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Use of Protein A in the Serum-in-Agar Diffusion Method in Immune Electron Microscopy for Detection of Virus Particles in Cell Culture

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Abstract A modified technique using protein A in the serum-in-agar (SIA) method for immune electron microscopy (IEM) was presented. Grids coated with staphylococcal protein A were floated on samples mounted on agar containing 2% antiserum and incubated at 37 C, for 60 min. After washing and staining, the grids were observed in an electron microscope. The effects of protein A on virus detection were evaluated using poliovirus and bovine rotavirus infected cell culture fluids. The results showed that the technique using protein A (PA-SIA) had at least 10-fold higher sensitivity for virus detection than the original SIA. The optimal concentration of protein A was 1 to 10 μg/ml for coating the grids to trap virus particles. The PA-SIA method was also compared with immunosorbent electron microscopy (ISEM). The former showed higher or at least the same sensitivity and some advantages in detecting antigen-antibody reaction than the latter method. These results indicate that our PA-SIA method may be superior to other IEM techniques presented previously for the detection and identification of viruses.

Immune electron microscopy (IEM) which permits morphological identification and immunological identification of the virus simultaneously (2) was first presented in 1941 by Anderson and Stanley (4). Since then IEM has been used for many purposes by several investigators (2, 6, 7, 10, 12–15, 35, 36). These studies were carried out by mixing virus with specific antiserum, and detecting the formation of aggregates of virions as a result of the antigen-antibody reaction. However, classical IEM has several disadvantages. It requires time consuming procedures such as incubation overnight, and high speed centrifugation to sediment the antibody-virus complexes (25). Furthermore, positive serological reactions are indicated by formation of aggregates, but the reactants must be in optimal proportions or aggregates will not be formed (2, 19), and aggregated virions may be originally present in clinical specimens, especially in fecal samples from patients with diarrhea (26).

To eliminate or minimize these problems, several modifications in IEM have been proposed (16, 18, 25). One of them is the method of Derrick (8), in which viruses were trapped on electron microscope grids coated with specific antibody.

This method has been called immunosorbent electron microscopy (ISEM) (17, 27, 29) or solid-phase immune electron microscopy (SPIEM) (1, 19, 21, 33, 34), and used extensively for the identification and quantitative analysis of various viruses. This technique has been further improved by Shukla and Gough (32) to increase the sensitivity by treating the grids with staphylococcal protein A before coating them with specific antiserum. Many investigators have confirmed the effect of protein A on the sensitivity in ISEM (11, 17, 21, 27, 28, 33, 34). Recently, Katz and Straussman (17) proposed the new name "Protein A coated grid technique" (PA-CGT) for this ISEM method with protein A.

Another modification of IEM is the method devised in 1971 by Kelen et al (20) using agar surface to adsorb impurities and trap the virus-antibody complex. This method was further improved by Anderson and Doane (3) for practical identification of enteroviruses. The practical value of their serum-in-agar diffusion (SIA) method was confirmed by Lamontagne et al (22) and Berthiaume et al (5).

It seems that SIA is more practical than the ISEM for the identification or serotyping of virus because SIA can easily observe the serological reactions indicated by clumping (2) and decoration (25) of virus particles with antibody, whereas the ISEM including PA-CGT requires post-treatment of grids with antibody after trapping the virus to obtain the decoration (25, 29), and no virus aggregation is obtained with ISEM unless the specimen is prepared by a special technique described by Almeida et al (1).

However, Katz et al (18) have shown that SIA is less sensitive in detecting virus particles than other IEM techniques. The author devised a new technique using protein A in SIA (PA-SIA), and obtained better results in the sensitivity and specificity of virus detection.

MATERIALS AND METHODS

Virus. The Sabin strain of poliovirus type I and the Lincoln strain of neonatal calf diarrhea virus (NCDV; bovine rotavirus) used were kindly supplied by I. Ohishi, Osaka Prefectural Institute of Public Health, Osaka, Japan. Adenovirus type 5 was the author's clinical isolate. NCDV was inoculated into rhesus monkey kidney cells. Poliovirus and adenovirus were inoculated into Vero cells. The infected cell cultures were incubated at 37 C. When extensive cytopathic effects were observed, the cells were harvested and frozen and thawed three times. After low-speed centrifugation ($800 \times g$, for 20 min), the supernatant fluids were stored at 4 C until used. Infectivity titers of these viral preparations used for IEM experiments ranged from 6.8 to 8.0 $TCID_{50}$ per ml.

