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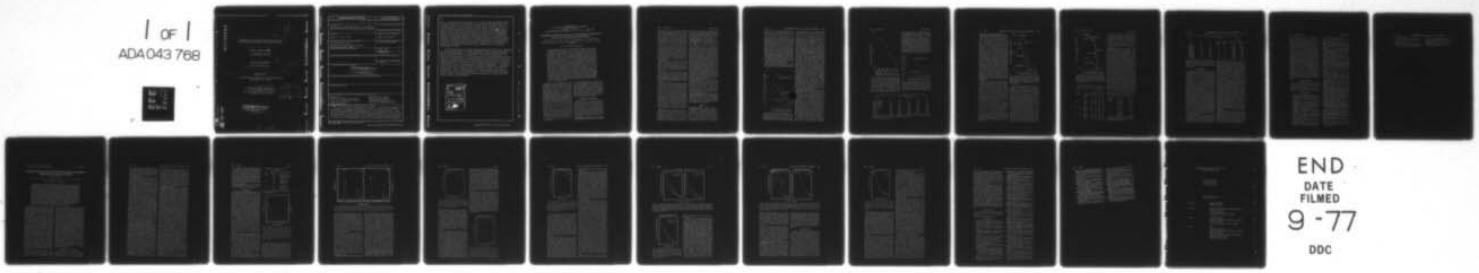

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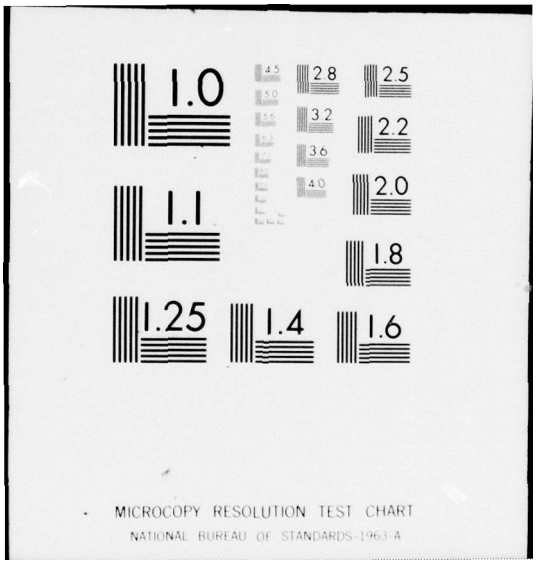
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A micro-solid-phase radioimmunoassay (SPRIA) is described for quantitation of antibodies to purified flaviviruses as well as to the purified envelope glycoprotein and 80,000-molecular-weight viral nonstructural protein. Sera from mice experimentally infected with Saint Louis encephalitis (SLE) virus or from humans after a primary SLE virus infection reacted more specifically with the major viral envelope protein in the SPRIA test than with antigens conventionally used in the complement fixation (CF) and hemagglutination inhibition

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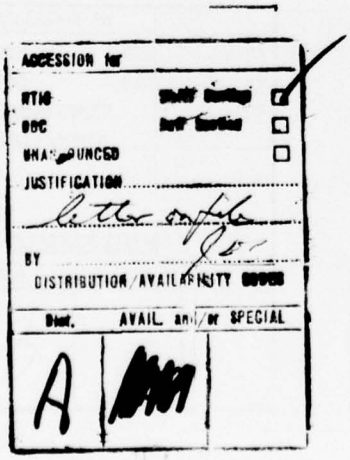
tests. A high degree of correlation ($P < 0.05$) was observed between SPRIA antiimmunoglobulin G binding values with the 80,000-molecular-weight nonstructural protein of SLE virus and antibody titers obtained by plaque reduction neutralization and CF with the nonstructural protein. In five of seven human sera in which CF antibody titers to the nonstructural protein were 4 or less, SPRIA testing revealed significant titers of IgG immunoglobulin reactive with this viral protein. The SPRIA test for antibodies reactive with group B togavirus nonstructural protein is as specific and sensitive as the plaque reduction neutralization test for titrating viral antibody in human and animal sera. Antibodies reactive with viral envelope proteins are broadly cross-reactive by the SPRIA technique, demonstrating both group- and complex-reactive antigenic determinants. The SPIRA test, using wells precoated with antigen, can be adapted to use in testing a large number of sera.

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.



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Solid-Phase Radioimmunoassay for Antibodies to Flavivirus Structural and Nonstructural Proteins

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A micro-solid-phase radioimmunoassay (SPRIA) is described for quantitation of antibodies to purified flaviviruses as well as to the purified envelope glycoprotein and 80,000-molecular-weight viral nonstructural protein. Sera from mice experimentally infected with Saint Louis encephalitis (SLE) virus or from humans after a primary SLE virus infection reacted more specifically with the major viral envelope protein in the SPRIA test than with antigens conventionally used in the complement fixation (CF) and hemagglutination inhibition tests. A high degree of correlation ($P < 0.05$) was observed between SPRIA anti-immunoglobulin G binding values with the 80,000-molecular-weight nonstructural protein of SLE virus and antibody titers obtained by plaque reduction neutralization and CF with the nonstructural protein. In five of seven human sera in which CF antibody titers to the nonstructural protein were 4 or less, SPRIA testing revealed significant titers of IgG immunoglobulin reactive with this viral protein. The SPRIA test for antibodies reactive with group B togavirus nonstructural protein is as specific and sensitive as the plaque reduction neutralization test for titrating viral antibody in human and animal sera. Antibodies reactive with viral envelope proteins are broadly cross-reactive by the SPRIA technique, demonstrating both group- and complex-reactive antigenic determinants. The SPRIA test, using wells precoated with antigen, can be completed in 1 day, providing a rapid, highly sensitive test which can be adapted to use in testing a large number of sera.

