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IMMUNOSORBENT ELECTRON MICROSCOPY FOR DETECTION OF VIRUSES

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I. Introduction

There is a wealth of methods in immunoelectron microscopy (IEM) as a diagnostic tool in virology. In all these methods the main step is the observation in an electron microscope of the interaction between viruses and their antibodies. This procedure permits, therefore, not only the direct visual recognition of the virion by its morphology but it also permits a specific identification of the virus via the antibody reacting with the virus. This combination of immunological and morphological method has an advantage over the other methods, where immunological reactions provide only a clue to the identity of the virus.

In this review, we shall only deal with immunoelectron microscopy (IEM) of viruses in suspension (or body fluids); we shall not consider the identification of viruses in histological sections by means of antibodies labeled with ferritin (Rifkind, 1976) or enzymes (Kraehenbuhl and Jamieson, 1976; Kurstak *et al.*, 1977). Clinical application of electron microscopy in medical virology has been reviewed by Field (1982).

The first IEM observation of a virus–antibody interaction was demonstrated by Anderson and Stanley in 1941 who used tobacco mosaic virus (TMV). The popularity of this method increased when Brenner and Horne (1959) introduced the simple principle of negative staining. In 1969 Almeida and Waterson (1969a) showed how agglutination of viruses by their antibodies could be demonstrated in the electron microscope and

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ISBN 0-12-039829-X

be used in the specific diagnosis of the virus. They called this phenomenon "Clumping." Milne and Luisoni (1975) showed the possibility of specific viral diagnosis based on the halo that antibodies formed around individual viruses or clumps of viruses and called this type of reaction "Decoration." These two main methods were employed in a number of variations: Optimal conditions for the reactions were found by varying the relative concentrations of virions and antibodies and by removing impurities from the reaction mixture either by centrifugation or by deposition of the complexes on agar (Kelen *et al.,* 1971; Anderson and Doane, 1973). These methods permitted the detection and identification of the viruses of rubella, corona, rhino, hepatitis A and B, rota, adeno, Norwalk, papilloma, etc. (Best *et al.,* 1967; Pensaert *et al.,* 1981; Kapikian *et al.,* 1972a,b; Feinstone *et al.,* 1973; Almeida *et al.,* 1971; Flewett and Boxall, 1976; Almeida and Waterson, 1969a).

These "classical methods," including the agar technique, were amply reviewed by Almeida (1980), Doane *et al.* (1974), Flewett and Boxall (1976), Doane and Anderson (1977), Almeida and Waterson (1969a), Milne and Luisoni (1977a,b), and van Regenmortel (1981a,b) and will not be described here. We shall, however, describe in detail IEM methods that are based on the principle introduced by Derrick (1973).

Derrick coated the electron microscope grid with antibodies so as to specifically trap from the suspension the viruses deposited on the grid. Since Derrick's method resembles solid phase immunoassays such as SPRIA (Catt, 1969) and ELISA (Engvall and Perlmann, 1971), it has been suggested that his method be called immunosorbent electron microscopy (ISEM) (Roberts *et al.,* 1982). In our opinion, Roberts' definition of ISEM is too narrow and should include not only methods with antibody-coated grids, but all other methods where a solid absorbent participates in the antibody-virus interaction, like the method of Milne and Luisoni (1975), where the virus is absorbed on the grid, or our own method (Katz *et al.,* 1980) in which the virus is absorbed to antibody-coated *Staphylococcus aureus.*

ISEM methods were used relatively more in plant virology than in animal virology. In this review, we shall discuss the newer modifications of ISEM methods in both plant and animal virology that were not covered by the review of Milne and Luisoni (1977b); we shall also present some of our own unpublished results in this field.

II. ISEM Methods

ISEM methods in this review include all the techniques where the "solid phase principle" is essential in a way similar to other solid phase immun-

oassays. For the sake of simplicity and uniformity, we propose to replace the older names and acronyms by new ones (see Table I).

Thus the Derrick technique (Derrick, 1973) will be named the antibody-coated grid technique (AB-CGT), the method of Shukla and Gough (1979) the protein A-coated grid technique (PA-CGT), the method of Katz *et al.* (1980) using protein A containing *S. aureus* bacteria the protein A-coated bacteria technique (PA-CBT), and the "decoration" technique as proposed by Milne and Luisoni (1975) the antigen-coated grid technique (AG-CGT).

TABLE I

PROPOSED NEW NAMES FOR IMMUNOSORBENT ELECTRON MICROSCOPY (ISEM) TECHNIQUES

Method first described by	"Old" names	References[a]	Proposed new name
Derrick (1973)	Derrick's method	2,5,8,9	Antibody-coated grid technique (AB-CGT)
	SSEM	1,3	
	ISEM	6,16,22	
	STREM	10	
	Trapping method	11,12	
	D-method	13	
	SPIEM-DAT	18	
	On grid IEM technique	20	
Milne and Luisoni (1975)	Decoration	2,4,5,22	Antigen-coated grid technique (AG-CGT)
Shukla and Gough (1979)	Shukla's method	7,14	Protein A-coated grid technique (PA-CGT)
	Serological trapping	12	
	STREM	10	
	ISEM	17	
	D + A method	13	
	SPIEM	19	
Katz *et al.* (1980)	SPIEM	15	Protein A-coated bacteria technique (PA-CBT)
	ISEM	21	

[a] (1) Derrick (1973); (2) Milne and Luisoni (1975); (3) Derrick and Brlansky (1976); (4) Milne and Luisoni (1977a); (5) Milne and Lesemann (1978); (6) Roberts and Harrison (1979); (7) Shukla and Gough (1979); (8) Lesemann *et al.* (1980); (9) Lesemann and Paul (1980); (10) van Regenmortel *et al.* (1980); (11) Nicolaieff and van Regenmortel (1980); (12) Nicolaieff *et al.* (1980); (13) Milne (1980); (14) Gough and Shukla (1980); (15) Katz *et al.* (1980); (16) Kerlan *et al.* (1981); (17) Obert *et al.* (1981); (18) Giraldo *et al.* (1982); (19) Kjeldsberg and Mortensson-Egnund (1982); (20) Rubinstein and Miller (1982); (21) Nicolaieff *et al.* (1982); (22) Roberts *et al.* (1982).

A. The Antibody-Coated Grid Technique (AB-CGT)

This technique, described by Derrick in 1973, was the first application of the principle of solid phase immunoassays to immunoelectron microscopy. Parlodion-carbon-coated grids were floated on drops of anti-tobacco mosaic virus (TMV) and anti-potato virus Y (PVY) (1:10 dilution) rabbit antisera. The grids were then washed to remove residual unattached antibodies and incubated for 1 hour with homologous and heterologous viruses derived from crude leaf extracts. Free viruses, as well as impurities, were washed off. The grids were dried and shadowed with metals. It was shown that 40 to 50 times more TMV particles were "trapped" on anti-TMV coated grids as compared to anti-PVY coated grids, while PVY was trapped 20 times more efficiently on anti-PVY coated grids than on anti-TMV coated grids. This AB-CGT was also shown to be suitable for quantitation, since the log of the number of virus particles specifically absorbed to grids decreased linearly with the virus dilution. Longer incubation times and higher temperature were found to increase the sensitivity of the AB-CGT.

