JOURNAL OF CLINICAL MICROBIOLOGY, June 1990, p. 1367–1374
0095-1137/90/061367-08$02.00/0

Vol. 28, No. 6

Comparison of Rotavirus Immunoglobulin A Coproconversion with Other Indices of Rotavirus Infection in a Longitudinal Study in Childhood

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Received 7 November 1989/Accepted 14 February 1990

In order to determine the sensitivity and reliability of antirotaviral fecal immunoglobulin A (IgA) as an indicator of rotavirus reinfection, the antibody responses to rotavirus of 44 infants with severe rotavirus gastroenteritis recruited on admission to a hospital were studied. Feces were collected daily during hospitalization and weekly thereafter, and sera were obtained every 4 months, for 6 to 32 months (median, 17 months). Antirotaviral IgG, IgA, and IgM were measured by enzyme immunoassay in all samples. Rotavirus antigen, rotavirus-neutralizing antibody, and total IgA were measured in feces. The results showed that use of an IgA index (ratio of specific IgA to total IgA) was unnecessary to identify copro-IgA conversion to rotavirus. The other markers of rotavirus infection tested showed a high level of predictive accuracy of coproconversion in rotavirus-neutralizing antibody. Copro-IgM, serum IgM, and virus in feces were insensitive measures of neutralizing antibody coproconversion. Seroconversion in IgG or IgA was detected in 46% of neutralizing coproconversions. The most sensitive marker, present in 92% of neutralizing coproconversions, was antirotaviral fecal IgA conversion. This correlation of fecal IgA with fecal neutralizing antibody suggests that coproconversions in IgA represent true elevations in antirotaviral IgA with neutralizing capacity. A coproconversion in IgA appears to indicate genuine rotavirus infection. Copro-IgA conversions in feces collected weekly are likely to be more sensitive markers of rotavirus reinfection than are seroconversion and virus detection combined in epidemiological studies of acute diarrhea in children and in rotavirus vaccine trials.

Rotaviruses are a major cause of severe acute diarrhea in humans and animals. These viruses infect the mature epithelial cells on the villi of the small intestine (14), and animal studies have identified a local intestinal antibody as the most important factor in passive host protection against rotaviral disease (24, 30). Vaccine programs have evaluated orally administered, live rotaviruses as vaccine candidates. These animal rotaviruses appear to be naturally attenuated in humans but share antigenic characteristics with human rotaviruses (31, 34). Clinical trials of these vaccine candidates have shown that the protection conferred against disease is serotype specific (9, 19), suggesting that successful vaccination may require controlled exposure of young children to rotavirus of more than one serotype.

During vaccine trials, predictions of efficacy on the basis of monitoring of seroresponses by complement fixation, enzyme immunoassay (EIA) for immunoglobulin G (IgG) and IgA, and neutralizing antibody have been less than the vaccine efficacy measured by clinical protection against diarrhea (32). Thus, it appears that serum immune responses may not be the most sensitive measure of vaccine take, at least when animal (heterologous) vaccines are given.

It is likely that antibodies produced by cells in the gut mucosa in response to infection and secreted into the gut lumen are the best markers of an immune response. However, the antibody response of the intestine in vaccinated children has not been studied extensively (20, 33). In natural, presumably primary, severe acute rotavirus infection, we have shown that seroconversions in antirotaviral IgM and IgG are sensitive indicators of an immune response and reflect the response in duodenal fluid. Both fecal IgA and serum IgA showed a high predictive accuracy of duodenal IgA levels at 1 and 4 months postinfection, but the stronger correlation of duodenal IgA was with fecal IgA (16). Thus, the measurement of copro-IgA most accurately reflects the duodenal antibody response to primary rotavirus infection.

The aim of this study was to analyze in detail the ongoing intestinal and serum antibody responses to rotavirus in infants and young children monitored longitudinally for at least 1 year following severe acute rotavirus infection. In particular, the study was designed to determine the relevance and reliability of changes in rotavirus-specific copro-IgA, rotavirus-neutralizing coproantibody, and serum IgG, IgM, and IgA as sensitive markers of secondary rotavirus infection. To this end, rotavirus antibody levels were measured in stools collected weekly and in serum collected at least every 4 months. Measurement of rotavirus-specific antibodies in feces by using EIA detected many significant increases in copro-IgA and neutralizing coproantibody not accompanied by virus excretion or by seroconversions. It is postulated that IgA coproconversion may be a reliable and sensitive marker of rotavirus reinfection in young children.

MATERIALS AND METHODS

Patients and samples. Patients were 44 children, 3 weeks to 39 months old, admitted with acute rotavirus diarrhea to the infectious diseases ward of the Royal Children's Hospital, Melbourne, Australia, between April 1984 and September 1985. The clinical, demographic, and laboratory findings of these children while hospitalized have been described previously (16). Approval for the study was given by the Human

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Ethics Committees of the Royal Children's Hospital and the World Health Organization. Informed signed consent was obtained from the parents of the children prior to enrollment in the study.

