kim, R. G. Wyatt, W. L. Cline, J. O. Arrobio, C. D. Brandt, W. J. Rodriguez, D. A. Sack, R. M. Chanock, and R. H. Parrott. 1976. Human reovirus-like agent as the major pathogen associated with "winter" gastroenteritis in hospitalized infants and young children. *N. Engl. J. Med.* **294**:965-972.
- Keller, R., and J. E. Dwyer. 1968. Neutralization of poliovirus by IgA coproantibodies. *J. Immunol.* **101**:192-202.
- Konno, T., A. Imai, H. Suzuki, and N. Ishida. 1975. Mercaptoethanol-sensitive antibody to reovirus-like agents in acute epidemic gastroenteritis. *Lancet* **ii**:1312.
- Lafferty, K. J. 1963. The interaction between virus and antibody. I. Kinetic studies. *Virology* **21**:61-75.
- Mach, J. P., and J. J. Pahud. 1971. Secretory IgA, a major immunoglobulin in most bovine external secretions. *J. Immunol.* **106**:552-563.
- McNeill, T. A. 1968. The neutralization of pox viruses. I. Evidence for antibody interference. *J. Hyg.* **66**:541-547.
- Middleton, P. J., M. D. Holdaway, M. Petric, M. T. Szymanski, and J. S. Tam. 1977. Solid-phase radioimmunoassay for the detection of rotavirus. *Infect. Immun.* **16**:439-444.
- Ogra, P. L. 1973. Immunologic aspects of hepatitis-associated antigen and antibody in human body fluids. *J. Immunol.* **110**:1197-1205.
- Ogra, P. L., R. B. Wallace, G. Umana, S. S. Ogra, D. K. Grant, and A. Morag. 1974. Implications of secretory immune system in viral infections, p. 271-282. *In* J. Mestecky and A. R. Lawton (ed.), *Immunoglobulin A system*. Plenum Publishing Corp., New York.
- Pahud, J. J., and J. P. Mach. 1970. Identification of secretory IgA, free secretory piece and serum IgA in the ovine and caprine species. *Immunochemistry* **7**:679-686.
- Peterson, M. W., R. S. Spendlove, and R. A. Smart. 1976. Detection of neonatal calf diarrhea virus, infant reovirus-like diarrhea virus, and a coronavirus using the fluorescent virus precipitin test. *J. Clin. Microbiol.* **3**:376-377.
- Rodger, S. M., R. D. Schnagl, and I. H. Holmes. 1975. Biochemical and biophysical characteristics of diarrhea viruses of human and calf origin. *J. Virol.* **16**:1229-1235.
- Saif, L. J., E. H. Bohl, and R. K. P. Gupta. 1972. Isolation of porcine immunoglobulins and determination of the immunoglobulin classes of transmissible gastroenteritis viral antibodies. *Infect. Immun.* **6**:600-609.
- Snodgrass, D. R., W. Smith, E. W. Gray, and J. A. Herring. 1976. A rotavirus in lambs with diarrhea. *Res. Vet. Sci.* **20**:113-114.
- Tufvesson, B., and T. Johnsson. 1976. Immunoelectrophoresis for detection of reo-like virus: methodology and comparison with electron microscopy. *Acta Pathol. Microbiol. Scand. Sect. B* **84**:225-228.
- Vaerman, J. P., and J. F. Heremans. 1972. The IgA system of the guinea pig. *J. Immunol.* **108**:637-648.
- Walker, W. A., S. N. Abel, M. Wu, and K. J. Bloch. 1976. Intestinal uptake of macromolecules. V. Comparison of the in vitro uptake by rat small intestine of antigen-antibody complexes prepared in antibody or antigen excess. *J. Immunol.* **117**:1028-1032.
- Walker, W. A., K. J. Isselbacher, and K. J. Bloch. 1973. Intestinal uptake of macromolecules. II. Effect of parenteral immunization. *J. Immunol.* **111**:221-226.
- Watanabe, H., and I. H. Holmes. 1977. Filter paper solid-phase radioimmunoassay for human rotavirus surface immunoglobulins. *J. Clin. Microbiol.* **6**:319-324.
- Watanabe, H., and K. Kobayashi. 1974. Peculiar secretory IgA system identified in chickens. *J. Immunol.* **113**:1405-1409.
- Wilsnack, R. E., J. H. Blackwell, and J. C. Parker. 1969. Identification of an agent of epizootic diarrhea of infant mice by immunofluorescent and complement-fixation tests. *Am. J. Vet. Res.* **30**:1195-1204.
- Woode, G. N., J. Bridger, G. A. Hall, J. M. Jones, and G. Jackson. 1976. The isolation of reovirus-like agents (rotaviruses) from acute gastroenteritis of piglets. *J. Med. Microbiol.* **9**:203-209.
- Yolken, R. H., H. W. Kim, T. Clem, R. G. Wyatt, A. R. Kalica, R. M. Chanock, and A. Z. Kapikian. 1977. Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. *Lancet* **ii**:263-266.