Alphavirus Neurovirulence: Monoclonal Antibodies Discriminating Wild-Type from Neuroadapted Sindbis Virus

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Wild-type Sindbis virus strain AR339 (SV) and a neurovirulent mutant (NSV), derived by neonatal and weanling mouse brain passage, both cause acute fatal encephalitis in neonatal mice, but NSV alone kills adult mice. NSV cannot be distinguished from SV by immune sera or simple biochemical tests. To localize the molecular changes associated with neuroadaptation, we used a new array of 30 anti-SV monoclonal antibodies to probe for differences between SV and NSV in four tests: immunoprecipitation, enzyme-linked immunosorbent assay binding, neutralization, and hemagglutination inhibition. Seventeen monoclonal antibodies detected differences. Both E1 and E2 glycoprotein gene products were altered during neuroadaptation, but the preponderance of changes was clustered on E2. The capsid protein C was not measurably altered. Mapping of both viruses with these monoclonal antibodies showed that during neuroadaptation SV topography substantially shifted, masking and unmasking biologically important neutralization and hemagglutination inhibition sites. These conformational rearrangements, predominantly on E2, coincided with the acquisition of increased neurovirulence and new lethality for adult mice.

The molecular biology of viral neurovirulence is poorly understood. By contrasting virulent viruses to genetically similar, but attenuated mutants we may learn what biochemical properties allow a virus to invade, infect, inflame, and injure the mammalian brain (18). The fewer the genetic changes detected and the more dissimilar the neurovirulence displayed, the more soundly any molecular rearrangements may be linked to acquired or lost neurovirulence.

Sindbis virus (SV) is the prototype alphavirus, the genus which includes the arthropod-borne viruses of eastern, western, and Venezuelan equine encephalitis (29, 38). Upon intracerebral (i.c.) inoculation, SV causes an acute inflammatory encephalitis with an age-dependent mortality (16, 19). Newborn mice invariably die; weanling and adult mice show vigorous central nervous system virus growth, yet no paralysis or death. Even immunosuppressed adult mice always recover from infection (26).

A strain of SV highly lethal for weanling and adult mice was isolated after six alternating i.c. passages of wild-type SV strain AR339 in neonatal and weanling mice (17). This "neuroadapted" Sindbis virus (NSV) is genetically stable after many tissue culture passes. NSV replicates 10- to 50-fold better in the brain than SV and kills adult mice at an i.c. 50% lethal dose of 2 to 20 PFU. NSV and SV are otherwise indistinguishable.

The simple structure of SV offered the hope that the genetic changes of neuroadaptation might be localized. SV contains a single molecule of positive-stranded RNA, which encodes three structural proteins: a 30-kilodalton (30K) icosahedral nucleocapsid protein (C), and two distinct, roughly 50K envelope glycoproteins, E1 and E2 (32, 37). The glycoproteins are post-translationally cleaved into PE2 (62K) and E1, distinctly glycosylated in the rough endoplasmic reticulum, and transported together through the golgi. PE2 is trimmed to E2 and the tightly, noncovalently bound E1-E2 heterodimer is inserted into the host's cell membrane, through which the virus buds to acquire its outer envelope and glycoprotein spikes (15). The envelope glyco-

proteins mediate cell tropism, infectivity, fusion, hemagglutination, and hemolysis (32). Monospecific antiserum to isolated E1 glycoprotein inhibits hemagglutination and antiserum to E2 neutralizes infectivity (10). Recent monoclonal antibody (mab) studies have shown that antibodies against either glycoprotein can block hemagglutination or infectivity (5, 6, 33, 34).

We reasoned that dissection of the SV-NSV dichotomy might begin to localize the molecular determinants of alphavirus neurovirulence. Here we first show that simple biochemical tests and immune sera cannot differentiate SV from NSV. Then, using a new array of 30 anti-SV mabs to distinguish SV from NSV, we show that both the E1 and the E2 glycoprotein gene products were altered during neuroadaptation, with the preponderance of protein changes clustered in E2. Finally, we construct E1 and E2 biological domain maps that suggest how the topography of the Sindbis viral envelope changed in the acquisition of neurovirulence for adult mice.

MATERIALS AND METHODS

Mice. Four-week-old BALB/c AnNrIBR mice of either sex were used (Charles River Breeding Laboratory, Inc. Wilmington, Mass.).