Blood and fecal specimens were collected from each child at 2 to 7 days (median, 5.5 days), 24 to 66 days (median, 33.5 days), and 100 to 282 days (median, 122 days) after the onset of symptoms and at approximately 4-month intervals thereafter. Although collection intervals varied from child to child, for each individual child, blood and feces were collected on the same day. Additional fecal specimens were collected daily while each child was in the hospital and at 7- to 10-day intervals from 39 (89%) children for more than 1 year and 13 (30%) children for more than 2 years. Overall, the children were monitored for 6 to 32 months (median, 17 months) after the onset of symptoms. All families were in telephone contact every 3 weeks with a research nurse (J.S.L.), who visited their homes each month, at which time a clinical history of the child was obtained. All of the mothers were requested to keep a diary of the health of the child, and approximately one-third did so for the duration of the study. Parents were asked to contact J.S.L. or a pediatrician (K.G.) immediately when episodes of acute diarrhea occurred. Extra fecal specimens were collected during diarrheal episodes. Additional blood samples were obtained 2 to 6 weeks following the diarrhea in 39 (35%) of the episodes.

Feces were refrigerated on collection and stored at −70°C within 4 h, if obtained in the hospital. Feces collected at home every 7 to 10 days by mothers were immediately frozen at −4°C in domestic freezers, collected each month, transferred to the hospital in a car freezer, and then stored at −70°C. A 10% (wt/vol) suspension of feces (0.1 g in 1.0 ml) in phosphate-buffered saline (pH 7.2) was prepared on the day of assay for antiviral antibody. After centrifugation of the suspension at 2,000 × *g* for 10 min, the resulting supernatant was kept on ice until tested and then was stored immediately at −70°C. This supernatant was assayed for rotavirus antigen by monoclonal EIA with EDTA treatment (13). Following Lyphogel concentration, the supernatant was examined for rotavirus by electron microscopy (EM) as previously described (13).

Viruses and cells. Human rotavirus strains used in all assays included RV-4 (serotype 1), RV-5 (serotype 2), and RV-3 (neonatal; serotype 3) previously adapted to culture in our laboratory (1); Wa (serotype 1) and ST-3 (neonatal; serotype 4) were provided by R. G. Wyatt (Bethesda, Md.); KU (serotype 1) was provided by T. Kutsuzawa (Japan). Simian rotavirus SA11 was supplied by H. Malherbe (San Antonio, Tex.).

All viruses were propagated in MA104 cells in the presence of trypsin as previously described (11).

EIAs for rotavirus antibodies. Sera and feces were examined for the presence of antirotaviral IgA, IgM, and IgG by EIA, with SA11 virus as the antigen (22). Serum-specific IgG was measured at a single dilution by direct EIA and was expressed in units derived from a standard curve (5). In serum, specific IgM titers were estimated by capture EIA with affinity-purified goat anti-human IgM (TAGO Immunoglobulins) as the coating reagent and monoclonal antibody to rotavirus VP6 (13), conjugated to horseradish peroxidase to detect virus bound via specific IgM. This eliminated the problem of false-positive results that were observed with some sera by using the direct IgM EIA (12). Serum IgA and fecal antibodies (IgM and IgG) were titrated by a modification of a direct EIA (22) in which sample dilutions were incubated in virus-coated plates overnight at 4°C, skim milk powder replaced bovine serum albumin to block background reactions, and bound enzyme substrate was detected with 3,3′-5,5′-tetramethylbenzidine. Fecal extracts did not show false-positive IgM titers by direct EIA. Fecal antirotaviral IgA was estimated at a single dilution (1:100) of feces by direct EIA and was expressed in units derived by computer from log-logit analysis of data obtained from titration of a reference fecal extract. These EIA systems for IgA, IgM, and fecal IgG to rotavirus were described previously (12).

Definitions of immune conversions to rotavirus. IgG seroconversion was defined as an increase in EIA units per milliliter of more than 30% between consecutive samples from the same child (5). Seroconversion in IgA to rotavirus was defined as a fourfold or greater rise in titer between sequential samples. A positive IgM response in serum or feces was defined as a titer equal to or greater than 1:200. An IgA coproconversion was defined as a threefold increase in EIA units per milliliter, to at least 100 units. These definitions of conversions in IgA and IgM have been validated (12).

Total IgA determination in feces. This assay was identical to that described previously for measurement of total IgA in neonatal breast milk (25). In brief, affinity-isolated, goat anti-human IgA (TAGO) was employed as a coating reagent and bound IgA was detected with the coating antibody, which had been peroxidase conjugated (TAGO). The Australian Serum Protein Standard, ASPS 78-1, a reference preparation of human serum immunoglobulins, was tested in serial fivefold dilutions on each plate. This data was used to express the total IgA level in feces in units of micrograms per milliliter, derived by computer from log-logit analysis, as described previously for rotavirus-specific IgA estimation in feces (12).

Calculation of specific IgA index. The ratio of the antirotaviral IgA level in units per gram of stool to the total IgA level in micrograms per gram of stool was designated as the specific IgA index.

