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Measurement of Rotavirus-Neutralizing Coproantibody in Children by Fluorescent Focus Reduction Assay

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A fluorescent focus reduction assay suitable for the measurement of rotavirus-neutralizing antibodies in the feces of children was developed. Of 408 stools tested, 7% showed false-positive neutralization, and the number of rotavirus serotypes neutralized by a fecal extract was proportional to the levels of antirotaviral immunoglobulin A in the extract.

Rotavirus is the major etiologic agent of acute infantile gastroenteritis worldwide, and the development of a safe and effective vaccine has a high priority.

As immunity to rotavirus is assumed to relate mainly to the presence of neutralizing antibodies in the intestine at the time of infection, the measurement of rotavirus-neutralizing antibody levels in the jejunal fluid of adults during two vaccine trials has been attempted (8, 9). In children with severe rotavirus gastroenteritis, we have shown that fecal antirotaviral immunoglobulin A (IgA) levels accurately represent the intestinal immune response to rotavirus (6), abrogating any need for duodenal intubation of children.

We have modified a fluorescent focus reduction neutralization (FFN) assay previously described for serum and hybridoma supernatant testing (2) for use on fecal extracts. This report describes the problems found and modifications required to produce a reliable and sensitive FFN assay particularly suitable for testing feces and a comparison of specific neutralizing and IgA antibody levels in feces.

Feces collection. As part of a longitudinal study of the immune responses to primary and secondary rotavirus infections in 44 children recruited during hospitalization for acute rotavirus gastroenteritis (6), stools from each child were collected at 7- to 10-day intervals. From a subset of 19 children, 408 stools were collected (mean, 21 per child) and analyzed for units of rotavirus-specific IgA (3) by an enzyme immunoassay (EIA) and for titers of rotavirus-neutralizing antibodies to rotaviruses RV-4, Wa, Ku, RV-5, RV-3, and ST-3. Rotavirus-specific IgM or IgG was not detected by the EIA in these stools (4).

FFN assay. Rotavirus stocks were propagated in MA104 cells with 10 μg of porcine trypsin (Sigma Chemical Co., St. Louis, Mo.) per ml to activate the virus and in maintenance medium with 0.5 μg of trypsin per ml as described previously (2). Virus stocks were also activated with trypsin prior to incubation with fecal extracts. Stocks were diluted to contain 2.5×10^4 fluorescing cell-forming units per ml of rotavirus and 5 μg of trypsin per ml in Dulbecco modified Eagle medium. After incubation at 37°C for 30 min, the activated virus stocks were 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) heat-inactivated fetal calf serum found to be free of rotavirus antibodies by the EIA and FFN assay. To prepare 10% (wt/vol) fecal extracts, we vortexed 0.1 g of stool with 1.0 ml of phosphate-buffered saline (pH 7.2) (PBS) by using glass beads and clarified the extracts by centrifugation at $2,000 \times g$ for 10 min. All fecal processing was carried out on ice. The diluted virus was mixed with an equal volume of fecal extract diluted 1:20 and 1:200 in Dulbecco modified Eagle medium–1 μg of trypsin per ml–1% heat-inactivated fetal calf serum in duplicate, giving final dilutions 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, the plates were washed once with PBS, and the monolayers were fixed in 70% (vol/vol) acetone for 5 min and air dried. Rabbit hyperimmune antiserum to SA11 (50 μl) at an optimal dilution (1:500) in PBS was added to each well, and the plates were incubated at 37°C for 30 min. The plates were washed in PBS before the addition to each well of 25 μl of fluorescein isothiocyanate-labeled sheep anti-rabbit IgG $F(ab')_2$ (Silenus, Melbourne, Australia) diluted 1:100 in PBS. After 30 min at 37°C, the plates were washed and air dried, and the wells were examined for specific fluorescence as described previously (2). The neutralization titer of each fecal sample was expressed as the reciprocal of the dilution giving a 50% reduction in the number of fluorescing cells.

Development of the FFN assay. The additional activation of virus stocks with trypsin prior to mixing with fecal extracts was necessary to obtain the full infectious potential of the rotaviruses. At 2×10^4 to 4×10^4 fluorescing cell-forming units per ml, treatment with 10 μg of trypsin per ml for 30 min gave peak titers for four rotaviruses tested (Wa, RV-5, RV-3, and ST-3). Higher or lower trypsin concentrations led to a 1.4- to 3-fold reduction in virus titers. Five of the stools collected were found to contain trypsin at levels of 80 to 160 μg/g of stool, measured by gelatin film digestion with purified porcine trypsin (type IX; Sigma) as the standard. These and 18 other specific IgA-containing fecal samples showed little or no rotavirus-neutralizing activity when the virus inoculum was not fully trypsin activated. Residual stool trypsin may have activated the rotavirus added during the FFN assay and given these probably false-negative results.