Derrick and Brlansky (1976) applied the AB-CGT to the diagnosis of other plant viruses as well as to the corn stunt mycoplasma. In this work, the grids were positively stained with 1% uranyl acetate in 50% ethanol. The authors claimed that this stain was superior to phosphotungstate or ammonium molybdate negative staining. They also noticed that Formvar-coated grids with or without carbon coating were not suitable for the AB-CGT, since proteins did not absorb to Formvar. They obtained best results in their work with carbon fronted parlodion-coated grids. Derrick and Brlansky also noticed that the addition of 0.4 *M* sucrose to the washing buffer (Tris buffer) markedly reduced the amount of debris on the grids.

In distinction from the previous work (Derrick, 1973), Derrick and Brlansky (1976) coated grids with high dilutions of antisera. The degree of dilution of the antiserum (except at very high dilution) had no effect on the number of virions trapped on the antibody-coated grids.

In their review, Milne and Luisoni (1977b) described not only the original papers of Derrick and Brlansky but proposed a few modifications, such as using carbon-fronted Formvar-coated grids and the use of shorter incubation times of antiserum and virus. Diluted antisera (1:10 or 1:100) in phosphate buffer (PB) were incubated on grids for 5 minutes. After washing with PB, drops of virus were incubated for 15 minutes on grids, washed with water, and stained with aqueous uranyl acetate. They preferred this negative staining upon the positive staining, obtained with ethanolic uranyl acetate that was used by Derrick and Brlansky (1976). With the

negative staining technique, better resolution was obtained and the viral capsids were well preserved, though better contrast was obtained by positive staining. Another modification proposed was an improved way for preparing and storing antiserum-coated grids. In this procedure, grids were adsorbed with antiserum, washed with PB and water, and then dried and stored (temperature not stated). Before use, the grids were wetted with PB, drained, and incubated with the virus. Results were only slightly better with "fresh" as compared to the stored grids.

In the same review, Milne and Luisoni proposed to combine the AB-CGT with "decoration." In this procedure, the trapped viruses (by the AB-CGT) were incubated with antiserum diluted 1:100 for 15 minutes, washed with PB and then with water, and stained with uranyl acetate. A virus was considered specifically trapped only if it was also "decorated" by the second layer of antibodies. In the authors' view, decoration is the best proof for a specific immune reaction, since with all other methods, clumping or trapping may occur nonspecifically, due to factors not entirely understood.

Milne and Lesemann (1978) confirmed the data of Derrick (1973) and Derrick and Brlansky (1976) that larger numbers of viruses were trapped on antibody-coated grids than on untreated grids or on control grids treated with normal serum. However, in disagreement with Derrick and Brlansky, Milne and Lesemann stated that in order to obtain maximal trapping, optimal dilutions of sera had to be used. The most effective dilutions were between 1:800 and 1:3200. At low dilutions of serum an inhibition of trapping occurred due to serum proteins competing with the antibodies for sites on the grid. This assumption was elegantly confirmed: The addition of increasing amounts of normal serum or bovine serum albumin (BSA) to an antiserum diluted 1:600 progressively inhibited the trapping of the homologous viral particles.

Roberts and Harrison (1979) used the AB-CGT for the detection of potato leafroll and potato mop-top viruses (PLRV and PMTV, respectively). The grids freshly coated with carbon only were incubated for 1 hour at 37°C with diluted antiserum (dilution 1:1000 in PB pH 6.5), washed, and further incubated on drops of virus (leaf extracts or aphid extracts) at 4°C for 1 to 72 hours. PMTV were stained with 1 or 2% sodium phosphotungstate or 2% ammonium molybdate. These stains were unsatisfactory for PLRV. The only stain with which good contrasts were obtained and did not disrupt PLRV was uranyl formate sodium hydroxide at pH 4.8, diluted 1:3 with distilled water.

With this modified AB-CGT of Roberts and Harrison (1979), at least a thousand times more virus particles were trapped on antibody-coated grids than on untreated grids. The viruses were more evenly distributed when

incubated at 4°C than at higher temperatures. To confirm the specificity of the AB-CGT, viruses bound to the grids were incubated on drops of antibody for 1 to 3 hours at room temperature, stained, and observed for antibody coating ("decoration"). The AB-CGT of Roberts and Harrison thus enables examinations and measurements of fragile viruses (PMTV) which are found in low amounts in fresh leaf extracts, without the need of purification and centrifugation steps.

Lesemann *et al.* (1980) studied various parameters of the AB-CGT which influence specific (serological) and nonspecific binding of tymovirus particles to electron microscope grids. Carbon–Formvar-coated grids were treated by the glow discharge procedure, and floated for 5 minutes on antiserum, normal serum, or buffer (PB). Grids were then washed with PB and floated for 15 minutes on virus drops. Nontrapped viruses were washed away with water. The grids were then stained with 2% aqueous uranyl acetate. With this procedure, purified viruses adsorbed to buffer-treated grids to the same degree as to antibody-coated grids. Different viruses exhibited different degrees of nonspecific binding. The nonspecific binding was inhibited by coating grids with normal serum or by diluting the virus with crude plant sap. Specific binding, however, was not inhibited by the plant sap. In agreement with Milne and Lesemann (1978) as well as with Roberts and Harrison (1979), Lesemann *et al.* (1980) declared that optimal antiserum dilutions have to be found for maximal trapping efficiency. There was a linear relationship between the log virus concentration and the log virus particle count, up to a virus concentration of 10 μg/ml. At higher concentrations, the grids were saturated.

Top and bottom components of 10–40% sucrose gradients, used for the purification of the virus, were used to determine the strength of binding of the virus onto buffer and antiserum-treated grids. They were distinguished in the electron microscope (EM) by their different staining. Nonspecifically bound components (on buffer-treated grids) could be easily removed, while specifically bound particles on antiserum-coated grids were tightly bound. Heterologous viruses could not be replaced by homologous viruses on antiserum-coated grids (and vice versa).

Nicolaieff and van Regenmortel (1980) determined the conditions of specific trapping using the AB-CGT for three isometric viruses [turnip yellow mosaic virus (TMYV), tomato bushy stunt virus (TBSV), and cauliflower mosaic virus (CaMV)] and five strains of TMV. They used Formvar–carbon-coated grids and PB for the dilution of antisera and viruses. Grids were floated for 4 minutes on diluted antiserum, washed with PB, and again floated for various periods of time on drops of purified viral preparations. Visualization of the adsorbed virions was done either

by platinum shadowing or by positive staining with 1% uranyl acetate in 45% ethanol.

In agreement with the results of others, Nicolaieff and van Regenmortel showed that optimal conditions for the specific serological trapping should be defined for each virus. High serum concentrations were inhibitory, but the degree of inhibition was different with different viruses. Crude sap inhibited adsorption of viruses to untreated grids, but not to antibody-coated grids. By using the AB-CGT at carefully defined conditions, the serological distance between TMV strains could be determined since the extent of serological trapping on the grid was proportional to the serological distance between the strains. For the determination of weak cross reactions of distant viruses, purified antibody preparations, diluted in 1 : 1000 normal serum or in 5% of BSA solution, were used for coating grids.

The suitability of the AB-CGT for the differentiation of TMV strains was examined and compared to an indirect ELISA in another work (van Regenmortel *et al.*, 1980). The best method for the detection of serologically distant strains of TMV using one single antiserum is the indirect ELISA. With the AB-CGT, several serotypes could also be detected. However, fewer strains could be detected by the AB-CGT than by indirect ELISA. The authors conclude that the AB-CGT is highly sensitive and offers many advantages over other diagnostic procedures.

The need of optimal, usually high, dilutions of antiserum for use in the AB-CGT stressed by Milne (1980) has been confirmed by others. However, Milne reported that out of 15 plant viruses tested, the AB-CGT did not work for 8 viruses. These viruses were representatives of three main groups: potyviruses, cucumoviruses, and nepoviruses. Milne suggested that their specific trapping on the antibody-coated grids was blocked by soluble coat viral antigens.