Definition and validation of significant increases in the IgA index. Both specific and total IgA levels were estimated by EIA in 795 fecal specimens collected from a subgroup of 19 children recruited into the study during 1984. These specimens covered the first 101 to 495 (median, 420) days of follow-up only. Twelve of these children showed a significant rise (threefold increase to ≥100 units) in specific IgA levels during the acute phase of their rotavirus gastroenteritis. The IgA index in these 12 children increased from 4- to 39-fold (median, 16-fold) to a minimum of 8.1. In the seven children in whom a rotavirus-specific IgA response was not detected, the IgA index decreased up to 10-fold or increased up to 3.8-fold (median, 5-fold decrease). Thus, an increase in the IgA index from ≥4-fold to ≥9-fold was defined as significant in demonstrating later rotavirus infections.

Five additional episodes of diarrhea during which rotavirus reinfection was detected occurred in the group of 44 children during surveillance. The fecal samples in which total IgA was estimated covered three of these. In addition, five episodes of diarrhea during which *Giardia lamblia* (2), adenovirus (2), and an unidentified "small virus" (1) were present were also identified. In each of the three episodes of rotavirus diarrhea, specific IgA units, total IgA units, and the specific IgA index increased significantly by 1 week following the time of the diarrhea and virus detection. The specific IgA units showed a greater increase (12- to 262-fold) than did the total IgA units (1- to 10-fold), so that the IgA index was elevated.

In contrast, no significant changes were detected in the

specific IgA units or in the specific IgA index during the five nonrotaviral episodes of gastroenteritis, although the children infected with *G. lamblia* and adenovirus all showed increases in their total IgA levels.

Fluorescent focus reduction neutralization assay in feces. The fluorescent focus reduction neutralization method was adapted from that described previously for estimation of rotavirus-neutralizing antibody levels in hybridoma supernatant fluids (11), and its development will be described elsewhere (B. S. Coulson, and P. J. Masendycz, J. Clin. Microbiol., in press). Rotavirus stocks (RV-4, Wa, Ku, RV-5, RV-3, and ST-3) were activated with trypsin prior to being incubated with fecal extracts. All extracts were reacted with each of the rotaviruses listed above. Stocks were diluted to contain 2.5×10^4 fluorescing cell-forming units per ml of rotavirus and 5 μg of porcine trypsin (Sigma Chemical Co.) per ml in Dulbecco modified Eagle medium. After incubation at 37°C for 30 min, the activated virus was further diluted to 2.0×10^3 fluorescing cell-forming units per ml in Dulbecco modified Eagle medium containing 1 μg of trypsin per ml and 1% (vol/vol) fetal calf serum found to be free of rotavirus antibodies by EIA and fluorescent focus reduction neutralization assay (Dulbecco modified Eagle medium-trypsin-fetal calf serum). The diluted virus was mixed with an equal volume of fecal extract diluted 1:20 and 1:200 in Dulbecco modified Eagle medium-trypsin-fetal calf serum in duplicate, giving a final dilution of stool of 1:200 and 1:2,000, respectively. The mixture was incubated at 37°C for 1 h, and 50 μl per well was inoculated in duplicate onto washed confluent monolayers of MA104 cells in microdilution plates. After centrifugation at $1,200 \times g$ for 30 min, the plates were incubated at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity overnight. The cell supernatants were removed, and the monolayers were fixed in 70% (vol/vol) acetone for 5 min and then were air dried. Rabbit hyperimmune antiserum to SA11 (50 μl) at optimal dilution (1:500) in phosphate-buffered saline was added to each well, and the plates were incubated at 37°C for 30 min. The plates were washed in phosphate-buffered saline before addition of 25 μl of fluorescein isothiocyanate-labeled sheep anti-rabbit IgG $F(ab')_2$ per well (Silenus, Australia) diluted 1:100 in phosphate-buffered saline. After 30 min at 37°C, the plates were washed and air dried and wells were examined for specific fluorescence, as described previously (11). The neutralization titer of each fecal sample was expressed as the reciprocal of the fecal dilution giving 50% reduction in the number of fluorescing cells. Fecal extracts ($n = 40$) containing no antirotaviral IgA, IgM, or IgG by EIA all gave reciprocal titers <200 by the fluorescent focus reduction neutralization assay. Samples giving titers below 200 were therefore considered to be negative for neutralizing antibody to the rotavirus being tested. A significant elevation in neutralizing antibody, indicating rotavirus infection, was considered to be a fourfold increase in titer to at least 1:400.

Statistical methodology. The paired Student *t* test was used to test the significance of elevations in IgG and IgA levels over time. Correlation between total IgA, specific IgA, and the IgA index was tested by using Pearson's test on logarithmically transformed data.

RESULTS

Of the 44 children recruited for follow-up, 4 withdrew from the study after 6 months, so their data are not included in this report. The remaining 40 children were monitored for 12 to 32 months (mean, 21 months). Samples were collected from 26 (65%) children until the end of the first rotavirus season after their initial infection (12 to 24 months; mean, 17 months) and from the remaining 14 children until the end of their second rotavirus season following recruitment (24 to 32 months; mean, 29 months). For the purposes of this study, the rotavirus season is considered to cover the six colder months, i.e., from May to October.