Sera. Specific antisera were purchased commercially. Antisera to polio or rotavirus were from Denka Seiken (Tokyo) and adenovirus type 5 antiserum was from ISUMUNIT Diagnostic Division (Rome). The titers of antisera to polio, rota, and adenovirus were 1: 640, 1: 32, 1: 3,200, respectively. These sera were heat-inactivated at 55 C for 30 min to eliminate complications arising from the presence of complement (2).

Preparation of protein A coated grids. Collodion carbon-coated supporting membrane on the electron microscope grids (400 mesh) was previously glow discharged (25) with JEOL Ion Sputter JFC-1100 at 500 v, 2 mA, for 60 sec, and the grids were floated for 20 min on drops of protein A (Sigma Chemical Co., St. Louis, Mo., U.S.A.) at a concentration of 10 μg/ml in phosphate buffered saline (PBS) pH 7.4, washed by continuous transfer on three drops of PBS, and used immediately without drying.

Preparation of serum-in-agar. Antiserum (0.1 ml) was mixed with 5 ml of 1.5% Noble agar (Difco) in a 55 C water bath, and 0.3 ml of the mixture was dispensed into each well of a disposable polystylene plate for differentiation of bacteria in the BBL Minitek System (BD, Baltimore, Md., U.S.A.) and solidified.

SIA procedure. The SIA technique of Anderson and Doane (3) was slightly modified in this study. Briefly, 30 μl of the samples was layered on agar containing serum in each well. Then the grids coated with protein A were floated on samples with the coated side down, and incubated at 37 C. After 60 min, about 0.1 ml of distilled water was carefully added to each well to float up the grids from the surface of the agar. Each grid was then taken out with fine forceps, washed by floating three times on distilled water and then negatively stained on a drop of 2% phosphotungstic acid (PTA), pH 7.4. All specimens were examined in a JEOL JEM 100C electron microscope at a magnification of 20,000 to 50,000×. For quantitative determinations, virus particles trapped on five grid squares were counted and the average numbers per grid square were calculated (31).

PA-CGT procedure. PA-CGT was performed as described by Svensson et al (34). Protein A coated grids prepared as above were floated on drops of diluted antiserum for 20 min. These antibody-sensitized grids were washed with PBS, blotted again, and floated on 20 μl of sample for 60 min at room temperature. After washing with 10 drops of PBS and 10 drops of distilled water, the grids were stained with PTA and examined by electron microscopy.

RESULTS

To determine the effect of protein A on the recovery of virus particles on the grids, culture supernatants of poliovirus and NCDV were serially diluted and examined (Table 1). It was shown that about 10 times more poliovirus particles were trapped on protein A coated grids than on the grids without protein A, and about 30 times more efficiently in case of NCDV.

Positive antigen-antibody reactions indicated by clumping (30) or decoration (25) were observed in all examinations on grids with and without protein A.

Although the number of aggregated poliovirus particles trapped on the grids coated with protein A decreased at higher dilutions of the sample (Table 2), the degree of decoration was regularly remarkable (Fig. 1). The decoration of NCDV was insufficient for identification, but its clumping was constantly observed (Fig. 2).

Nonspecific adsorption of virus on the grids treated with protein A was compared with that on the grids without protein A using agar containing no serum,

Table 1. Effect of protein A on the sensitivity of virus detection by the serum-in-agar (SIA) method

Virus	Serum in agar	Protein A coating	Number of virus particles trapped per grid square[a]				
			Dilution of sample				
			UD[b]	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Polio	antipolio	+[c]	3,200	650	70	3	—[d]
		−	330	40	3	—	—
NCDV	antirota	+	2,300	330	23	1.2	—
		−	71	10	0.4	—	—

[a] The counts were carried out in three separate experiments and five grid squares each and averaged.

[b] Undiluted culture fluid containing $10^{6.8}$ $TCID_{50}$/ml of poliovirus or $10^{8.0}$ $TCID_{50}$/ml of NCDV.

