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Antigenic Characterization of Flavivirus Structural Proteins Separated by Isoelectric Focusing

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Isoelectrofocusing of nonionic-detergent-disrupted flaviviruses separated the envelope glycoprotein of 53,000 to 58,000 daltons and the nucleocapsid protein of 14,000 daltons. The envelope protein and nucleocapsid protein were isolated at isoelectric points of pI 7.8 and 10.3, respectively. The antigenic determinants of St. Louis encephalitis, Japanese encephalitis, and dengue virus envelope and nucleocapsid proteins were examined by solid-phase competition radioimmunoassay. By the appropriate selection of antiserum and competing proteins, it was possible to distinguish type-specific, complex-reactive and flavivirus group-reactive antigenic determinants. The envelope glycoproteins of St. Louis encephalitis, Japanese encephalitis, and dengue viruses were found to contain each of these three classes of antigenic determinants. Most of the determinants on the envelope protein were type specific, some were complex reactive, and a small fraction were flavivirus group reactive. The nucleocapsid protein contained only flavivirus group-reactive antigenic determinants.

Purified flaviviruses contain three classes of antigenic determinants: type specific, complex reactive, and flavivirus group reactive. Type-specific antigens, which distinguish between different flaviviruses, have been studied by virus neutralization (7, 8, 18, 25, 40, 41) and hemagglutination inhibition (HI) with adsorbed sera (7-9). Complex-reactive antigens can be demonstrated by complement fixation (CF), immunodiffusion, or neutralization tests (7, 8, 13, 18, 25, 27, 40, 43). Japanese encephalitis (JE), St. Louis encephalitis (SLE) and West Nile viruses form one serocomplex of viruses, which share antigens that are immunologically distinct from viruses in the dengue or yellow fever serocomplexes (5). Each virus within a complex can be considered as a serotype containing type-specific determinants that react only with homologous antibody and complex-reactive antigens common to viruses within the complex (7-9). Antigenic cross-reactivity among the flaviviruses appears to be due to group-reactive antigens shared by all flaviviruses. These broadly reactive determinants have been demonstrated in CF tests with reagents that do not differentiate between different flaviviruses but clearly distinguish between togaviruses of the alpha-virus and flavivirus genera (4).

The major envelope protein of JE and SLE viruses, released from the virions or infected cells after detergent treatment, binds to erythrocytes (17, 34), reacts with antibodies in the CF and immunodiffusion tests (13, 17, 24, 42), and induces the formation of neutralizing antibodies (12, 26, 27). In both CF and immunodiffusion tests, this isolated glycoprotein is serologically broadly cross-reactive (19, 26, 27). Dengue virus envelope glycoprotein isolated by concanavalin A chromatography is type specific by CF and does not react with neutralizing antibodies (34). We now report additional studies on the antigenic properties of purified flavivirus proteins: the large glycosylated envelope protein with a molecular weight of 53,000 to 58,000, and the nucleocapsid protein with a molecular weight of about 14,000 (32-39, 41, 42). By using ^{125}I-labeled purified protein antigens in an inhibition solid-phase radioimmunoassay, we were able to analyze these viral components for each of their antigenic determinants. The envelope glycoprotein contains multiple antigenic determinants: strongly reactive type-specific and complex-reactive antigens, as well as the flavivirus group determinants. The flavivirus nucleocapsid protein is broadly cross-reactive within the flavivirus group, and its serological specificity appears comparable to group-reactive proteins of the alphaviruses (10, 11) and influenzavirus (21, 30).

MATERIALS AND METHODS

Viruses and cells. Virus strains used in these experiments were the Tampa Bay human-28 strain of SLE and the Nakayama, JaGAr-01, and Yokoshiba strains of JE viruses. Four prototype dengue

viruses were used: dengue 1 (DEN-1) (Hawaii), dengue 2 (DEN-2) (TR-1751), dengue 3 (DEN-3) (H-87), and dengue 4 (DEN-4) (H-241). Milligram quantities of SLE, JE, and dengue viruses were propagated in roller bottle cultures of porcine kidney (PS) cells or rhesus monkey kidney (LLC-MK_2) cells as previously described (38).

Preparation of purified virus. Tracer amounts of virus labeled with radioactive amino acids were prepared by incubating infected cells in medium 199 containing 1/10 the normal concentration of amino acids and 5% dialyzed calf serum. Mixtures of radioactive amino acids (New England Nuclear Corp.) were added to a final concentration of 10 μCi/ml for ^{3}H-labeled amino acids or 2 μCi/ml for ^{14}C-labeled amino acids at 12 h postinfection. After 48 h, the virus-containing media were pooled and clarified by centrifugation at 10,000 $\times$ g for 30 min. All purification procedures were carried out at 4°C. The virus was concentrated by glycol precipitation (23) and collected by centrifugation at 10,000 $\times$ g for 30 min. The precipitates were suspended in one-fifth the original volume of TNE buffer (0.01 M Tris [pH 8.0], 0.15 M NaCl, and 0.001 M EDTA), homogenized in a tissue grinder, and clarified by centrifugation at 5,000 $\times$ g for 10 min. The resulting supernatant fluid was layered over a discontinuous sucrose gradient (15 ml of 15% [wt/vol] sucrose in TNE buffer resting on 5 ml of 65% [wt/vol] sucrose in the same buffer) and centrifuged at 80,000 $\times$ g for 3.5 h in a Spinco SW27 rotor. The visible virus band at the interface of the two sucrose solutions was collected, diluted with an equal volume of TNE buffer, and layered on a 25-ml gradient of potassium tartrate (48%, wt/vol) and glycerol (30% vol/vol) in TNE buffer (24). Virus preparations were centrifuged to equilibrium at 25,000 rpm for 15 h, and the virus band was removed and recycled through a second similar isopycnic centrifugation. The virus band was collected and dialyzed for 3 h against 0.2 M Tris buffer (pH 8.0) to remove the potassium tartrate and glycerol.