Kerlan *et al.* (1981) applied the AB-CGT for the detection of plum pox virus (PPV) and chlorotic leaf spot virus (CLSV). Carbon-coated grids were coated by floating, for 5 minutes, on antisera, diluted 1 : 100, washed, and floated for 15 minutes on extracts from infected plants. Staining was with aqueous 2% uranyl acetate. In most experiments, grids before staining were further incubated with homologous rabbit antiserum for decoration, as proposed by Milne and Luisoni (1977b) and in some experiments, a double decoration was performed by a second incubation of the grids with sheep anti-rabbit immunoglobulin (IgG). For simple and double decorations antisera were diluted 1 : 100.

The AB-CGT that was even more sensitive than ELISA detected 5 to 10 ng/ml of the viruses. The sensitivity was attributed to the enhanced trapping efficiency of the antibody-coated grids. The single decoration

step was used for confirmation of specificity while the second decoration (with sheep anti-rabbit IgG) increased the sensitivity of the AB-CGT. With double decoration, lower magnification in the EM could be employed, since the width of the virions was increased 3-fold as compared to the untreated viruses.

The authors concluded that the AB-CGT provided a useful diagnostic tool and could be used as an alternative for ELISA.

Cohen *et al.* (1982) studied the effect of the pH of virus extracts and antiserum on the trapping efficiency in the AB-CGT. Four plant viruses were examined: cucumber mosaic virus (CMV), lily symptomless virus (LSV), potato virus Y (PVY), and carnation mottle virus (CarMV). The technique was as described by Milne and Luisoni (1977a). The carbon-fronted Formvar-coated grids were incubated with optimally diluted antisera at different pH values, and then reacted with viruses extracted from infected leaves with PB at different pH values. The efficiency of trapping of the AB-CGT was compared to the trapping of viruses on untreated grids, and to control grids that were coated with normal sera.

The results of Cohen *et al.* (1982) indicated that the pH of the virus extract had a marked effect on the efficiency of trapping, yet each of the viruses behaved differently. The optimal pH for LSV and for CarMV was 7.0. LSV had one sharp peak at pH 7.0 while CarMV was trapped only slightly less efficiently at pH values of 5.0 and 6.0; pH 8.0 was not satisfactory for both viruses. CMV was trapped most efficiently at pH 8.0 and about 2-fold less at pH 5.0 and 7.0; the worst pH was 6.0. PVY had two optimal pH values for trapping: 6.0 and 8.0, while pH values of 5.0 and 7.0 were less satisfactory.

The effect of the pH of the antiserum on the trapping efficiency was smaller though still significant.

The reasons for the pH dependence of trapping that was demonstrated with the AB-CGT in this work are not understood and the conclusion is that optimal pH conditions should be determined for each virus separately.

The first to apply the AB-CGT to the diagnosis of an animal virus (rotavirus) were Nicolaieff *et al.* in 1980. The results of their work with AB-CGT as compared to the PA-CGT, are reviewed in Section II,B.

Giraldo *et al.* (1982) applied the modified AB-CGT combined with decoration of Milne and Luisoni (1977b) for the detection of BK virus (BKV), a member of the human papovaviruses. In their optimal procedure they incubated for 5 minutes a drop of 1:500 dilution of rabbit antiserum, on a Formvar–carbon-coated grid. After washing with PB saline (PBS) a drop of virus was incubated on the grid for 15 minutes, washed again, and incubated for decoration with a drop of an 1:2500 antiserum dilution. After a fixation step by 0.8% glutaraldehyde the grid was again washed

with PBS and water and stained with 1% aqueous uranyl acetate. The specificity of the reaction was confirmed by cross experiments with polyoma virus. Similar number of viruses were trapped with a wide range of capture-antibody dilutions (1:500 to 1:10,000). The decoration step facilitated viewing of the virions. Antibody-coated grids trapped 17 to 28 more viruses as compared to untreated grids. As few as 10^2 to 10^3 plaque forming units per ml (PFU/ml) were detected specifically within 1 hour. The method is regarded by the authors as rapid, sensitive, and specific and is recommended for the detection of viruses in clinical specimens.

Rubinstein and Miller (1982) compared as ELISA method to an EM procedure and to the AB-CGT for the detection of rotaviruses. In their AB-CGT, Formvar-carbon-backed grids were incubated for 15 minutes on drops of antiserum diluted 1:2000 in PBS, washed on three drops of PBS, further incubated for 15 minutes on virus drops, and washed again. Negative staining was done with 2% phosphotungstic acid. The ELISA and AB-CGT were equally sensitive while the classical EM procedure was at least 9 times less sensitive. It was estimated that the ELISA and AB-CGT were able to detect approximately 10^6 simian rotavirions and 10^7 particles of human rotavirions. Out of 455 clinical specimens (stools from children with diarrheal diseases) 197 were positive by the AB-CGT while 193 of the 197 were also positive by ELISA. Of the 258 negative samples by the AB-CGT, 238 were negative by ELISA. For 18 specimens that were positive by ELISA and negative by the AB-CGT a confirmatory blocking test showed that all of them were "true" positives. The failure to detect the viruses in the ELISA positive samples by the AB-CGT can be partially explained by the presence of viral debris which may block the adsorption of intact particles to the antibody-coated grids. However, a few samples that were positive by the AB-CGT were negative by ELISA. The reason for this phenomenon is not quite understood.

B. The Protein A-Coated Grid Technique (PA-CGT)

In 1979 Shukla and Gough suggested coating of grids with protein A before coating them with specific antiserum to improve the trapping capacity of such grids. They found that with this PA-CGT they could detect 339(!) times more sugarcane mosaic virus (SCMV) and 5 times more TMV than on untreated grids, and 67 and 7 times more, respectively, with grids treated with antiserum alone (AB-CGT). The optimal procedure in their report was as follows: 5 μl of 0.1 mg/ml protein A was deposited on grids for 10 minutes followed by 1:20 dilution of TMV antiserum for 10 minutes. Five microliters of infected plant sap was then added to grids for 10 minutes. This was followed by "decorating" antibody (anti-TMV 1:100,

anti-SCMV 1:5) for 10 minutes and staining with 2% aqueous uranyl acetate, pH 4.5. After each step (except the first) the grids were washed with 20-30 drops of buffer. It was important not to let the grids dry except after staining.

In a later paper Gough and Shukla (1980) became aware that the results of their PA-CGT were sensitive to the serum concentration and found that the optimal serum concentration for protein A-coated grids was 1:100 or less, while with grids coated with antiserum alone, the serum at 1:1000-1:2000 gave best results. With this modified method the increase in the number of particles on grids treated with protein A and antiserum over those treated with antiserum alone (each at its optimal concentration) was 25-fold for SCMV and about 2-fold for erysimum latent virus and for TMV.

Gough and Shukla also found that protein A and antiserum-coated grids could be stored up to 6 months at 4°C while still retaining 25% of their trapping activity. Storage at room temperature did not give good results. Protein A could be stored at least 18 months in a frozen state.

Milne (1980) compared the performances of the AB-CGT and the PA-CGT for the trapping of two plant viruses (ryegrass cryptic virus (RCV) and grapevine stem pitting-associated virus (GSP-AV). For both techniques carbon-Formvar-coated grids were used. The carbon coating was performed immediately before use. For the AB-CGT a drop of serum was placed on the grid for 10 minutes, rinsed with 20 drops of PB, and drained. A drop of the virus preparation was then placed on the grid for 15 minutes. The grid was then rinsed with 20 drops of water and stained with 5 drops of 2% aqueous uranyl acetate.For the PA-CGT, grids were first covered with a drop of 0.1 mg/ml protein A for 10 minutes, rinsed with PB, and processed as for the AB-CGT.

