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Solid-Phase Radioimmunoassay for the Detection of Rotavirus

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A solid-phase radioimmunoassay method has been developed for the detection of rotavirus in the form of a purified antigen and in stool. The parameters of the radioimmunoassay were examined and optimized to give high sensitivity and same-day results. Compared with electron microscopy, the assay is up to 10 times as sensitive for detection of the virus in stool and up to 128 times as sensitive for detection of a purified virus antigen. In a field study on stool specimens it was at least as efficient as electron microscopy.

The development of radioiodination processes (5, 8) and the finding that gamma globulin was adsorbed by polystyrene tubes (1) were the essential techniques of the clinically usable solid-phase radioimmunoassay (RIA). In virology, the RIA was developed to detect such agents as the hepatitis B surface antigen (4), the hepatitis A antigen (13), and herpesvirus (4), as well as antibodies to virus (3, 7).

Generally, the method involved attachment of a specific antibody to a solid phase such as polystyrene substrate, capture of the antigen by this antibody, and detection of the antigen by another specific antibody with a radioactive tracer.

We have applied this method to the detection of rotavirus (otherwise known as IGV, human reovirus-like agent, duovirus), which is a highly significant cause of gastroenteritis in our community (10). RIA studies so far have dealt with detection of the virus in serum, in tissue culture, or in a partially purified state, so our attempt to detect the virus directly in stools was a novel approach. Accordingly, we reexamined the parameters of the RIA and, based on our findings, developed a usable assay for the detection of rotavirus in stools.

MATERIALS AND METHODS

(i) Preparation of solid phase. The internal surfaces (1.5 cm depth) of polystyrene tissue culture tubes (12 by 75 mm, clear; Falcon Plastics) were coated with rabbit anti-rotavirus globulin by a method similar to that of Catt et al. (2). A 0.5-ml amount of the above globulin at a protein concentration of 12 μg/ml was added to each tube. The tubes were rolled at an angle of 5° in a modified Wedco cell culture tube rolling apparatus for 6 h at room temperature. After removing the unattached globulin, 0.6 ml of crystalline bovine plasma albumin (BPA) (Connaught Laboratories) at a concentration of 15 mg/ml was added to each tube. These tubes were further rolled under above conditions for 20 h to block unreacted sites. The albumin was then removed, and the tubes were rinsed with 2 ml of phosphate-buffered saline (PBS) and dried in a National vacuum incubator at room temperature. They were then stored at 4°C overnight before use.

(ii) Assay proper. A total volume of 0.5 ml of antigen was added to each coated tube. If the antigen was a stool, it was suspended in 5 volumes of PBS, containing 0.02% (wt/vol) phenol red as pH indicator, 0.01% NaN_3, and 0.1% BPA, and centrifuged at 2,000 × g for 15 min, and the supernatant was used in the assay. If the preparation was too acidic, it was brought to pH 7 by adding a few drops of $NaHCO_3$ solution. If the antigen was purified, rotavirus of known concentration in terms of absorbancy at 260 nm (A_{260}), it was made up in PBS containing 0.1% BPA and 0.01% NaN_3. The tubes containing the antigen were incubated at an angle of 15° in the rolling apparatus for 2 h, and then the contents were removed and the tubes were washed with 3 (2 ml) volumes of PBS. Next 0.5 ml of guinea pig anti-rotavirus globulin at a concentration of 10 μg/ml was added to each tube; the tubes were rolled at a 15° angle for 1 h and washed with 5 (2 ml) volumes of PBS. Finally, 0.4 ml of ^{125}I-labeled rabbit anti-guinea pig globulin was added to each tube. After 1 h of incubation as outlined above, each tube was washed with 5 (2 ml) volumes of PBS and counted in a Searle gamma counter.

(iii) Ingredients of the assay. (a) Rotavirus used as antigen in this study was purified by CsCl gradient centrifugation as outlined by Petric et al. (11).

