Detection of Rotavirus by Serological Trapping on Antibody-Coated Electron Microscope Grids

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A serological trapping technique for detecting rotaviruses is described which involves coating electron microscope grids with protein A and specific rotavirus antiserum. The presence of a layer of antibodies on the grid increases the number of rotavirus particles that can be visualized. Thirty-five crude fecal extracts from infants suffering from diarrhea were examined by the serological trapping technique and by standard electron microscopy. When the specimens were deposited on antibody-coated grids, 71% of them were found to contain virus particles, compared with 20% on standard uncoated grids. The method is simple and rapid and does away with the need to concentrate the specimens.

The trapping of viruses on electron microscope grids coated with specific antiserum was first described by Derrick (3). The method increases the number of virions that adhere to grids and has been used extensively for the identification and quantitative analysis of plant viruses (13, 14). Recently the sensitivity of the method was further increased by coating the grids with protein A from *Staphylococcus aureus* before coating them with specific antiserum (18). In the present report, we show that the serological trapping technique can also be applied successfully to the rapid diagnosis of human rotavirus infections.

Direct electron microscopic examination of fecal material has become a standard diagnostic technique for rotavirus infections (7, 9, 10). Unfortunately, the virus concentration in the average stool sample is insufficient for direct visualization of virions on the grids, and it is necessary in most cases to first concentrate the virus by ultracentrifugation. This makes diagnosis by electron microscopy a rather laborious procedure. The serological trapping method described here eliminates the need for concentrating the specimens and is a more sensitive method of diagnosis than is standard electron microscopy.

Other techniques used for detecting rotavirus in feces include counterimmunoelectroosmophoresis (6, 19), enzyme-linked immunosorbent assay (4, 17), radioimmunoassay (8, 11), and enzyme-linked fluorescence assay (20) as well as a solid-phase system with erythrocytes coated with rotavirus antibodies (2).

MATERIALS AND METHODS

Specimens. A total of 35 stool specimens were obtained from 31 infants admitted to the hospital in

Strasbourg from October to December, 1979. Twenty-six of these infants were suffering from diarrhea.

Purification of rotavirus. The procedure described by Rodger et al. (16) was applied to a human stool which had been found by electron microscopy to contain many rotavirus particles. Virus was first extracted with fluorocarbon and then concentrated with 8% (wt/vol) polyethylene glycol. The concentrated virus was layered on a preformed 40 to 55% (wt/vol) cesium chloride gradient in 0.2 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.5). After centrifugation (18 h at $180,000 \times g$ in an SW41 rotor), the viral bands located in the lower half of the gradients were harvested and dialyzed overnight against 0.002 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.5). The particles were then pelleted and used for immunization.

Immunization. Rabbits were immunized by four weekly injections of 300 μ g of purified rotavirus mixed with complete Freund adjuvant. The rabbits were bled 1 week after the last injection. When tested by the microcomplement fixation test against tissue culture-adapted bovine rotavirus, the antiserum had a titer of 1/512.

Laying hens were immunized by the same procedure used for rabbits. Chicken immunoglobulins were not obtained from serum but were extracted from the yolks of immunized hens (A. Polson, M. B. von Wechmar, and M. H. V. Van Regenmortel, *Immunol. Commun.*, in press).

Standard electron microscopy. For electron microscope observations, 4 volumes of distilled water were added to 1 volume of fecal material. The mixture was homogenized, and after low-speed centrifugation ($5,000 \times g$ for 20 min), the supernatant fluid was applied to a carbon-coated electron microscopic grid (300 mesh). After staining with 2% phosphotungstic acid (pH 7.0), the grids were screened for 20 min for the presence of rotavirus particles in a Philips EM 300 electron microscope at a magnification of $39,000\times$. If a single particle was found, the specimen was scored as positive (see Table 3).