Milne concluded that an optimum serum dilution is required in both methods. High antiserum concentrations were inhibitory, although somewhat less in the PA-CGT than in the AB-CGT. Protein A treatment of grids did not lead to a dramatic increase of the number of virions trapped over grids treated with antiserum alone. At optimal conditions for both techniques, PA-CGT trapped only 2 to 3 times more virions than the AB-CGT. However, at low virus concentrations similar numbers of virions were trapped on antiserum coated grids with or without protein A. Protein A may thus be of advantage in cases where the number of antibodies is a limiting factor but not when the numer of virions is limited.

Lesemann and Paul (1980) studied the effect of various conditions for the use of protein A in the PA-CGT and compared the results with the AB-CGT. They used pioloform-carbon-coated grids after exposure to glow discharge. The grids were floated for 5 minutes on drops of protein A

(10 μg/ml or more) diluted in PB, washed with 20 drops of PB, drained on filter paper, and floated again for 5 minutes on drops of diluted antiserum or normal serum. After washing with 20 drops of PB, the grids were transferred for 15 minutes onto drops of virus. The grids were then washed with 40 drops of water and stained with 2% aqueous uranyl acetate. The work was done with a purified preparation of maize chlorotic mottle virus (MCMV) and with eggplant mottled crinkle virus (EMCV) in the form of crude plant extracts.

Lesemann and Paul concluded that protein A at concentrations less than 10 μg/ml was not sufficient to bind antibodies in saturating amounts, and that higher concentrations of protein A did not improve the test. In their opinion the main advantage of the protein A in the PA-CGT is that it allows the use of sera at high concentrations which are inhibitory in the AB-CGT. The use of higher antiserum concentrations enhances trapping capacity of the grids. Under these conditions six times more particles (from high virus concentrations) were trapped in the PA-CGT as compared to the number trapped in an optimized AB-CGT. Other advantages of using high serum concentrations in the PA-CGT are (1) nonspecific binding on normal serum coated grids is depressed; (2) low titered antisera can be used; and (3) high concentration of antibody on the grid permits the detection of weak heterologous reactions. However, when the trapping efficiency of the two tests was compared under conditions where the virus was present at low concentrations the PA-CGT did not show any advantage over the AB-CGT (see also Milne, 1980).

Two explanations were proposed for these results: (1) that not all of the immunoglobulins are absorbed (like IgM) on protein A-coated grids, and thereby some antibody activity is lost; and (2) grids coated with diluted antiserum alone are not completely covered and some of the virions attach nonspecifically.

Nicolaieff *et al.* (1980) used the PA-CGT for the trapping of animal viruses such as rotaviruses. In their study the virus specimens were fecal extracts from infants suffering from diarrhea. The grids were first coated by flotation on 50 μl drops of 25 μg/ml of protein A for 4 minutes, and after transfer through drops of PB the grids were floated on 50 μl drops of 1 : 500 diluted antiserum (10 minutes), followed by rinsing in PB. Such grids were put overnight on drops of stool extracts diluted in PB. The grids were then washed in distilled water and stained with 1% uranyl acetate in 45% ethanol for 2 minutes.

Using that method, Nicolaieff *et al.* (1980) found virus particles in 71% of specimens as compared to only 20% on uncoated grids. The protein A layer improved the trapping 5- to 10-fold as compared to grids coated only with rabbit antiserum. Equivalent results were obtained on grids coated

with purified immunoglobulins from immunized chickens at optimal concentration of 0.1 mg/ml.

The trapping of rotavirus by the PA-CGT was highly specific in that coronavirus and other 27 nm particles detected by standard electron microscopy were not seen on the protein A-treated grid.

In a more extensive work from the same laboratory (Obert *et al.*, 1981), the sensitivity of detection of rotavirus in human stools by the PA-CGT was compared to direct EM, counterimmunoelectrophoresis (CIEP), and ELISA. While EM and CIEP detected rotaviruses in 36 and 38%, respectively, of the 63 specimens tested, ELISA and PA-CGT detected 59 and 61%, respectively. Both ELISA and PA-CGT were equally sensitive and could detect about 2 ng/ml of the virus. However, the direct visualization of particles in the PA-CGT provided an advantage over ELISA, since no confirmatory tests were necessary.

With most samples, particles were visible by the PA-CGT after 60 minutes incubation on grids, yet for maximum sensitivity the overnight incubation was routinely used. The authors pointed out that with PA-CGT, about 80 samples a day could be handled by one person. This method, extensively used in plant virology, is also likely to find many applications in the diagnosis of animal viruses.

Another detailed comparison of direct EM, PA-CGT, and ELISA for the detection of rotaviruses was performed by Kjeldsberg and Mortensson-Egnund (1982) on 115 fecal samples from children with gastroenteritis. For optimal results in the PA-CGT they used grids coated with 10 μg/ml of protein A and antiserum at a dilution of 1:640–1:2560. The coated grids were then incubated on the specimen drops for 18 hours at room temperature.

Rotavirus was found in 36% of samples by both the PA-CGT and ELISA without false positives, while by direct EM the virus was found in only 30%. The advantage of direct EM in this study was that (1) in addition to rotavirus, in 8 samples also adenovirus, astrovirus, and calicivirus particles were observed, and (2) the examination time was shorter. On the other hand this is the least sensitive method and usually requires centrifugation of the fecal extract.

Though PA-CGT and ELISA were equally sensitive, ELISA lends itself better to mass screening than electron microscopy. In one microplate up to 22 crude fecal extracts can be set up, and several specimens may be examined in a single test, whereas in electron microscopy each specimen has to be observed separately; therefore assuming even that the time allowed for one specimen is 4–5 minutes, it would require about 2 hours to achieve a result equivalent to 1–2 minutes reading time of ELISA.

The PA-CGT technique has also been used by us for the detection of

Sindbis virus as a model for arboviruses. One of us (DK) has determined the optimal conditions for trapping Sindbis virus and for its visualization by electron microscopy (D. Katz and Y. Straussman, unpublished results, 1982).

Best results were obtained with commercial 400 mesh carbon-coated grids (Polaron Equipment Ltd Watford, England). For trapping Sindbis virus these grids were first coated with 1 μg/ml of protein A in PBS for 15 minutes and then after washing with PBS, with Sindbis rabbit antiserum at a dilution of 1:500 for 15 minutes. Grids were then washed with 0.1% bovine serum albumin (BSA) in PBS and incubated for 1–3 hours on droplets of virus suspension. At no time in the procedure until after staining were the grids allowed to dry. The grids were positively stained for 3 minutes with 2% uranyl acetate in 47% ethanol. This stain gave better results than an aqueous solution of uranyl acetate or phosphotungstate.

Our results showed, in agreement with other investigators, that significantly more virus particles were trapped on grids treated according to the PA-CGT as compared to the number trapped on nontreated grids.

We found that 1 μg/ml of protein A used for coating grids was as good as 5 and 25 μg/ml. However, grids that were coated with antiserum only trapped less virions than grids coated with protein A at any of the concentrations tested at antiserum (dilutions 1:100 and 1:500). At antiserum dilution 1:2500 the differences were not significant. Under best conditions the PA-CGT trapped about 1.5 times more viruses than the AB-CGT (grids treated with antiserum only). In accordance with Lesemann and Paul (1980) we also found that the main advantage of the PA-CGT over the AB-CGT is that the former is less dependent on antiserum dilution.