(b) Stools were collected from patients aged 1 week to 2 years who had been admitted to The Hospital for Sick Children with acute gastroenteritis or who had developed gastroenteritis in the hospital.

(c) Rabbit anti-rotavirus antibody was prepared by injecting a total of 1 ml of 2× CsCl gradient-purified rotavirus in equal amounts of incomplete Freund adjuvant into the back muscles of a rabbit in three weekly divided doses. The virus A_{260} was 1.0

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units. One month after the first injection, the rabbit was exsanguinated and the serum was preserved at −20°C.

(d) Guinea pig anti-rotavirus antibody was prepared in a similar manner to that outlined above, except that the antigen was injected into the hind legs as described by Middleton et al. (10). Both serum preparations were precipitated with a 40% (vol/vol) final concentration of ammonium sulfate, resuspended in PBS, and desalted by passing through a 20-cm Sephadex G-25 column. They were referred to as anti-rotavirus globulin. Both the rabbit and guinea pig antisera had anti-rotavirus titers of 2,048 complement-fixing units per 25 μl (9).

(e) Rabbit anti-guinea pig globulin was purchased from Flow Laboratories and was made monospecific by affinity chromatography. The rabbit globulin was reacted with guinea pig globulin attached to cyanogen bromide-activated Sepharose 4B (Pharmacia) and was subsequently eluted with glycine-hydrochloride buffer at pH 2.8 in accordance with the method outlined in the Pharmacia brochure (12). The eluted globulin was reacted with $Na^{125}I$ (protein iodination grade) purchased from New England Nuclear by the lactoperoxidase technique of Marchalonis (8). The iodine was removed by filtration through a Sephadex G-25 column, and the lactoperoxidase was removed by filtration through a Sephadex G-150 column. The elution pattern of the latter was determined by monitoring the absorbance of each fraction at 412 and 280 nm, as well as its radioactivity. The first peak that had a maximum A_{280} and radioactivity but a low A_{412} was taken as the iodinated globulin. Specific radioactivity was 3×10^6 cpm/μg.

(iv) **Comparative assays.** Electron microscopy (EM) and complement fixation (CF) were done as outlined in a previous publication (9).

RESULTS

To simplify reporting, we refer to the rabbit anti-rotavirus antibody attached to the polystyrene tubes as the "capture antibody" (CA). The guinea pig anti-rotavirus antibody that binds to the immobilized antigen is referred to as the "detector antibody" (DA), and the ^{125}I-labeled rabbit anti-guinea pig antibody is referred to as the "indicator antibody" (IA). The initial part of our experiment established the optimal conditions under which the above antibodies were used to detect the purified rotavirus antigen and rotavirus in stool. In each experiment only one variable was examined in reference to its effect on yield of the assay. The yield was considered as the amount of radioactivity immunospecifically bound to the tube for a given antigen preparation.

(i) Parameters of RIA. (a) CA. In the initial studies on coating of polystyrene tubes for the RIA, Catt et al. (2) found that a 2- to 3-h period of exposure to the CA at pH 9.6 was optimal. In our study we have reexamined these parameters using purified rotavirus antigen at a concentration of 10^{-2} and 10^{-3} A_{260} units, as well as stool preparations positive and negative for rotavirus by negative-contrast stain EM. The effect on the yield of the RIA of coating at different pH's is shown in Fig. 1. Tubes were coated with CA at 12 μg/ml at pH 9.6, 7.5, and 5.5 for 0 to 24 h. They were then used in an RIA with 10^{-3} A_{260} units of purified rotavirus antigen. The yields did not vary greatly between the different pH's. The coating was nearly complete after 1 h as judged by the yield that did not rise beyond 6 h of incubation. Lowest backgrounds were obtained at pH 9.6.

For a virus-positive stool as antigen, coating tubes at pH 9.6 proved far superior to pH 5.5 and 7.5. Hence, in the assay proper, tubes were coated with CA at pH 9.6 for 6 h.