Serodiagnosis and seroepidemiology of group B arbovirus (togavirus) infections are often complicated by the development of antibodies that exhibit extensive cross-reactions with group-reactive antigens present in conventional serological reagents (2, 11, 14-16, 22). Type-specific serodiagnosis is usually possible in primary infections by plaque reduction neutralization (PRNT); however, identification of the infecting agent by the more rapid hemagglutination inhibition (HI) or complement fixation (CF) tests is frequently difficult because of the development of antibodies that are broadly cross-reactive (11, 12, 14, 23, 26, 28). During secondary infections with related flavi-viruses, patients often develop antibodies that are equally reactive by HI, CF, and PRNT, with conventional antigens representing flaviviruses to which the patient has never been exposed (11, 12, 14, 15, 22, 25, 26). The need for type-specific antigens and rapid highly sensitive techniques for measuring virus-specific antibodies has stimulated the development of a solid-phase radioimmunoassay (SPRIA) procedure using purified flavivirus proteins. We report here homologous and heterologous antibody reactions between St. Louis encephalitis, (SLE), Japanese encephalitis (JE), and West Nile (WN) viruses, isolated viral envelope proteins, and nonstructural antigens using the SPRIA procedure and have correlated these results with conventional serological analysis of serum antibody from SLE patients.

MATERIALS AND METHODS

Viruses. Virus strains used in these experiments were the Tampa Bay Human-28 strain of SLE, the Ar-248 strain of WN, and the Nakayama strain of JE viruses obtained from the Center for Disease Control, Atlanta, Ga. The New Guinea B strain of dengue-2 (DEN-2) virus, adapted to replicate in cultures of LLC-MK_2 cells, were obtained from Leon Rosen of the Pacific Research Section, National Institutes of Health, Honolulu, Hawaii. Viruses were propagated in either porcine kidney (PS) or rhesus monkey kidney (LLC-MK_2) cells as previously described (18, 19).

Preparation of solubilized antigens from infected cells. Infected PS or LLC-MK_2 cells were suspended

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and washed three times with phosphate-buffered saline (PBS) containing calcium and magnesium ions (7) and resuspended to 10% (wt/vol) in 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 7.8. The cell suspension was frozen and thawed three times, and Triton X-100 was added to give a final concentration of 0.5%. The mixture was held at 4 C for 1 h and sonicated at 20 kcycles for 5 min, and the particulate material was removed by successive centrifugation at 27,000 × *g* for 20 min and at 100,000 × *g* for 1 h at 4 C. Solubilized antigens in the supernatant fluid were concentrated fivefold by ultrafiltration (Diaflo XM-50) and dialyzed overnight against tris(hydroxymethyl)aminomethane buffer. Purification of solubilized antigens from the crude cell culture antigen suspension was accomplished by ion exchange chromatography and organic solvent extraction as previously described (18, 19). Virions were purified from cell culture fluids by polyethylene glycol precipitation and repeated density gradient centrifugation in sucrose and potassium tartrate gradients (18).

Antisera. Antisera to group B arboviruses (togaviruses) were prepared in young adult mice using Sarcoma 180 cells for the induction of ascites (18). Mice were inoculated subcutaneously with a 20% suspension of virus-infected mouse brain suspension on days 1 and 3, and intraperitoneally on days 25 and 28. Ascitic fluids were harvested on day 45 and stored at −70 C.

Young adult New Zealand white rabbits were injected subcutaneously in each flank with 0.5 ml of a 20% suspension of infected mouse brain (ISMB) and simultaneously in each footpad with 0.20 ml of complete Freund adjuvant. Secondary subcutaneous injections of ISMB were given after 21 days, followed by test bleedings and ISMB injections at 10-day intervals.

Rabbit heavy-chain specific antiserum to mouse immunoglobulin G (IgG) and gamma-chain-specific antisera to human IgG were purchased from Behring Diagnostics, Somerville, N.J. The IgG fraction of goat anti-human IgG and anti-mouse IgG sera was prepared by precipitation from whole serum with 0.5 volume of saturated ammonium sulfate solution (pH 7.6). After centrifugation at 10,000 × *g* for 30 min at 4 C, the precipitate was resuspended in an equal volume of one-half-saturated, neutralized ammonium sulfate. The precipitate was collected by centrifugation and resuspended in 0.002 M phosphate buffer, pH 8.0. The precipitated globulin was dialyzed against this phosphate buffer for 2 to 5 days at 4 C with frequent changes. The globulin was further purified by chromatography on diethylaminoethyl-cellulose (9). Fractions that contained IgG were concentrated to the original serum volume by ultrafiltration (Diaflo XM-50) and stored at −70 C.

Iodination of proteins. Purified IgG-specific immunoglobulins were iodinated with radioactive ^{125}I according to the procedure of Hunter and Greenwood (13), as modified by Purcell et al. (17). Iodinated globulin was applied to a column (1.5 by 50 cm) packed with Sephadex G-25 and eluted with PBS that contained 0.1% sodium azide and 10% normal rabbit serum (PBSRS). Fractions contained in the first peak of radioactivity were pooled, diluted with an equal volume of normal rabbit serum, and stored at 4 C. Antibody preparations obtained by this procedure had an average specific activity of 10 μCi of globulin protein per μg. Just before use, the labeled antiglobulin was diluted in PBSRS to a final concentration of 50,000 counts/min per 50 μl.

Microtiter SPRIA. The microtiter SPRIA techniques of Purcell et al. (17) and Rosenthal et al. (21) were modified for use with Linbro U-bottom polyvinyl microtiter plates, which had been treated for cell culture growth (Bellco Glass, Inc.). Seventy-five microliters of rabbit antivirus IgG immunoglobulin containing 500 μg of protein in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6, were placed in the bottom of each microtiter well. The plate was incubated at 4 C for 4 h, the unadsorbed globulin was removed by aspiration, and the wells were washed twice with PBS. To each well was then added 200 μl of PBSRS, and the plate was incubated for 1 h at 37 C. The unadsorbed rabbit serum solution was removed, and 50 μl of virus antigen diluted in PBS was added to each well. The plates were then incubated at 4 C overnight. Unadsorbed viral antigen was removed by aspiration, and 50 μl of specimen antibody diluted in PBSRS was added to each well and the plates were incubated at 37 C for 4 h. Unreacted antibody was removed by aspiration, the wells were washed twice with PBS, and 50 μl of ^{125}I-labeled anti-IgG or anti-IgM globulin was placed in each well. Plates were then incubated at 37 C for 4 h. Unbound ^{125}I-labeled antiglobulin was removed and, after being washed three times with PBS, the microtiter wells were separated with scissors and placed in tubes and assayed for radioactivity in a Beckman 310 gamma spectrometer.