Isoelectric focusing of viral structural proteins. Ten milligrams of purified flavivirus in 2 ml of 0.2 M Tris buffer (pH 8.0) was mixed with 100 mg of Triton N-101 and 0.1 M dithiothreitol, and the mixture was held at room temperature for 15 min. The solubilized virus was isoelectric focused on an LKB-8101 (110 M1) Ampholine column (LKB-Produckter AB). Linear 0 to 65% sucrose gradients were prepared with ultrapure sucrose (Schwarz/Mann), 1% Ampholine (LKB-Produckter AB), and 0.5% Triton N-101. Application of the virus sample to the gradient and conditions of electrophoresis were essentially the same as described by Dalrymple et al. (10). Column fractions were collected and immediately analyzed for pH and radioactivity. Peak fractions were pooled, dialyzed against 0.05 M Tris (pH 8.0) for 3 to 4 days to remove sucrose and ampholytes and to reduce the detergent concentration, and stored at −70°C. The protein concentration of the antigen preparations was determined on acetone-precipitated samples (21) and analyzed for purity by polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE. The discontinuous polyacrylamide gel system used was basically that described by Laemmli (20). The resolving gel contained 13% (wt/vol) acrylamide and 0.62% (wt/vol) N,N^1-diallytartardiamide. Before electrophoresis, proteins were concentrated by acetone precipitation, suspended in sodium dodecyl sulfate (SDS) sample buffer (0.0625 M Tris, pH 6.8; 1% SDS, 10% glycerol; 0.001% bromophenol blue; 0.1% 2-mercaptoethanol), and solubilized by heating at 100°C for 10 min. Gels were manually sliced into 1-mm sections, dispensed into vials, and incubated in scintillation cocktail at 56°C for 15 h. The liquid scintillation counting cocktail consisted of 4% NCS (Amersham Searle Corp.) and 0.4% Omnifluor (New England Nuclear Corp.) in scintillation-grade toluene.

Solid-phase radioimmunoassay and antiserum preparation. The solid-phase microtiter SPRIA test for detection of flavivirus antibodies was previously described (38). This procedure was been modified to provide a sensitive inhibition test in which antibody sites are blocked by increasing amounts of unlabeled antigen before the addition of a constant amount of ^{125}I-labeled antigen. Seventy-five microliters of rabbit antivirus immunoglobulin G (IgG) containing 500 μg of protein in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6) was placed in the wells of each Linbro U-bottom polyvinyl microtiter plate (Bellco Glass Inc.) that had been treated for cell culture growth. The plates were then incubated at 37°C for 2 h, the unadsorbed globulin was removed by aspiration, and the microtiter well was washed twice with phosphate-buffered saline (PBS) (12). To each well was then added 200 μl of PBS that contained 10% normal rabbit serum, and the plate was incubated for 1 h at 37°C. The unadsorbed rabbit serum was removed by aspiration, and the wells were washed three times. In the inhibition test, 50 μl of unlabeled antigen diluted in PBS was added in increasing amounts of antibody-coated wells, which were then incubated 37°C for 3 h and refrigerated at 4°C for 4 h. The unbound antigen was removed, and each well was charged with 50 μg of ^{125}I-labeled antigen in 50 μl of PBS containing 2% rabbit serum. The plates were incubated at 4°C overnight and washed 10 times with PBS, and the microtiter wells were separated with scissors and placed in scintillation vials. Radioactivity was measured in a Beckman 310 spectrometer. The average count from three replicate samples was determined, and the radioactivity bound without inhibitor minus the radioactivity bound with inhibitor was divided by the counts bound in the absence of inhibitor and multiplied by 100 to obtain the percent binding.

Hyperimmune mouse ascitic fluids were prepared by the method of Brandt et al. (2). Rabbit antisera to viral antigens were prepared as previously described (39). Rabbit anti-viral IgG globulin used to coat the solid phase was prepared by precipitation from whole serum with ammonium sulfate and chromatography on DEAE-cellulose (15, 39). Column fractions that contained IgG were concentrated to the original serum volume by ultrafiltration (Diaflo XM-50) and stored at −70°C.

Iodination of virus and isolated viral components. Virions and viral structural proteins isolated by isoelectric focusing were extrinsically iodinated

by a modification of previously described techniques (32). Iodination was achieved with lactoperoxidase (15 μg/500 μl) in the presence of H_2O_2 (1.0 M) and ^{125}I (500 μCi/500 μl). After incubation at 25°C for 15 min, a second portion of H_2O_2 was added and the reaction was continued for an additional 15 min. After iodination, the free iodine was separated from the antigens by filtration through a Sephadex G-25 column equilibrated in PBS with 0.0% Triton N-101. The peak fractions were pooled, and a portion was removed for trichloroacetic acid precipitation and counting of radioactivity.

Protein determination. Protein was determined by the modified Lowry method (22), with crystalline bovine serum albumin used as a standard.

RESULTS

Structural protein nomenclature. Flaviviruses contain three structural proteins originally designated V-1, V-2, and V-3 (Table 1) by various authors (33, 35, 39, 41, 42). Although this nomenclature does provide a general identification, it fails to describe any of the now known chemical, physical, or immunological properties of the proteins. To more completely describe the flavivirus structural proteins used in this study, we designate the envelope glycoproteins by the letters "gp" followed by the molecular weight in thousands. Virus abbreviations approved by the American Committee on Arthropod-Borne Viruses are used to designate individual viruses (14). The envelope glycoprotein of SLE virus is thus designated SLE gp 53 (E), and analogous JE and dengue virus subunits are designated JE gp 58 (E) and DEN gp 58 (E), respectively. Similarly, the nucleocapsid component shall be designated "p" 14 (N); SLE p 14 (N), JE p 14 (N), and DEN p 14 (N). The nonglycosylated membrane proteins of the flavivirus will be designated "p" 7 (M). Such a system of nomenclature is now widely used to describe proteins of the oncogenic RNA viruses and provides a concise and unambiguous method of describing viral structural components (1).

Isoelectric focusing of disrupted SLE virions. Radioactive SLE virions labeled with ^3H-amino acids disrupted with Triton N-101 and dithiothreitol were isoelectrically focused in a pH-range ampholine gradients of 5 to 8 and 9 to 11, respectively, without any substantial 7.8 and 10.3, respectively (Fig. 1). A minor peak of radioactivity was observed at pH 3.8. The two major peaks of radioactive protein focused at pH 7.8 and 10.3 were refocused on narrow-pH-range ampholine gradients of 5 to 8 and 9 to 11, respectively, without any substantial change in their isoelectric point.

