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Filter Paper Solid-Phase Radioimmunoassay for Human Rotavirus Surface Immunoglobulins

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A filter paper solid-phase radioimmunoassay has been developed. Filter paper disks adsorbed a large amount of rotavirus and serum globulin and gave small mean variation of coating and low background binding. The rotavirus isolated from stools from infants with acute enteritis 1, 3, and 4 days after onset of symptoms was shown to be already covered with immunoglobulin G (IgG), IgA, and IgM antibodies by this radioimmunoassay, by immunoelectrophoresis, and by immune electron microscopy. The immunoglobulins covering the virus particle were partially separated during ^{125}I labeling and eluted at the position expected for IgG during Sephadex G-200 gel filtration. Rabbit antiserum prepared against purified fecal rotavirus contained not only rotavirus antibodies but also a fairly large amount of immunoglobulin antibody, reflecting the antibodies on the rotavirus particle surface.

Immune electron microscopy and complement fixation tests for determining rotavirus antibodies in human sera have been used (7), but these serological methods have characteristic shortcomings in sensitivity and reliability. Radioimmunoassay (RIA) methods may offer an improved alternative method, particularly the solid-phase RIA method, which is simple, economical, speedy, and reproducible. Microtiter plates or plastic tubes have often been used for the solid phase, coated with antisera (5, 11, 13), *Echinococcus* antigens (10), or cultured cells infected with vaccinia virus (19). The chief difficulty in the solid-phase method is associated with separation of the adsorbed protein during incubation and washing. Although filter paper was used as a solid phase in a previous RIA test (16), in that case the paper was activated with cyanogen bromide in order to bind protein. We now describe an improved solid-phase RIA method in which filter paper disks are used as the solid surface without activation.

Human and calf rotaviruses were discovered in the gut and feces of young children and newborn calves with acute enteritis (1-3, 9). Preliminary experiments in our laboratory on detection of rotavirus in stools by serological means gave unsatisfactory results. We tried rocket immunoelectrophoresis, Sepharose solid-phase RIA, and radioimmunoprecipitin tests, using rabbit rotavirus antiserum. The results obtained were not consistent with electron microscopic observations, in that very weak reactions were sometimes produced by samples containing large numbers of rotavirus particles, and false-positive reactions also occurred. These findings suggest that the virus particles were covered with something inhibiting combination of the particles with the antibodies or at least that an inhibitor was present in feces. In the present communication, analyses of the inhibitory substance, done by the filter paper disk RIA method, are described.

MATERIALS AND METHODS

Viruses. Stools collected from infants with acute enteritis 1, 3, and 4 days after onset of symptoms were kindly supplied by I. D. Gust, Fairfield Hospital, Melbourne, Victoria, Australia, and pooled. Rotavirus was purified from the stools according to the method described by Rodger et al. (12). This procedure involved extraction with fluorocarbon and rate zonal centrifugation. The resultant virus-containing bands were layered onto cesium chloride gradients. After centrifugation, the virus-containing bands were harvested and dialyzed against 0.05 M phosphate buffer, pH 7.5.

SA-11 (vervet monkey) rotavirus was propagated in primary cultures of cynomolgus monkey kidney cells. Infected cells were freeze-thawed once, sonically treated for 10 s in a 10-kc MSE sonic oscillator, and centrifuged for 20 min at 13,000 × *g*. The resultant supernatant was again centrifuged to pellet virus for 80 min at 75,000 × *g*, and the pellet was resuspended in phosphate buffer. This partially purified virus was used as cultured rotavirus in the following experiments.

Antiserum and immunoglobulin preparations. An antiserum to rotavirus was prepared in rabbits with fecal rotavirus, purified as described above, as antigen. Human serum globulin fraction containing immunoglobulin G (IgG), IgM, and IgA, kindly supplied by Commonwealth Serum Laboratories, Mel-

bourne, was used to prepare antiserum in rabbits. Human serum IgG, IgM, and IgA were purchased from Meloy Laboratories, Springfield, Va. (lot no. D10152712, D10252238, D10352709, respectively), and rabbit anti-human γ-chain, μ-chain, and α-chain sera were obtained from Behringwerke AG, Marburg/Lahn, Germany (lot no. 2622H, 2454N, 2716K, respectively).