Nonspecific binding of ^{125}I-labeled antiglobulin was determined by control tests in which six normal human sera or mouse ascitic fluids, shown not to contain virus antibody by PRNT, were incubated in triplicate wells, which received viral antigen. Serum titers were determined and expressed as the reciprocal of the serum dilution at which 50% of the maximal specific binding of ^{125}I-labeled antiglobulin occurred. All serum titers are expressed as the average of triplicate determinations.

Antiviral antibody titrations by other methods. PRNT tests for SLE virus antibodies were performed as described by Dalrymple et al. (3) using monolayers of PS cells. CF tests for titration of antigen and antibody were carried out by the LBCF procedure (27). Hemagglutination (HA) and HI tests were done as described by Casals and Clark (2).

RESULTS

The indirect SPRIA. Unpurified SLE virus-solubilized intracellular antigens, purified intracellular viral envelope protein (antigen I), and purified nonstructural viral protein (antigen III) (18, 19) were immunologically bound to the solid phase. Tenfold serial dilutions of mouse anti-SLE hyperimmune ascitic fluid were prepared, and antibodies in each dilution reacted with antigen bound to the solid phase.

The amount of ^{125}I-labeled anti-mouse IgG that reacted with viral antibody bound to the solid phase was largely proportional to the concentration of antibody in the ascitic fluid at dilutions between 1,000 and 100,000 (Fig. 1). A marked prozone was usually observed at serum dilutions lower than 1:100. The data indicated that antibody of the IgG class in ascitic fluid reacted specifically with each of the viral antigens at serum dilutions greater than 1,000. The 50% end point titers in the ascitic fluid for antibodies to the SLE envelope protein, nonstructural protein, and crude antigen were 6,000, 10,000, and 4,000, respectively. Although 50% end point titers were similar, maximum binding of iodinated antiglobulins was consistently lower when purified antigens were used in the test. Fifty percent antibody end point titers to the purified nonstructural antigen were usually slightly higher than those obtained with crude antigens or envelope protein.

From preliminary investigations, it was clear that not only the type of antigen, but also the amount of viral antigen used to sensitize the immunoglobulin-coated solid phase, played an important role in the sensitivity of the test. The amount of viral antigen necessary for optimal sensitivity in antibody titrations and the antigenic reactivity of different antigen preparations in the test were determined (Fig. 2). A purified SLE virion preparation, which contained 6 mg of protein, 2048 HA units, and 3×10^8 plaque-forming units/ml was serially diluted and used to sensitize a series of wells coated with SLE virus immune globulin. The data are expressed as the counts per minute of anti-mouse IgG bound to the plate when a dilution of 1:1,000 of hyperimmune mouse ascitic fluid was reacted with different concentrations of antigen bound to the solid phase. The amount of radioactive indicator antiglobulin that reacted with the mouse IgG bound to the solid phase decreased linearly when the amount of antigen used to sensitize the solid phase decreased from 64 to 8 CF units of viral antigen (Fig. 2). At virus antigen concentrations greater than 256 HA units, representing 64 CF units and 4×10^8 plaque-forming units, the counts per minute of antiglobulin that bound to the solid-phase fixed antigen did not increase. Three other purified virion preparations and three soluble antigens were evaluated for both specificity and reactivity by using this procedure (Fig. 2). All six of the preparations tested for antigenic reactivity gave ^{125}I-labeled antiglobulin binding values close to the straight line previously established for purified virion antigens. Tests using antigens at concentrations lower than 8 HA units were more variable than those using 256 HA units, which appeared to saturate the immunologically reactive sites on the buond antigen.

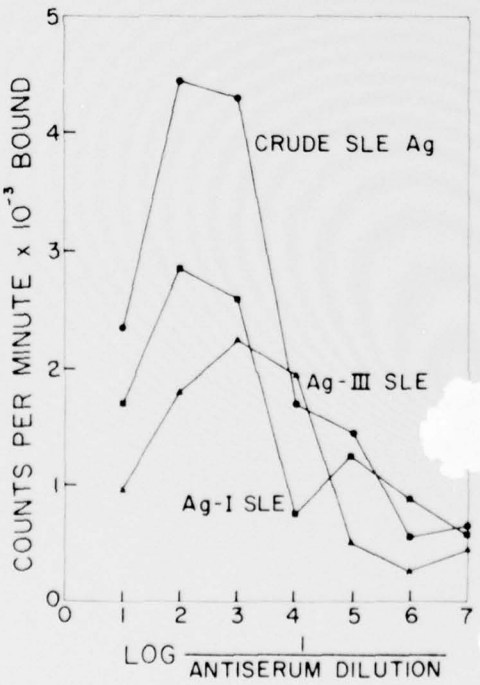

FIG. 1. *Determination of binding activity of a mouse anti-SLE ascitic fluid with SLE antigens by the SPRIA procedure. The lines represent Brij-58 detergent-solubilized antigens from infected cells (●), purified intracellular nonstructural protein (▲), and purified intracellular envelope glycoprotein (■).*

The relationship between the 50% serum dilution SPRIA titers and the HA units of virion antigen used in the test was determined. RIA titers of both human and mouse sera e approximately twofold higher when anti- n equivalent to 8 HA units was used in the est (Table 1). Use of more than 8 HA units of antigen did not substantially change the SPRIA 50% end point. The total amount of radioactivity bound to the solid phase when less than 16 HA units were used was quite low, necessitating long counting periods to obtain significant values (Fig. 2). From these observations it was concluded that, for best results, immunoglobulin titrations should be done using 32 HA units of purified virus antigen or 16 CF units of solubilized purified viral protein.

