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Immunochemical Studies of Sindbis Virus Variants

By

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Summary

We have demonstrated that the small plaque (SP) variant of Sindbis virus is more extensively neutralized by complete antibody and serum IgG fractions than is the large plaque (LP) variant. The Fc portion failed to demonstrate any neutralizing potential and did not block subsequent neutralization by IgG or Fab fragments. The LP and SP variants of Sindbis gave identical patterns upon isoelectric focusing. Treatment of the virus with intact serum IgG or isolated Fab fragments was found not to have an irreversible effect on the net charge of the virus population. The selective processes involved in the maturation of the nucleocapsid implicate the incorporation of charged host determinants which are then reflected in the charge of the virion. It is suggested that the virus core has a preferential affinity for those components of the plasma membranes which have an isoelectric point at pH 3.6.

1. Introduction

Sindbis, a group A arbovirus is composed of an inner ribonucleocapsid and an outer lipoglycoprotein envelope. It contains two major virus-induced structural proteins (33), lipids and carbohydrate derived from the host of origin (7, 24), and the ribonucleic acid. Plaque variants of this virus, first reported by Hannoun *et al.* (15) have been shown to differ in various biological characteristics such as pathogenicity for baby mice, the effects of temperature upon virulence, and elution from calcium phosphate columns (7, 15, 24, 33). This paper reports investigations of the characteristics of the large plaque (LP) and small plaque (SP) variants of Sindibs virus, using immunological and electrophoretic techniques.

2. Materials and Methods

2.1. Media and Diluents

Eagle's medium (13) buffered with 0.001 M tris-(hydroxymethyl)-aminomethane (Tris) and modified by the substitution of Hanks's basal salt solution (14) for Eagle's

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salt solution, was used for the growth and maintenance of chick embryo cells (CE). The medium was supplemented with penicillin (100 units/ml), streptomycin (50 mcg/ml), mycostatin (50 units/ml), and 5% calf serum (EC-5). For solid overlay, Eagle's medium containing 3% calf serum (EC-3) was jelled with 1% Bactoagar or Ionagar. Hanks's basal salt solution (BSS) plus 1% inactivated calf serum (BSSC) was used for washing cell cultures and as a diluent for all virus and tissue suspensions.

2.2. Cell Cultures

Chick embryo monolayers were prepared from decapitated 10-day-old embryos (12). The monolayers were used routinely after 24 to 32 hours of incubation.

2.3. Virus

Sindbis virus strain A339 (35) was obtained from the American Type Culture Collection. The ATTC stock contained two plaque types. Isolation of large (LP) and small (SP) plaque variants was performed by clone selection and sequential passage of the two types.

Cloned stocks were grown in CE monolayers and harvested after 12 hours to minimize the effect of heat inactivation upon the virus (28). Stock virus titers were generally between 10^8 and 10^9 PFU/ml and contained from 4 to 64 hemagglutinating units/ml.

2.4. Virus Purification

Clarified virus was centrifuged at 21,000 r.p.m. (59,000 $\times$ *g*) in the Spinco 21 rotor. Each tube contained a 5 ml 15% sucrose cushion. After centrifugation for 2.5 hours, the pellets were resuspended in a minimal amount of BSSC, pooled and agitated on a VDRL shaker for at least 2 hours at 4° C in order to disperse clumps. This suspension was layered on a 28 ml linear sucrose gradient (15—45%) and centrifuged for 2 hours at 25,000 r.p.m. (63,500 $\times$ *g*) in the Spinco 25.1 rotor. The tubes were punctured at the bottom and 1 ml fractions were collected by gravity flow. Fractions containing peak infectivity were concentrated by centrifugation for 1.5 hours at 39,000 r.p.m. (115,000 $\times$ *g*) in the Spinco 39 rotor. The pellet was suspended in a minimal amount of BSSC, as described above, and was stored at —70° C.

2.5. Membrane Preparation

Cells, labeled in their phospholipid moiety with P^{32}, were harvested after 24 hours growth, and were washed twice with Eagle's medium. Plasma membranes were prepared by the method of ROSENBERG (30). After centrifugation the plasma membranes were collected from the interface between the 41% and 37% sucrose fractions.