Immunoelectrophoresis. Fecal rotavirus was electrophoresed in barbital buffer, pH 8.6, $\mu = 0.05$, containing 0.7% agarose for 20 min at 4.3 mA/cm. After the electrophoresis, rotavirus antiserum and IgG, IgM, and IgA were added to the wells on the anode side.

Immune electron microscopy. Equal volumes of rotavirus and antiserum were mixed and incubated for 30 min at 37°C. A microdrop of the mixture was put on 0.8% agarose in distilled water and covered with a grid. After the grid was negatively stained, it was observed with a Hitachi HU11A electron microscope.

Radiolabeling. Fecal rotavirus and rotavirus antiserum, precipitated to prepare the globulin fraction by 45% saturation of $(NH_4)_2SO_4$ at pH 7.2, were labeled with ^{125}I by the chloramine-T method (8). A ratio of 1 μCi of ^{125}I per μg of protein was used, and unreacted iodine was separated from the radiolabeled protein by gel filtration on Sephadex G-200 (2.3- by 15-cm column) with 0.05 M phosphate buffer containing 0.5% bovine serum albumin (BSA), pH 7.5.

Solid-phase RIA. Filter paper MN 615 (Macherey-Nagel Co., Düren, Germany), Whatman filter paper no. 1 (W. & R. Balston Ltd., England), Millipore filter PH (Millipore Corp., Bedford, Mass.), and flexible polyvinyl microtiter plates (Cook Engineering Co., Alexandria, Va.) were used as supports for the virus or globulin fraction and prepared as follows. A disk having a diameter of 1 cm was cut out of a filter paper, washed with distilled water, and dried. The disk was placed on a plastic wrap, and 25 μl of specimen, the diluent of which consisted of 0.05% BSA in phosphate buffer, was dropped on and dried at room temperature. Plastic plates were similarly treated. Three drops (approximately 75 μl) of 10% formaldehyde in phosphate buffer, ice cold, were then added to fix protein onto the disk, and the disks were left 5 min at room temperature. Three drops of phosphate buffer were then dropped onto the disk and removed. This washing was done four times. The disk coated with protein was moved into a plastic tube with a diameter of 1.3 cm and a flat bottom (Johns Professional Products, Cheltenham, Victoria, Australia), which also contained 1 ml of 1% BSA in phosphate buffer to saturate any remaining binding sites. After overnight incubation at 4°C, the disk was washed with phosphate buffer once. A 0.2-ml amount of 0.05% BSA in phosphate buffer and 25 μl of ^{125}I-labeled virus or antiserum, containing 0.5% BSA, were then added to the tube. After incubation at 4°C for 1 day, the disk was washed with phosphate buffer four times and counted with a Packard auto-γ scintillation spectrometer. Since no mammalian sera were considered to be completely negative for rotavirus antibody in preliminary random tests, disks coated with 0.05% BSA but no specimen were used as controls for nonspecific binding in almost all experiments. Specific binding was estimated by the subtraction of nonspecific binding from total binding. All tests were performed in duplicate, and the results were averaged.

RESULTS

Comparison of four kinds of solid surfaces. To measure the protein-binding ability of the solid surfaces, Macherey-Nagel filter, Whatman filter, Millipore filter, and microtiter plates, serial twofold dilutions of the stock solutions of ^{125}I-labeled rotavirus (15,100 cpm, 0.09 μg of protein per 25 μl) or anti-rotavirus serum globulin (29,800 cpm, 0.2 μg of protein per 25 μl) were placed on disks or in wells, dried, and Formalin fixed as described above. After the final washing, the percentage of coating was shown by (counts per minute remaining/input counts per minute) × 100 (Fig. 1). In most samples, the percentage of protein adsorbed increased with increasing dilution, although the actual amount adsorbed was greater in the higher protein concentrations. The relative percentage of coating of rotavirus and serum globulin to the solid surfaces decreased in the order Millipore filter > MN 615 filter > Whatman filter > microtiter plate. The approximate area of the plate surface in contact with the solution was 0.5 cm^2, compared with the 0.8-cm^2 area of the disk. If the two faces of the disk are considered, the plate surface area was only 31% of that of the disk, no doubt contributing to the lower binding of the plate. Millipore filters and microtiter plates bound less ^{125}I in the form of labeled rotavirus than in the form of labeled serum globulin, but MN 615 and Whatman filters adsorbed similar amounts of rotavirus and serum globulin. Mean variations of the averaged counts