To determine the optimal concentration of CA, tubes were coated with 1 to 100 μg of globulin per ml and subsequently were used in an RIA with 10^{-2} and 10^{-3} A_{260} units of purified rotavirus antigen. As shown in Fig. 2, the highest yields were obtained using the CA at about 12.5 μg/ml. Both higher and lower concentrations gave substantially reduced yields. Hence, in the final assay tubes were coated with 0.5 ml of CA at pH 9.6 at 12 μg/ml.

Unreacted sites on the polystyrene tube were blocked best by coating with crystalline BPA at 15 μg/ml, pH 9.6, for periods between 6 h to overnight. BPA proved superior to bovine serum albumin fraction V in this function. In addition, all further reactions were carried out in a buffer of PBS + 0.1% (wt/vol) BPA. The BPA that had been used to block the tubes was found adequate for this purpose.

A limited study on the temperature of incubation (room temperature, 37°C, 45°C) on coating revealed that room temperature was best.

(b) Antigen. Purified rotavirus antigen at a concentration of 10^{-2} and 10^{-3} A_{260} units was incubated in an RIA for 1 to 25 h as shown in Fig. 3. Maximum yield of radioactivity was reached after 16 h of incubation. However, to keep the assay brief, a 2-h period of incubation which had about 80% of the 16-h yield was adopted. The effect of changing the incubation time had a minimal effect on the background counts.

The optimal pH for incubation of the antigen and subsequent reactions was between 6.5 to 7.0. Hence, all reactions after coating were carried out in PBS at this pH. To ensure that stool specimens had the correct pH, phenol red at a 0.02% final concentration was added, and the color was adjusted by adding a dilute solution of sodium bicarbonate.

(c) DA. Concentrations of DA of 200, 100, 50, 25, 12.5, 6.25, 3.12, and 1.6 μg/ml were used in

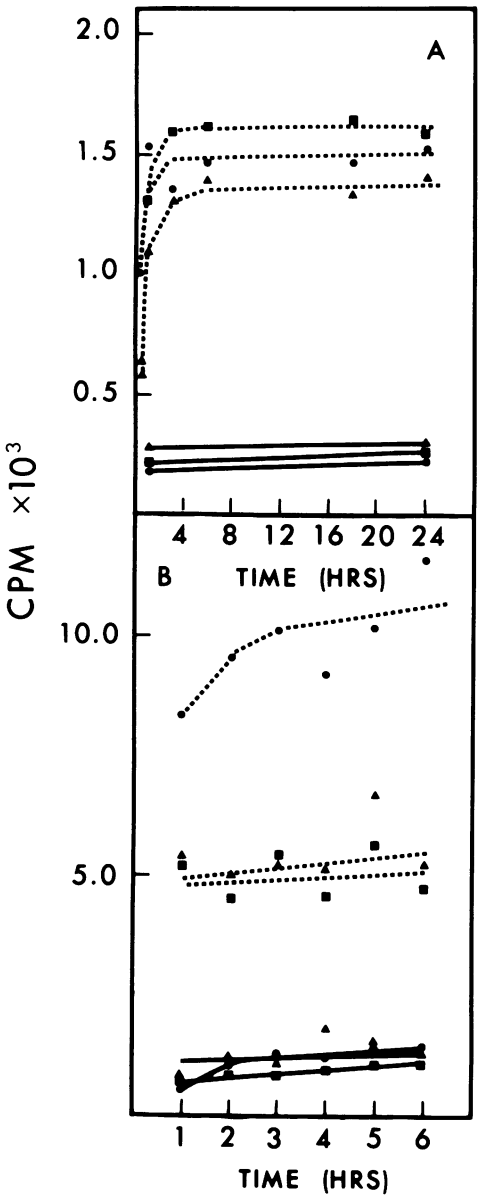

FIG. 1. *Effects of pH and time of incubation of the CA on the yield of the RIA. Symbols: (▲) pH 5.5, (■) pH 7.5, and (●) pH 9.6. (A) Rotavirus antigen (10^{-3} A_{260} units) was assayed in tubes coated for the indicated periods of time at the indicated pH. Symbols: Input IA was 21,000 cpm. Symbols: (---) rotavirus antigen; (——) PBS control. (B) Virus-positive (---) and -negative (——) stool preparations were assayed in tubes coated for the indicated periods of time at the indicated pH. Input IA was 180,000 cpm.*