[c] Grids were treated with protein A (10 μg/ml) for 20 min at room temperature.

[d] No virions were observed on five grid squares.

Table 2. Number of clumps of poliovirus detected on a grid by the serum-in-agar method using protein A (PA-SIA)[a]

Sample[b] dilution	Number of particles in a clump					Number of single particles[c]
	2–4	5–10	11–20	21–50	>50	
UD	218	52	18	13	18	564
10^{-1}	45	4	1	1	1	350
10^{-2}	2	0	0	0	0	23

[a] Grids treated with 10 μg/ml of protein A and agar containing 2% antiserum to poliovirus (Titer 1: 640) were used.

[b] Poliovirus infected culture fluid ($10^{6.8}$ $TCID_{50}$/ml).

[c] The counts were carried out on a representative grid square.

fetal bovine serum (FBS) or heterologous antiserum. The results obtained are shown in Table 3.

In the absence of serum in the agar, protein A coated grids showed about eight-fold fewer poliovirus and about five-fold fewer NCDV particles than those trapped on grids without coating. In the presence of FBS or heterologous antiserum in the agar, except in the case of examinations of NCDV and agar containing antiserum to poliovirus, the number of virus particles trapped on both grids with and without protein A was less than that in the absence of serum in the agar. In these cases, protein A coated grids exhibited three to 14-fold greater decreases in nonspecific adsorption than grids without protein A. In the case of NCDV and agar containing antiserum to poliovirus, a considerable number of NCDV particles was trapped on the grids coated with and without protein A. These trapped NCDV particles were occasionally aggregated (Fig. 3). This positive reaction was not detected when FBS or antiserum to adenovirus was used. Therefore, it was considered that anti-

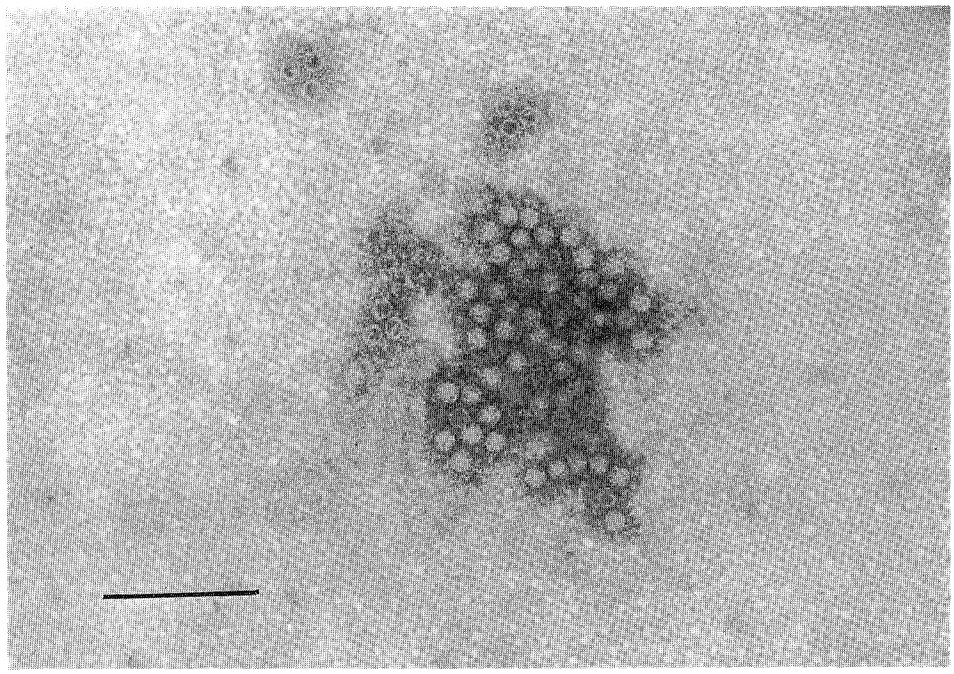

Fig. 1. Poliovirus particles trapped on a grid coated with 10 μg/ml of protein A by modified serum-in-agar (PA-SIA) method. The bar represents 200 nm.

serum to poliovirus obtained commercially may contain certain titers of antibody to rotavirus (NCDV).