2.6. Hyperimmune Sera

Antisera of high titer were prepared by the immunization of New Zealand strain rabbits with freshly harvested and partially purified virus variant preparations. Antiviral sera were adsorbed extensively with normal CE fibroblasts to remove cross-reacting antibodies. Plaque neutralization tests were performed using 1×10^7 PFU/ml of infectious virus.

2.7. Isolation of Rabbit IgG and Fragments

Gamma globulin (IgG) was prepared from rabbit sera by the procedure outlined by KECKWICK (16) and STRAUSS *et al.* (32). Papain digestion of rabbit IgG was performed according to PORTER (25) as modified by MANDY *et al.* (18) Fc crystals formed upon dialysis of reaction mixture and Fab fragments were isolated from the soluble dialyzed portion of the papain-digested IgG, by carboxymethylcellulose column chromatography. Fragments were tested for homogeniety by ultracentrifugation in the Beckman model E analytical centrifuge.

2.8. Gel Diffusion Technique

Seventy-five hundredths percent agarose (Nutritional Biochemical Corporation), buffered with sodium veronal (8.98 g sodium barbitol, 0.5 g sodium azide, 64.5 ml of 0.1 M HCl, water to one liter) pH 8.6, ionic strength 0.1, was used in all gel diffusion

studies, following Ouchterlony (23). The slides were stained for 10 minutes in a solution of amido black (17.0 g amido schwarz 10B, 10.0 ml glacial acetic acid, 1250 ml ethanol, 3850 ml water) and rinsed in a bath of the same solution without the dye.

2.9. Immunoelectrophoresis

Immunoelectrophoresis was performed according to Scheidegger (31) in 1% buffered agarose (pH 8.6) with a current of 25 mA for 2 hours.

2.10. Block Electrophoresis

Electrophoresis of the purified virus was performed horizontally in a leucite electrophoresis apparatus ($1.5 \times 2.5 \times 45$ cm) containing a block of Pevikon (a copolymer of polyvinyl chloride and polyvinyl acetate which gives rise to negligible electroosmotic cathodal flow and which is a non-swelling medium showing little tendency to adsorb proteins) as described by Müller-Eberhard (20). The buffer was 0.1M borate saline, pH 9.0, and a current of 400V was applied for 48 hours. After electrophoresis, 1.0 cm sections were cut from the block and the virus was eluted by the addition of 2 ml of borater-saline buffer. The eluates were analysed spectrophotometrically at 260 and 280 nm and hemagglutination and infectivity titers determined.

2.11. Isoelectric Focusing

Isoelectric focusing was performed according to Swensson (34). A 110 ml capacity column (L.K.B., Uppsala, Sweden) was employed. The 1% carrier ampholyte solutions covered the pH range of 3 to 10 or 3 to 5. A sucrose density gradient of 0 to 40% (w/v) was used to prevent convection. The electrodes were protected with dilute phosphoric acid at the anode and with ethylene-diamine at the cathode. With the wider pH gradient (pH 3.0 to 10.0) the potential was 600 volts and equilibration was reached within 24 hours. Experiments in the pH range 3.0 to 5.0 were carried out for 48 hours at 700 volts. The temperature was maintained at 5° C by circulating chilled water. Fractions of approximately 1 ml were collected and assayed for pH (Corning model 12 Research pH meter with a Corning semi-micro combination electrode), radioactivity (Ansitron liquid scintillation counter) and infectivity in CE monolayers.

2.12. Labeling with Radioisotopes

H^3-glycine was used to label Sindbis virus in CE monolayers. The growth medium was removed from the cells and was replaced with Eagle's medium containing 20 μCi of the labeled amino acid. After 3 hours incubation, the labeled medium was removed and 10 PFU of virus was added per cell. The virus was adsorbed for 45 minutes and the cells washed once with BSS. The labeled amino acid medium was added back to the monolayers after the attachment period. Virus was harvested after 12 hours and purified as described above.