An important finding was that the washing of the grids in PBS alone (after incubation with antiserum and with virus) contributed to nonspecific trapping of virions. When, however, the washing solution was replaced by PBS-BSA (BSA, 0.1%) the test became very specific and the ratio of specific to nonspecific counts on the grids was about 40:1. These results are summarized in Table II.

Time and temperature influence the amount of virus trapped on grids. This was concluded from an experiment in which a 1:150 dilution of Sindbis virus (1.3×10^7 PFU/ml) was incubated with coated grids for 1, 2, 3, and 5.5 hours at 24°C (room) and 37°C. The optimal time of incubation was 3 hours; longer or shorter incubation times were less efficient (Fig. 1). At 37°C (3 hours incubation) about 1.5 more viruses were trapped as compared to the amount trapped at 24°C. However, 3 hours of incubation at 37°C caused damage to the virions and the surfaces of the grids were covered with debris (Fig. 2).

TABLE II

PRESENCE OF BSA IN BUFFER AFFECTS THE SPECIFITY OF VIRUS TRAPPING

	Grids washed after serum coating with		
	Serum dilution[a]	PBS	PBS-BSA
Antiserum	1:500	ND[b]	241 ± 43[c]
	1:2500	140 ± 39	193 ± 15
Normal serum	1:500	ND	6 ± 9.6
	1:2500	178 ± 48	17 ± 27

[a] Serum incubated on grids previously coated with protein A (1 μg/ml).

[b] Not done.

[c] Number of viruses trapped per unit area.

Figure 3 shows that the $\log_{10}$ number of virus trapped on grids was proportional to the virus concentration.

The minimum detectable amount of virus was at about 10^6 PFU/ml.

C. *The Protein A-Coated Bacteria Technique (PA-CBT)*

In the last 2 years we have described (Katz *et al.*, 1980; Nicolaieff *et al.*, 1982) another ISEM method where the trapping device is a strain of

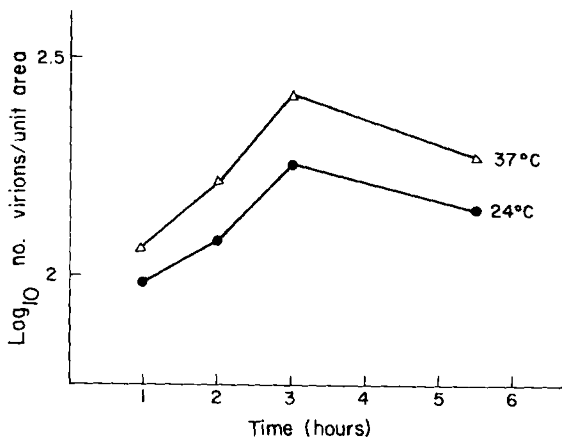

FIG. 1. The effect of time and temperature on the trapping efficiency of the PA-CGT. Carbon-coated grids, were coated with 1 μg/ml of protein A followed by anti-Sindbis rabbit serum (1:500). Such grids were then incubated at the indicated temperature and time with Sindbis virus stock, diluted 1:150.

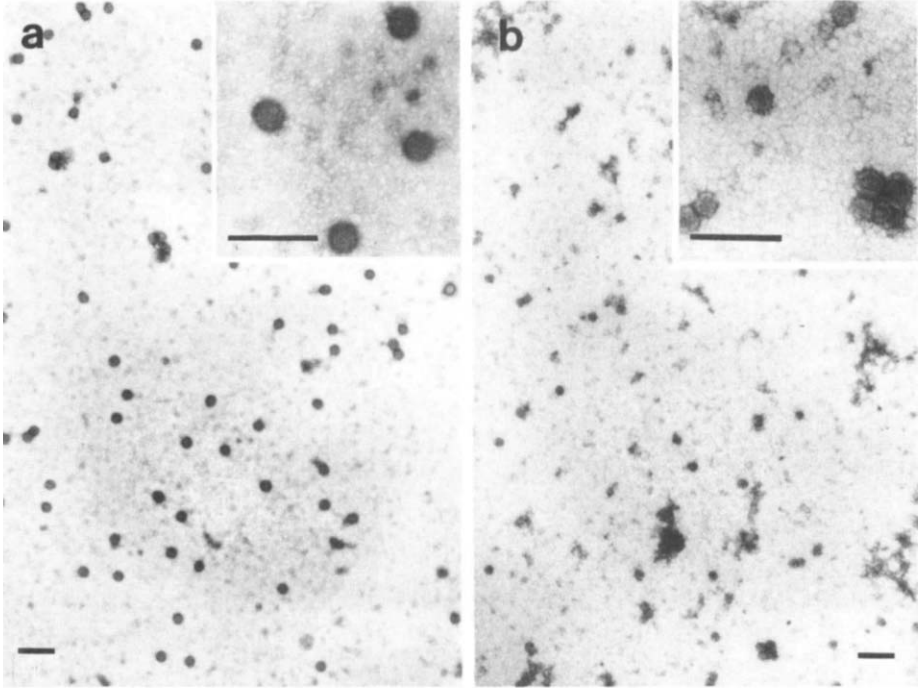

FIG. 2. Trapping of Sindbis virus (1:50 diln) on grids treated as described in Fig. 1. (a) Incubation of virus on grids at 24°C; (b) incubation of virus at 37°C. Note that the virions in (b) are damaged and there are many debris. Bars = 200 nm.

Staphylococcus aureus that contains protein A on its surface and can therefore easily be coated with specific antibody directed against a given virus. Such coated bacteria can be then used to "collect" the target virus from a suspension. The virions trapped on the surface of the bacteria can then be visualized in the electron microscope. We have used this technique (PA-CBT) for trapping of Sindbis virus (Katz *et al.*, 1980) and of plant viruses such as tomato bushy stunt (TBSV), turnip yellow mosaic (TYMV), and tobacco mosaic (TMV) viruses (Nicolaieff *et al.*, 1982).

The procedure for Sindbis virus was as follows: A suspension of *S. aureus* (3×10^8 cells/ml in PBS) was mixed with rabbit anti-Sindbis virus diluted 1:10 in PBS containing 0.02% sodium azide (PBS + Az) and incubated at 37°C for 15 minutes. The suspension was then centrifuged in an Eppendorf centrifuge for 1.5 minutes (3200 *g*) and washed similarly in PBS + Az. The pellet was resuspended in 1 ml of a target virus suspension for 40 minutes in a 37°C bath (or incubator) with shaking; after centrifugation for 2 minutes the pellet was resuspended in 0.1 ml of

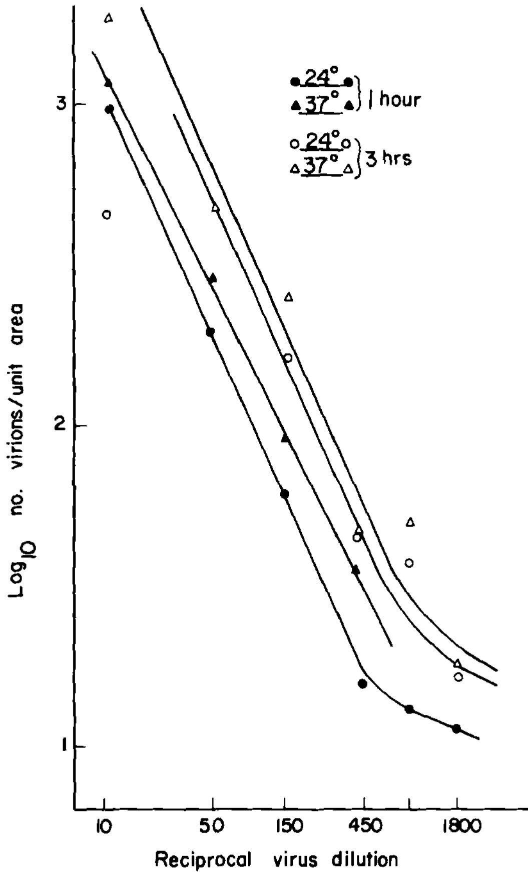

FIG. 3. Trapping of Sindbis virus at 24 and 37°C by the PA-CGT. Grids coated with 1 μg/ml of protein A and antiserum (1:500) were incubated on droplets of virus suspension at 24 and 37°C for 1 hour. The virus was applied to grids at various dilutions. Note the linear relationship between the log number of virions observed per unit area and their concentration in the suspension.