These results indicate that protein A further enhances specificity in trapping homologous virus by eliminating the nonspecific adsorption of heterologous viruses on the grids. The ratio of specific trapping and nonspecific adsorption on grids coated with protein A was 3,200: 2 to 10 for poliovirus and 2,300: 8 to 20 for NCDV. (Tables 1 and 3). The inhibition of nonspecific adsorption was also effective in preventing the contamination. When observing the grids, cellular debris and other contaminants were scarcely seen on the grids treated with protein A, whereas a certain level of contaminants remained on the grids without protein A.

The optimal concentration of protein A for trapping virus specifically seemed to be 1 to 10 μg/ml. A concentration of 50 μg/ml caused a decrease in the number of trapped particles to some degree (Table 4). Consequently, grids treated with 10 μg/ml of protein A were used in the following investigations.

To determine the optimal incubation time, samples of 10-fold diluted poliovirus were incubated for 30, 60, 90, and 120 min (Table 5). The maximal number of particles was trapped after 60 min, and longer or shorter incubation periods resulted in a marked decrease of trapping efficiency. However, the degree of decoration was markedly increased by prolonged incubation (Fig. 4).

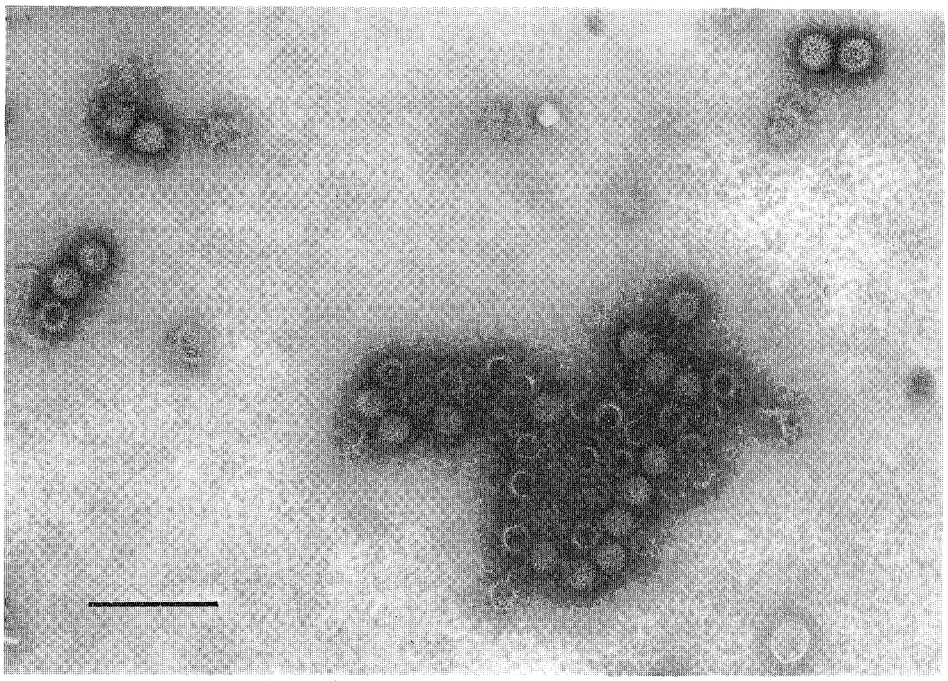

Fig. 2. Bovine rotavirus (NCDV) particles trapped on a grid coated with 10 μg/ml of protein A by PA-SIA method. The bar represents 300 nm.

Table 3. Effect of protein A on the nonspecific adsorption of virus in the SIA method

Virus[a)]	Serum in agar	Protein A coating	Number of virus particles trapped per grid square[b)]
Polio	absent	−	50
		+	6.8
	FBS[c)]	−	33
		+	9.7
	antirota	−	27
		+	2.0
NCDV	absent	−	98
		+	20
	FBS	−	65
		+	8.3
	antiadeno	−	28
		+	8.0
	antipolio	−	230
		+	850

a) Undiluted culture fluids presented in Table 1 were used.

b) The counts were carried out in two separate experiments and five grid squares each, and averaged.

c) Fetal bovine serum.