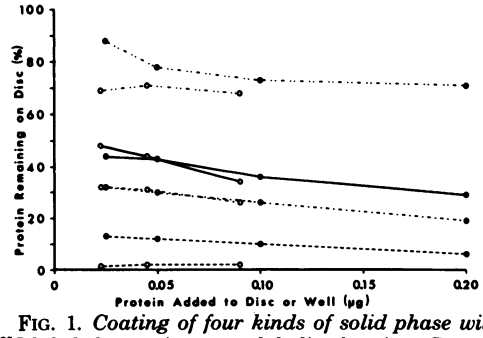

FIG. 1. *Coating of four kinds of solid phase with ^{125}I-labeled rotavirus or globulin fraction. Protein remaining on the disk or plate after incubation and washing was shown by (remaining counts per minute/added counts per minute) × 100. Solid phase:* (——) *Filter MN 615;* (——·——) *Whatman filter no. 1;* (——··——) *Millipore filter PH;* (-----) *polyvinyl microtiter plate. Coating material:* (○) *Rotavirus;* (●) *globulin fraction.*

were calculated from these results: ±2.3, ±2.1, ±2.4, and ±8.5% of MN 615 filter, Whatman filter, Millipore filter, and microtiter plate, respectively.

To determine the effect of washing to remove nonspecific binding, 15,100 cpm of ^{125}I-labeled serum globulin was added into tubes containing MN 615 filter paper disks. The disks were precoated with 0.05% BSA in phosphate buffer. After incubation at 4°C for 1 day to complete nonspecific binding, the disks were washed and counted after each washing; four washes were considered to be enough (Table 1). Nonspecific binding to the various solid surfaces was investigated by adding 15,000 to 18,000 cpm of labeled serum globulin as described above. Millipore filters showed very high background binding, 6.1% of total input radioactivity, compared with the other solid surfaces; MN 615 filters, Whatman filters, and microtiter plates bound 1.3, 1.6, and 1.2%, respectively.

On the basis of these results, the MN 615 filter was used as solid phase in the following experiments.

Rotavirus antibodies in fecal extracts. A pool of stools containing rotaviruses was treated with fluorocarbon and centrifuged. The resultant supernatant was used to coat disks, to which serially diluted ^{125}I-labeled rotavirus, 12,000 to 600 cpm, was added to examine binding. To measure background binding, coated disks were reacted with anti-immunoglobulin serum to cover all possibly present antibodies before addition of labeled rotavirus. The nonspecific binding was determined for each dilution of labeled virus. The specific binding percentage was shown by (counts per minute remaining − nonspecific counts per minute/counts per minute in the input sample) × 100. With decrease of the input of labeled rotavirus, specific binding increased rapidly, 0.2 to 32% (Table 2), showing the presence of rotavirus antibodies in the extract.

Anti-immunoglobulin activity in rabbit anti-rotavirus serum. In a gel following immunoelectrophoresis (Fig. 2), this serum formed a single precipitin line with samples of human IgG, IgA, or IgM and a major diffuse and minor lines with fecal rotavirus, suggesting the presence of not only rotavirus antibodies but also immunoglobulin antibodies in the rabbit antiserum. To quantify each sort of antibody in the antiserum, quantitative binding tests were done, in which disks coated with serially diluted samples of each class of immunoglobulin or with increasing amounts of cultured rotavirus (SA-11) were used to absorb specific antibodies from a preparation of ^{125}I-labeled globulin prepared from this serum containing 16,200 cpm per sample. Equivalence points (17) were measured, and the level of specific binding at these points are shown in Table 3. There was little difference in the amounts of antibody reacting with each class of immunoglobulin demonstrated in the antiserum, ranging from 4.1 to 4.4%, whereas 5.2% of the total globulin consisted of rotavirus antibodies. Since these percentages of anti-immunoglobulins were considered to be too high to be produced by free immunoglobulins in purified fecal rotavirus preparations and formation of the rotavirus precipitin lines was apparently influenced by IgG, IgA, and IgM as antigens, we