PBS + Az by vigorous mixing (vortex). A drop of the suspension was applied to Formvar-carbon-coated grids, drained, and stained for 1 minute with 2% phosphotungstate pH 7.3.

The virions could be seen attached to the surface of the bacterium as single particles or as a continuous layer according to the virus concentration (Fig. 4a). The minimum concentration of Sindbis virus that could be detected as single virions per cell was 10^5–10^6 PFU/ml.

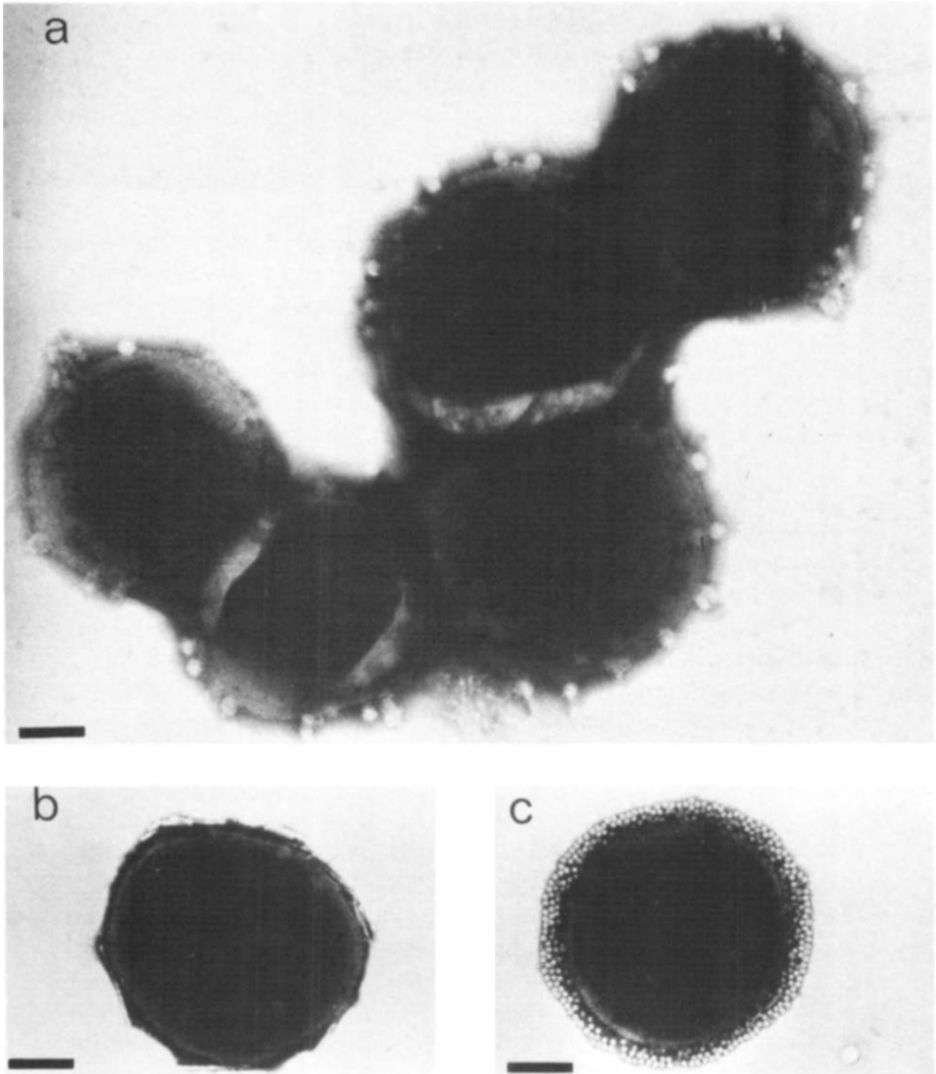

FIG. 4. Trapping of viruses on *Staphylococcus aureus*. *S. aureus* suspensions were coated with specific antiserum (PA-CBT) against (a) Sindbis virus (10^8/ml), (b) tobacco mosaic virus (TMV) (100 ng/ml, and (c) TYMV (turnip yellow mosaic virus) (500 ng/ml). Bars = 200 nm.

A similar procedure of the PA-CBT was used for plant viruses (Nicolaieff *et al.*, 1982), however, it was found that higher dilutions of antiserum (1:10,000) were preferable for coating of bacteria (Fig. 4b and c). By this method (method 1) 20–50 ng ($2-5 \times 10^9$ virus particles)/ml of TYMV and TBSV could be detected. The specificity of the PA-CBT expressed as a ratio between the number of homologous virions to heterologous ones was 20:1 for TBSV/TYMV and 43:1 for TYMV/TBSV.

The sensitivity of the PA-CBT was increased about 4-fold when a smaller number of coated bacteria was used for trapping (2×10^6/ml), the final centrifugation step was omitted, and the suspension allowed to settle on the microscope grid for 4.5 hours (method 2).

Both techniques, PA-CGT and the PA-CBT, have similar sensitivities, however the PA-CBT is less reproducible because of various technical problems such as bacterial clumping and heterogeneity.

The PA-CBT was used by Lee *et al.* (1981) for serotyping of herpes virus and for the demonstration of adenovirus–antibody immunocomplexes. For serotyping, 0.1 ml of packed, heat killed, formalin-fixed, *S. aureus* containing protein A bacteria was mixed with 0.4 ml of a 1:400 dilution of rabbit anti-herpes virus antiserum and incubated at 37°C for 1 hour. The cells were spun down at 2000 *g* for 10 minutes, washed twice with PBS, and resuspended in 12 ml PBS of the herpes virus suspension, incubated, and layered on 2 ml of a 5% sucrose layer for centrifugation at 2000 *g* for 15 minutes. The pellet was resuspended in 1 ml and processed for electron microscopy by the pseudoreplica technique. As in the PA-CBT described by us, in case of a positive result viruses are seen adsorbed to the surface of the bacteria.

For demonstration of immunocomplexes a model system was used in which radioactively labeled adenovirus was complexed with its antibody. The complexes were precipitated in the cold with 4% polyethylene glycol (PEG), pelleted at 2000 *g* for 20 minutes, washed again with 4% PEG, centrifuged as before, and resuspended in PBS. The immunocomplexes were then mixed with 3% of protein A-containing bacteria, incubated for 2 hours at 37°C and centrifuged on a 5% sucrose cushion, 2000 *g* for 10 minutes at 4°C. The adsorbed viruses were then eluted from the pelleted bacteria with KCl–HCl buffer, pH 2.5, and after another centrifugation at 2000 *g* for 10 minutes, the supernatant was processed for electron microscopy by the pseudoreplica technique. It was calculated that 60% of the radioactive-labeled virus was present in the final eluate. In this procedure the bacteria are used merely as an intermediate immunoadsorbent: a positive reaction is indicated by the appearance of single virions in the electron microscope.