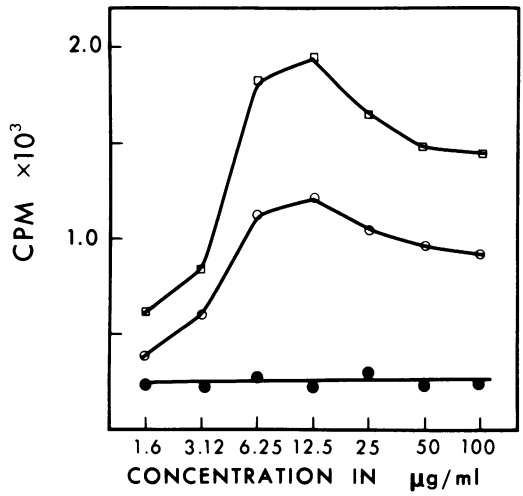

FIG. 2. *Effects of concentration of CA on the yield of the RIA. Tubes coated with the indicated amount of CA were used to assay 10^{-2} (□) and 10^{-3} (○) A_{260} units of rotavirus antigen; (●) PBS control. Input IA was 23,000 cpm.*

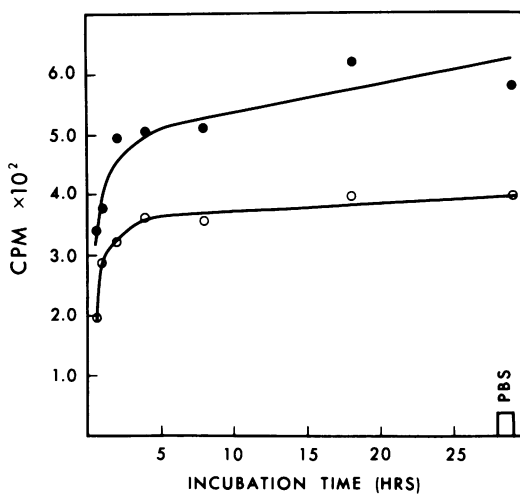

FIG. 3. *Effects of time of incubation of rotavirus antigen on the yield of RIA. Symbols: (●) 10^{-2} A_{260} and (○) 10^{-3} A_{260} units of rotavirus antigen. Bar marks counts with a PBS control. Input IA was 5,000 cpm.*

an RIA on 10^{-2} and 10^{-3} A_{260} units of rotavirus antigen. As shown in Fig. 4, the highest yields were obtained with 200 µg of DA per ml. However, the background also increased with an increasing concentration of DA. This actually led to a lower positive to background ratio (P/B ratio) with the high DA concentrations. From results of three such experiments, a standard concentration of 10 µg of DA per ml was used in the final assay.

In a similar experiment, a fixed amount of DA was used for incubation times varying from 0.5 to 2 h. Again, the highest counts were obtained at the longest incubation time, but in

order to keep backgrounds counts low and to minimize the time needed to do the assay, a 1-h incubation time was selected. This gave 85 to 89% of the yield of the 2-h incubation period.

(d) IA. As with DA, the optimal incubation time was selected as 1 h. Again, brevity of the assay was important.

To determine the optimal concentration of IA the RIA was performed with 10^{-3} A_{260} units of the purified virus antigen and with virus-positive and -negative stools. As shown in Fig. 5, the highest input count of IA gave the highest yield and the highest P/B ratio. Accordingly, IA was used in the final assay at 20,000 to 180,000 cpm per tube.