Longitudinal analysis of rotavirus-specific antibody isotypes in serum. Antirotaviral antibody fluctuations detected in serum during the first 4 months of follow-up have been previously reported (16). Since these fluctuations are considered to relate to the rotavirus infection which brought the child to hospital, they are not considered in this analysis, which describes antirotaviral antibody fluctuations observed during the later 5 to 32 months of follow-up.

Of the 40 children monitored until the end of the first rotavirus season after that in which they were recruited, 33 (83%) showed one ($n = 23$), two ($n = 9$), or three ($n = 1$) seroconversions after the first 4 months of follow-up. These seroconversions were detected in antirotaviral IgG only (43%), IgA only (30%), or both IgG and IgA simultaneously (27%). The overall seroconversion rate was 1.0 per child per year. Of the 14 children monitored until the end of their second rotavirus season, 9 (64%) showed a single seroconversion. The antibody isotypes involved were both IgG and IgA (56%) and IgG only (44%). The overall seroconversion rate for the 14 children in the 12 to 18 months including their second season was 0.7 per child per year. The overall seroconversion rate for the 40 children during the 2 to 3 years including two seasons was 0.8 per child per year.

Antirotaviral IgM was detected in only one child on one occasion and was accompanied by a seroconversion in IgA.

Overall, 35 of the total 54 seroconversions were associated with an episode of diarrhea (65%), including only 3 (5%) in which rotavirus was detected in stools by EIA or EM. Rotavirus was found in fecal specimens from two other children without seroconversion (see below).

The geometric mean titers (IgA) and units (IgG) of antirotaviral antibodies measured in the serum samples of all 40 children studied are shown at 4-month intervals from recruitment (Fig. 1). Large elevations in both IgG and IgA occurred following the severe, presumably primary, episode of rotavirus gastroenteritis. Further significant elevations in IgG occurred between 12 and 16 months and between 24 and 28 months after the severe gastroenteritis (paired *t* test; $P < 0.025$ and $P < 0.05$, respectively). As the children were recruited during the period from April to September in two successive years, the increases in geometric mean titer of specific IgG from 12 to 16 months and from 24 to 28 months corresponded to the ends of the two successive winters (rotavirus seasons) following the severe episode of rotavirus gastroenteritis. A significant rise in the geometric mean titer of specific IgA was recorded only between 4 and 8 months after the severe gastroenteritis (paired *t* test; $P < 0.05$). The rise in geometric mean titer of specific IgA from 12 to 16 months was not statistically significant (paired *t* test; $P > 0.05$).

Comparison of antirotaviral IgA units and the specific IgA index for longitudinal analysis of rotavirus coproantibody. There were 795 fecal specimens from 19 children in which specific and total IgA levels were measured and the IgA index was calculated. There was a weak but significant correlation between specific and total IgA levels for each fecal specimen ($r_s = 0.348$; $P < 0.01$). However, the stronger correlation was between specific IgA and the IgA index ($r_s = 0.743$; $P < 0.005$). These results show that the measured

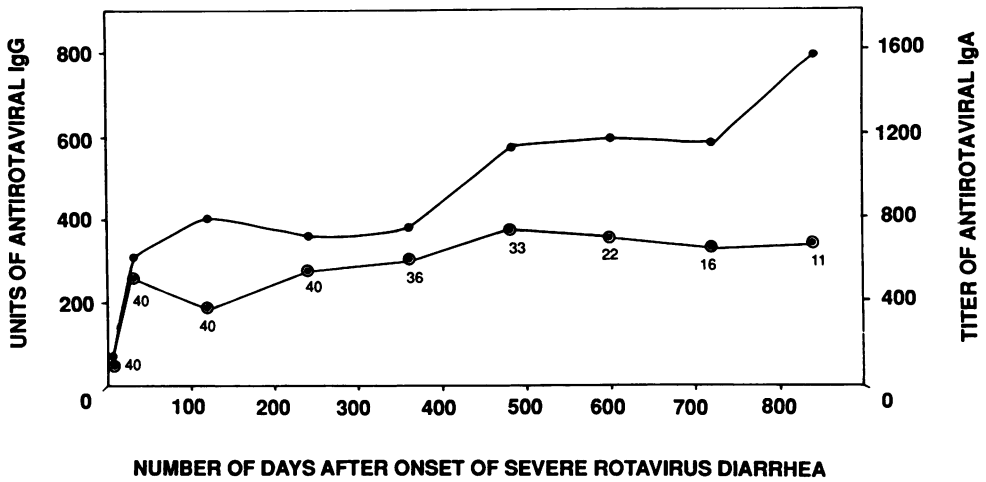

FIG. 1. Geometric mean titers (IgA) and geometric mean units (IgG) of antirotaviral antibodies in sera of children at each 4-month interval following hospitalization with rotavirus gastroenteritis. Symbols: ●, IgG; ⊙, IgA. Numbers refer to number of serum samples tested at each time point.

variations in specific IgA in feces were not the result of coincidental changes in total IgA concentration.