The specificity of the ^{125}I-labeled anti-IgG reaction with serum IgG that had reacted with viral antigens on the solid phase is shown in Fig. 3. Serial twofold dilutions of unlabeled

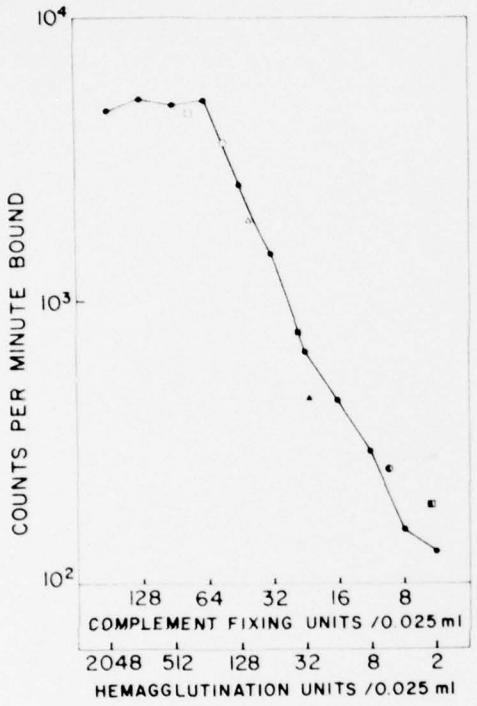

FIG. 2. *Correlation of antigenic mass used to sensitize the solid phase with antiviral globulin binding. Seven separate preparations of SLE virus with varying specific activities were compared in the SPRIA test at a constant 1:1,000 dilution of SLE antiserum. A single preparation was examined at twofold dilutions of the virus (●). The HA activity of the virus or CF activity of the solubilized SLE antigen preparations were as follows: (○) 77 HA units; (△) 54 HA units; (◗) 9 complement-fixing units (CFU); (□) 80 CFU; (▲) 20 CFU; (■) 25 CFU; and (▮) 6 CFU.*

rabbit anti-mouse IgG or human IgG serum were added to wells coated with immunologically bound SLE virus, and then ^{125}I-labeled anti-mouse IgG was added. It can be seen that at dilutions of 1:20 or less unlabeled anti-mouse serum inhibited binding of labeled anti-IgG immunoglobulin by 82 to 92%. An inhibition of approximately 50% was produced by a 1:80 dilution of the unlabeled anti-IgG serum. Anti-human IgG did not significantly block the reaction of ^{125}I-labeled anti-mouse IgG at any dilution. These data indicate that the reaction of ^{125}I-labeled anti-IgG with serum IgG that had previously been reacted with viral antigen on

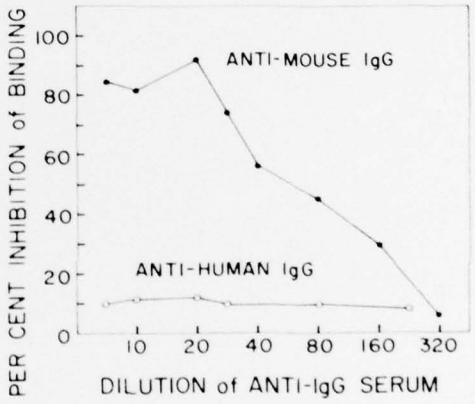

FIG. 3. *Determination of the specificity of the anti-IgG reaction for IgG bound to the solid phase. Serial twofold dilutions of anti-mouse or anti-human IgG serum were added to wells immunologically coated with SLE virus prior to addition of ^{125}I labeled anti-mouse IgG. Data are expressed as percentage of binding obtained in wells not treated with anti-IgG globulin.*

TABLE 1. *Effect of antigen concentration on the SPRIA 50% end point titration of antibody*

Serum	50% SPRIA end point titer[a]				
	4[b]	8	16	32	64
Human, SWD/18	450	950	1,050	1,000	1,150
Human, SWD/24	400	780	820	850	825
Human, R1614	925	1,270	1,250	1,200	1,175
Human, R1728	940	2,680	2,650	2,580	2,500
Human, R1423	350	900	875	920	950
Mouse, 1	2,500	4,700	4,650	4,570	4,400
Mouse, 2	4,000	6,200	6,100	6,050	6,000
Mouse, 3	3,200	7,200	7,300	7,100	7,000
Mouse, 4	7,000	11,000	9,000	10,050	9,400
Mouse, 5	6,200	12,600	13,500	12,700	13,500

[a] Titer expressed as the serum dilution at which 50% of the maximal binding of ^{125}I-labeled antiglobulin occurs using the specific amount of antigen indicated in the test.

[b] HA units of purified virus used.

the solid phase was highly specific.

Homologous and heterologous SPRIA reactions. A comparison of SPRIA titrations for IgG immunoglobulins in homologous and heterologous ascitic fluids reactive with SLE and JE virions immunologically bound to the solid phase is presented in Fig. 4 and 5. For this series of experiments, all ascitic fluids were adjusted to have homologous PRNT of 10,000. Homologous and heterologous reactions with SLE virions (Fig. 4) indicated that homologous reactions occurred at serum dilutions that gave 50% end point titers approximately 10-fold higher than did end point reactions with the heterologous sera. The amount of antiviral IgG in heterologous JE and WN ascitic fluids of equal homologous antibody activities, which reacted with SLE virions fixed to the solid phase, was usually of equal magnitude at lower serum dilutions. Antibodies of the IgG class in DEN-2 hyperimmune ascitic fluid did not react extensively with SLE virions, indicating a difference in heterologous group-reactive antigenic determinants present on the surface of JE subgroup viruses. The subgroup determinants are apparently either absent or present in only small amounts in the DEN-2 virion. The 50% serum dilution end point for the DEN-2 ascitic fluid using SLE virions as the heterologous antigen was about the same as that obtained for the JE and WN sera.