(ii) Sensitivity. Dilutions of pure rotavirus antigen and of stools positive and negative for rotavirus were tested in parallel by RIA, CF, and EM (9), and the limits of sensitivity of each system were obtained. These are indicated in Fig. 6. The rotavirus antigen had a starting concentration of 10^{-2} A_{260} units; the rotavirus-positive stool showed numerous virus particles per EM field, whereas the negative stool had no virus detectable by EM.

Purified antigen can just be detected at 2×10^{-2} A_{260} units by CF, at 2.5×10^{-3} A_{260} units by EM, and at 4×10^{-5} A_{260} units by RIA (Fig. 6). Hence, for the purified rotavirus antigen the RIA is 128 times as sensitive as the EM. In repeated experiments using different lots of purified rotavirus antigen it was shown that RIA was between 32 to 128 times as sensitive as EM, which is seen here to be about 8 times as sensitive as CF. For purified virus the cutoff point for the RIA has been taken as a P/B ratio of 2 or greater.

Similarly, virus could be detected in the positive stool at a dilution of 2.5×10^{-3} by EM and at a dilution of 3.1×10^{-4} by RIA. CF proved very insensitive in this case, being limited to a dilution of 1×10^{-1}. Hence, for detection of virus in stool the RIA is 8 times as sensitive as EM and 300 times as sensitive as CF. In similar experiments using different stool preparations,

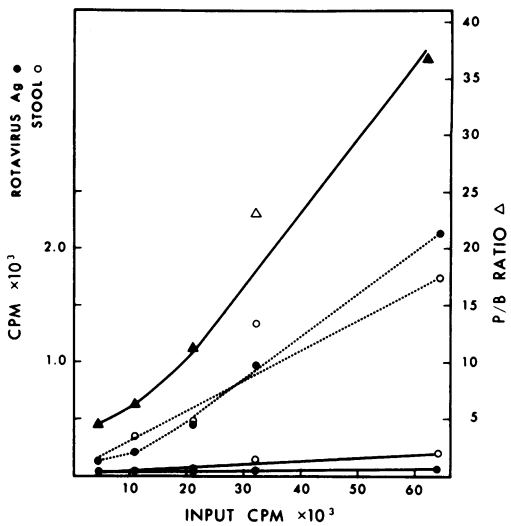

FIG. 5. *Effects of input IA on the yield of RIA. Positive (--○--) and negative (—○—) stool preparations and 10^{-3} A_{260} units of antigen (--●--) were assayed using the indicated amounts of IA; (—●—) PBS control.*

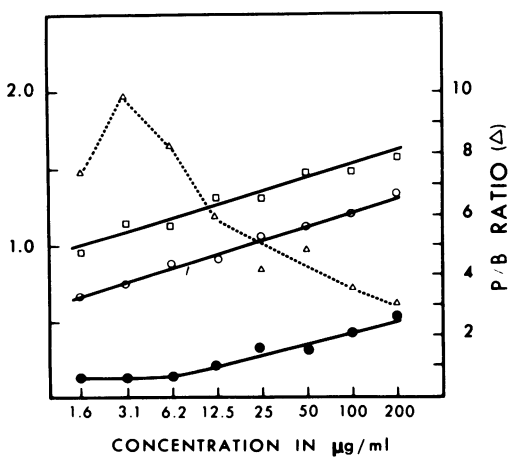

FIG. 4. *Effects of concentration of DA on the yield of the RIA. The yield of 10^{-2} (□) and 10^{-3} (○) A_{260} units of antigen were assayed using the indicated concentrations of DA. PBS control (●). The P/B ratio of 10^{-2} A_{260} of antigen to PBS control is also plotted (△). Input IA was 21,000 cpm.*