P^{32}-labeled virus was prepared using phosphate-free Eagle's medium (all phosphate-containing compounds were omitted from Hanks's BSS) with 0.5 M Tris buffer, pH 7.4. To obtain labeled plasma membranes or virus with the P^{32} predominantly in the phospholipid, the cells were incubated for 8 hours with carrier-free Eagle's medium to which 25 μCi/ml P^{32} had been added. This labelling period was followed by 2 hours incubation with complete Eagle's medium with unlabeled phosphate in order to decrease the amount of P^{32} in the intracellular pool. The cells then were washed with BSS, and 10 PFU of virus was added per cell and adsorbed for 45 minutes. The cells were incubated in Eagle's medium for 12 hours and the virus was harvested and purified as described above.

The method of McConahey and Dixon (19) using Chloramine T, was used to label IgG or Fab fragments with I^{125}.

2.13. Radioactivity Assay

Radioactivity was assayed in an Ansitron liquid scintillation counter with the background radioactivity automatically subtracted. The activity of a sample was determined by adding an aliquot, not exceeding 1 ml, tp 15 ml Bray's solution (1).

3. Results

When cloned plaque types of Sindbis virus were tested against variant specific sera it was found that the SP variant was neutralized to a greater extent than the LP variant by either homologous or heterologous antisera. To extend these observations we added anti-Sindbis LP or anti-Sindbis. SP sera to a mixed population containing equal amounts of the LP and SP variants. The results in Figure 1 show that the number of unneutralized LP variants are three to four

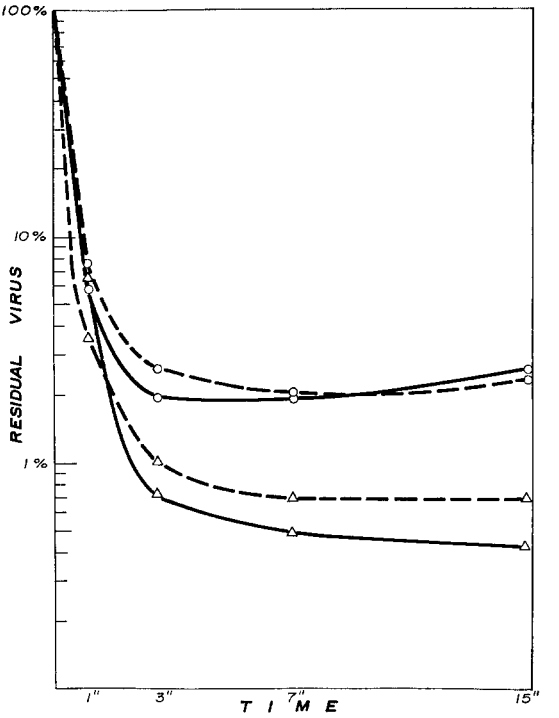

Fig. 1. Results of the neutralization of a mixed population of Sindbis LP and SP virus variants by anti-Sindbis LP and anti-Sindbis SP sera
△——△ SP variant neutralized by anti-LP serum; △- - -△ SP variant neutralized by anti-SP serum; ○——○ LP variant neutralized by anti-LP serum; ○ - - - ○ LP variant neutralized by anti-SP serum

times greater than the number of SP variants plaquing in such mixed neutralization studies. The greater neutralization of the SP variant was observed in serum titrations carried out over a 10,000-fold range of dilutions. This was also reflected in the neutralization rate constants (8) of the anti-Sindbis sera. Both anti-LP and anti-SP sera demonstrated higher K values for the SP variant of Sindbis virus.

The possibility that the antisera affected the size of the plaques was considered. Measurements of the plaque sizes of both variants after exposure to antibody are shown in Table 1. It can be seen that the size of plaques formed by the variants was unaffected by neutralization. In addition, it was determined that the increase in plaque size which may be achieved by manipulation of the overlay medium does not increase the titer of the virus population surviving neutralization. The Sindbis LP and SP variants were neutralized as described earlier. Plaque titrations of the

surviving fractions were performed using ionagar, ionagar supplemented with 0.2% protamine sulfate, and agarose. The mean plaque sizes of both variants increased. The virus titers, however, were unaffected. This shows that the mechanism by which the plaque size is increased affects both variants of Sindbis virus equally.