Reactions of purified JE virions with IgG antibodies in homologous and heterologous sera are presented in Fig. 5. The amounts of homologous and heterologous IgG that bound to the JE virions at serum dilutions of 1:100 to 1:10,000 were approximately equal. The 50% end point SPRIA titers for antivirus in the SLE and WN sera were equal and approximately 5- to 10-fold less than that of the anti-JE IgG reaction with homologous JE virion. IgG antibodies in DEN-2 serum reacted at very low levels with JE virions bound to the solid phase. The reactions of anti-DEN-2 IgG with SLE virions (Fig. 4) and JE virions (Fig. 5) are very similar.

These observations suggest that the high-level cross-reactions observed by the SPRIA test at low serum dilutions between SLE, JE, and WN viruses are measuring complex-reactive antigens, which have determinants common to viruses in the JE-WN serological complex. Low-level cross-reactions observed between JE and SLE viruses with DEN-2 at low serum dilutions could therefore be designated as flavivirus group-reactive determinants. Alphaviruses and Bunyaviruses do not cross-react with flaviviruses by the SPRIA technique (unpublished data).

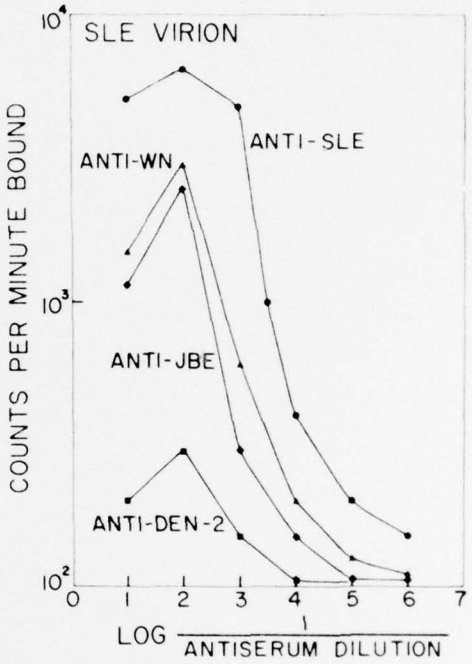

FIG. 4. *Determination of homologous and heterologous SPRIA reactions of SLE virions. Serial 10-fold dilutions of immune ascitic fluids prepared against SLE, JE, WN, and DEN-2 viruses were tested against 32 HA units of SLE immunologically bound to the solid phase.*

Using the 50% serum dilution end point of ^{125}I-labeled anti-IgG binding as an estimate of antibody titer, homologous and heterologous SPRIA tests using solubilized envelope proteins of SLE, JE, WN, and DEN-2 were compared with antibody titers obtained by conventional HI, CF, and virus neutralization tests. The data presented in Table 2 indicate that the SPRIA test using solubilized group B viral envelope protein is at least 10-fold more sensitive for assay of viral antibody than either the HI or CF test. The SPRIA test is as sensitive as viral neutralization for detection of flavivirus antibody and approximately as specific as the HI test for IgG-reactive immunoglobulin.

The specificity and sensitivity of the SPRIA for detecting IgG immunoglobulins reactive with the flavivirus nonstructural protein were determined (18–20). Sera used in these tests were collected from mice 15 days after challenge with 2×10^5 plaque-forming units of purified SLE, JE, or WN viruses (Table 3). The reaction of immunologically bound virus nonstructural protein (column 1) with IgG present

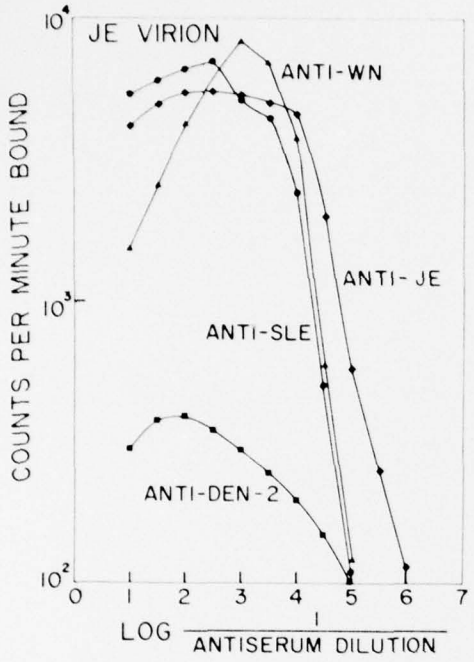

FIG. 5. *Determination of homologous and heterologous SPRIA reactions of JE virions. Serial 10-fold dilutions of immune ascitic fluids prepared against JE, SLE, WN, and DEN-2 viruses were tested against 32 HA units of JE virus immunologically bound to the solid phase.*

TABLE 2. *Comparison of the sensitivity and specificity of the SPRIA with conventional serology using purified group B virus envelope glycoprotein*

Antiserum	Test	Antibody titer against:			
		SLE	JE	WN	DEN-2
Anti-SLE	SPRIA	10,500	6,200	7,400	3,200
	PRNT	8,000	40	20	<10
	CF	128	32	16	8
	HI	512	128	128	64
Anti-JE	SPRIA	8,700	12,700	9,400	5,600
	PRNT	160	6,000	80	20
	CF	32	128	32	16
	HI	128	256	64	16
Anti-WN	SPRIA	5,900	5,200	7,200	5,300
	PRNT	80	40	8,000	40
	CF	16	32	128	16
	HI	128	128	256	8
Anti-DEN-2	SPRIA	1,800	2,100	2,600	6,400
	PRNT	10	40	20	4,200
	CF	64	64	128	512
	HI	128	128	64	1,024

in the mouse sera (column 2) was assayed using ^{125}I-labeled anti-mouse IgG sera. Reactions of SLE, JE, and WN nonstructural antigen preparations with IgG antibody in homologous sera were approximately 40-fold greater than with antibodies in sera from mice infected with heterologous viruses. Antibodies in the sera to each of the nonstructural proteins were relatively specific and did not show low-level cross-reactions.