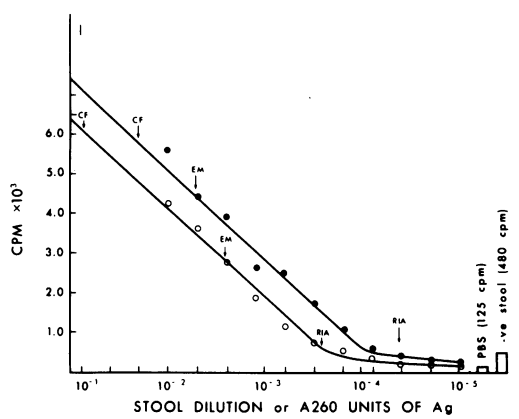

FIG. 6. *Effects of dilution of rotavirus antigen (●) and rotavirus-positive stool (○) on the RIA. Abscissa represents the dilution of stool or the concentration of antigen in A_{260} units. Arrows indicate the limit of sensitivity of each system.*

the RIA proved to be up to 10 times as sensitive as EM and 100 times as sensitive as CF. For stool, the limits of RIA were taken as counts that had a P/B ratio of 4 or greater. Negative stools gave counts that were always below 4 times background and usually about 2 times background when diluted 1:5.

(iii) Neutralization. A box titration of the antigen and antibody such as used in the CF test was tedious to perform with the RIA system. Accordingly, a fixed amount of antigen both as a stool, positive for rotavirus, and as purified rotavirus was exposed to varying dilutions of rabbit anti-rotavirus antibody for 1 h, and the mixtures were examined by RIA. The effect of diluting the antibody on the yield is seen in Fig. 7A. The antibody at a dilution of 1:80 completely neutralized 10^{-3} A_{260} units of antigen and had a similar effect on a virus-positive stool preparation. The yield was decreased from 3,400 to 750 cpm. A 50% neutralization was reached with the antibody diluted to about 1:1,000. This is approximately the titer of the serum used by CF. When the system was examined from the alternative perspective, a fixed amount of antibody (1:20 dilution) was reacted with varying amounts of antigen and these mixtures were examined by RIA. The above antibody neutralized up to 10^{-3} A_{260} units of antigen and gave up to 80% neutralization of 10^{-2} A_{260} units of the pure antigen (Fig. 7B). A similar pattern of neutralization of the positive stool was observed. Human convalescent sera that had anti-rotavirus activity by CF were also found to neutralize 10^{-3} A_{260} units of rotavirus antigen in the RIA.

(iv) Application. Ninety-seven stools of gastroenteritis patients previously examined by EM were examined by the RIA. As shown in Table 1, all stools negative by RIA were negative by EM. All stools with other viruses by EM were negative by RIA. With one exception, all stools positive by RIA were positive for rotavirus by EM. The P/B ratios for negative stools fell between 0.68 and 3.63, with a mean of 1.91. For positive stools the P/B ratios were between 5.41 and 16.91, with a mean of 11.18. Hence, we feel that our decision to use four times the PBS count as the cutoff limit was justified when screening stools for the presence of rotavirus antigen.