Table 1. *Size of Sindbis Plaques after Neutralization*

	Normal serum control (100 plaques) mean diameter	Anti-Sindbis LP (100 plaques) mean diameter	Anti-Sindbis SP (100 plaques) mean diameter
Sindbis LP	3.91 mm	3.93 mm	3.84 mm
Sindbis SP	1.85 mm	2.10 mm	1.91 mm

Studies using purified antibody IgG (100 mg/ml)were performed. The reactions showed the same preferential neutralization of the SP variant. An additional manipulation of the experiment involved the neutralization of the variants with isolated Fab fragments of antibody. The rationale for this experiment was the

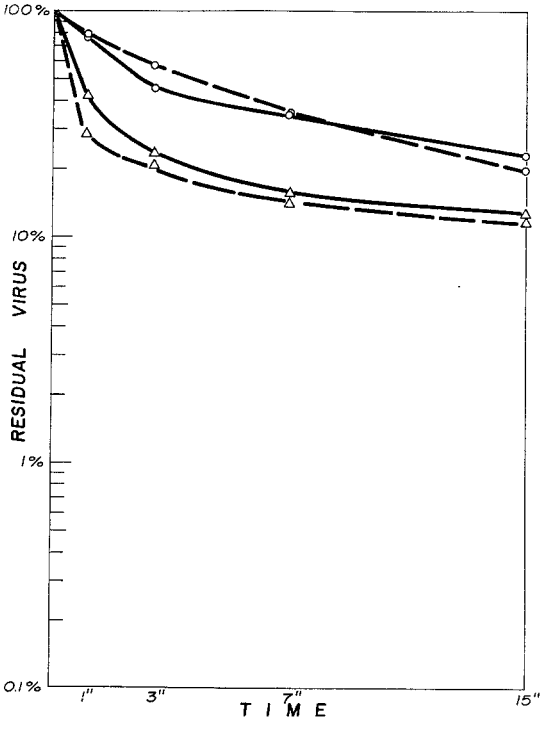

Fig. 2. Results of the neutralization of Sindbis LP and SP virus variants by Fab fragments isolated from anti-Sindbis LP and anti-Sindbis SP sera
△———△ SP variant neutralized by anti-LP_{Fab}; △———△ SP variant neutralized by anti-SP_{Fab}; ○———○ LP variant neutralized by anti-LP_{Fab}; ○ — — ○ LP variant neutralized by anti-SP_{Fab}

possibility that Fab fragments would not demonstrate preferential neutralization of the SP variant in the absence of lattice formation. Although not as efficient as the divalent IgG or whole serum, Fab fragments neutralized the virus (Fig. 2).

Again, the SP variant was neutralized more completely than the LP variant. Therefore, although enhanced by lattice formation, differences in neutralization are not explained exclusively on the basis of third order antigen-antibody complexes. It was found that the Fc fragments did not neutralize the virus nor did the Fc portion block sites which would interfere with subsequent neutralization by IgG or Fab fragments.

Adsorption of antisera, carried out in two stages using 1×10^{10} PFU/ml each time, showed that the neutralizing capacity of the anti-Sindbis sera could be eliminated by a population of homologous or heterologous virions. These results are in agreement with the report by BOSE and SAGIK (4) that neutralizing antibodies are directed against the virion envelope.

Iodine^{125}-labeled IgG was used to quantitate the binding of viral antibodies to virus particles seeking possible differences between Sindbis LP and SP variants. After labeling, the specific activity of the anti-Sindbis LP IgG was 1.212×10^9 molecules/CPM while that of the anti-Sindbis SP IgG was 1.054×10^9 molecules/CPM. Equal volumes of virus (2.0×10^9 PFU/ml) and antiserum were incubated at 37° C for 1 hour, then at 4° C overnight. The neutralized virus was centrifuged through a 30% sucrose cushion and the pellets were counted in a Nuclear-Chicago Gamma Well Scintillation Counter (model 8725). The results, corrected for background and control values, are presented in Table 2.

Table 2. *Molecules of IgG Bound per Virus Particle*

	Reactants			
	LP + LPIgG	SP + LPIgG	LP + SPIgG	SP + SPIgG
Specific Counts Pelleted	84	59	72	70
Molecules IgG Pelleted	1.019×10^{11}	7.151×10^{10}	7.759×10^{10}	7.378×10^{10}
PFU Virus Neutralized	1.26×10^9	1.22×10^9	1.06×10^9	1.02×10^9
Mol IgG/PFU	80.8	58.6	71.6	72.3

The results of this experiment, and an additional experiment using adsorbed IgG, show that between 60 and 80 molecules of IgG were bound per PFU when 96% of the virus population was neutralized. No difference was seen between the Sindbis LP and SP variants in numbers of antibody molecules bound.