Radioimmunoassay and serological correlations. Antibody titers to purified viral antigens were determined by the SPRIA test, using polyvalent anti-human IgM and IgG indicators, and compared to titers obtained by conventional CF, HI, and neutralization tests. Sera used in this study were collected from patients with documented cases of SLE virus infection approximately 1 year after the diagnosed acute infection. Results presented in Table 4 show a high degree of correlation ($P < 0.05$) between HI and neutralization titers with SPRIA titers using the nonstructural proteins in the test. CF titers with envelope glycoprotein did not show a significant correlation with the other serological data. Antibodies to SLE envelope protein and nonstructural protein were demonstrable by both CF and radioimmunoassay in 7 of the 10 sera examined. CF antibody to both envelope glycoprotein and the nonstructural viral protein were present in high titer only in sera that had neutralization titers greater than 760. In two convalescent sera, 79 and 207, CF antibodies to envelope protein were present without significant CF or radiochemically detectable antibody to the nonstructural protein. The lack of correlation between CF-reactive antibody with HI or neutralizing antibody of either IgG

TABLE 3. *Analysis of the sensitivity and specificity of the SPRIA test using the 80,000-molecular-weight flavivirus nonstructural protein*

Flavivirus nonstructural protein	Antiviral serum	SPRIA titer[a]
SLE	Anti-SLE	10,500
	Anti-JE	140
	Anti-WN	230
JE	Anti-SLE	260
	Anti-JE	15,600
	Anti-WN	320
WN	Anti-SLE	230
	Anti-JE	210
	Anti-WN	15,200

[a] Micro-SPRIA titers expressed as the reciprocal of the dilution at which 50% of the maximal ^{125}I-labeled antiglobulin was bound.

TABLE 4. *Comparison of the SPRIA with conventional serology for detection of SLE virus antibody in convalescent human sera*

Patient no.	HI	PRNT	CF		SPRIA	
			SLE envelope glycoprotein	SLE nonstructural protein	SLE envelope glycoprotein	SLE nonstructural protein
SWD-17	80	926	16	4	12,500	16,000
SWD-22	80	870	32	16	32,000	16,000
SWD-31	10	640	4	4	8,000	8,000
SWD-37	40	460	4	4	16,000	4,000
SWD-50	40	480	4	<4	4,000	1,000
SWD-68	160	1,270	32	16	64,000	32,000
SWD-79	40	380	16	<4	4,000	500
SWD-91	20	420	4	4	8,000	1,000
SWD-207	10	327	32	<4	2,500	500
SWD-239	80	760	16	16	32,000	8,000

or IgM antibody detected by radioimmunoassay indicated that these sera, collected approximately 1 year after SLE virus infection, contained negligible CF antibody of either the IgM or IgG class.

DISCUSSION

Serological diagnosis of flavivirus infection is usually established on the basis of CF, HI, and neutralization tests using unpurified antigen preparations derived from infected suckling mouse brain (1, 2, 9, 10, 14). These tests exhibit varying degrees of group- and type-specific reactivity (1, 9, 10, 14, 15). The immunological interpretation of classical serological results are therefore often complicated by the intragroup antigenic cross-reactivity observed between antigenically related flavivirus present in the same geographic region (9, 13, 14, 19). The preparation of purified flavivirus CF antigens has given impetus for investigation of antibody responses to specific viral proteins in order to establish means for more specific serodiagnosis of infection. These purified proteins only possess CF activity, which severely limits their usefulness as serological reagents, because CF antibodies develop later than HI and neutralizing antibodies and decline to barely detectable levels within a few months after acute infection and only IgG-class immunoglobulins fix complement with group B arbovirus (togavirus) antigens (11, 14, 20, 23). The SPRIA test has resolved these problems and has provided a system for quantitation of virus antibodies using structural and nonstructural proteins as purified antigens.

Immunoglobulins in mouse and human sera that reacted with viral envelope glycoprotein by SPRIA were cross-reactive, indicating the presence of group- and complex-reactive determinations as well as type-specific determinants on the virion surface. Usually, heterologous SPRIA reactions with envelope proteins occurred at lower serum dilutions than did homologous reactions. The antigenic makeup of the group B virus envelope protein appears to include group-, complex-, and type-specific determinants. The SPRIA test appears to differentiate clearly between viruses in distinct serological complexes and not between members of the complex. The flavivirus SPRIA is not as specific as the radioimmunoprecipitation test for alphavirus virions or envelope proteins (3, 4). The SPRIA analysis of mouse and human patient sera for antibodies to the nonstructural flavivirus nonstructural protein confirms our previous results, which indicated that this protein is antigenically type specific (17–19). CF and SPRIA antibody titers to the nonstructural protein were lower than those reactive with the envelope protein, indicating that the major immunological response is directed toward the virion surface antigens. Antibodies to DEN virus soluble nonstructural protein (SCF) are found in convalescent sera from patients with secondary DEN infections but not in sera collected from patients with primary DEN (10). In both SLE and DEN infections, antibodies to nonstructural protein were found at low levels, indicating that these proteins are poor immunogens or that insufficient amounts of the nonstructural viral proteins are released from the infected cell to effectively stimulate antibody production.

The SPRIA test for immunoglobulins of the IgG class in sera from convalescent human SLE patients has a high correlation with both HI and PRNT tests (Table 3). Less correlation was observed between ^{125}I-binding values and serum titers determined by the CF test using envelope proteins or virions. Daugharty et al. (5) reported that solid- and liquid-phase ra-

dioimmunoassay for influenza antibody correlated well with titers measured by the HI test but not with titers determined by the CF test. Mumps virus radioimmunoassay titers obtained with anti-IgG indicator globulin correlated well with HI and PRNT test results; however, the data most closely correlated with CF analysis (6). The specificity and sensitivity of the liquid-phase radioimmunoprecipitation test for IgG antibody to group A togaviruses showed a high correlation between radioimmunoprecipitation and PRNT titers. The radioimmunoprecipitation and the SPRIA tests for group A arbovirus (togavirus) (3) and rabies virus (29) antibodies and results described in this report for flavivirus antibody indicate that titers obtained by immunochemical procedures are approximately 10-fold higher than those obtained by PRNT and 50- to 100-fold higher than those obtained by the HI test. The SPRIA test described here is highly sensitive, with a viral specificity intermediate to that of the HI and PRNT test when virions or enveloped proteins are used and as specific as PRNT when type-specific antigens are used. The technique is simple and rapid and could be used to measure viral reactive immunoglobulins of different classes.