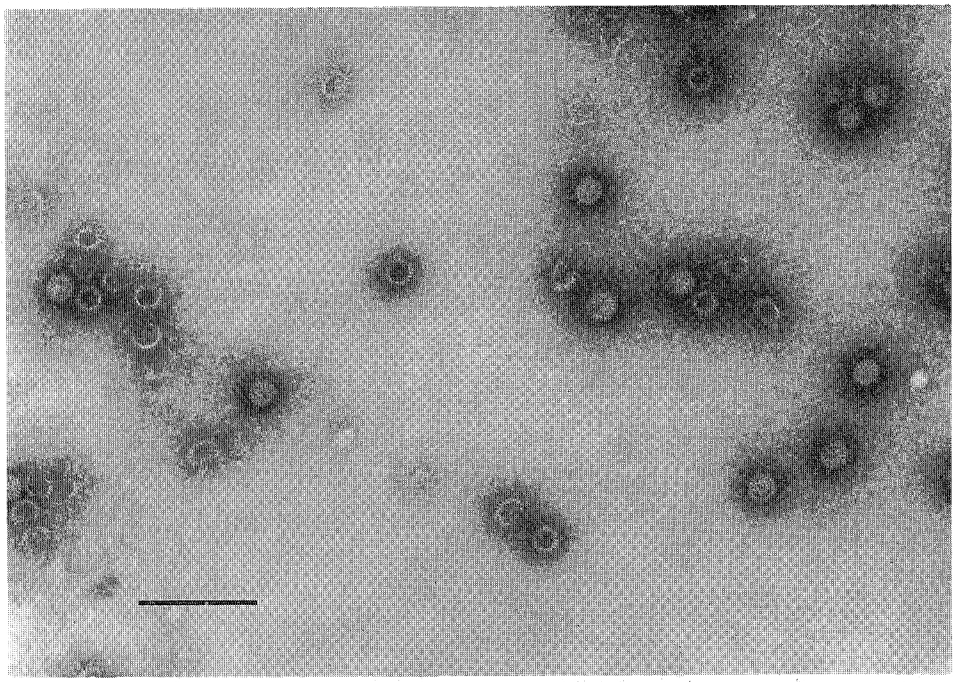

Fig. 3. NCDV particles trapped on a grid coated with protein A in the presence of antiserum to poliovirus which is supposed to contain certain titer of specific antibody to rotavirus. The bar represents 300 nm.

Table 4. Trapping of virus particles on electron microscope grids coated with varying protein A concentrations

Virus[a)]	Serum in agar	Protein A (μg/ml)	Number of virus particles trapped per grid square[b)]
Polio	antipolio	0	64
		1	600
		5	740
		10	640
		50	330
NCDV	antirota	0	18
		1	150
		5	160
		10	290
		50	180

a) Culture fluids presented in Table 1 were diluted 10 fold and used.

b) The counts were carried out in two separate experiments and five grid squares each, and averaged.

Table 5. Trapping of virus particles on electron microscope grids with protein A at varying incubation periods[a)]

Incubation time (min)	Number of virus particles trapped per grid square[b)]
30	81
60	250
90	77
120	97

[a)] Ten-fold diluted culture fluid of poliovirus was examined by the PA-SIA method.

[b)] The counts were carried out in two separate experiments and five grid squares each, and averaged.

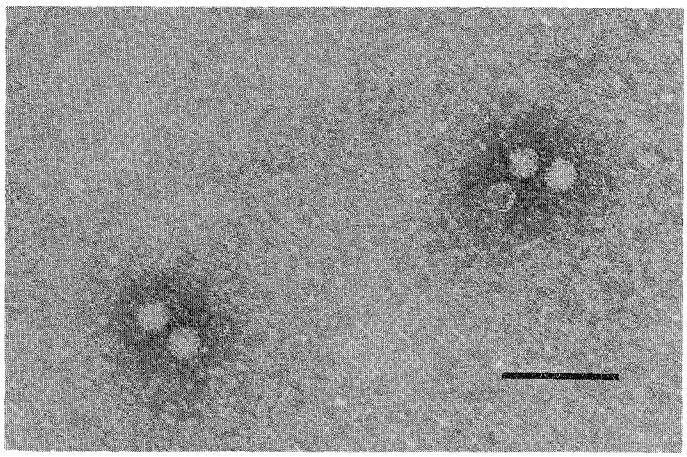

Fig. 4. Decoration of poliovirus after 120 min of incubation. Note the difference of appearance around the particles. The bar represents 100 nm.