Cells and virus. BHK-21 cells and chicken embryo fibroblasts were grown as described before (16, 19). Wild-type SV strain AR339 (39) and NSV, isolated by alternately passing SV three times each in neonatal and weanling mouse brains (17), were twice plaque purified from BHK-21 cells. Stock SV and NSV contained 1.5×10^9 and 3.0×10^9 PFU/ml, respectively. NSV killed 4-week-old mice with a 50% lethal dose of 2 PFU upon i.c. inoculation. Up to 10^7 PFU of SV inoculated i.c. were never fatal. Virus was purified by sedimentation to equilibrium density on 15 to 40% (wt/wt) linear potassium tartrate gradients (34), dialyzed extensively against phosphate-buffered saline, pH 7.4 (PBS), and assayed for protein (25).

Isolation of mabs. (i) **Immunization.** Female BALB/c mice were inoculated intravenously (10^7 PFU) and subcutaneously (10^6 PFU) with live SV AR339 once or twice at 1- to

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4-month intervals and were boosted intravenously 3 days prior to fusion. SV hyperimmune sera were obtained from the mice used for fusions.

(ii) **Hybridomas.** The fusion protocol of Kennett et al. (21) was modified. Briefly, 1×10^8 to 2×10^8 spleen cells were fused to 1×10^7 to 5×10^7 P₃NS1-Ag-4-1 (NS1) (22) myeloma cells, using 50% polyethylene glycol 1540 (BDH Laboratories, Poole, U.K.) and grown in hybridoma selective medium: Dulbecco modified Eagle medium, 20% fetal bovine serum, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1.3 μ M bovine zinc insulin (Sigma Chemical Co., St. Louis, Mo.), 4.5 mM pyruvate, 1.1 mM oxaloacetate, 2 mM glutamine, vitamins, 100 μ M nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 μ g/ml). Fused cells were sometimes mixed with 10^7 BALB/c mouse peritoneal cells (>95% esterase positive), freshly isolated by lavaging the peritoneal cavity with 5 ml of sterile 0.34 M sucrose. Cells were screened by enzyme-linked immunosorbent assay (ELISA), and those secreting anti-SV antibodies were cloned once or twice by limiting dilution in soft 0.25% (wt/vol) Bacto-Agar (Difco Laboratories, Detroit, Mich.) (14). Pristane-primed (Sigma Chemical Co.) BALB/c mice were injected intraperitoneally with 5×10^6 cloned hybridoma cells to produce ascites fluid, which was clarified by centrifugation at $300 \times g$ for 15 min and filtered through 0.2- μ m Nalgene filters (Nalge Co., Rochester, N.Y.).

(iii) **Anti-SV virus ELISA.** Purified SV or NSV were coated overnight onto 96-well polystyrene plates (Immulon II; Dynatech Laboratories, Alexandria, Va.) at a final concentration of 1 μ g of protein per well in 200 μ l of either 1.6 g of Na₂CO₃-2.9 g of NaHCO₃ per liter of H₂O (pH 9.5) or PBS. Hybridoma supernatant fluid, ascites, or immune serum was incubated in duplicate for 2 to 4 h at room temperature, washed, and detected by horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum (R α M Ig) (U.S. Biochemical Corp., Cleveland, Ohio, or DAKO Corp., Santa Barbara, Calif.). Color was developed by adding 200 μ l of substrate (6.75 mg of *o*-phenylenediamine, 25 μ l of 30% H₂O₂, in 100 mM citrate-phosphate buffer, pH 5.1) and was measured at 492 nm in a Titertek Multiscan. The ELISA titer was the greatest dilution of ascites or immune serum yielding an optical density at 492 nm of ≥ 0.5 after background had been subtracted.

(iv) **Isotype.** Hybridoma antibody class was determined by ELISA. Plates were coated with purified SV in bicarbonate buffer, washed, and incubated at 4°C with hybridoma culture medium, followed by immunoglobulin subclass-specific horseradish peroxidase-conjugated R α MIg (immunoglobulin A [IgA], IgM, IgG1, IgG2A, IgG2B, IgG3, kappa and lambda light chains; reference 27 or Miles Laboratories, Naperville, Ill.), developed, and read as above. About 20% of the hybridomas were isotyped by substituting unconjugated isotype-specific R α M Ig (Miles Laboratories) for purified SV in the initial coating step.

(v) **Radioimmunoprecipitation.** Either L-[³⁵S]methionine (>600 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) or L-[4,5-³H]leucine (40 to 60 Ci/mmol; Amersham Corp.) was added to BHK-21 or chicken embryo fibroblast cells infected with SV or NSV (0.5 mCi/10⁷ cells). Labeled virus was purified and then lysed for 1 h at 37°C in 25 mM Tris hydrochloride buffer, pH 7.6, containing 0.5 M NaCl, 1% Zwittergent 3-14 (Calbiochem-Behring, La Jolla, Calif.), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories, Richmond, Calif.), and 1 mM

disodium EDTA (lysate buffer). Cell lysates were prepared by adding lysate buffer to infected and sham-infected BHK-21 or chicken embryo fibroblast cells, labeled for 30 min to 3 h with [³H]leucine or [³⁵S]methionine (0.5 mCi/10⁷ cells).