The specific IgA level and the IgA index calculated as described previously are shown in Fig. 2 and 3 for two representative children. The child represented in Fig. 2 showed very high levels of antirotaviral copro-IgA within 1 week of the onset of acute severe rotavirus gastroenteritis. A peak was also detected in the total IgA levels, but at a lower level, so the IgA index for this period was also significantly elevated. Similarly, the elevation in antirotaviral copro-IgA at day 80 was reflected in both the total IgA levels and the IgA index.

Fluctuations in the levels of total IgA occurred from day 90 to day 460, unaccompanied by significant variations in the other parameters. An extreme elevation in copro-IgA and IgA index at 480 days was due to rotavirus-associated diarrhea but was not accompanied by any alteration in total IgA levels.

Figure 3 represents a child, aged 21 months at hospitalization, in whom no copro-IgA conversion or specific copro-IgA was detected during hospitalization with acute rotavirus gastroenteritis. Two secondary boosts in copro-IgA occurred. The first, at day 80, was accompanied by a rise in the IgA index, although total IgA levels were stable. The second copro-IgA boost from days 250 to 260 was accompanied by diarrhea and a significant elevation in both total IgA levels and the IgA index.

Specific IgA coproconversions as defined here (see Materials and Methods) were detected in 24 instances from the 795 specimens collected from 19 children. These events were

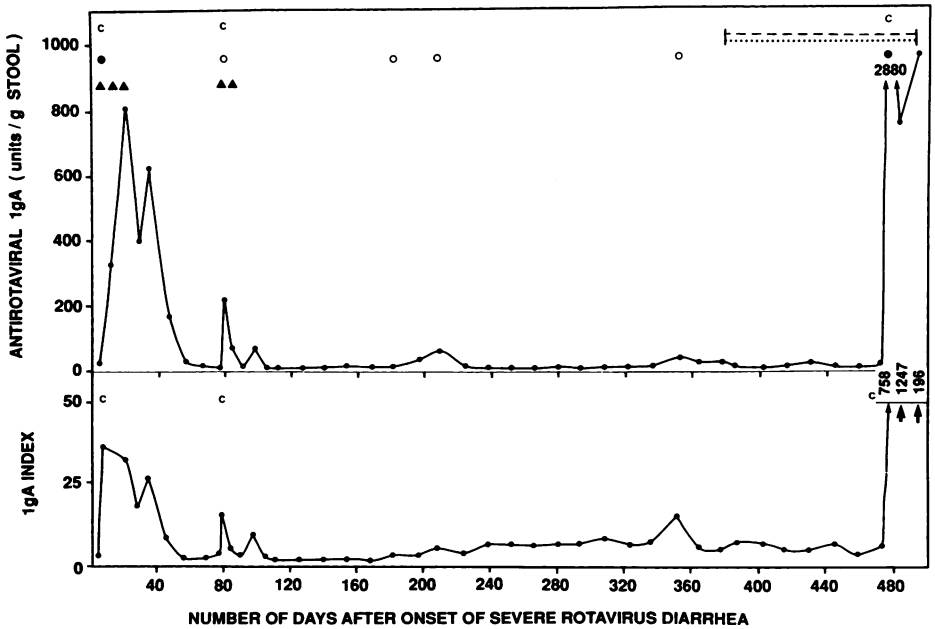

FIG. 2. Comparison of the profiles of antirotaviral IgA units, IgA indices, and levels of total IgA in stools of a 3-month-old child. C, IgA coproconversion; O, diarrhea recorded. Symbols: ●, diarrhea with rotavirus present in the stools; ▲, IgM detected in the stools. -----, IgG seroconversion; ·····, IgA seroconversion.

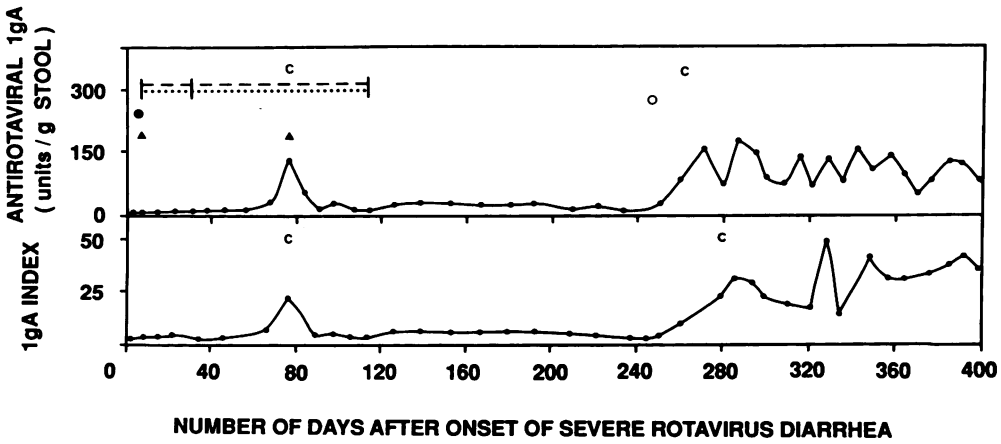

FIG. 3. Comparison of the profiles of antirotaviral IgA units, IgA indices, and levels of total IgA in the stools of a 21-month-old child. Symbols are described in the legend to Fig. 2.