TABLE 1. *Effect of washing to remove nonspecific binding after addition of 15,100 cpm of ^{125}I-labeled serum globulin into the tubes containing filter paper disks*

Washing	Radioactivity remaining on disk (%)
1	3.7
2	2.1
3	1.7
4	1.3
5	1.2

TABLE 2. *Anti-rotavirus activity in a pooled extract of feces containing rotavirus*[a]

Labeled rotavirus input (cpm)	Total binding (cpm)	Background binding (cpm)	Specific binding of labeled rotavirus (%)
12,919	728	696	0.2
2,849	337	165	6.0
596	204	13	32

[a] The fecal supernatant obtained after fluorocarbon treatment was applied to MN 615 filter paper disks. Background binding was measured on disks treated with anti-immunoglobulin serum before addition of the dilutions of labeled rotavirus.

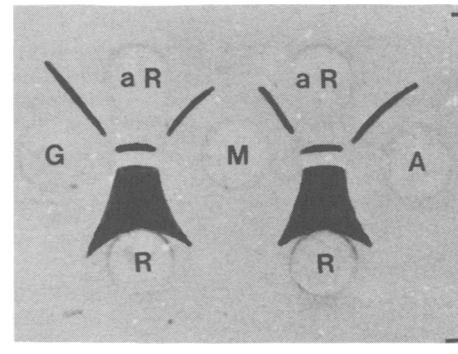

FIG. 2. *Immunoelectrophoresis of fecal rotavirus. Rabbit antifecal rotavirus serum and IgG, IgA, and IgM were added into the wells after electrophoresis of fecal rotavirus, in barbital buffer, pH 8.6, μ= 0.05, containing 0.7% agarose, for 20 min at 4.3 mA/cm. Abbreviations: R, Fecal rotavirus; aR, antifecal rotavirus; G, M, A, IgG, IgM, IgA.*

concluded that fecal rotavirus particles in the preparations with which the rabbits were immunized must have carried firmly bound immunoglobulin molecules of all three classes.

Immunoglobulins on the surface of virus particles. Preliminary experiments indicated that some commercial rabbit anti-human immunoglobulin sera contained rotavirus antibodies, cross-reacting with human rotavirus, as follows.

First, filter paper disks coated with rotavirus SA-11 from cell culture were used to absorb rabbit anti-γ-, anti-α-, and anti-μ-chain sera. When these disks were then tested for binding of ^{125}I-labeled anti-rotavirus globulins, it was found that prior exposure to the anti-H-chain sera resulted in considerable inhibition of the reaction. As a control, SA-11 disks were treated with a rabbit serum that was previously shown to be negative or very low in rotavirus antibodies (0.6% specific binding of ^{125}I-labeled rotavirus) before exposure to the ^{125}I-labeled rotavirus antiserum. Such control disks showed no inhibition of specific binding by the "normal" rabbit serum (Table 4). In this experiment, the levels of specific binding to untreated (SA-11 coated) or control disks were about 5% (actually 4.9 and 5.3%, respectively).

TABLE 3. *Binding of ^{125}I-labeled rabbit anti-rotavirus serum globulin to disks coated with immunoglobulins or cultured rotavirus at equivalence point*

Protein added to disks (μg)	Specific binding of radioactivity (%)
IgG (7.4)	4.3
IgA (13)	4.4
IgM (0.9)	4.1
Cultured rotavirus, three disks[a]	5.2

[a] More than one disk coated with undiluted virus was required to attain equivalence.