For identifying the solubilized SLE viral proteins that were separated by isoelectric focusing, portions of protein that focused at pI 7.8 and 10.3 and purified virus that focused at pI 4.8 (data not shown) in sucrose-ampholine gradients were examined by PAGE (Fig. 2). Electropherograms of purified SLE virions labeled with ^{14}C-amino acids exhibited the three polypeptide patterns characteristic of flaviviruses: an envelope glycoprotein of 53,000 (gp 53), nucleocapsid protein of 14,000 (p 14), and membrane protein of approximately 7,000 (p 7). The viral protein that focused at pI 7.8 appeared as

TABLE 1. *Flavivirus structural protein nomenclature*

Virus	Previous nomenclature	Proposed nomenclature[a]
DEN-2	V-3	DEN-2 gp 58 (E)
	V-2	DEN-2 p 14 (N)
	V-1	DEN-2 p 7 (M)
SLE	V-3	SLE gp 53 (E)
	V-2	SLE p 14 (N)
	V-1	SLE p 7 (M)
JE	V-3	JE gp 58 (E)
	V-2	JE p 14 (N)
	V-1	JE p 7 (M)

[a] For an explanation of the nomenclature, see Results and reference 1.

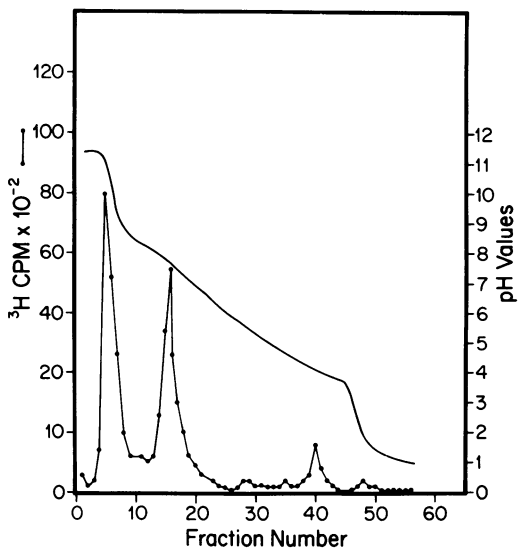

FIG. 1. *Isoelectric focusing profile of SLE viral proteins. Purified virus intrinsically labeled with ^3H-amino acids was disrupted with Triton N-101 and dithiothreitol and isoelectrically focused (pH 3 to 10) in the presence of 0.1% detergent. Electrophoresis was continued for 24 h at 900 V after the current dropped to less than 4 mA. Two-milliliter fractions were collected from the bottom of the column, and pH measurements were performed immediately. ^3H radioactivity* (●).

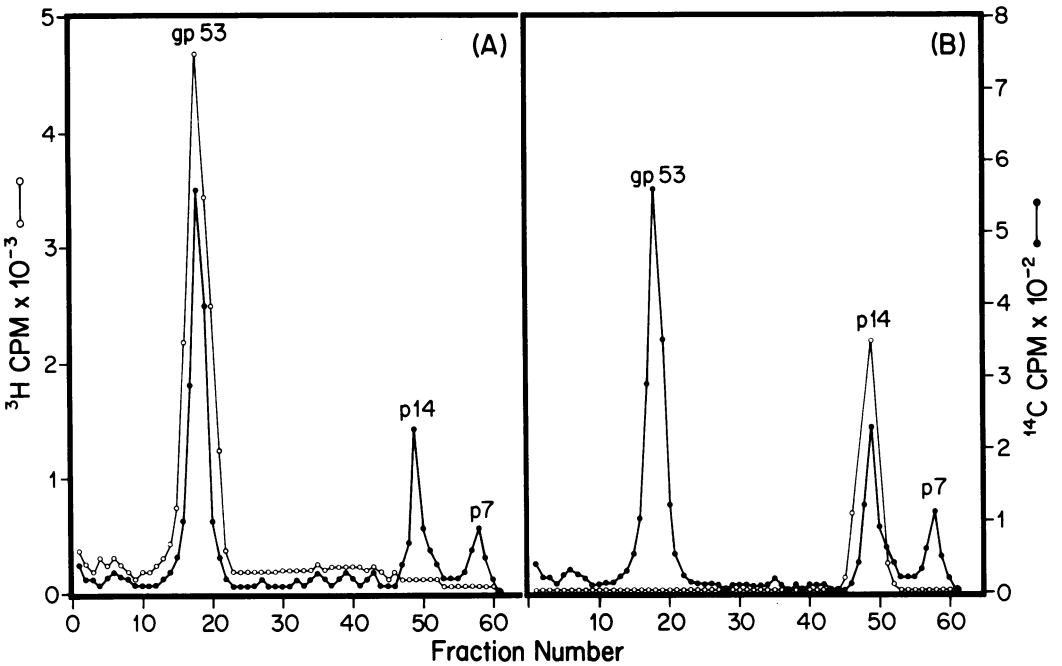

FIG. 2. *PAGE of SLE virions and proteins separated by isoelectric focusing. (A) Coelectrophoresis of ^{14}C-amino acid-labeled virion proteins and ^{3}H-amino acid-labeled SLE protein from pH 7.8 region of electrofocusing gradients; (B) coelectrophoresis of ^{14}C-amino acid-labeled virion proteins and ^{3}H-amino acid-labeled proteins from the pH 10.3 region of an electrofocus column separation of detergent-disrupted virus.*

a single polypeptide, which migrated in coincidence with the envelope glycoprotein gp 53 (Fig. 2A). The pI 10.3 protein migrated in coincidence with the nucleocapsid protein p 14 (Fig. 2B). The low-molecular-weight membrane protein p 7 was not detected in either the glycoprotein or nucleocapsid peaks, which appeared to be essentially free from contamination by each other and homogeneous by PAGE. The small amount of material that focused at pI 3.8 consisted of a mixture of envelope and nucleocapsid proteins that apparently were not dissociated by detergent treatment.

Dengue and JE virus nucleocapsid and envelope proteins that were isolated by isoelectric focusing of detergent-dissociated virus had pI values similar to those of SLE virus. Envelope glycoproteins JE gp 58 and DEN-2 gp 58 had isoelectric points of 7.6 and 7.8, respectively. The nucleocapsid proteins of these viruses had isoelectric points of 10.2 to 10.4. Intact JE and DEN-2 virions focused with pI values of 5.2 and 5.0, respectively. Isolated envelope glycoproteins of each virus studied did not hemagglutinate gander erythrocytes; however, they did bind to erythrocytes at pH values of 6.4 to 6.8 and serologically reacted in CF and immunodiffusion tests.