analyzed to determined whether or not specific IgA coproconversion was as sensitive a measure of rotavirus infection as was the IgA index. Of 24 specific IgA coproconversions, 20 (83%) were associated with a significant rise in the IgA index. Of these 20, 18 (90%) were also associated with other measures of rotavirus infection (at least one of rotavirus in stools, copro-IgM, seroconversion, or neutralizing antibody coproconversion). Three of the four IgA coproconversions not showing an elevated IgA index were also associated with other markers of rotavirus infection. These coproconversions did not show an elevated IgA index, since the total IgA level was also increased. The false-negative and false-positive rates for specific copro-IgA conversion as a measure of rotavirus infection were 5% (1 of 21) and 4% (1 of 24), respectively. The IgA index showed a false-negative rate of 17% (4 of 24) and a false-positive rate of 5% (1 of 21). We concluded that specific copro-IgA measurement alone was more sensitive an estimate of rotavirus infection than was the IgA index. Overall analysis of the longitudinal data was thus performed with specific copro-IgA unit values only. This avoided the need for total IgA estimation in all stools collected.

Comparison of IgA coproconversion with conventional measures of rotavirus infection during rotavirus reinfection. Rotavirus-specific copro-IgA units were estimated in all stool specimens (3,150) collected from the 40 children (mean, 79 specimens per child) in whom antibody isotypes in serum were studied. These specimens were also analyzed for the presence of rotavirus by EM and EIA and for elevated levels of copro-IgM to rotavirus. A subset of 422 fecal specimens collected from 10 children (mean, 22 per child) was also titrated for neutralizing antibody to rotaviruses RV-4, Wa, Ku, RV-5, RV-3, and ST-3.

The 115 coproconversions recorded in the 3,150 stools tested were accompanied by seroconversion in 61 cases (53%), comprising IgG seroconversion alone (46%), IgA seroconversion alone (28%), IgG and IgA seroconversion (25%), and conversion in IgG, IgA, and IgM (1%). Seroconversion in the absence of IgA coproconversion or any other marker occurred on three occasions. The incidence of IgA coproconversion with accompanying seroconversion was not age related.

On all of the five occasions when copro-IgA conversions were associated with the presence of rotavirus in stools, mild to moderate diarrhea was recorded. Four cases were accompanied by seroconversion or copro-IgM. All 11 antirotaviral IgM-containing stools also showed copro-IgA conversion. Six (55%) were associated with seroconversion, and two (18%) were associated with the presence of rotavirus in stools. Copro-IgG to rotavirus was not detected in any stool sample tested.

The relation of significant increases in neutralizing coproantibody against at least one rotavirus serotype to the other markers of rotavirus infection tested is shown in Table 1. Although seroconversion, rotavirus detection, and coproconversion in IgM all showed a high predictive accuracy of coproconversion in neutralizing antibody (92 to 100%), they were insensitive markers, being present in only 4 to 46% of neutralizing antibody coproconversions. In contrast, rotavirus coproconversion in IgA was both a highly predictive (92%) and sensitive (92%) marker of coproconversion in rotavirus-neutralizing antibody. Two neutralizing antibody conversions and one IgA coproconversion were not accompanied by any other marker of rotavirus infection.

Longitudinal analysis of rotavirus-specific IgA in stools. Levels of specific copro-IgA units were estimated in stool specimens collected from 40 children, the same population in which antibody isotypes in serum were studied. Of the 40 children monitored for 12 to 18 months, including one rotavirus season, 36 (90%) showed at least one coproconversion. Up to 6 coproconversions per child were recorded,

TABLE 1. Comparison of rotavirus-neutralizing coproantibody conversions with other markers of rotavirus reinfection

Source	Marker	% (No. positive/no. tested)	
		Sensitivity[a]	Predictive accuracy[b]
Serum	Specific IgG conversion	29 (7/24)	88 (7/8)
	Specific IgA conversion	33 (8/24)	100 (8/8)
	Specific IgM conversion	4 (1/24)	100 (1/1)
	Specific IgA, IgG, or IgM conversion	46 (11/24)	92 (12/13)
Feces	Rotavirus detected (EM or EIA)	4 (1/24)	100 (1/1)
	Specific IgM conversion	13 (3/24)	100 (3/3)
	Specific IgA conversion	92 (22/24)	92 (22/24)

[a] Sensitivity, Percentage of children with a neutralizing coproantibody conversion who also had a particular marker at the given site.