3.1. Gel Diffusion Reactions

Variant-specific hyperimmune sera were utilized in two-dimensional gel diffusion tests to establish the degree of antigenic identity of the Sindbis variants. Specific anti-Sindbis LP and anti-Sindbis SP sera formed one band of identity in gel when tested against either plaque variant of Sindbis virus. This band, located near the antigen well, was shown to contain the intact viral antigen by staining with acridine orange (9). Neither normal rabbit serum nor anti-chick embryo fibroblast serum reacted with the viral antigen. BOSE *et al.* (6) have shown that the

peptides of the LP variant were identical regardless of the host of origin, whereas the peptides of chick embryo and baby hamster kidney cell membranes were different from each other. Antisera directed against the LP variant propagated in BHK-21 cells or in mouse brain showed reactions of identity with sera directed against the LP variant propagated in CE fibroblasts. This would indicate that the intact virions have similar, if not identical, envelope determinants regardless of the host of origin. Antiserum directed against mouse brain hemagglutinin also reacted with the Sindbis LP variant grown in CE fibroblasts, confirming the envelope origin of this antigen.

3.2. Electrophoretic Studies

In viral purification studies utilizing calcium phosphate columns, Bose *et al.* (5) found that Sindbis LP and SP variants could be eluted differentially, suggesting possible charge differences between the variants. Immunoelectrophoresis carried out at pH 8.6 showed that both variants migrated toward the anode at the same rate. When the Sindbis virus variants were coelectrophoresed in a horizontal Pevikon block at pH 9.0, the LP and SP variants again migrated toward the anode at indistinguishable rates (Fig. 3) manifesting a considerable heterogeneity

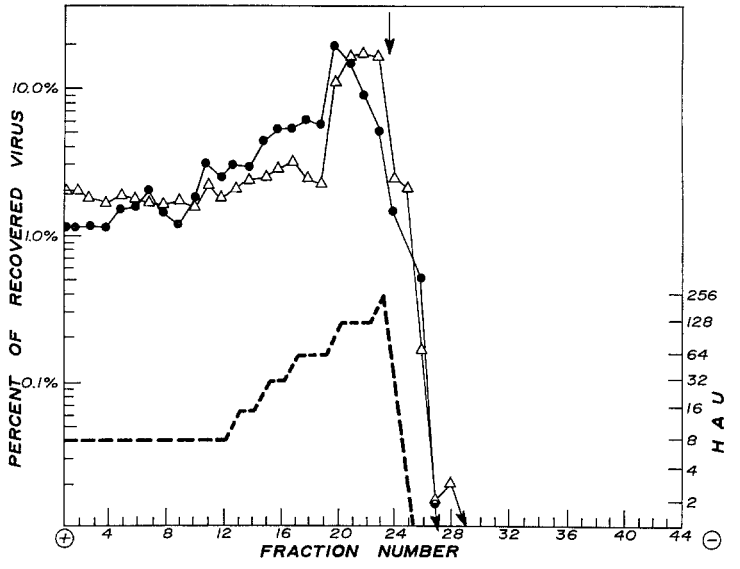

Fig. 3. Electrophoresis of a mixed population of Sindbis LP and SP virus variants in a Pevikon starch block. Arrow denotes the origin
●———● LP variant; △———△ SP variant; – – – Hemagglutinating units (HAU)

in the charge of the populations. In an experiment utilizing only the LP variant, infectious virus was isolated from the extreme anode end of the zone of migration and was used as seed for a new virus stock. Upon electrophoresis, the same heterogeneity was observed in the progeny as in the parental populations.