ACKNOWLEDGMENTS

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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 × *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 × *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 × *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 × *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'*-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.

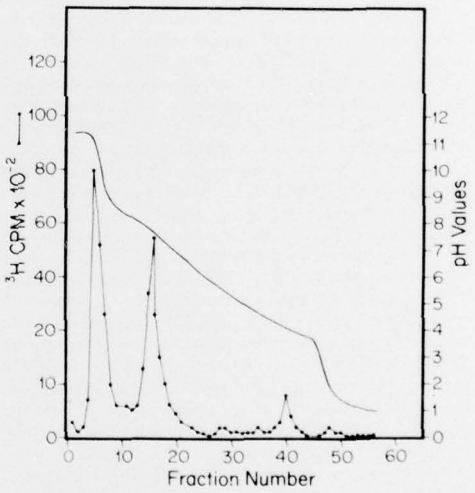

FIG. 1. *Isoelectric focusing profile of SLE viral proteins. Purified virus intrinsically labeled with ^3H-amino acids was disrupted with Triton N-101 and dithiothreitol and isoelectrically focused (pH 3 to 10) in the presence of 0.1% detergent. Electrophoresis was continued for 24 h at 900 V after the 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 (●).*

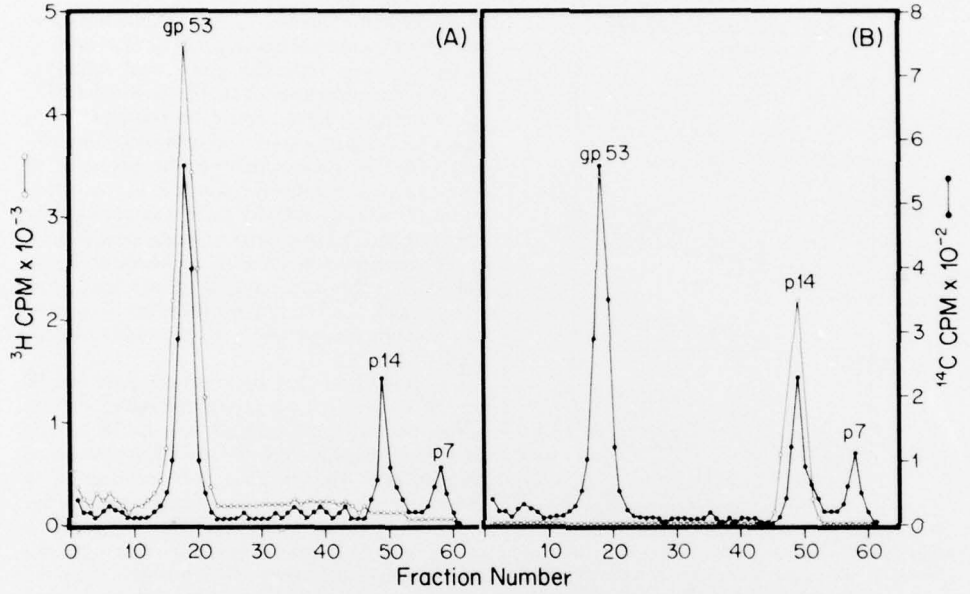

FIG. 2. *PAGE of SLE virions and proteins separated by isoelectric focusing. (A) Coelectrophoresis of ^{14}C-amino acid-labeled virion proteins and ^{3}H-amino acid-labeled SLE protein from pH 7.8 region of electrofocusing gradients; (B) coelectrophoresis of ^{14}C-amino acid-labeled virion proteins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

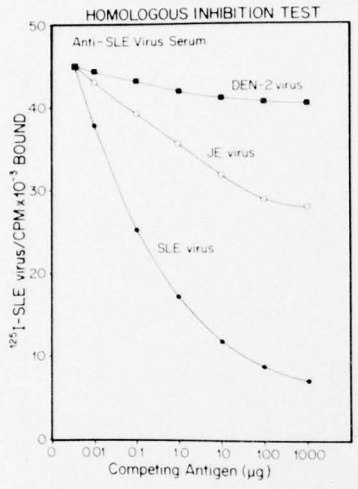

FIG. 3. *Analysis of serologically related flaviviruses by competition solid-phase radioimmunoassay. Comparison of the adsorption of SLE, JE, and DEN-2 virions to microtiter wells coated with anti-SLE IgG and subsequently incubated with saturating amounts of ^{125}I-labeled SLE virions.*

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.

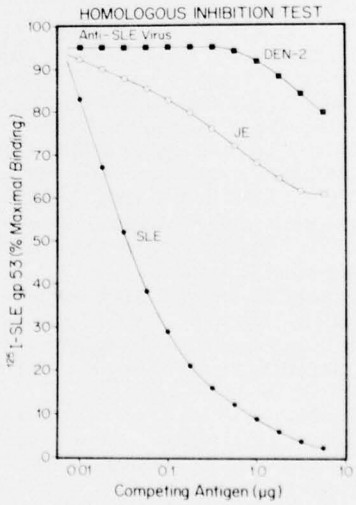

FIG. 4. *Analysis of type-specific, complex-reactive, and group-reactive flavivirus antigenic determinants on envelope glycoproteins by homologous competition solid-phase radioimmunoassay. Comparison of the adsorption of SLE, JE, and DEN-2 envelope glycoproteins to microtiter wells coated with anti-SLE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