The sensitivity of PA-SIA was also compared with that of PA-CGT of ISEM for three different species of viruses (Table 6). Although the comparisons were not performed under optimal conditions for both methods, only slight differences in the number of trapped adenovirus and NCDV between the two methods were observed. However, poliovirus showed a marked difference. Poliovirus particles were not seen in any specimens prepared by PA-CGT using 50- to 100-fold diluted antiserum to poliovirus, and only a small number of virus particles, which was less than that obtained nonspecifically in PA-SIA, could be detected on grids coated with protein A and 500- to 1,000-fold diluted antiserum, whereas a number of scattered and aggregated poliovirus particles could be seen in the specimens of the PA-SIA method.

Moreover, in the PA-SIA method, positive antigen-antibody reaction was recognized by the presence of aggregated particles or decoration of viruses, whereas in the PA-CGT method, virus particles trapped on the grids were scattered indi-

Table 6. Comparison of serum-in-agar diffusion method using protein A (PA-SIA) and protein A coated grid technique (PA-CGT) of immunosorbent electron microscopy (ISEM) for detecting virus in cell culture

Samples		Number of virions per grid square[a]	
Virus	Dilution	PA-SIA	PA-CGT
Polio	UD	>1,000	—[b]
	−1	310	—
	−2	70	—
Adeno	UD	27	9.0
	−1	7.2	1.7
	−2	0.2	—
NCDV	UD	>1,000	>1,000
	−1	70	170
	−2	10	7

[a] The counts were carried out on five grid squares and averaged.

[b] No virions were observed on five grid squares at serum dilutions of ×50 and ×100, however, seven particles (mean count) per grid square were detected at serum dilutions of ×500 and ×1,000.

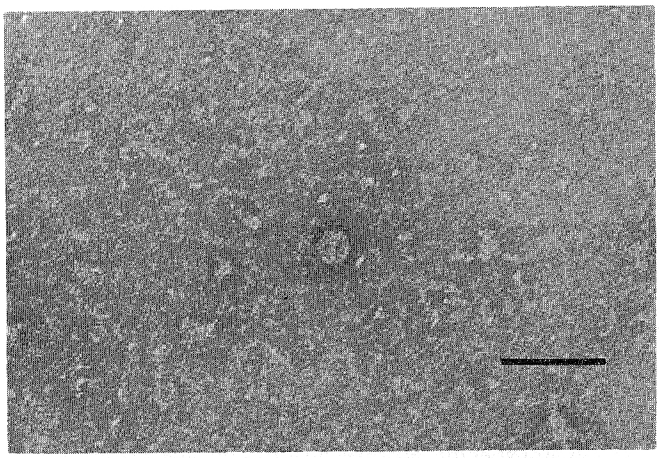

Fig. 5. Poliovirus particles trapped on a grid coated with protein A and specific antiserum. Note the absence of decoration as in Fig. 4. The bar represents 100 nm.

vidually, and no detectable decoration with antibody was seen (Fig. 5). Furthermore, the degree of contamination with cell debris was remarkably less in the PA-SIA method than in the PA-CGT method.

DISCUSSION

Protein A which is a cell wall protein produced by strains of *Staphylococcus aureus* has the ability to bind specifically to the Fc region of immunoglobulin (IgG) molecules from most mammalian species (9), and it has been applied in many immuno-

logical assays (23). In ISEM, protein A has been used for anchoring of specific antibodies to the grids, and its value has been recognized by many investigators (11, 17, 21, 27).