D. The Antigen-Coated Grid Technique (AG-CGT)

In the AG-CGT the grid is directly coated with the virus (antigen) which is specifically identified by the decoration of a specific antibody. This technique was extensively used by Yanagida and Ahmad-Zadeh (1970) and Yanagida (1972) for the localization of gene products and identification of antigenic precursors in bacteriophage T4.

Other authors used this technique for similar purposes. Wrigley *et al.* (1977) studied the binding sites of antibodies to isolated hemagglutinin and neuraminidase of influenza virus, Norrby (1969) identified antigen specificities at the surface of adenovirus, and Vernon *et al.* (1981) studied the localization of herpes simplex virus nucleocapsid polypeptides.

The first who used the AG-CGT for plant viral diagnosis and not for morphological localization of antigens were Milne and Luisoni (1975). In their procedure carbon-fronted Formvar-coated grids were touched to the virus suspension for a few seconds, rinsed with 30 drops of PB, drained, and further incubated with a drop of diluted antiserum for 15 minutes at room temperature in a humid chamber. The grids were then washed with 20 drops of PB and with 50 drops of distilled water and finally stained with 2% of aqueous uranyl acetate. Some viral suspensions were either purified preparations in dilute buffer, or preparations from CsCl or sucrose bands. Others were infected leaf extracts. The washing steps which were essential to remove efficiently salts, sugars, and many impurities did not detach the viruses. Once a homologous antiserum was used, the interaction with the virus was evident as a halo of antibodies surrounding the virus. All the viruses tested (TMV, TRV (tobacco rattle virus), CVMV (carnation vein mottle virus), TBSV, and CMV remained intact after the AG-CGT except for MRDV (maize rough dwarf virus). For this virus a fixation step with 2% glutaraldehyde was included before washing and proceeding to the antiserum coating step. In the same paper a short timed clumping method was compared to the AG-CGT. The authors conclude that whenever adequate numbers of viruses are present, the AG-CGT is preferable because it is quick and simple: when the virus concentration is low, either the AB-CGT (Derrick, 1973) or the "clumping method" (Milne and Luisoni, 1977a) should be used.

The AG-CGT was successfully used for a detailed serological analysis of fractions from MRDV virions and for the determination of the serological relationship to a cross reacting rice black streaked dwarf virus (Luisoni *et al.*, 1975).

Milne and Lesemann (1978) compared three methods, AG-CGT, clumping, and AB-CGT, in a study of oat sterile dwarf and related viruses.

Their conclusion was that the AB-CGT was the most reliable for the study of serological relationships between the viruses since in principle only one particle was sufficient to obtain reliable results.

III. Discussion

In all immunosorbent electron microscopical (ISEM) methods like in the ELISA or SPRIA methods, one of the components of the system is adsorbed to a solid phase. We have discussed in this review four methods. In three of them (AG-CGT, PA-CGT and AB-CGT) one of the reagents is adsorbed to an electron microscopic grid, while in the fourth (PA-CBT) protein A is naturally present on the surface of a bacterium, which serves as a solid support. For the sake of uniformity of nomenclature we have suggested that these methods be given new names, that would identify both the support as well as the adsorbed reagent. Inspection of Table I would indicate how urgent is the need to unify the nomenclature of the ISEM methods.

The method of Derrick (1973) (AB-CGT) and its modifications are a real breakthrough in immunoelectron microscopy. At optimal conditions they give highly specific and reproducible results, and their sensitivity is similar to that of the "classical IEM methods" that were based on the phenomenon of clumping (Almeida and Waterson, 1969b). The AB-CGT method is attractive also because there is no need to purify the samples examined, and because it permits good identification of virions present in crude extracts of infected secretions or excretions. This fact is emphasized by Narang and Codd (1981) who studied acute nonbacterial gastroenteritis: differential centrifugation of fecal samples actually led to loss of some viral flora. While in untreated fecal samples examined by direct EM methods adenovirus, astrovirus, rotavirus, and "small round" viruses were seen, in the centrifuged samples some of these viruses were lost, most probably because some viruses, already clumped by indigenous antibodies in faeces, are removed by centrifugation. The presence of such clumps may be mistakenly interpreted as due to the effects of specific immune serum added during the process of preparation of the sample for IEM.

In the ISEM methos viruses cannot only be identified but also counted and their concentration may be numerically expressed as number of virions per unit of area, and can therefore be statistically evaluated. With these methods, therefore, one can quantify the effects of quality of the supporting grid, the time of adsorption, the pH, the presence of salts, and the type of staining, and thus optimize the results of the test.

1. Grid Coating

It is now quite clear that the critical parameter in all ISEM methods is the quality of the grid and its coating. Various investigators employed in their studies carbon-backed Formvar-coated grids (Rubinstein and Miller, 1982), carbon-fronted Formvar grids (Milne and Luisoni, 1977b), carbon-coated grids only (Roberts and Harrison, 1979), and numerous other variations. Lesemann and Paul (1980) as well as Milne and Luisoni (1977b) advocate the pretreatment of grids with glow discharge, but Milne and Leseman (1978) state that in the case of the AB-CGT this treatment has no particular advantage. Each laboratory should, therefore, test the grids and their coating for the particular system and viruses which it is investigating. The same consideration also applies to staining: one stain may be suitable for one group of viruses, but deletorious to another (Roberts and Harrison, 1979).

2. Antisera and Buffers

Another parameter which deserves consideration is the dilution of the antiserum used for coating the grids. In some cases, undiluted serum may actually inhibit the adsorption of virions. This fact is also recognized in other solid phase immunoassays. One has, therefore, to determine the optimal serum dilution for every virus (especially in the AB-CGT) (Nicolaieff *et al.*, 1980, 1982; Lesemann *et al.*, 1980; Milne, 1980).

Other factors of importance in the ISEM methods are the quality of the antiserum and the composition of the adsorption and washing buffers. So, for instance, the addition of a protein (BSA) to the washing buffer and to the virus suspension improved the specificity of the results, i.e., increased the differential counts on antiserum-coated grids as compared to those obtained on grids coated with normal serum (control) (Table II, Section II,B). In many studies, however (Milne and Luisoni, 1977b; Nicolaieff *et al.*, 1980), good specific results were obtained, though protein was not present in the washing buffer. In those cases the procedure involved washing with some 20–50 drops between each incubation step; when BSA was present in the buffer, 6 drops were sufficient to achieve an equivalent degree of specificity.

3. Incubation Time

The time of incubation of the virus sample on the antiserum-coated grid affects the number of virions trapped (Nicolaieff *et al.*, 1982; Kjeldsberg and Mortensson-Egnund, 1982). We observed that the maximal number of Sindbis virions adsorbed after 3 hours; longer incubation periods resulted in a decrease of the number of virions on the grid. It is feasible that

this finding may be true for other sensitive viruses, but not necessarily for viruses which are refractory to prolonged incubation.

4. Temperature of Incubation

In most of the studies, reviewed here, the virus samples were incubated on the grids at room temperature. In our own studies with Sindbis virus, incubation at 37°C led to an increase in the number of virions seen on the grid, but their structure was impaired (Fig. 2, Section II,B).