Mab ascites (5 to 25 μ l), hybridoma supernatant fluid (1 ml), or immune mouse serum (5 to 25 μ l) was preincubated for 2 h at room temperature with 25 to 35 μ l of a 50% suspension of protein A coupled to Sepharose CL4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). To allow immunoprecipitation by IgA and IgM mabs, some protein A-Sepharose beads were first mixed 1:1 (vol/vol) with R α M Ig, IgA, or IgM (DAKO) in the presence of excess cold SV and NSV to reduce nonspecific virus binding. Labeled cell lysate (10⁶ cpm) or virus (2×10^5 cpm) was added and the mixture was incubated at 4°C for 12 to 18 h with constant shaking. Beads were pelleted ($12,000 \times g$ for 3 min) and washed four times in lysate buffer. The precipitated proteins were boiled for 3 to 5 min in 40 μ l of 0.05 M Tris hydrochloride, pH 6.9, with 2% SDS, 1.5% dithiothreitol, 2% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue and were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 14-cm vertical slab Laemmli gels (24), using a 10.8% acrylamide resolving gel and a 3.0% acrylamide stacking gel. Radioactive proteins were detected by fluorography (3). For clarity, mabs recognizing capsid protein have been numbered 1 to 99; those recognizing E1, 101 to 199; those binding E2, 201 to 299; and those binding both E1 and E2 (or PE2), 301 to 399.

(vi) **Protein immunoblot.** Purified SV (about 500 μ g of protein) was separated by SDS-PAGE and transferred electrophoretically (50 V for 16 to 20 h; Trans-blot cell; Bio-Rad) to nitrocellulose paper (0.45 μ m; Schleicher & Schuell Co., Keene, N.H.). Transfer buffer contained 0.02 M Tris hydrochloride, 150 mM glycine, and 20% (vol/vol) methanol. All viral proteins were transferred, but the transfer of the 30K capsid protein C was greatly reduced without the addition of 0.05 to 0.1% SDS. SDS unpredictably altered mab binding and so was not routinely used. One nitrocellulose strip was stained for protein with 1% amido black-1% aniline blue in 45% methanol-10% acetic acid. Nitrocellulose strips were blocked in PBS-3% bovine serum albumin (blocking buffer) and then incubated for 4 h at room temperature or 16 h at 4°C with 2 to 3 ml of mab supernatant fluid, ascites (diluted 1:50 to 1:100 in blocking buffer), or mouse immune sera (1:100 in blocking buffer). After extensive washing in PBS-1% bovine serum albumin, strips were incubated with excess horseradish peroxidase-coupled R α M Ig (DAKO), washed, and developed by adding 4-chloro-1-naphthol substrate (15 mg of 4-chloro-1-naphthol dissolved in 5 ml of methanol and mixed with 25 μ l of PBS, pH 7.4, plus 12 μ l of 30% H₂O₂).

(vii) **Plaque reduction neutralization.** SV and NSV (50 to 100 PFU/0.5 μ l of (Dulbecco modified Eagle medium-1% fetal bovine serum) were preincubated in duplicate with twofold dilutions of mab ascites or immune mouse sera for 30 min at 37°C, and residual infectious virus was measured by plaque assay in triplicate on BHK-21 cells (20). The plaque reduction neutralization 80% titer was the highest dilution of mab ascites or serum reducing the number of plaques by 80%.

HI. For hemagglutination inhibition (HI), the method of Clarke and Casals (7) was adapted, using goose erythrocytes at pH 6.0. Hemagglutinin was concentrated from SV- or NSV-infected BHK-21 cells by polyethylene glycol precipitation (10% in 0.5 M NaCl) and was added at 4 U per well. The HI titer was the highest dilution of ascites or serum

FIG. 1. Immune precipitation by selected anti-SV mabs. [^{35}S]-methionine-labeled SV (A) or SV-infected BHK-21 cell extracts (B) were precipitated by sera or mabs bound to protein A-Sepharose beads and separated by SDS-PAGE on 3 to 10.8% Laemmli gels. Composite fluorographs from four experiments. Lanes 1 and 7, SV hyperimmune mouse serum; 2 and 9, normal mouse serum; 3 and 9, anti-visna virus mab; 4, mab 1; 5, mab 101; 6, mab 202; 10, mab 2; 11, mab 104; 12, mab 106; 13, mab 107; 14, mab 108; 15, mab 109; 16, mab 208; 17, mab 201; 18, mab 301; 19, mab 303. SV structural proteins are marked.

causing complete inhibition of hemagglutination. Normal serum and anti-visna virus mabs caused some nonspecific HI; therefore, titers of $<1:320$ were considered negative.