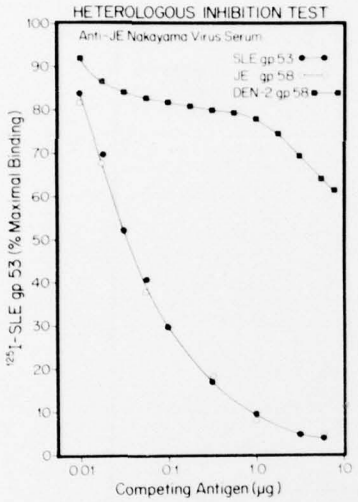

FIG. 5. *Analysis of complex-reactive and group-reactive antigenic determinants on the envelope protein by heterologous competition solid-phase radioimmunoassay. Comparison of adsorption of SLE, JE, and DEN-2 envelope glycoproteins to microtiter wells coated with anti-JE IgG, which were subsequently 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

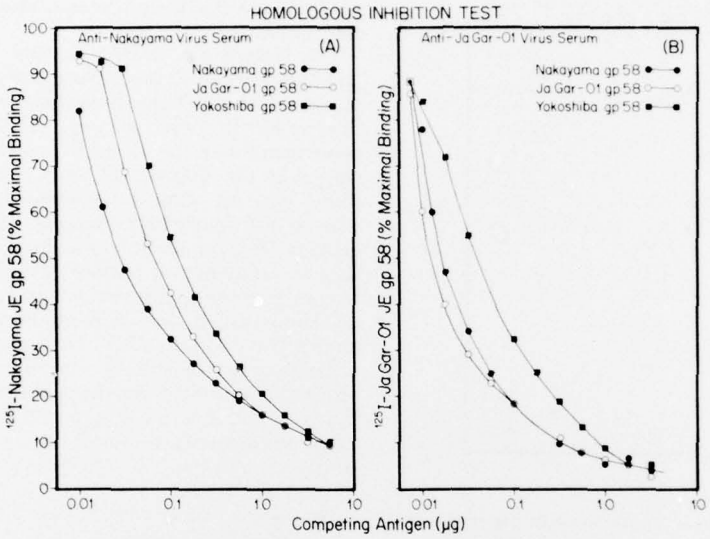

FIG. 6. *Analysis of type-specific and group-reactive antigenic determinants of JE gp 58 proteins by homologous competition solid-phase radioimmunoassay. (A) Comparison of the adsorption of Nakayama, JaGar-01, and Yokoshiba gp 58 proteins to microtiter wells coated with anti-Nakayama IgG and subsequently 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.*

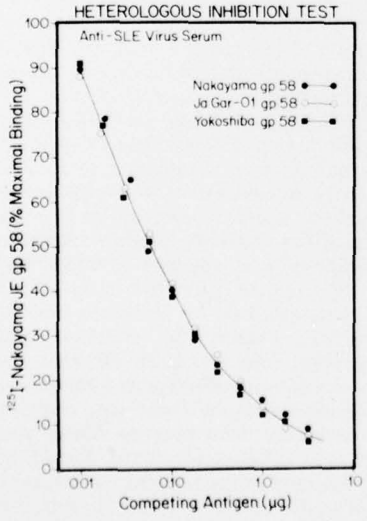

FIG. 7. *Analysis of complex-reactive antigenic determinants of JE gp 58 proteins by heterologous competition radioimmunoassay. Comparison of the adsorption of Nakayama, JaGar-01, and Yokoshiba gp 58 proteins to microtiter wells coated with anti-SLE IgG and subsequently saturated with ^{125}I-labeled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

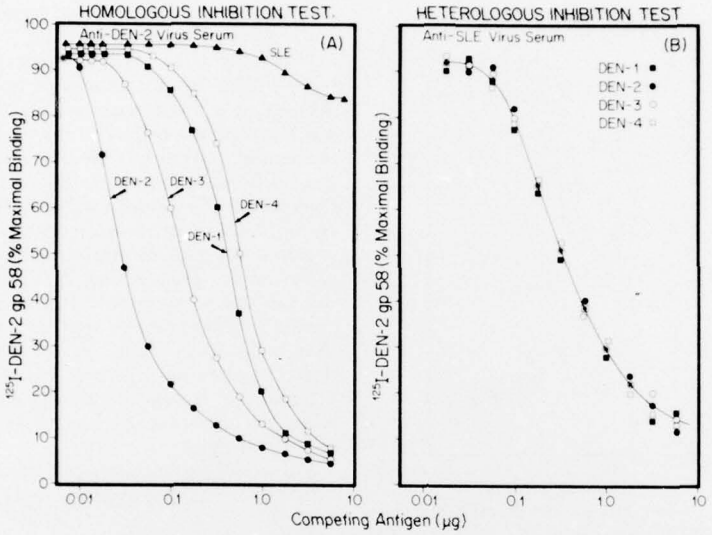

FIG. 8. *Analysis of type-specific, complex-reactive, and group-reactive antigenic determinants of DEN gp 53 protein from serotypes 1 through 4 by homologous and heterologous competition radioimmunoassay. (A) Comparison of the adsorption of DEN-1, -2, -3, and -4 gp 58 proteins to microtiter wells coated 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

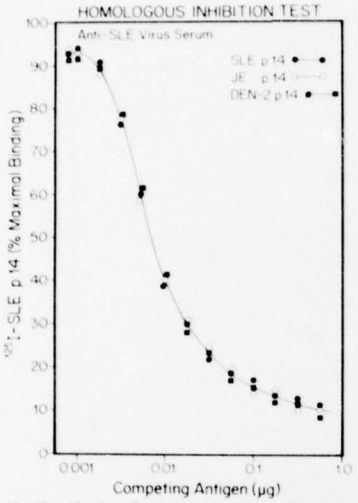

FIG. 9. *Analysis of group-reactive antigenic determinants of flavivirus nucleoprotein p 14. The competition radioimmunoassay was performed by incubating SLE, JE, and DEN-2 p 14 proteins with anti-SLE IgG-coated microtiter wells and subsequently with saturating amounts of ^{125}I-labeled SLE gp 53 protein.*

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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