In this study, the author first adopted protein A in SIA to increase the sensitivity of the method and obtained good results similar to those in ISEM. However, the mechanism of enhancement for trapping viruses by protein A in the PA-SIA method is somewhat different from that in PA-CGT of ISEM. In PA-CGT, protein A plays a role in binding the antibody molecules to the grids and inhibiting the serum proteins which are adsorbed on the grids competitively with antibody, and it allows the use of sera at high concentrations which are inhibitory in the ISEM without protein A (16). In PA-SIA, protein A plays a role in binding the viruses adsorbed with homologous antibody which is involved in the agar and gradually diffused into the sample. This action of protein A in SIA significantly increased the sensitivity for trapping homologous viruses. Results obtained in this study showed that about 10-fold the number of poliovirus particles and about 30-fold the number of NCDV particles were trapped on grids treated with protein A compared with the number of virus trapped on nontreated grids. It is not certain why this enhancement effect occurs, but one possibility may be as follows; in original SIA, adsorption of viruses on the grids is always nonspecific and may be inhibited by the presence of serum proteins or contaminating materials in the sample (24). On the other hand, in the PA-SIA, viruses are adsorbed with homologous antibody at first and then specifically bound to the grids through protein A. The inhibition of binding to protein A by such a contaminating material does not occur in this case. This difference in the two techniques may affect the trapping efficiency.

Blocking of the binding site on a layer of protein A by free antibodies may occur when an extremely high amount of IgG molecules is present in the reaction system, but these antibodies also have the ability to bind virus serologically, and give specific trapping of viruses. Furthermore, as the concentration of antibody in the reaction system gradually increases, the possibility of blocking by free antibody may be eliminated.

The results presented in Table 3 also indicate that protein A has a marked effect on eliminating nonspecific adsorption of heterologous viruses. This effect of protein A, in association with the enhancement of trapping, greatly increased the relative specificity of SIA. Indeed, in the presence of homologous antibody, grids coated with protein A trapped about 100- to 1,600-fold more virus particles than in the absence of homologous antibody (Tables 1 and 3). Since comparisons of specific trapping and nonspecific adsorption were performed on undiluted samples, the number of specifically trapped virus particles may have been saturated (24) and thus shown lesser values. Therefore, a higher degree of relative specificity would have been obtained if suitably diluted samples had been used for comparisons. If this is not the case, the degree of this relative specificity would be greater than the results using ISEM obtained by Katz and Kohn (16), who showed a ratio of specific to nonspecific trapping on grids of about 40: 1. This extremely high specificity of PA-SIA has an advantage in identification of viruses which have been identified

only by the detection of clumping in original SIA because the clumping is reduced at higher dilutions of sample (Table 2).

The reduction of nonspecific adsorption was also effective for eliminating contamination on the grids. In observing the grids, cellular debris and other contaminants were scarcely seen on grids treated with protein A, whereas a certain amount of contaminants remained on the grids without protein A. Therefore, only three washings with distilled water were required to obtain good results in PA-SIA while 20 or more washings have been required in other ISEM methods including PA-CGT (16).

Other than using protein A, PA-SIA and the original method differ in the manner of removal of the grids from the surface of the agar after incubation. In PA-SIA, grids mounted on samples in microwells containing serum-in-agar are removed from the agar surface by adding a small amount of distilled water to the wells and floating the grids spontaneously up to the surface of the water, while in the original method, the grids are removed directly from the agar surface with forceps. This PA-SIA procedure was very effective in obtaining a constant number of trapped virions, and the counting became more quantitative.

Concerning to optimal conditions for PA-SIA, investigatio ns performed in this study are insufficient, especially with respect to serum concentrations in agar. The decoration of NCDV was extremely weak compared to that of poliovirus. It was assumed that the reason for this phenomenon was the low titer of antibody (1 : 32) in the antiserum to rotavirus compared with the titer of antiserum to poliovirus (1 : 640). However, the trapping efficiency on rotavirus has been significantly enhanced by this antiserum. Consequently, such titers of antibody in the antiserum may be sufficient for detecting virus by the PA-SIA method.

In a comparison of sensitivity between PA-SIA and PA-CGT, similar results were obtained in trapping NCDV and adenovirus. However, the sensitivity of PA-CGT in trapping poliovirus was extremely low under conditions thought to be suitable. The reason for this is not clear. These results indicate that PA-SIA is superior to PA-CGT for detection of viruses.

Another advantage of SIA is being able to concentrate samples by means of agar such as lyphogel which has been used for concentrating virus samples by Whitby and Rodgers (37). Thus, it may be possible to enhance the sensitivity more than that obtained in this study by increasing the amount of sample and agar.

Attempts at further increasing sensitivity and applying this technique to the direct detection of viruses in clinical and environmental specimens are underway.

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