RESULTS

Biochemical and immunological relatedness of SV and NSV.

SV and NSV were not distinguished by simple biochemical tests or immune sera. [^3H]uridine-labeled SV and NSV banded at the same buoyant density. By SDS-PAGE SV and NSV showed the same three structural proteins: E1 (50K), E2 (48K), and C (30K). SV- and NSV-infected cell extracts contained indistinguishable precursor P98 and PE2 (or P62) proteins (Fig. 1 and data not shown). SV- and NSV-immune sera failed to distinguish SV from NSV by immunoprecipitation, ELISA binding, immunohistochemical staining of virus-infected BHK cells, neutralization, or HI (data not shown).

Isolation of mabs. A total of 275 anti-SV hybridomas were isolated from five separate fusions, using mice immunized with live SV AR339. About 50 hybridoma clones, most with high ELISA titers, were biologically characterized further; 30 are described here (Table 1). All isotypes were represented; 13% were IgA; 13%, IgM; 7%, IgG1; 40% IgG2A; 10%, IgG2B; and 17%, IgG3. All contained kappa light chains.

The antigen specificity of each mab was determined by immune precipitation of radiolabeled purified SV or NSV or infected cell extracts. Selected fluorographs are shown in Fig. 1 and results summarized in Table 2. Four mabs (13%) bound C, 11 (37%) bound E1, 12 (40%) bound E2, and 3 (10%) precipitated both E1 and E2.

Three mabs (301 to 303) coprecipitated the two distinct envelope glycoproteins in the presence of nonionic detergents and 0.1% SDS. Since E1 and E2 are tightly complexed as a heterodimer, we confirmed coprecipitation results by reacting these antibodies with purified SV which had first been lysed, separated by SDS-PAGE, and electrophoretically blotted onto nitrocellulose (immunoblot). Composite

TABLE 1. Anti-SV hybridomas: isotypes and antigen specificities

Clone	Isotype	Immunoprecipitation	Immunoblot	Immunoprecipitation preference ^a
1	IgG2A	C	C	
2	IgG2A	C		
3	IgM	C	C	
4	IgM	C		
101	IgG2A	E1	E1	
102	IgG2A	E1	E1	
103	IgG2A	E1		
104	IgG2A	E1		SV
105	IgG3	E1		SV
106	IgG2B	E1	E1	
107	IgG2B	E1		
108	IgG1	E1	E1	
109	IgG2A	E1		
110	IgG2B	E1	E1	
111	IgG2A	E1		
201	IgG3	PE2, E2	E2	NSV
202	IgG3	PE2, E2	E2	NSV
203	IgG2A	E2	E2	
204	IgM	PE2, E2		NSV
205	IgG2A	PE2, E2		NSV
206	IgG2A	PE2, E2		NSV
207	IgA	PE2, E2		NSV
208	IgA	PE2, E2		
209	IgG3	PE2, E2		NSV
210	IgG3	PE2, E2		NSV
211	IgA	E2		
212	IgG1	E2		
301	IgG2A	PE2, E2, E1	PE2, E2, E1	
302	IgM	PE2, E2, E1		
303	IgA	PE2, E2, E1	E2, E1	

^a Immunoprecipitation preference was judged by inspection of fluorographs.