[b] Predictive accuracy, Percentage of children with a particular marker at the given site who also had a neutralizing coproantibody conversion.

with a rate of 1.6 per child per year. Of the 14 children monitored for 2 to 3 years, including two rotavirus seasons, 12 (86%) showed IgA coproconversions to rotavirus in the 12 to 18 months including the second season, ranging from 1 to 4 per child, with a rate of 1.7 per child per year.

The episode of severe rotaviral gastroenteritis which provided the starting point for longitudinal follow-up in the children was probably a primary infection (16) as judged by finding low or no rotavirus antibodies in acute-phase specimens and by detecting IgM serum antibody in 100% of the children. The peak level of specific copro-IgA was identified where possible for each child producing copro-IgA following the primary infection and each later copro-IgA conversion (representing reinfection). Of the 97 pairs of primary and reinfections, 79 (81%) showed greater peak copro-IgA levels during the reinfections than during the primary infection. In six of seven children with five to eight reinfections, the peak copro-IgA level to rotavirus increased by two- to eightfold, in a stepwise fashion from the first to the fourth or fifth reinfection. Peak levels were lower during the last three or four copro-IgA conversions, which occurred during the second season of follow-up.

DISCUSSION

Infection of children with rotavirus is currently considered proven if rotavirus particles or antigen are detected in stools or if seroconversion in IgG, IgM, IgA, or neutralizing antibody can be demonstrated. In severe acute gastroenteritis where infection is usually primary, these criteria are consistently met (5, 6, 10, 16, 17, 26, 29). However, rotavirus infection is often mild or asymptomatic (5, 8, 15). In this study, although all children excreted rotavirus during their episode of acute rotaviral gastroenteritis, virus was detected at the time of later IgA coproconversions on only five occasions and was never found during IgA coproconversions not accompanied by diarrhea. Concentration of fecal extracts by 10-fold and pretreatment with EDTA failed to increase this number. All children studied had antirotaviral IgA and IgG in serum, and rotavirus was not found in stools containing high levels of antirotaviral copro-IgA. It is likely that preexisting immunity may have kept virus growth in the intestine to levels below the limits of detection by EIA and EM. One of the rotavirus-positive diarrheal episodes occurred during the brief hospitalization of the child for other reasons, when all stools passed were obtained. Collection of all stools during each diarrheal episode may improve virus detection. When more sensitive antigen assays, perhaps based on probes for viral RNA, are available, the fecal samples of this study could be retested. In calves, experimental secondary rotavirus infections resulted in immune conversions in serum and feces but viral excretion was not detected (3).

Seroconversion following rotavirus reinfection was detected on 81 occasions, much more frequently than was rotavirus antigen in stools. The major isotype involved was IgG (58%), followed by IgA (41%) and IgM (1%). IgG and IgA seroconversions occurred simultaneously in 75% of the cases, and these antibodies persisted for prolonged periods. In contrast, specific IgM was detected only once. All of these children had shown an IgM immune response in serum during their primary infection, with 91% responding in IgG and 68% responding in IgA (16). The IgM levels during primary infection peaked at least 6 days after onset and often fell to baseline within 1 month. Sera may not have been collected near enough to the time of rotavirus infection to detect specific IgM in rotavirus reinfections not temporally associated with diarrhea. However, serum was collected less than 4 weeks after diarrhea in 44%, and less than 10 days after diarrhea in 15%, of reported diarrheal episodes. Children showed increasing numbers of rotavirus seroconversions with increased length of follow-up, but these rarely involved specific IgM.

At the end of each of the two successive rotavirus seasons (winters) during which the children were studied, their geometric mean levels of specific IgG rose significantly. This suggests that reinfection with rotavirus also tended to be a seasonal event and reflects our finding that most of the children in the population studied seroconverted to rotavirus each season. Similar IgG and IgA profiles with increasing age were shown in the Federal Republic of Germany (7).

The children monitored for one complete rotavirus season after recruitment showed a higher seroconversion rate (1.0 per child per year) than those monitored during their second season (0.7 per child per year). The overall seroconversion rate of 0.8 per child per year is similar to rates of infection (0.3 to 1.2 episodes per child per year) reported in other longitudinal studies in both developed and developing countries. These rates were calculated by using rotavirus detection and seroconversion in IgG or IgA or both by EIA (6, 26, 27, 29), rotavirus detection and seroconversion by indirect immunofluorescence (17), and rotavirus detection alone (19, 21).

The weekly collection of stools from 44 children for 6 to 32 months has enabled us to study childhood antirotaviral coproantibody profiles in great detail. Specific IgM was rarely detected, and specific IgG was never found. Marked fluctuations in specific copro-IgA, which were not the result of coincidental changes in total IgA concentration, occurred in many children, although total IgA levels were sometimes elevated during proven rotavirus infections. Thus, use of the IgA index, although giving similar results to specific copro-IgA alone, was shown to be unnecessary to identify copro-IgA conversion to rotavirus. In a small study, Hjelt et al. (18) came to the same conclusion. The IgA index has been used for rotavirus copro-IgA measurement without prior verification (4, 23).