Serological trapping technique. Electron microscope grids, coated with a film of Formvar-carbon, were used for the trapping procedure. Antiserum, immune globulins, and protein A were diluted in 0.1 M sodium phosphate buffer (pH 7.0). Grids were floated on 50- μ l drops of protein A solution (usually 26 μ g/ml) for 4 min and were then placed on drops of phosphate buffer. Thereafter, the grids were floated on 50- μ l drops of dilute antiserum, usually for 10 min. After an intermediate rinse on a drop of phosphate buffer, the antibody-coated grid was left overnight on a drop of stool extract diluted with an equal volume of 0.2 M phosphate buffer. When a purified preparation of chicken immune globulins (0.2 mg/ml) was used instead of antiserum for coating grids, a period of 4 min was allowed for adsorption. No protein A was used in this case, since it has no affinity for avian immunoglobulin G. After the trapping reaction, the grids were rinsed by placing them on a series of drops of distilled water. Virions were visualized by positive staining with 1% uranyl acetate in 45% ethanol for 2 min. A more even staining was obtained in this way as compared with negative staining. Grids were examined at an instrumental magnification of 4,000 $\times$. Four areas were photographed with 70-mm film. Particle counts were made with a binocular magnifier on four 68- by 73-mm micrographs and were recorded as the mean number of particles.

RESULTS

Earlier findings obtained in several laboratories have shown that optimal conditions for the specific serological trapping of different viruses can vary considerably (1, 12, 14, 15). Therefore, preliminary experiments were conducted to determine the optimal protein A concentration, antiserum dilution, and virus-antibody interaction time suitable for detecting rotavirus in fecal extracts. Some of the results obtained with a particle-rich stool extract are presented in Table 1. An intermediate layer of protein A (26 μ g/ml) led to a 5- to 10-fold increase in the number of trapped virions, compared with grids coated only with rabbit antiserum. Optimal trapping conditions were achieved when a 1/500 dilution of antiserum was adsorbed for 10 min on the protein A-coated grid. The specimens were placed on the grids in the evening and examined the next morning. The specificity of the trapping reaction was demonstrated by the low counts observed when normal serum was used instead of rotavirus antiserum.

Anti-rotavirus globulin obtained from the yolks of immunized chickens was also an effective reagent for specifically trapping virus particles. Highest particle counts were reached when a 0.1-mg/ml globulin preparation was adsorbed for 10 min on the microscope grid (Table 2).

In addition to the specificity of trapping which enhanced the reliability of the diagnosis, another advantage of the serological trapping procedure

TABLE 1. Serological trapping of rotavirus on electron microscope grids coated with protein A and specific antiserum^a

Protein A concn (μ g/ml)	Dilution of rotavirus serum	Time of trapping of rotavirus on grids (h) ^b	Mean rotavirus counts at:			
			1 min	4 min	10 min	60 min
0	1/500	18	2	21	46	22
2.6	1/100	1	2	— ^d	12	—
	1/500	1	1	—	13	—
26	1/100	1	4	—	16	—
	1/500	1	6	—	13	—
		18	131	185	220	138
	1/5,000	18	53	112	126	155
260	1/100	1	14	—	16	—
	1/500	1	19	—	13	—
		18	95	144	179	121
	1/5,000	18	81	82	65	168
26	1/500 (Normal serum)	18	1	1	3	1

^a A crude extract from a virus-positive stool was used as the antigen. When observed by standard electron microscopy, this specimen showed one to two particles per 68- by 73-mm micrograph. The antiserum was obtained by immunizing a rabbit with a purified rotavirus preparation.

^b This was the final step of the procedure, i.e., it was performed after the antiserum had been added to the protein A-coated grid.

^c The numbers represent mean counts on four 68- by 73-mm micrographs taken at an instrumental magnification of 4,000 $\times$ at the indicated times of antiserum adsorption on protein A-coated grids.

^d —, Not done.