TABLE 2. SV versus NSV: comparative mab ELISA, neutralization, and HI titers^a

Antibody	ELISA titer		80% PRNT		HI titer	
	SV	NSV	SV	NSV	SV	NSV
Anti-capsid (C)						
1	>5.7	>5.7	<1.0	<1.0	—	—
2	ND	ND	<1.0	<1.0	ND	ND
3	>5.7	>5.7	<1.0	<1.0	—	—
4	4.0	4.0	≤1.0	≤1.0	—	—
Anti-E1						
101	5.0	5.0	1.0	1.0	3.4	3.4
102	5.7	5.7	4.1	1.0	—	—
103	5.0	4.0	4.1	2.6	—	—
104	>5.7	>5.7	≤1.0	≤1.0	3.7	4.9
105	>5.7	>5.7	>3.5	>3.5	4.9	5.7
106	>5.7	>5.7	3.8	4.1	3.7	4.0
107	>5.7	>5.7	3.8	3.2	2.8	3.4
108	>5.7	>5.7	3.5	3.6	—	—
109	>5.7	>5.7	2.3	≤1.0	—	—
110	>5.7	>5.7	2.3	2.6	4.9	ND
111	>5.7	>5.7	ND	ND	4.3	4.8
Anti-E2						
201	2.0	>5.7	<1.0	>3.2	—	4.9
202	3.0	>5.7	1.0	>3.8	—	4.9
203	5.7	5.7	2.3	<1.0	ND	ND
204	>5.7	≤1.0	3.5	<1.0	—	—
205	4.0	4.0	2.3	2.3	—	2.8
206	>5.7	>5.7	>4.4	≤1.0	4.6	4.8
207	5.7	5.7	2.6	≤1.0	—	—
208	4.0	4.0	2.9	2.3	—	—
209	>5.7	>5.7	>4.4	>4.2	5.5	6.0
210	<2.0	5.0	<1.0	3.5	—	2.8
211	5.0	5.0	<1.0	<1.0	ND	ND
212	ND	ND	2.6	<1.0	ND	ND
Anti E1-E2						
301	5.0	5.0	<1.0	3.5	—	—
302	>5.7	>5.7	3.2	<1.0	—	—
303	>5.7	>5.7	<1.0	<1.0	—	—
Controls						
Anti-visna virus mabs						
IgA	—	—	<1.0	<1.0	—	—
IgM	—	—	<1.0	<1.0	—	—
IgG subclasses	—	—	<1.0	<1.0	—	—
SV immune mouse sera	5.7	5.0	3.9	3.8	3.4	3.7
Normal mouse sera	—	—	<1.0	<1.0	—	—

^a All titers are $^{-}\log_{10}$ dilutions of mab ascites or mouse sera. Anti-visna virus mabs of matched isotypes served as negative controls. HI titers of ≤ 2.5 were considered negative (—). PRNT, Plaque reduction neutralization; ND, not done.

immunoblots are shown in Fig. 2 and summarized in Table 1. Mabs 301 and 303 clearly bound both E1 and E2 as well as two SV-specific higher-molecular-weight proteins. Binding by mabs 3, 108, and 201 to single determinants is shown for comparison. About one-third of the antibodies reacted positively on immunoblot, including many which neutralized virus and inhibited hemagglutination.

SV versus NSV. (i) Preferential immune precipitation. We used these 30 mabs to probe for differences between SV and NSV. Ten mabs preferentially immunoprecipitated either SV or NSV (Table 1). Though equal amounts of labeled SV, NSV, or infected cell lysates were added to each protein A-Sepharose-bound antibody, greatly unequal amounts of the radioactive proteins were precipitated. A composite

fluorograph from two experiments is shown in Fig. 3. Immune serum (lane 1) and two-thirds of the mabs (lanes 3, 6, and 8) precipitated the proteins of both strains equally. Surprisingly, 8 of 10 differentially immunoprecipitating antibodies preferred NSV (lanes 4 and 5). All were targeted against E2. Only two antibodies preferred SV (both anti-E1, lane 7), the strain against which the mabs were originally synthesized. Anti-C antibodies bound SV and NSV without preference (lane 9). Adding capture R α M Ig serum to the precipitation sandwich (protein A bead-R α M Ig-mab-³⁵S-labeled virus) did not equalize viral protein precipitated (Fig. 3, lanes 1 to 6). Differential precipitation of SV and NSV polypeptides by these 10 mabs in the presence of three detergents suggested that the primary or secondary structures of both envelope glycoproteins (E2 > E1) had been changed in NSV.

(ii) ELISA, neutralization, and HI. We reasoned that biological properties such as infectivity and hemagglutination might be more differentially sensitive to inhibition by these 30 antibodies. We tested each mab and isotype matched anti-visna virus mabs for their abilities to: (i) bind purified SV or NSV on ELISA at physiological pH; (ii) neutralize SV or NSV infectivity; and (iii) inhibit SV- or NSV-induced hemagglutination. Results are summarized in Table 2.

Twenty-five mabs (83%), like SV- and NSV-immune sera, bound both viruses similarly. Typical ELISA binding curves are shown for five antibodies in Fig. 4. Five mabs bound one strain 10 to 10,000 times better than the other; four of these five were directed against E2. To illustrate, mab 204 preferred SV and mab 210 preferred NSV (Fig. 4B and C). Anticapsid antibodies bound both viruses equally. ELISA results suggested at least three mutations in NSV: a small change in one epitope of E1 (antibody 103) and a greater shift in at least two epitopes of E2 (since 204 preferred SV and 201, 202, and 210 favored NSV).