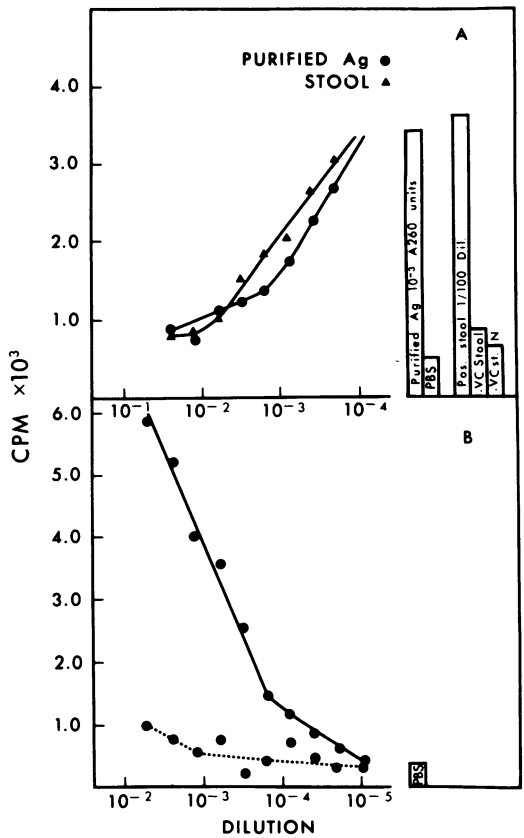

FIG. 7. *Effects of preincubation of rotavirus antigen (●) or rotavirus-positive stool (▲) with reference rabbit anti-rotavirus antibody on the yield. (A) Varying dilutions of antibody were incubated with a constant amount of antigen and positive stool. (B) Varying dilutions of rotavirus antigen were incubated with (---) and without (——) a constant amount (1:20 dilution) of rotavirus antibody.*

TABLE 1. *Comparison of EM and RIA for detection of rotavirus*

Results	No. of stools	%	P/B ratio limits	P/B ratio avg
Negative by EM for virus	53	54.6		
Positive by EM for virus other than rotavirus	12	12.4		
Positive by EM for rotavirus	32	33		
Negative by RIA	64	66	0.68–3.63	1.91
Positive by RIA	33	34	5.41–16.91	11.18
Total	97	100		

DISCUSSION

The RIA technique is becoming increasingly important in virology due to its sensitivity and relative efficiency. Other than isolation of the virus in cell culture, it is the most sensitive technique for the detection of viral antigens. When viruses cannot be readily isolated in cell culture, such as with hepatitis B virus and rotavirus, it is the most sensitive technique available (6).

As previous RIA procedures were developed to examine virus in serum, in tissue culture fluid, or in a partially purified state (4, 6, 13), we felt that it was necessary reexamine the parameters of the RIA to make it suitable for the detection of virus in stools. In addition, it was imperative that the assay be able to give same-day results, so it should be at most 5 h long. In this respect the incubation was optimized both for maximal yield and minimal times.

From an examination of the parameters of the assay, the following observations were made. The pH for coating the tubes with CA was 9.6 and was used in other RIAs. The CA concentration was found to be optimal at about 12.5 μg/ml. Beyond this, the actual yield of the assay decreased. Other workers have also made this observation (13). A balance was developed with the IA and DA concentrations when it was seen that the DA beyond 12 μg/ml will lead to high backgrounds, thus lowering the P/B ratio. On the other hand, the IA did not give marked rises in background when used in high concentrations. Accordingly, it was decided to use DA in lower than saturation amounts and increase the IA to obtain sufficiently high counts. This gave higher P/B ratios for a given preparation of antigen. Backgrounds were also kept down by including 0.1% of BPA in all incubation mixtures.

Initial studies showed that the pH of the stools from gastroenteritis patients varied and led to RIA results that were discordant with EM. When such stools were brought to a neutral pH with sodium bicarbonate, such discordant results disappeared. If stools were diluted less than 1:5 with PBS, some virus-negative preparations by EM showed unduly high counts. It was on this basis that we decided on the 1:5 dilution.

Neutralization studies have shown that the antigen activity can be blocked by specific antibody. This confirms the specificity of the assay.

In the diagnosis of stool specimens, the RIA gave comparable results to the EM despite its greater sensitivity. In this regard, it was difficult to be certain of a positive RIA diagnosis when the EM diagnosis was negative. Further studies using neutralization are in progress to resolve this matter.

This assay is reliable if high-titer specific sera are used in concentrations as we have outlined and stool specimens are prepared as indicated. If these principles are not adhered to, false positives and negatives may be generated.

RIA has three outstanding advantages. (i) Test results can be obtained on the same day, (ii) the system is more sensitive than existing techniques, and (iii) it lends itself to a high-volume operation.

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