Preliminary experiments showed that in the absence of virus, feces diluted 1:200 to 1:2,000 usually destroyed the MA104 cell monolayer. The addition of rotavirus antibody-free, heat-inactivated serum to the fecal extract-virus mixture prevented this destruction. The minimum effective concentration of normal rabbit serum or fetal calf serum

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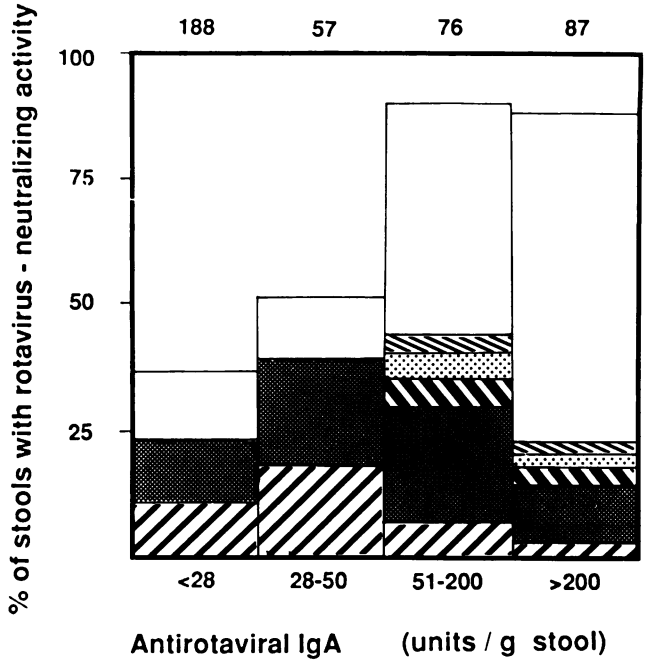

FIG. 1. Distribution and range of rotavirus-neutralizing activity and levels of antirotaviral IgA in 408 stools collected from 19 children. Symbols: ▨, RV-3 rotavirus only; ▩, one serotype 1 rotavirus and one serotype 3 rotavirus; ▤, one serotype 1 rotavirus; ▥, one serotype 3 rotavirus and one serotype 4 rotavirus; ▧, two serotype 1 rotaviruses; □, at least three rotaviruses.

(Flow Laboratories, Sydney, Australia) was 1% (vol/vol). However, serum addition resulted in high levels of background fluorescence unless the MA104 cell monolayers were washed once with PBS before being fixed with acetone. It is likely that trypsin inhibitors in the serum (particularly α_1-antitrypsin) were preventing trypsin destruction of the cell monolayer by the fecal extract and that the resulting precipitation produced the nonspecific immunofluorescence.

Validation of the FFN assay and comparison with the IgA EIA. The distribution and range of rotavirus-neutralizing activity in the 408 fecal specimens tested in relation to units of antirotaviral IgA present per gram of stool are illustrated in Fig. 1. Stools containing more than 50 units of IgA per g were also more likely to contain rotavirus-neutralizing activity directed to three or more rotaviruses than were stools with lower levels of specific IgA. An unexpected finding was the neutralization activity present in 69 stools containing less than the positive-negative cutoff of 28 units of specific IgA per g of stool (3). Fourteen (20%) of these stools, collected from two children, showed reactivity to the MA104 cell control in the EIA, and 13 of these neutralized RV-3 rotavirus only (Table 1). Absorption of 10% (vol/vol) extracts from four of these stools with an equal volume of packed MA104 cells for 1 h at 37°C removed the MA104 cell reaction in the EIA and the neutralization of RV-3 rotavirus. This result suggests that the binding of an unknown fecal factor to MA104 cells prevented RV-3 rotavirus entry, giving a false-positive neutralization result. It is possible that the acetylated sialic acids reported to inhibit rotavirus replication (10) were involved.

A large proportion (60%) of the 69 stools showing neutralization activity in the absence of specific IgA were collected within 7 days of a coproconversion to rotavirus. The EIA for rotavirus-specific antibodies is likely primarily to detect

TABLE 1. FFN reactivity to rotaviruses of 69 stools negative for antirotaviral IgA in the EIA

Stool characteristic	No. of stools with positive FFN titer to rotavirus:			
	RV-3	RV-4	RV-3 and RV-4	Three rotaviruses
MA104 cell reactive	13[a]	0	1	0
Collected within 7 days of IgA coproconversion	5	1	11	24
Neither of the above	3	0	8	3

[a] Twelve of these stools were collected from one child. Overall, 28 stools from this child were tested, and all were negative for antirotaviral IgA in the EIA. Of 16 MA104-reactive stools, 12 neutralized RV-3 rotavirus. None of 12 MA104-nonreactive stools neutralized any rotavirus tested.

antibody directed to the group antigen, VP6, whereas neutralization is a property of antibodies directed to VP4 and VP7 (5). Possibly, the immune responses to these viral proteins did not peak in synchrony. Antibody produced early in rotavirus infection is of low avidity (7) and can be missed by the EIA (1), so IgA levels in stools collected just prior to the recorded copro-IgA boosts may be underestimated. In the remaining 20% of the 69 stools, no clear explanation of the neutralization activity was evident. The FFN assay may be more sensitive than the EIA or these may have been additional false-positive FFN results.

In summary, we have developed an FFN assay suitable for the measurement of rotavirus-neutralizing antibodies in stools from infants. The inclusion of fetal calf serum and complete trypsin activation of virus were required for reliable neutralization titer determination. Stools containing moderate to high levels of specific IgA were likely to neutralize at least three of six rotaviruses tested. Of 408 stools tested, a maximum of 28 (7%) showed proven nonspecific neutralization, related in part to MA104 cell reactivity. This assay is relatively simple to perform and has facilitated our understanding of the neutralizing coproantibody response to rotavirus infection, which appears to differ from the serum neutralizing antibody response in some children (unpublished data). This test should be of value in assessing the efficacy of candidate rotavirus vaccines in children in field trials, in particular, when ethical considerations preclude the collection of duodenal fluid.

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