FIG. 2. Composite immunoblots. Lane V, SV on Coomassie blue-stained gel strip before blot; 1, SV hyperimmune mouse serum; 2, normal mouse serum; 3, anti-visna virus mab; 4, mab 3; 5, mab 108; 6, mab 201; 7, mab 301. Immune serum and mab 301 bound several unidentified SV-specific >60K polypeptides not visible in lane V.

FIG. 3. Preferential immunoprecipitation by mabs of [35 S]methionine-labeled SV-infected (s) or NSV-infected (n) BHK-21 cell extracts. Composite fluorograph of two experiments. Lane 1, SV-immune mouse serum; 2, anti-visna virus mab; 3, mab 1; 4, mab 209; 5, mab 210; 6, mab 205; 7, mab 104; 8, mab 107; 9, mab 102. Mabs 209 and 210 preferred NSV; mab 104 preferred SV. RαM Ig serum was added as a capture antibody to protein A beads before the mabs in experiment 1 (lanes 1 to 6). Several proteolytic degradation bands of the C protein are seen below 30K. SV proteins are marked.

By 80% plaque reduction many anti-E1 and anti-E2 mabs neutralized both viruses effectively (Table 2). All isotypes, including IgA, showed the ability to neutralize without added complement. Seven neutralizing antibodies bound either E1 or E2 on protein immunoblot after SDS-PAGE, indicating that these antibodies could recognize critical primary amino acid sequences, not just tertiary conformation. Typical plaque reduction curves are illustrated in Fig. 4. Thirty-eight percent of neutralizing mabs, like immune sera, neutralized SV and NSV equally. Thirteen of 21 neutralizing mabs (62%) inhibited either SV or NSV infectivity preferentially. Nine of these 13 neutralized SV far better than NSV. Eight differentially neutralizing antibodies were targeted against E2, three against E1, and two against the E1-E2 complex. Eight of 13 mabs which detected differences between SV and NSV on plaque reduction failed to separate the strains on ELISA; neutralization was a more sensitive assay of viral changes.

By combining immunoprecipitation, ELISA, and plaque reduction data, we deduced that during neuroadaptation viral neutralization epitopes suffered one of four fates. They were preserved intact, obscured, unmasked, or uncoupled (Table 3). Mab 106 (Fig. 4A) was the prototype antibody directed against a preserved site. Like six other anti-E1 and three anti-E2 antibodies, it strongly bound and neutralized both viruses. Mab 204 (Fig. 4B) was typical of antibodies directed against a subsequently obscured epitope. It avidly bound and neutralized SV, but lost both abilities in parallel against NSV. The NSV neutralization epitope was hidden or blocked, not destroyed, since mab 204 continued to immunoprecipitate NSV. Mab 210 (Fig. 4C) illustrated antibodies to a viral neutralization epitope unmasked or enhanced during neuroadaptation. Like antibodies 201, 202, and 301, it bound but did not neutralize SV. It bound NSV better and displayed the new ability to strongly neutralize NSV. Mab 102 (Fig. 4D) was typical of six antibodies directed against SV neutralization epitopes physically preserved on NSV, but uncoupled from neutralization. These six antibodies strongly and equally bound SV and NSV and neutralized

SV, but lost the ability to neutralize NSV. Antibody 102 continued to bind an accessible site on NSV, but this binding was somehow disconnected from subsequent neutralization.

Table 3 summarizes the differences between SV and NSV revealed by these 30 anti-SV mabs. Preferences are weighted on the assumption that the greater the differential mab reactivity, the greater the NSV mutation revealed. On 31 of 110 tests this array of anti-SV mabs revealed differences between SV and NSV. Both the E1 and the E2 envelope glycoproteins showed mutations. The preponderance of changes (75%) and the greatest changes were in E2. No alterations in capsid protein C were demonstrated.

DISCUSSION

Mabs are powerful probes of protein fine structure (23, 41). They can exquisitely discriminate between closely related virus strains and can detect antigenic variation unseen by routine serological methods (13, 40). Sequencing of influenza virus hemagglutinins has proven that mabs can detect grand surface changes in tertiary protein conformation or even a single, conservative amino acid substitution, such as tyrosine for phenylalanine (4, 42).

We have used 30 anti-SV mabs to compare SV and a neurovirulent derivative NSV in a battery of four tests: immunoprecipitation, ELISA binding, neutralization, and HI. Seventeen mabs to the surface glycoproteins E1 and E2 detected differences between the two strains. No differences were detected in the core protein. Both the E1 and the E2 gene products were altered, but by far the preponderance of changes was clustered on E2. These mabs have allowed us to map the biological domains of the envelope glycoproteins and to suggest how the topography of SV shifted during its mutation to NSV.