3.3. Isoelectric Focusing of Sindbis Virus

After preliminary studies with the LP variant, a mixed population of Sindbis LP and SP variants was subjected to isoelectric analysis. The LP variant was labeled with H^3-glycine and 1 ml, containing 2.4×10^8 PFU and 14,800 CPM, was mixed with 1 ml of the SP labeled with P^{32}, containing 1.8×10^8 PFU and 13,900 CPM. The results obtained by isoelectric focusing for 48 hours are shown in Figure 4. The peaks of isotope activity and infectivity for both variants were

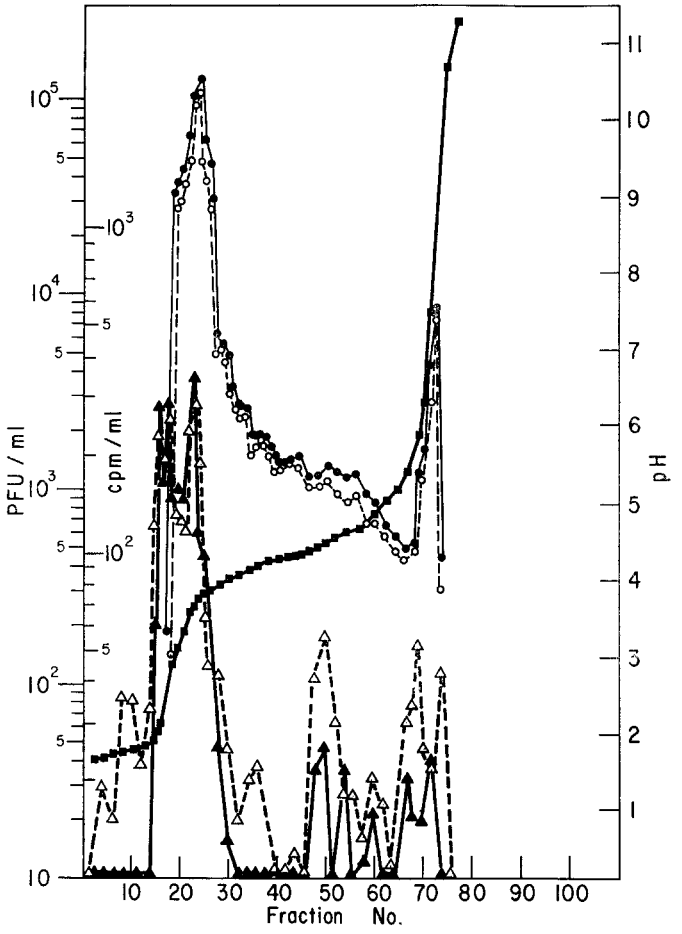

Fig. 4. Isoelectric focusing of a mixed population of Sindbis LP (H^3 glycine label) and Sindbis SP (P^{32} label) virus variants
△ – – –△ SP c.p.m./ml; ○ – – –○ SP PFU/ml; ▲———▲ LP c.p.m./ml; ●———● LP PFU/ml; ■———■ pH

located at pH 3.6. A minor peak of infectivity was located at pH 7.0. The variants were not separated by this technique and the net charge of the variants would appear to be equivalent. Uninfected fibroblasts were labeled with P^{32} and the results of isoelectric focusing of the plasma membranes showed a major peak, in uninfected cells, at pH 2.25 and a lesser peak at pH 3.6.

3.4. The Isoelectric Point of Antibody-Neutralized Virus

If the virus titer was reduced by 98.5% with anti-Sindbis IgG the major virus peak was found, as before, at pH 3.6, and a minor band was observed at pH 5.22 (Fig. 5). These results suggest that a majority of the virus-IgG complex had been