TABLE 4. *Inhibition of the reaction between SA-11 (cultured) rotavirus on disks and ^{125}I-labeled anti-rotavirus serum globulin by prior exposure to anti-H-chain sera (rabbit)*

Dilution of anti-H-chain serum[a]	Inhibition (%)
Anti-γ chain	
10^{-1}	100
10^{-2}	69
Anti-α chain	
10^{-1}	80
10^{-2}	38
Anti-μ chain	
10^{-1}	90
10^{-2}	58
Normal rabbit serum 10^{-1}	0

[a] Final dilutions were 6.7×10^{-2} and 6.7×10^{-3} of 10^{-1} and 10^{-2}, respectively. See text for details of the experiment.

Immune electron microscopy was then carried out with human (fecal) and SA-11 (cultured) rotaviruses and samples of the anti-H-chain sera before and after absorption. The original sera reacted more strongly with the fecal rotavirus than with the virus of cell culture origin, and adsorption with SA-11 virus reduced the reaction, but agglutination of fecal rotavirus was still evident with two of the three sera (Table 5). These results suggest that fecal rotavirus but not cultured rotavirus was covered with antibodies.

Antibodies separated from fecal rotavirus. The iodinated preparation of fecal rotavirus was gel filtered through Sephadex G-200. There were three peaks of radioactivity in the resultant elution profile (Fig. 3). The elution positions of the first (peak 1) and the second (peak 2) peaks

TABLE 5. *Immune electron microscopy of rotaviruses with anti-H-chain sera before and after absorption with SA-11 rotavirus of cell culture origin*

Anti-H-chain serum	Degree of agglutination[a]	
	Fecal rotavirus	Cultured rotavirus
Before absorption		
Anti-γ chain	++	+
Anti-α chain	+++	+
Anti-μ chain	++	++
After absorption		
Anti-γ chain	−	−
Anti-α chain	+	+
Anti-μ chain	++	−

[a] Symbols: −, no clumps; +, mainly in clumps, up to 10; ++, most of particle in clumps, up to 10 to 20; +++, massive clumps.

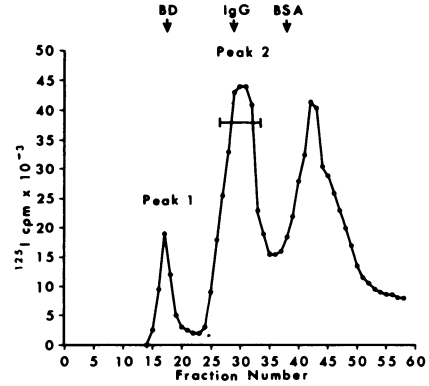

FIG. 3. *Gel filtration on a Sephadex G-200 column of a ^{125}I-treated preparation of fecal rotavirus. Abbreviations and symbol: BD, Blue dextran; BSA, bovine serum albumin; ⊢—⊣, pooled.*

were about the same as those of blue dextran and IgG, respectively. Peak 1 was considered to contain labeled rotavirus particles, and the third peak was considered to contain free ^{125}I. Peak 2 was always about twice as high as peak 1 in replicate treatments of fecal rotavirus with iodine, and it appeared most probable that it consisted of either rotavirus fragments or immunoglobulins dissociated from the particles during the iodination reactions.

The labeled peak 2 components bound to disks coated with either fecal or cultured rotavirus (Table 6); the percentage binding to cultured rotavirus was slightly greater than that binding to fecal rotavirus. Binding of the peak 2 fraction to the cultured rotavirus disks was greater than that of labeled rotavirus antiserum globulin. This high binding capability of the peak 2 fraction suggests that the fraction was rich in antibodies that were separated from the virus particles during iodination.

To confirm the immunoglobulin nature and class of the peak 2 material, the following inhibition test was done. ^{125}I-labeled rabbit anti-rotavirus serum, known to contain rotavirus, IgG, IgM, and IgA antibodies, was absorbed with disks coated with peak 2 components. The supernatant was removed, and binding of residual antibodies was measured on disks prepared with IgG, IgM, IgA, and cultured rotavirus, the amounts of which were adjusted to the equivalence point quantities shown in Table 3. Inhibition of specific binding at the equivalence points caused by the preabsorption with peak 2 material is shown in Table 7. Binding of the labeled antibodies with each class of immunoglobulin was highly inhibited. These results make us confident that peak 2 consisted mainly of immunoglobulins and possibly a minor quantity of rotavirus fragments.