Topography and functions of E1 and E2. Mabs targeted against E1 and E2 have been mapped to neutralization, HI, or only ELISA binding sites on SV (Fig. 4). Each glycoprotein was segregated into three immunodominant regions.

The E1 glycoprotein has historically been considered the viral hemagglutinin since monospecific antiserum to E1

FIG. 4. Comparative ELISA and neutralization titers. Mab ascites tested against SV (●) and NSV (○). (A) mabs 101 and 106; (B) mab 204; (C) mab 210; (D) mab 102. OD, Optical density.

alone blocks hemagglutination (10). Our mabs distinguished three regions on SV E1: a neutralization site (I), an HI site (III), and a region of overlap mediating both neutralization and HI (II). Eight anti-E1 mabs of diverse IgG isotypes neutralized SV infectivity without added anti-mouse globulins or complement; half of these also inhibited hemagglutination. Schmaljohn et al. (33, 34) and Chanas et al. (5, 6) have also reported two anti-E1 SV mabs that neutralized virus; both also blocked hemagglutination. Our data show that anti-E1 mabs can neutralize and inhibit hemagglutination and that the sites for each of these viral functions may be distinct or may overlap.

The E2 glycoprotein has traditionally been considered the site for neutralization since monospecific antiserum to E2 alone blocks infectivity (10, 38). Nine of 11 previously published anti-SV-neutralizing mabs have been directed against E2 (6, 9, 30, 33, 34). Schmaljohn et al. (34) reported that all six of their neutralizing anti-E2 mabs also inhibited hemagglutination. By competitive binding they mapped all of their anti-E2 mabs to a single antigenic site, but because three of the mabs did not neutralize NSV, they suggested that this site might contain more than one discrete epitope. Our mabs distinguished three regions on SV E2 with different biological functions: a neutralization site (I), an overlapping neutralization-HI site (II), and a site accessible for ELISA binding only (III). Antibodies to site III on SV-E2 did not neutralize infectivity or inhibit hemagglutination. Our mabs distinguished a strict neutralization domain (I) from the overlapping neutralization-HI domain (II), into which most previously reported anti-E2 mabs would seem to fall; six mabs neutralized SV without blocking hemagglutination.

Our data suggest that antibodies to either E1 or E2 can independently inhibit SV infectivity and hemagglutination and that both glycoproteins contain at least three distinct functional domains. This assumes that the antiviral activity mediated by a mab depends upon the actual antigenic site to which it binds (41, 42). Clearly, steric and allosteric phenomena reduce the resolution of this approach. Because E1 and E2 are tightly complexed as heterodimers, conformational shifts in one glycoprotein might well alter the other. Clegg et al. (8) demonstrated that an anti-E1 mab may enhance the binding of anti-E1 antibodies at a different site and that mab binding may actually change the conformation of E1 to cause its release from PE2 or E2. Competitive mab mapping of the envelope glycoproteins of Venezuelan equine encephalitis virus has shown that steric and allosteric interactions may even extend across proteins. Several anti-gp56 monoclonal antibodies blocked the binding of an anti-gp50 antibody (31). We have assumed here that most mabs work directly and not at a distance. This would tend to underestimate the actual number of physical antigenic sites modulating neutralization and HI; each functional domain may consist of more than one discrete epitope.

TABLE 3. Neutralization epitopes after neuroadaptation

Mab prototype	ELISA		80% PRNT ^a		Fate of SV neutralization epitope
	SV	NSV	SV	NSV	
106	+	+	+	+	Preserved
204	+	-	+	-	Obscured
210	+/-	+	-	+	Unmasked
102	+	+	+	-	Uncoupled

^a PRNT, plaque reduction neutralization.TABLE 4. SV versus NSV: summary of significant differences by anti-SV mabs^a

CLONE	Immunoprecipitation	ELISA	80% PRNT	HI
Anti-C				
1				
2				
3				
4				
Anti-E1				
101				
102			S***	
103		S	S	
104	S			N
105	S			
106				
107				
108				
109			S	
110				
111				
Anti-E2				
201	N	N***	N**	N**
202	N	N**	N**	N**
203			S	
204	N	S****	S**	
205	N			
206	N		S***	
207	N		S	
208				
209	N			
210	N	N****	N**	
211				
212			S	
Anti-PE2-E1				
301			N**	
302			S**	
303				

^a Antibodies preferring SV (S) and NSV (N) by at least 10-fold on ELISA, neutralization or HI are marked. Each asterisk (*) represents on log₁₀ difference in antibody titer. Immunoprecipitation differences were graded by inspection of fluorographs as very severe or negligible.