Analysis of the serum and mucosal antibody responses to severe acute rotavirus gastroenteritis in these children showed that copro-IgA, followed by serum IgA, were the best predictors of duodenal IgA at 1 and 4 months after infection (16). It is thus likely that the copro-IgA fluctuations measured subsequently in this study, accompanied by virus-neutralizing activity, reflect similar antibody changes in the duodenum. This is the site at which virus-neutralizing antibodies have been shown to exert protective capabilities in animal models (24, 30).

All of the other markers of rotavirus infection tested showed a high level of predictive accuracy in relation to conversion in neutralizing coproantibody (92 to 100%). However, the sensitivities of the markers in relation to neutralizing coproantibody varied widely. Detection of copro-IgM, virus in stools, or serum IgM was an insensitive measure of coproconversion in neutralizing antibody. Seroconversion in IgG or IgA or both was a moderately sensitive marker, being recorded in 46% of coproconversions. The most sensitive marker, present in 92% of neutralizing antibody coproconversions, was rotavirus-specific copro-IgA conversion.

Taken together with the validation of the unadjusted rotavirus-specific IgA level as a reliable indicator of fluctuations in rotavirus copro-IgA, the correlation of copro-IgA

with neutralizing coproantibody suggests that coproconversions represent true elevations in antirotaviral IgA with neutralizing capacity. A coproconversion in IgA appears to indicate genuine rotavirus infection. Without measurement of neutralizing coproantibodies, Bernstein et al. (4) concluded from a human rotavirus challenge study in adults that increases in fecal antibody may be a reliable indicator of rotavirus infection, even in the absence of detectable virus shedding or seroconversion.

Taking coproconversion in specific IgA or neutralizing antibody as the standard measure, this study shows that use of seroconversion and virus detection only as measures of rotavirus reinfection would lead to underestimation of virus infection by approximately 200%. On the basis of the incidence of coproconversion in IgA, the more accurate estimate of rotavirus infection in this study is 1.6 per child per year for the period covering the first rotavirus season and 1.7 per child per year for the period covering the second season after recruitment. In contrast to that observed for seroconversion rates, no drop in coproconversion rate in the second season was observed, although the peak copro-IgA levels recorded during the coproconversions were lower in the second season than the first. It is likely that seroconversions in the presence of already elevated serum IgA and IgG rotavirus antibody levels are difficult to detect. Copro-IgA levels often declined to baseline before a new coproconversion was observed. Overall, 1.7 rotavirus coproconversions per child per year were recorded in this study. This is in excess of the rates (0.3 to 1.2) determined in other reports (6, 17, 19, 21, 26, 27, 29), but coproconversions were not measured in these studies. Shinozaki et al. (28) also reported a rate of 1.7 rotavirus infections per child per year from copro-IgA titer increases to rotavirus in seven children tested monthly for 1 year.

Two of the five children excreting rotavirus and showing copro-IgA conversions during reinfection did not seroconvert. This suggests that the mucosal and systemic antibody responses to rotavirus can be unrelated, as was postulated by Angeretti et al. (2).

The preliminary results from the longitudinal study indicated that an anamnestic copro-IgA response, with higher peak copro-IgA levels, occurred in 81% of rotavirus reinfections. Anamnestic responses in copro-IgA have been documented previously in five children with secondary rotavirus infection (35) and in a larger group of children receiving rhesus rotavirus vaccine (33).

Sufficient virus for serotyping was obtainable only from one of the five children excreting rotavirus during reinfection, and this showed serotype 1 by monoclonal EIA (11; Fig. 2, day 480). The primary infecting virus was also serotype 1 (Fig. 2, day 3). It was not possible to determine the infecting serotype from the increases in antibody levels measured by EIA, as antigens of different serotypes (1 to 4) gave similar EIA units and titers (12). During reinfection, increases in neutralizing coproantibody titers usually occurred to more than one serotype simultaneously, so this method was also unsuitable for determining the serotype of the infecting virus during reinfection.

In conclusion, the results of the study presented here suggest that specific antirotaviral copro-IgA is a reliable indicator of rotavirus reinfection and that it is a more sensitive measure than is rotavirus detection or seroconversion. Confirmation of this finding in children studied longitudinally from birth in both developed and developing countries is required. Measurement of rotavirus-specific copro-IgA in stools, collected weekly during epidemiological studies and vaccine trials, is likely to provide an accurate estimation of rotavirus infection and may assist in the measurement of vaccine take and vaccine efficacy.

ACKNOWLEDGMENTS

This study was supported by grants from the National Health and Medical Research Council of Australia, the Diarrheal Diseases Control Program of the World Health Organization, and the Royal Children's Hospital Research Foundation.

The advice of Don Roberton is much appreciated. We are grateful for the assistance of Leanne Unicomb, Sandra Lawrance, Lorraine Adams, Neil Francis, Lynette Vaelioja, Jane Lee, Val Williams, and the nursing staff of the infectious diseases ward, Royal Children's Hospital.

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