5. Effect of pH on Trapping of Virions

The pH of the buffer may strongly affect the degree of adsorption of the virions to the grids in the AB-CGT (Cohen *et al.*, 1982). This pH dependence may explain the failure of Milne (1980) to trap efficiently cucumber mosaic virus (CMV). Milne worked with buffers at pH 7.0; Cohen *et al.* (1982) finds that for CMV the optimal pH was 8.0.

6. Protein A Coating of Grids

There has been a general agreement among the investigators cited in this review that treatment of grids with protein A (Shukla and Gough, 1979) before their coating with antiserum improved the results in comparison to the AB-CGT. Coating of grids with protein A subsequently permits use of a wider range of dilutions of the antiserum, and the inhibition of binding with undiluted serum is avoided. Because of that, one may safely use undiluted sera of low titer. The number of virions trapped on protein A–antiserum-coated grids is greater than that with the AB-CGT by a factor of least 2 (Milne, 1980), provided that the tests are done each at its optimal performance. With samples with small number of virions both methods were similar in their trapping efficiency.

The coating of grids with protein A obviates the need to find the optimal pH for the binding of immunoglobulins (antiserum) to the grid (Cohen *et al.*, 1982), since binding to protein A occurs as well at the neutral pH of buffers usually employed in most laboratories.

7. Decoration

Decoration was used in conjunction with AB-CGT (Milne and Luisoni, 1977b; Giraldo *et al.*, 1982; Roberts and Harrison, 1979) or with PA-CGT (Shukla and Gough, 1979). Kerlan *et al.* (1981) even employed a double decoration method so as to make the virions in the image increase in size due to the double antibody coating on them. All investigators agree that decoration aids the specificity of the tests and makes the identification of the virus easier. In our experience with Sindbis virus, the specificity of PA-CGT was equally high with or without decoration, presumably because

of the use of BSA in the buffer. It seems thus that the decoration method would be advantageous for very small viruses, or when there is a problem of background and contrast.

8. S. aureus - Protein A

In 1980 we suggested to use as a trapping agent *Staphylococcus aureus* which has on its surface protein A; we now call this technique PA-CGT (Katz *et al.*, 1980). We hoped that this method would be more sensitive than the other ISEM methods because (1) protein A is a natural product of the bacteria, and they adsorb large amounts of gamma globulins; (2) the bacteria added to a virus suspension trap viruses during their brownian movement in the suspension; (3) though freely moving, each bacterium performs as a solid phase; and (4) after the incubation of the bacteria with the viral suspension it is easy to spin down the bacteria even in a clinical centrifuge, and to resuspend them in a minute volume of buffer, suitable for the deposition on the EM grids.

Though the efficiency of trapping with the PA-CBT is higher than that of PA-CGT (Nicolaieff *et al.*, 1982), the sensitivity of PA-CBT is about equal to that of PA-CGT. The reason for this lack of improved sensitivity lies in the fact that in the EM one may see only the virions at the circumference of bacteria (Fig. 4), but not those adsorbed on top or underneath them (a calculation indicates that 45 times more bacteria are present on the bacterium than actually seen). In addition the bacteria tend to clump, and this clumping also impairs the observation in the EM.

The use of bacteria, however, by the method of Lee *et al.* (1981) may avoid some of the problems that our method poses. The improvement suggested by Lee *et al.* (1981) is to "peel off" the viruses trapped on the bacteria by elution at low pH, and thus to obtain a concentrated suspension of the virions, free of bacteria. This technique that also facilitates the detection of immunocomplexes may be of importance in diseases such as hepatitis B (Almeida and Waterson, 1969b).

9. Direct Trapping of Viruses on Grids

It seems to us that the AG-CGT as described by Milne and Luisoni (1975) is useful only if the quantity of the virus in the sample is very high and therefore that this method is more useful for the study of the antigenic structure of a virus rather than for diagnosis.

10. ISEM and Other Solid Phase Immunoassays

The sensitivities of PA-CGT or AB-CGT are equivalent to those of ELISA (Rubinstein and Miller, 1982; Nicolaieff *et al.*, 1982). The sensitivity of the methods is expressed either as limiting ng/ml or as the number

of infective units of virus (e.g., PFU/ml). ELISA is able to detect a few ng/ml of viral protein which is equivalent to 10^7–10^8 virions. Katz *et al.* (1980) found that the limit of detection of Sindbis virus with PA-CBT is 10^5–10^6 PFU/ml, while Giraldo *et al.* (1982), working with papova viruses, set the limit 10^2–10^3 PFU/ml. As long as the exact number of virions necessary for 1 PFU is not determined it is very misleading to compare various methods using PFU as a criterion. In respect to the Sindbis virus 1 PFU contains approximately 30 virions (A. Shapira and S. Lustig, personal communication). Therefore, the limiting detectable number of Sindbis virions by the PA-CGT would be 3×10^7. As to the data of Giraldo *et al.* (1982) concerning papovavirus we do not know how many virions there are in 1 PFU, and therefore it is impossible to state whether his method is more or less sensitive.

What are the comparative merits of ISEM methods in relation to SPRIA or ELISA?

The relative disadvantages of ISEM lie, first, in the requirement for an expensive instrument (electron microscope), second, in the small number of samples that can be visually processed in EM, and third, in the fact that the presence of soluble antigens in the sample may decrease greatly the sensitivity of the method.

On the other hand the advantages of ISEM are (1) direct and dependable identification of a virus based not only on the specific antigen–antibody reaction, but also on morphology; and (2) the preparation of samples for electron microscopy requires only 15–60 minutes for most of the ISEM methods.

11. Plant vs Animal Viruses

The number of publications describing the use of ISEM methods for the diagnosis of animal viruses is small in relation to the number of studies on plant viruses. We assume that this state of affairs is due to the historical fact that the ISEM methods were primarily developed by plant virologists and the transfer of methodology from the plant virus field to that of animal viruses might take some time. In distinction from animal viruses which are present only in scant numbers in body fluids and excretions, plant viruses are quite abundant in the tissues of infected plants, and their concentrations there may reach values as high as 1 mg/ml (Lesemann *et al.*, 1980). The finding and identification of plant viruses by EM is thus much easier than that of animal viruses. It is therefore understandable that quite a considerable effort has yet to be invested in the optimization of the ISEM methods for the diagnosis of animal viruses.

Plant viruses are not infective to animals, while any infective virus of man presents a potential hazard for the laboratory worker (Field, 1982). The existing methods for the inactivation of virus infectivity for ISEM

(that would not affect the integrity and characteristic morphology of the virus in question) are still in the stage of development.

Nevertheless, in those studies where ISEM methods had been applied to the diagnosis of animal viruses, satisfactory results have already been obtained. We believe therefore that diagnostic laboratories equipped with EM would do well to introduce and to perfect the ISEM methods, especially in those cases where other diagnostic methods are not yet satisfactory. The ISEM also permits a quite detailed study of antigenic variations in the same genus of virus, especially now when monoclonal antibodies could be used as the trapping or decorating γ-globulins, and thus would visually pinpoint the type or strain differences.

One has to bear in mind, however, that in the methods of the type of IEM, SPIRA, or ELISA, one examines at a time the presence of only one specific antigen. With the direct EM methods, one can distinguish several morphologically and antigenically distinct viruses (Kjeldsberg and Mortensson-Egnund, 1982). Berthiaume *et al.* (1981) suggest using commercial pools of γ-globulin as clumping antibodies, since such pools contain antibodies to a large number of common animal viruses. Such pools would thus be very useful to pick up from feces not only rotaviruses but other viruses such as astrovirus, adenovirus, and calicivirus. Berthiaume's method can be hopefully applied to the PA-CGT, as well as to other ISEM methods.

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