Serological reactivity of purified virions. The indirect-competition solid-phase radioimmunoassay test was used to antigenically differentiate SLE, JE, and DEN-2 virions. Purified SLE, JE, and DEN-2 virions were added in increments to wells coated with rabbit anti-SLE virus IgG. Adsorption of unlabeled virus was measured by adding 1.4 μg of ^{125}I-labeled SLE virus in 50 μl per well (Fig. 3). Differences between adsorption of SLE virus to the homologous bound antibody and the reactivity of heterologous JE and DEN-2 viruses were clearly evident. DEN-2 virus in concentrations of 0.1 μg to 10 mg was not appreciably adsorbed by anti-SLE IgG, whereas JE virus at high concentrations reacted with heterologous antibody to a limited extent. Homologous SLE virus at concentrations of 0.1 μg blocked the binding of labeled SLE indicator antigen by approximately 50%. Unlabeled JE virus at concentrations of 0.1 μg blocked ^{125}I-labeled SLE virus binding by about 31%. These results suggest that antigens on the surface of the SLE envelope contain specific antigenic determinants in addition to those complex-reactive components shared with HE but absent from DEN-2 virus.

Type-specific, complex-antigenic, and group-antigenic determinants of the flavivirus envelope protein. Antigenic analysis of the separated major envelope glycoprotein of SLE,

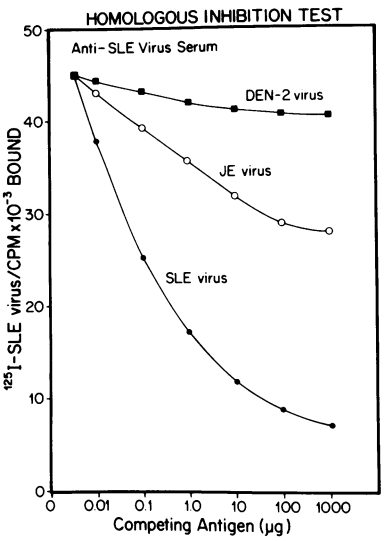

FIG. 3. *Analysis of serologically related flaviviruses by competition solid-phase radioimmunoassay. Comparison of the adsorption of SLE, JE, and DEN-2 virions to microtiter wells coated with anti-SLE IgG and subsequently incubated with saturating amounts of ^{125}I-labeled SLE virions.*

JE, and DEN-2 viruses was attempted with competition radioimmunoassay procedures. The experimental design consisted of reacting homologous antiserum and envelope glycoprotein in a binding assay and measuring the ability of increasing concentrations of heterologous virus envelope glycoprotein to interfere with the binding of homologous ^{125}I-labeled antigen. The ability of a heterologous virus glycoprotein to interfere with the binding of the radiolabeled envelope glycoprotein of the homologous virus was interpreted as evidence for complex- or group-reactive determinants on the competing molecule.

Such an experiment was carried out with labeled homologous SLE gp 53 and SLE antiserum. The competition of heterologous JE gp 58 and DEN-2 gp 58 was compared with that of the unlabeled homologous reaction of SLE gp 53 protein. Two characteristics of the competition assay are shown by the data presented in Fig. 4: (i) 5 μg of JE gp 58 protein, which gave maximal competition for the complex-reactive antibody, was approximately 100-fold greater than the amount of SLE gp 53 protein required for 53% competition of all antibodies; and (ii) the maximal competition by JE and DEN-2 virus proteins was about 30 and 10%, respectively.

To exclude the possibility that the JE and dengue antigens were immunologically inactive, we analyzed the competing proteins in a heterologous assay with labeled SLE indicator, using the same labeled JE and DEN-2 antigens and JE virus antiserum in place of SLE serum. In this system, only the group and complex-reactive determinants that JE gp 58 and SLE gp 53 antigen share should be reactive. Both SLE and JE gave equal and complete competition (Fig. 5), demonstrating the presence of comparable amounts of complex-reactive determinants, whereas DEN-2 gp 58 was not equally competitive. These results indicate that DEN-2 gp 58 contains few JE-SLE complex antigens but does exhibit flavivirus group antigens, which block the binding of indicator SLE antigen at high dengue competing protein concentrations.

To extend these findings and compare the JE and SLE proteins, we performed reciprocal experiments with ^{125}I-labeled JE gp 58 as the antigen and SLE and dengue glycoprotein as competitors. The results were analogous to those shown in the previous experiment (Fig. 5). Only a fraction of the anti-JE virus antibodies bound SLE gp 53 protein, and approximately threefold more SLE antigen than JE protein was required for 50% of the maximal observed competition. DEN-2 antigen at a concentration of 10 μg/ml bound less than 6% of the JE-SLE complex specific antibody present in the JE serum.

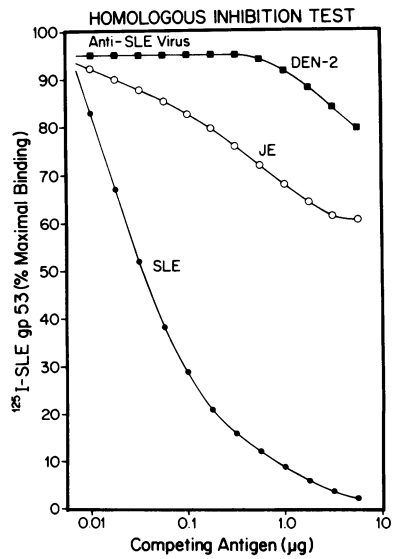

FIG. 4. *Analysis of type-specific, complex-reactive, and group-reactive flavivirus antigenic determinants on envelope glycoproteins by homologous competition solid-phase radioimmunoassay. Comparison of the adsorption of SLE, JE, and DEN-2 envelope glycoproteins to microtiter wells coated with anti-SLE IgG, which were subsequently incubated with saturating amounts of ^{125}I-labeled SLE gp 53 protein.*

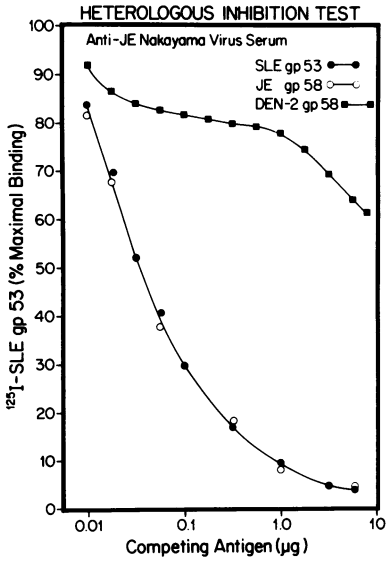

FIG. 5. *Analysis of complex-reactive and group-reactive antigenic determinants on the envelope protein by heterologous competition solid-phase radioimmunoassay. Comparison of adsorption of SLE, JE, and DEN-2 envelope glycoproteins to microtiter wells coated with anti-JE IgG, which were subsequently incubated with saturating amounts of ^{125}I-labeled SLE gp 53 protein.*

These experiments were repeated by adding Triton N-101-degraded virions as a competitor instead of purified envelope proteins. The results of these experiments were essentially the same as those shown in Fig. 4, except that 10- to 20-fold more purified viral polypeptide than dissociated virus was required to inhibit 50% maximal binding by the indicator SLE gp 53. This indicated either that the purified antigens were not as reactive as solubilized virus, or that other antigenic determinants on the membrane and nucleocapsid proteins were perhaps participating in the reaction. The data from these experiments did not permit resolution of these alternatives.