TABLE 6. *Binding of the peak 2 fraction shown in Fig. 3 to the rotavirus disk*

Labeled protein (μg)	Specific binding of radioactivity (%)	
	Fecal rotavirus	Cultured rotavirus
Peak 2 components		
0.15	5.4	5.7
0.038	7.0	7.3
0.0094	7.4	7.9
Anti-rotavirus serum globulin		
0.046		4.6
0.012		4.7

TABLE 7. *Inhibition of reaction of ^{125}I-labeled rabbit anti-rotavirus serum with immunoglobulins and cultured SA-11 rotavirus by prior absorption of the antiserum with material from peak 2 of Fig. 3*[a]

Amt of peak 2 components (μg)	Inhibition (%)			
	IgG	IgA	IgM	Cultured rotavirus
0.84	90	99	62	25
0.0084	77	70	38	10

[a] See text for details.

DISCUSSION

In comparison with previously described solid-phase supports, the paper disks were shown to bind rotavirus and serum globulin more effectively than plastic surfaces and to give small mean variation and low background binding. Microtiter plates were found to give more variable results, suggesting that the absorbed protein is easily detached from the plastic surface during incubation and washing. Millipore filters, applied as solid phase in a recent enzyme-labeled antibody method (14), bound a large quantity of rotavirus or serum globulin, but the background binding was approximately five times as high as that with filter paper.

Another merit of filter paper as solid phase is that since the paper is porous, the area participating in the antigen-antibody reaction is increased, and this may facilitate binding of larger molecules than can be held on plastic surfaces. Furthermore, paper disks coated with antigens (or antibodies) can conveniently be used to absorb antisera, like the insoluble beads used for affinity chromatography. This characteristic was effectively used in this study, when the rabbit rotavirus antibodies were absorbed from the rabbit anti-human H-chain sera. This application could be extended to absorption of several antibodies or antigens in one solution sample by addition of several disks, each coated with different antigens or antibodies.

Electron microscopy has been used for detection of rotaviruses in the intestinal contents or feces of man and various animals (2, 4, 9, 15, 18) and is still the standard procedure with which other tests must be compared. Naturally, efforts are being made to develop simple and reliable serological tests for rotavirus detection that could be undertaken in the many diagnostic laboratories lacking such specialized and expensive equipment. The present study illustrates two of the major problems likely to be encountered by serologists entering the rotavirus field.

First, there is the finding that rotavirus particles isolated from infants with acute enteritis only a few days after onset of symptoms were already partly covered with antibodies. We cannot be sure whether the antibodies were mater-

nal or were produced by the infants themselves, but the present results are suggestive; the affinity of the antibodies was high, since some were still attached to virus particles after fluorocarbon and cesium chloride treatments. It is unlikely that such high-affinity antibodies were produced in infants within a few days of the onset of disease. At any rate, the possibility of contamination of "purified" fecal rotavirus preparations with tightly bound antibodies must be considered if they are to be used as diagnostic antigens or for production of antisera.

Second, rotaviruses have now been identified in many different animal species, and those of different origins cross-react extensively (4, 6). Woode and Bridger (18) have drawn attention to the probable existence of rotaviruses of rabbits and of goats on the basis of detecting rotavirus antibodies in random sera, and our experience with the rabbit anti-immunoglobulin sera is similar. For example, the commercial rabbit anti-human H-chain sera mentioned in Table 4, on filter paper disks at a 10^{-1} dilution, showed 3 to 7% specific binding of ^{125}I-labeled SA-11 rotavirus. Rabbit rotavirus has recently been detected directly in the U.K. (T. H. Flewett, personal communication). In general, it cannot be assumed that any mammalian serum is free of rotavirus antibody until it has been checked. Such a check is routine in the preparation of antisera in laboratory animals, but less obvious sources of possible confusion are the use of "normal" sera in passive hemagglutination or RIA tests and of "normal" immunoglobulins for absorption of anti-immunoglobulins from other sera.

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