TABLE 2. Serological trapping of rotavirus on grids coated with purified immunoglobulin from immunized chickens^a

Globulin concn (mg/ml)	Mean rotavirus counts at:			
	1 min	4 min	10 min	60 min
Anti-rotavirus				
1	28	73	76	131
0.2	60	86	115	132
0.1	79	125	139	137
0.05	60	82	101	100
Normal				
1	0	1	0	1

^a Data were obtained with the same fecal extract used in Table 1. The reaction time for the trapping of rotavirus on globulin-coated grids was 18 h. Times shown are times of adsorption of globulin on grid.

was that the layer of serum proteins present on the grids considerably reduced the adsorption of debris and contaminating materials. A typical

electron micrograph obtained with a particle-rich stool extract is shown in Fig. 1.

The serological trapping method was applied to the examination of 35 fecal specimens that were also examined in parallel by standard electron microscopy. Of the 35 specimens deposited on antibody-coated grids, 20 showed a density of one to four virus particles on each 68- to 73-mm micrograph. Eighteen of these 20 specimens were negative by standard electron microscopy (after 20 min of screening and observation time), and only 2 were scored as positive. Five of the 35 specimens showed densities of 20 to 450 particles on each antibody-coated micrograph, and all of them were scored as positive by standard microscopy. Ten specimens were found to be negative by both techniques. The results summarized in Table 3 clearly establish the superiority of the serological trapping procedure, since 71% of the specimens were found to contain virus particles, compared to 20% by standard microscopy.

FIG. 1. Rotavirus particles in crude fecal extract. The specimen was deposited on a grid coated with rotavirus antiserum and stained with uranyl acetate in ethanol. The bar represents 300 nm. The inset shows rotavirus particles at double the magnification from the same specimen which had been negatively stained with sodium phosphotungstate.

TABLE 3. Comparison of standard electron microscopy and serological trapping microscopy for detecting rotavirus in 35 fecal specimens

No. of samples	Rotavirus detection by:	
	Standard EM ^a	Serological trapping EM
7	+	+
10	—	—
18	—	+

^a EM, Electron microscopy.

DISCUSSION

The technique of serological trapping of virions described in this report is a type of solid-phase immunoassay in which the antibodies are attached to electron microscope grids mainly through their Fc regions (5). The presence of protein A considerably increases the efficiency of trapping by the adsorbed antibodies since their binding sites are preferentially exposed. The results shown in Table 1 demonstrate that it is necessary to vary some of the parameters to determine the optimal conditions for the test. Studies with numerous viruses and antisera have shown that it is not possible to define a unique set of conditions (serum dilution, time of adsorption, etc.) that would be optimal for every virus-antiserum combination (13, 14).

Compared to the standard electron microscopical examination of specimens, the main advantages of the serological trapping procedure are its simplicity and rapidity. The time needed to examine one specimen was approximately 2 min. The method also obviates the need to concentrate crude extracts, and in addition, the specificity of binding increases the reliability of diagnosis. The layer of serum proteins present on the grids prevents the adsorption of contaminants and leads to a fairly clear preparation, even when the crude extract is allowed to react with the grid for 18 h. As a result, the presence of small numbers of particles can be more easily visualized.

When examined by standard microscopy, some of the specimens examined in the present work also revealed the presence of coronaviruses and other 27-nm virus-like particles. However, these particles were not trapped on the grids coated with rotavirus antiserum. The trapping method, therefore, is highly specific.

Several studies have shown that the serological trapping technique can detect virus concentrations as low as 10 ng/ml (1, 3, 15). This makes the technique as sensitive as other viral diagnostic procedures such as immunoenzymatic tests and radioimmunoassays. Additional work is required to establish whether the trapping technique is able to distinguish between different rotavirus serotypes. However, the fact that most specimens are likely to contain single-shelled rotavirus particles (which are not type-specific), together with the finding that a single antiserum used for trapping can detect a wide range of serotypes (14), suggests that the usefulness of the method is not restricted to the detection of a single serotype. The method should be applicable to the diagnosis of many human and animal viral infections.

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