The envelope glycoproteins were further characterized by testing the competition of these proteins from different strains of JE virus and serotypes of dengue virus. Antigenic differences among the Nakayama, JaGar-01, and Yokoshiba strains of JE virus were analyzed by testing competition in homologous assay systems containing ^{125}I-labeled Nakayama gp 58 protein and homologous antiserum. The complex and type-specific antigens should be distinguished since inhibitions greater than that of SLE gp 53 would indicate type-specific determinants common to the different JE virus strains. Failure to compete for all of the antibody should similarly indicate the presence of strain-specific determinants.

The Nakayama, JaGAr-01, and Yokoshiba gp 58 proteins all showed strong competition for antibodies to the Nakayama protein (Fig. 6A), thus confirming the presence of type-specific determinants on the JE gp 58 protein that are shared by the different JE virus strains. Since there was an almost complete competition when a sufficiently high concentration of heterologous JE virus proteins was used, it appears that the majority of antibodies in the Nakayama serum were directed at the type-specific determinants. It was evident, however, that among the different JE virus strains the concentration of type-specific determinants or the affinity of antibodies for these determinants in the Nakayama serum was not the same since increased amounts of protein were required for competition in the order of Nakayama, JaGAr-01, and Yokoshiba.

Analogous results were obtained from an experiment with anti-JaGAr-01 serum and heterologous ^{125}I-labeled Nakayama gp 58 protein (Fig. 6A). In this case, the assay measured determinants common to the three viruses, and it would be expected that they would give similar competition curves. It appears that determinants common to the Nakayama and JaGAr-01 viruses were not equally shared with the Yokoshiba virus since a greater concentration of these viral proteins was required for complete competition. Differences among the type-specific determinants present on the JE virus gp 58 protein are either in the number of type-specific determinants per molecule or in their affinity for the antibody to Nakayama gp 53 protein.

Experiments were attempted to determine whether the concentration of complex-reactive determinants was the same on gp 53 proteins from the different JE virus strains. A heterologous inhibition test was used in which each of the gp 58 proteins from the three JE virus strains was tested for its ability to inhibit the SLE antibody-Nakayama envelope protein cross-reaction (Fig. 7). Each JE virus gp 58 protein was equally effective as a competitor in the heterologous assay measuring complex-reactive antigenic determinants. These results suggest that differences among the JE virus strains detected in the homologous competition assay (Fig. 6A and B) are due to variations in the type-specific determinants in the gp 58 protein. These variations in type-specific antigens that are not shared by other strains of the type virus could be classified as subtype determinants.

Studies similar to these carried out with gp 58 protein of JE virus were performed with the

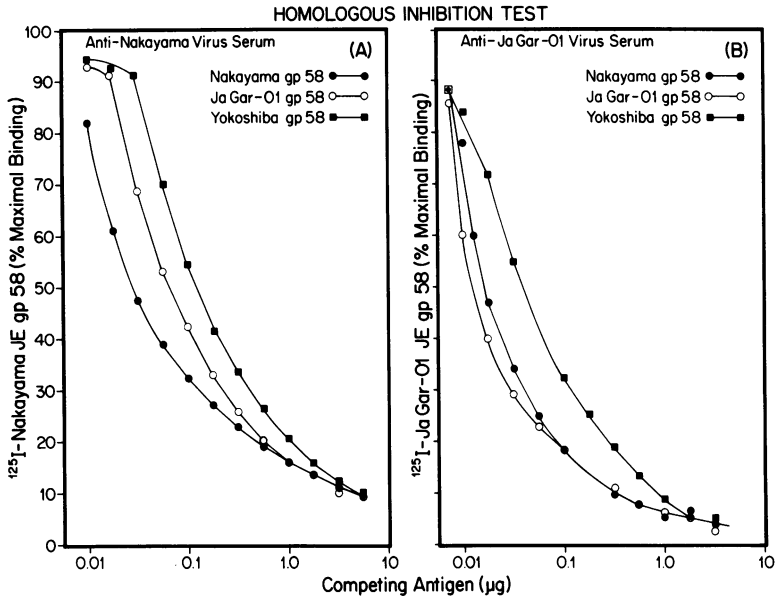

FIG. 6. *Analysis of type-specific and group-reactive antigenic determinants of JE gp 58 proteins by homologous competition solid-phase radioimmunoassay. (A) Comparison of the adsorption of Nakayama, JaGar-01, and Yokoshiba gp 58 proteins to microtiter wells coated with anti-Nakayama IgG and subsequently incubated with ^{125}I-labeled gp 58 Nakayama protein. (B) Comparison of adsorption of gp 58 proteins of JE virus strains to microtiter wells coated with anti-JaGar-01 IgG and subsequently saturated with ^{125}I-labeled gp 58 protein of JaGar-01.*

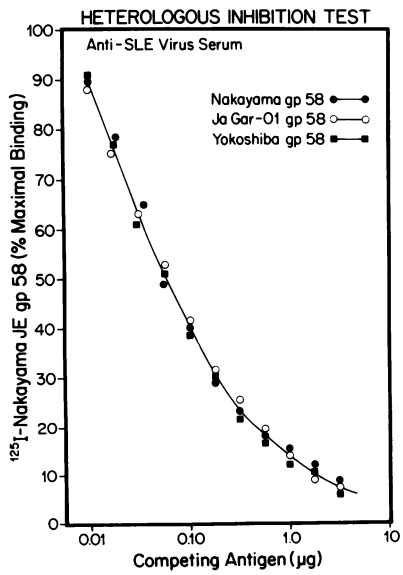

FIG. 7. *Analysis of complex-reactive antigenic determinants of JE gp 58 proteins by heterologous competition radioimmunoassay. Comparison of the adsorption of Nakayama, JaGar-01, and Yokoshiba gp 58 proteins to microtiter wells coated with anti-SLE IgG and subsequently saturated with ^{125}I-labeled Nakayama gp 58 protein.*

gp 58 envelope protein of dengue types 1 through 4.