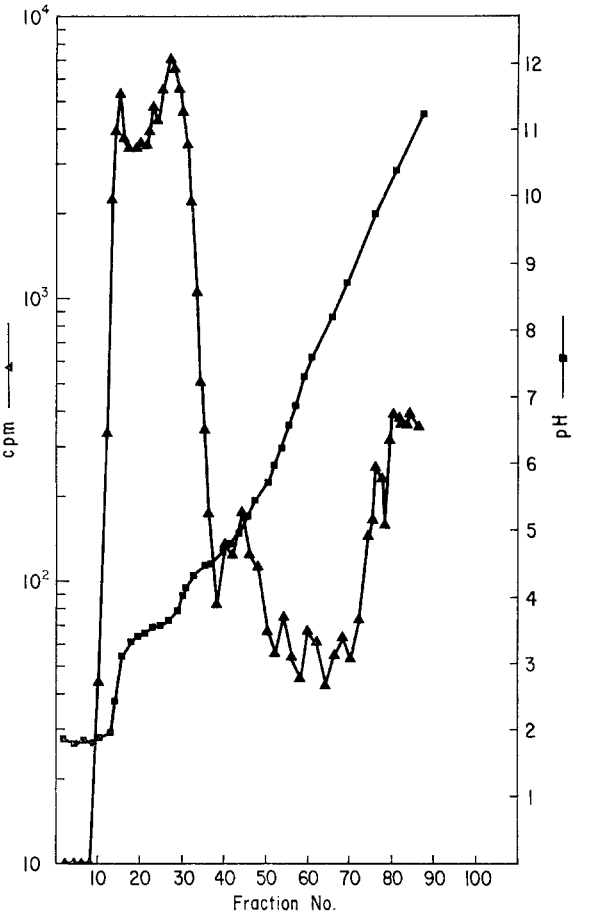

Fig. 5. Isoelectric focusing of P^{32}-labeled Sindbis SP neutralized by anti-Sindbis SP IgG

disassociated with no irreversible alteration of the net charge of the virus population. (The virus-IgG complex had been shown to disassociate when the neutralized virus was maintained at pH 3.6 for 24 hours.) The minor peak at pH 5.22 may represent that residual virus which remained complexed with serum IgG. When Fab fragments were employed in a similar experiment in order to avoid the more complex lattice effects encountered with intact IgG, only one peak, at pH 3.6, was observed. It is probable that the formation of lattices with complete antibody molecules served to maintain a small fraction of the virus-IgG complex even at low pH. This protection was apparently not afforded by the univalent Fab fragments which are incapable of forming a protective lattice.

4. Discussion

The SP variant of Sindbis virus was neutralized to a greater extent than was the LP variant. This differential neutralization was observed with whole serum, serum IgG, and isolated Fab fragments of IgG. We have found, in agreement with KLINMAN (17) and CREMER *et al.* (10) that the Fc fragment of serum IgG neither neutralizes the viruses tested nor interferes with the subsequent neutralization by serum antibody.

The differential neutralization of the two variants studied may reflect the relative structural stability of the two virus particles rather than different antibody binding capacities. Alternatively, the relative difference in the neutralization of the SP and LP variants of Sindbis virus may be a function of the greater sensitivity of the RNA of one variant to intracellular degradation. Studies with these plaque variants may provide such information by (a) the examination of infectious viral RNA extracted from the Fab-neutralized variants, (b) determination of the RNase sensitivity of the Fab-neutralized variants, (c) the subsequent membrane and/or lysosomal association of cell-associated Fab-neutralized particles.

As BOSE *et al.* (6) have demonstrated near identity of the constituent peptides of large and small plaque virions, the basis for differential elution from calcium phosphate columns, plaque variation, alteration of neuroinvasiveness may have to be sought at another, more subtle level than membrane peptides and antigenic determinants.

Plaque size variants of non-enveloped viruses such as polyoma (9, 36), foot-and-mouth disease (26), and encephalomyocarditis (2) have been studied by electrophoretic techniques, however in these investigations, the virus populations have been found to demonstrate considerable electrophoretic heterogeneity. Enveloped viruses characteristically have exhibited isoelectric points at low pH values (11, 22). As the membranes of the host cells used in our system demonstrated isoelectric peaks at pH 2.55 to 3.6, we feel that the nascent nucleocapsid may have a preferential affinity for those components of the cell plasma membrane which band at pH 3.6. We feel, too, that this peak, represents normal cell membrane components which influence the charge of the virus population when incorporated into the mature virion, or alternatively represents membrane moieties into which virus-induced protein may be substituted. It has been shown that Sindbis virus acquires lipids and glycopeptides from preformed cell material in the course of maturation (3, 7). The influence of the cell membrane upon the final charge of the virion is one obvious consequence of this action. This would explain the acquisition of its net charge by a virus population budding through those membranes. It would explain, too, the identity in charge of the large and small plaque variants of Sindbis virus. As other studies have also demonstrated an antigenic similarity in the lipid-containing envelope of plaque-size variants of vesicular stomatitis virus (27), it would appear that the selective mechanism which incorporates those virus-coded charged determinants of the host cell, gives rise to a rather stable, complex host-influenced virus-specific envelope.

Acknowledgements

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