In experiments carried out with the homologous assay system, ^{125}I-labeled DEN-2 gp 58 protein and anti-DEN-2 serum, the effects of competition by DEN-1, -2, and -3, and -4 were compared with the standard competition given by DEN-2 gp 58. As seen in Fig. 8A, a comparison of the DEN-2 protein competition with that of DEN-4 indicates that 20-fold more DEN-4 glycoprotein than DEN-2 glycoprotein was required to produce a 50% maximal competition with the ^{125}I-labeled DEN-2 indicator. By comparison, 18-fold more unlabeled DEN-1 protein and 2-fold more unlabeled DEN-3 protein were required to achieve 50% maximal competition than were required for the homologous DEN-2 reaction. SLE virus gp 53 protein at a concentration of 50 μg inhibited the homologous DEN-2 precipitation by 12%, indicating the reaction of flavivirus reactive determinants. It thus appears that the minor antigenic component present in the DEN-2 envelope is a group-reactive determinant that is also present in SLE and JE viruses. Complex and type-specific determinants of DEN-2 gp 58 protein were also revealed in this experiment. The more-effective competition by DEN-3 gp 58 protein than by

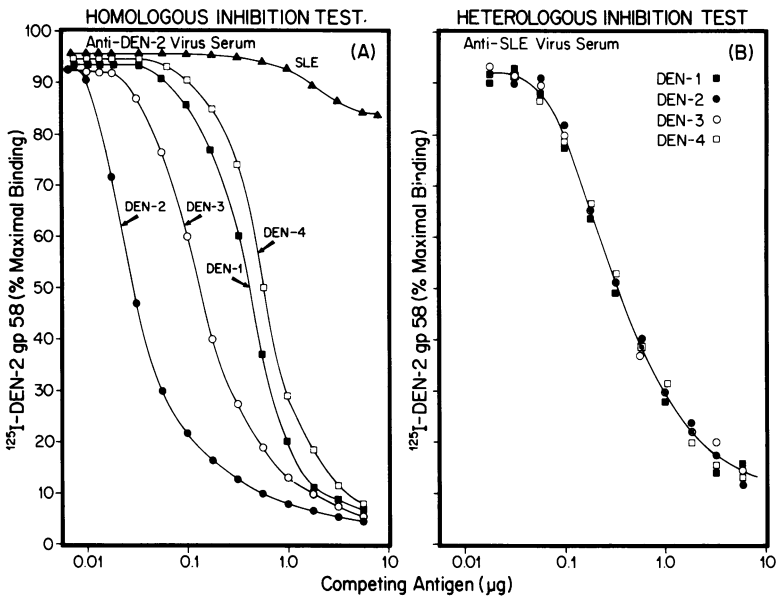

FIG. 8. *Analysis of type-specific, complex-reactive, and group-reactive antigenic determinants of DEN gp 53 protein from serotypes 1 through 4 by homologous and heterologous competition radioimmunoassay. (A) Comparison of the adsorption of DEN-1, -2, -3, and -4 gp 58 proteins to microtiter wells coated with anti-DEN-2 IgG and subsequently saturated with ^{125}I-labeled DEN-2 gp 58 protein. (B) Reactions of gp 58 proteins from dengue serotypes 1 through 4 with anti-SLE IgG bound to the solid phase and subsequently saturated with ^{125}I-labeled DEN-2 gp 58 protein.*

SLE clearly indicated the presence of dengue complex-specific determinants. The same competition curves give evidence that DEN-2 type-specific antigens are also present; greater amounts of DEN-3 than DEN-2 were required for the competition, and even high concentrations of DEN-3 gp 58 protein failed to compete for all of the antibodies binding the DEN-2 protein.

All of the envelope glycoproteins used in the dengue virus experiments were examined in the heterologous assay system for the presence of group-reactive antigens. There were no differences in the competition of the four dengue serotypes in their reaction with antibodies in the SLE serum (Fig. 8B), which indicates that the concentration of flavivirus-reactive determinants present in the envelope of all four serotypes of the dengue virus is the same.

Antigenic determinants of the flavivirus nucleoprotein. A series of experiments was done to analyze and characterize the antigenic determinants present on the nucleocapsid proteins of SLE, JE, and DEN-2 viruses. Radioactive, purified p 14 proteins of each of the viruses were prepared by isoelectric focusing of detergent-dissociated virions. A competition RIA experiment was carried out with ^{125}I-labeled SLE p 14 protein and anti-SLE serum in which we compared the competition of purified DEN-2 and JE p 14 proteins with the standard competition by unlabeled SLE p 14 (Fig. 9). Both JE and DEN-2 competing proteins gave equal and complete competition with the SLE nucleocapsid. This indicated that the nucleocapsid proteins contained no type-specific or complex-reactive determinants, but demonstrated that they contained equal amounts of group-reactive antigens.

DISCUSSION

Treatment of SLE, JE, and dengue virions with Triton N-101 and dithiothreitol dissociated the structural proteins, which were then separated by isoelectric focusing. Isoelectric focusing has been used to separate JE virus nonstructural CF antigen from other viral proteins (13). Dalrymple et al. (10) recently reported the use of a similar procedure for isolating alphavirus structural proteins in an antigenically active form. Ultracentrifugation of nonionic detergent-treated flaviviruses permits separation of the nucleocapsid from the glycoprotein and membrane but does not facilitate separation of the viral envelope components (19, 33, 34, 37, 38). SDS solubilization of JE virus permitted chromatographic separation of the envelope glycoprotein but did not resolve the membrane

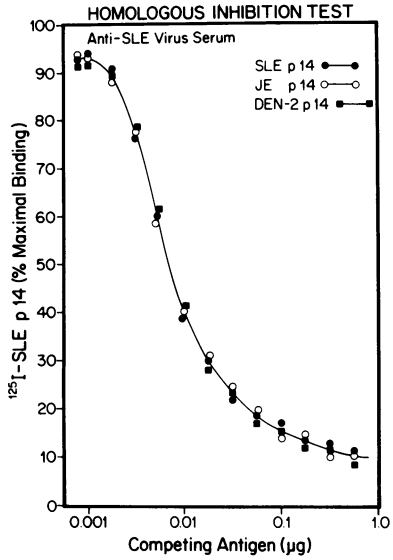

FIG. 9. *Analysis of group-reactive antigenic determinants of flavivirus nucleoprotein p 14. The competition radioimmunoassay was performed by incubating SLE, JE, and DEN-2 p 14 proteins with anti-SLE IgG-coated microtiter wells and subsequently with saturating amounts of ^{125}I-labeled SLE gp 53 protein.*

and nucleocapsid proteins (13). Treatment of JE virus with SDS destroyed hemagglutinating activity; however, the isolated glycoprotein was reactive by CF and immunodiffusion (13). Concanavalin A chromatography of detergent-solubilized antigen separated a form of DEN gp 58 that antigenically did not bind neutralizing antibody and reacted as a type-specific antigen by CF (34). Isoelectric focusing of flaviviral proteins that had been solubilized with nonionic detergent facilitated the isolation of purified antigens that biologically and antigenically are like intact virions, bind to erythrocytes, react by CF, immunoprecipitate, and are immunologically reactive in the solid-phase radioimmunoassay.

The observation that the envelope glycoprotein of flaviviruses contains at least three different antigenic determinants indicates that these proteins are immunologically complex. The antigenic cross-reactivity of isolated JE gp 58 and SLE gp 53 proteins by CF and immunodiffusion was reported previously (13, 26, 27). From our radioimmunoassay data, we previously suggested that the envelope glycoprotein contains group-reactive and type-specific antigens (38). Westaway et al. (40, 43) used different techniques, and their results also suggested that the surface glycoproteins have multiple antigenic sites. JE and dengue virus soluble complement-fixing nonstructural proteins contain both group-reactive and type-specific antigenic determinants (3, 13, 28).

From the data reported here it can be assumed that the differences in the amounts of competing protein required for 50% maximal competition are proportional to differences in the concentration of different antigens rather than differences in the affinity of antibody for the protein. These data indicate that there are equal amounts of complex-reactive and group-reactive antigens on the virus glycoproteins of each virus. This observation indicates that there is one primary sequence in the flavivirus envelope glycoprotein that is invariant and that a portion of that sequence is shared by different viruses. The variant portion of the glycoprotein composes the major portion of the molecule antigenically recognized as the type-specific antigen.

Flaviviral proteins are not unique in having multiple antigenic determinants on a single molecule as the major internal protein, and envelope glycoprotein of mammalian C-type RNA viruses also have multiple antigens (36). Antibody to the E_1 protein of Sindbis virus, which hemagglutinates, is cross-reactive with Western equine encephalitis virus (10), yet the alphavirus kinetic HI test permits antigenic distinction of closely related agents (4, 45). These data suggest that E_1 alaphavirus protein has both type-specific and complex-reactive antigens. The envelope glycoprotein and nucleocapsid proteins of SLE, dengue, and JE viruses share at least one common group antigen as measured by competition radioimmunoassay and immunodiffusion (13). The group determinants on these two proteins are probably responsible for the broad serological cross-reactivity observed upon repeated immunization or after multiple flavivirus infections (6, 7, 9, 16, 17, 37, 43, 44). Nucleoproteins of the alphaviruses (10, 11), influenzavirus (21, 30), and rabies group viruses (31), like the flaviviruses, contain group-reactive determinants.

The presence of complex-reactive and type-specific antigens on the flaviviruses glycoprotein explains the serological complexity of the immune response. Neutralizing antibody induced by the flavivirus glycoprotein is produced to the type antigen and is expressed in the HI reaction (7–9, 16, 17). Infection with one member of a complex induces complex-reactive HI antibodies, which do not provide immunity to infection with a second member of the complex (29, 40, 44). Adsorption of sera with a heterologous antigen makes the HI reaction type specific (8, 9, 16, 17). This indicates that the complex-reactive and type-specific determinants on the envelope glycoprotein are involved in the

flavivirus HI reaction. Variations within the type determinant differentiate viruses within a serocomplex and strains of virus that differ antigenically from the prototype (16, 17, 24, 25). Our studies with strains of JE virus confirm earlier antibody adsorption and HI and CF immunotyping of these strains (17, 24, 25) and clearly show variations within the JE type-specific determinants (25).

The structures of alphavirus and flavivirus envelopes are obviously quite different. The alphavirus membrane contains two surface glycoproteins distinctly different immunologically, chemically, and biologically (10, 11). The flavivirus envelope contains only one glycoprotein on its surface, which has multiple antigenic determinants and biological functions (19, 25-27, 33, 35, 39, 41-43). The diversity in alphavirus and flavivirus virion structure reflects basic differences in the mechanisms of viral-directed biosynthesis and morphogenesis. We are currently investigating the immunological specificities of the flavivirus proteins and the structural relationships of the envelope glycoproteins.

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LITERATURE CITED

1. **August, J. T., D. P. Bolognesi, E. Fleissner, R. V. Gilden, and R. C. Nowinski.** 1974. A proposed nomenclature for the virion proteins of oncogenic RNA viruses. Virology 60:595-601.
2. **Brant, W. E., E. L. Buescher, and F. M. Hetrick.** 1967. Production and characterization of arbovirus antibody in mouse ascitic fluid. Am. J. Trop. Med. Hyg. 16:339-347.
3. **Cardiff, R. D., W. E. Brandt. T. G. McCloud, D. Shapiro, and P. K. Russell.** 1971. Immunological and biophysical separation of dengue-2 antigens. J. Virol. 7:15-23.
4. **Casals, M. D.** 1964. Antigenic variants of eastern equine encephalitis virus. J. Exp. Med. 119:547-565.
5. **Casals, J.** 1957. The arthropod-borne group of animal viruses. Trans. N.Y. Acad. Sci. 19:219-235.
6. **Casals, J.** 1963. Relationships among arthropod-borne animal viruses determined by cross-challenge tests. Am. J. Trop. Med. Hyg. 10:587-596.
7. **Clark, D. H.** 1960. Antigenic analysis of certain group B arthropod-borne viruses by antibody adsorption. J. Exp. Med. 111:21-32.
8. **Clark, D. H.** 1962. Antigenic relationships among viruses of the tick-borne encephalitis complex as studied by antibody adsorption and agar gel precipitation techniques, p. 67-75. *In* H. Libikova (ed.), Biology of viruses of the tick-borne encephalitis complex. Proceedings of the Symposium of the Cezchoslovak Academy of Sciences, vol. 3. Academic Press Inc., New York.
9. **Clark, D. H.** 1964. Further studies of antigenic relationships among viruses of the group B tick-borne complex. Bull. W.H.O. 31:45-56.
10. **Dalrymple, J. M., S. Schlesinger, and P. K. Russell.** 1976. Antigenic characterization of two Sindbis virus envelope glycoproteins separated by isoelectric focusing. Virology 69:93-103.
11. **Dalrymple, J. M., S. N. Vogel, A. Y. Teramoto, and P. K. Russell.** 1972. Antigenic components of group A arbovirus virions. J. Virol. 12:1034-1042.
12. **Dulbecco, R., and M. Vogf.** 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. 99:167-182.
13. **Eckels, K. H., F. M. Hetrick, and P. K. Russell.** 1975. Virion and soluble antigens of Japanese encephalitis virus. Infect. Immun. 11:1053-1060.
14. **Executive Committee, The American Committee on Arthropod-Borne Viruses.** 1969. Arbovirus names. Am. J. Trop. Med. Hyg. 18:731-734.
15. **Fahey, J. L., and A. P. Horbett.** 1959. Human gamma globulin fractionation on anion exchange cellulose columns. J. Biol. Chem. 234:2645-2651.
16. **Hammon, H. M., D. H. Clarke, and W. H. Price.** 1965. Antigenic variation of West Nile virus in relation to geography. Am. J. Epidemiol. 82:40-55.
17. **Hammon, H. M., and W. H. Price.** 1966. Further observations on geographic variation in the antigenic character of West Nile and Japanese B viruses. Am. J. Epidemiol. 83:113-122.
18. **Hammon, W. McD., and G. E. Sather.** 1964. Problems of typing dengue viruses. Mil. Med. 129:130-135.
19. **Kitano, T., K. Suzuki, and T. Yamaguchi.** 1974. Morphological, chemical, and biological characterization of Japanese encephalitis virus virion and its hemagglutinin. J. Virol. 14:631-639.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
21. **Lief, F. S., and W. Henle.** 1956. Studies on the soluble antigen of influenza virus. I. The release of S antigen from elementary bodies by treatment with ether. Virology 2:753-771.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
23. **McSharry, J., and R. Beneinger.** 1970. Concentration and purification of vesicular stomatitis virus by polyethylene glycol "precipitation." Virology 40:745-779.
24. **Obijeski, J. F., A. T. Marchenko, D. H. L. Bishop, B. W. Cann, and F. A. Murphy.** 1974. Comparative electrophoretic analysis of the virus proteins of four rhabdoviruses. J. Gen. Virol. 22:21-33.
25. **Okuno, T., T. Okada, A. Kondo, M. Suzuki, M. Kabayashi, and A. Oya.** 1968. Immunotyping of different strains of Japanese encephalitis virus by antibody adsorption and complement-fixation tests. Bull. W.H.O. 38:547-563.
26. **Qureshi, A. A., and D. W. Trent.** 1973. Group B arbovirus structural and nonstructural antigens. I. Serological identification of Saint Louis encephalitis virus soluble antigens. Infect. Immun. 7:242-248.
27. **Qureshi, A. A., and D. W. Trent.** 1973. Group D arbovirus structural and nonstructural antigens. III. Serological specificity of solubilized intracellular antigens. Infect. Immun. 8:993-999.
28. **Russell, P. K., D. Chiewsilp, and W. E. Brandt.** 1970. Immunoprecipitin analysis of soluble complement-fixing antigens of dengue viruses. J. Immunol. 105:838-845.
29. **Sather, G. E., and W. McD. Hammon.** 1960. Protection against Saint Louis encephalitis and West Nile arboviruses by previous dengue virus (type 1-4) infection. Proc. Soc. Exp. Biol. Med. 135:573-578.
30. **Schild, G. C., and H. G. Pereira.** 1969. Characteristics of the ribonucleoprotein and neuraminidase of influenza A viruses by immunodiffusion. J. Gen. Virol. 4:355-363.

31. **Schneider, L. G., B. Dietzchoid, R. E. Dierrs, W. Matthaeus, P. J. Enzmann, and K. Strohmaier.** 1973. Rabies group-specific ribonucleo-protein antigen and a test system for grouping and typing rhabdoviruses. J. Virol. 11:748–755.
32. **Sefton, B. M., G. G. Wickus, and B. Burge.** 1973. Enzymatic iodination of Sindbis virus proteins. J. Virol. 11:730–735.
33. **Shaprio, D., W. E. Brandt, R. D. Cardiff, and P. K. Russell.** 1971. The proteins of Japanese encephalitis virus. Virology 44:108–124.
34. **Stholman, S. A., O. R. Eylar, and C. L. Wisseman, Jr.** 1976. Isolation of the dengue virus envelope glycoprotein from membranes of infected cells by concanavalin A chromatography. J. Virol. 18:132–140.
35. **Stollar, V.** 1969. Studies on the nature of dengue viruses. IV. The structural proteins of type 2 dengue virus. Virology 39:426–438.
36. **Strand, M., and J. T. August.** 1974. Structural proteins of mammalian oncogenic RNA viruses: multiple antigenic determinants of the major internal protein and envelope glycoprotein. J. Virol. 13:171–180.
37. **Tignor, H. G., and W. H. Price.** 1971. Antibody responses in spider monkeys following single and double infectious with group B arboviruses. Am. J. Epidemiol. 94:386–396.
38. **Trent, D. W., C. L. Harvey, A. Qureshi, and D. LeStourgeon.** 1976. Solid-phase radioimmunoassay for antibodies to flavivirus structural and nonstructural proteins. Infect. Immun. 13:1325–1333.
39. **Trent, D. W., and A. A. Qureshi.** 1971. Structural and nonstructural proteins of Saint Louis encephalitis virus. J. Virol. 7:379–388.
40. **Westaway, E. G.** 1965. The neutralization of arboviruses. I. Neutralization in heterologous virus-serum mixtures with four group B arboviruses. Virology 26:528–537.
41. **Westaway, E. G.** 1975. The proteins of Murray Valley encephalitis virus. J. Gen. Virol. 27:283–292.
42. **Westaway, E. G., and B. M. Reedman.** 1969. Proteins of group B arbovirus Kunjin. J. Virol. 4:688–693.
43. **Westaway, E. G., M. Shew, and A. J. Della-Porta.** 1975. Reactions of purified hemagglutinating antigens of flaviviruses with 19S and 7S antibodies. Infect. Immun. 11:630–634.
44. **Wisseman, C. L., Jr., B. H. Sweet, M. Kitaoka, and T. Tamiya.** 1962. Immunological studies with group B arthropod-borne viruses. I. Broadened neutralizing antibody spectrum induced by strain 17D yellow fever vaccine in human subjects previously infected with Japanese encephalitis virus. Am. J. Trop. Med. Hyg. 11:550–561.
45. **Young, N., and K. M. Johnson.** 1969. Antigenic variants of Venezuelan equine encephalitis virus: their geographic distribution and epidemiologic significance. Am. J. Epidem. 89:286–307.