Molecular changes of neuroadaptation. The genetically stable NSV strain was derived from wild-type SV by mouse brain passage. Neuroadaptation presumably selected mutations that favored growth in the central nervous system and thereby increased lethality. Because the mabs had been originally synthesized against SV, we expected most antibodies to prefer SV, as they did on the plaque reduction assay: 8 of 13 distinguishing mabs neutralized SV but not NSV infectivity. Nearly all anti-E1 mabs showing a preference preferred SV (Table 4). By contrast, many anti-E2 mabs showing a preference preferred NSV. Neuroadaptation enhanced antibody access to or avidity for broad NSV E2 regions. Changes in E2 amino acid sequence, conformation, glycosylation, or weakening of the NSV E1-E2 heterodimer could explain the preferential binding.

By mapping the biological properties of our mabs against NSV and comparing them with the SV map, we deduced that the topography of the NSV envelope had been substantially rearranged during neuroadaptation. Because the E1 and capsid proteins show considerable sequence homology across several alphaviruses (29, 38), we expected that

FIG. 5. SV and NSV topography. Biological domains of SV and NSV envelope glycoproteins E1 and E2 were mapped with mabs to neutralizing (N) and HI sites. Mabs binding virus but not neutralizing or blocking hemagglutination were marked E (ELISA binding only). Topographical changes in SV during neuroadaptation to NSV were localized to the hatched domains.

changes would occur primarily in the least conserved envelope glycoprotein E2.

Only one major topographical change was detected in NSV E1 (Fig. 5). Antibodies 102 and 109 (domain I_A), which neutralized SV, failed completely to neutralize NSV, though

they bound NSV to the same high ELISA titers. This suggested that the E1 glycoprotein could be divided into at least four distinct domains (I_A, I_B, II, and III). During neuroadaptation the I_A domain was split from the I_B. Antibody binding to the accessible I_A site no longer neutralized

NSV, as if the I_A site had been uncoupled from neutralization. Uncoupling has also been noted in mab studies of rabies virus variants (13) and the California serogroup of bunyaviruses (26a). Uncoupling suggests either that (i) the structure of some neutralization sites is so fastidious that even slight protein mutations, which do not alter mab avidity (as in the rabies virus case), may allow the virus to escape neutralization, or (ii) certain neutralization sites may be disconnected from a postulated chain of secondary neutralization steps which follow antibody binding at the neutralization epitope.

Three major topographical changes were detected in NSV E2 (Fig. 5). First, antibodies 203, 204, 207, and 212 (domain I_A), like antibodies to the E1-I_A domain, still bound but no longer neutralized NSV. The E₂-I_B domain continued to neutralize. Second, a small region of the overlapping neutralization-HI domain (II) bound by a single antibody (206) split away and after neuroadaptation only modulated hemagglutination. Third, the three antibodies to site III (201, 202, and 210), which neither neutralized SV nor inhibited hemagglutination by it, bound NSV to higher ELISA titer and acquired the new abilities of neutralization and HI. Neuroadaptation apparently shifted the E₂-III domain into a more accessible, biologically functional configuration. This suggests a partially constituted, covert, or blocked potential neutralization and HI site on SV E2. The E₂-III domain is pathogenically very important since we have found that mabs to this domain prevent death from established NSV encephalitis in adult mice (J. Stanley, S. J. Cooper, and D. E. Griffin, submitted for publication). Of the five distinct domains of E2, neuroadaptation modified I_A and III significantly and II_B partially. The symmetry of E1 and E2 topographical changes suggests three-dimensional reciprocity, with some polypeptide regions moving into neutralizing position (E2-III) while others are forced out (E2-I_A, E1-I_A). The bonding energy requirements for the E1-E2 heterodimer may require such reciprocal shifts.

Several studies on other neurotropic viruses have similarly concluded that the determinants of neurovirulence appear to lie largely on surface proteins. Rabies glycoprotein critically mediates its neurovirulence (11). Proteins of the mammalian reovirus outer capsid (the hemagglutinin σ 1 and μ 1C) have been linked to neuron tropism and entry (12, 36). Neuroinvasiveness in the California serogroup of bunyaviruses cosegregates with the middle-sized RNA segment encoding the G₁ and G₂ surface glycoproteins, mediators of cell attachment and fusion (35). Recently, Olmsted et al. (28) showed that SV mutants selected *in vitro* for the ability to rapidly penetrate BHK cells were much less deadly when injected into neonatal mice. Interestingly, the rapidly penetrating avirulent mutant had an altered E2 glycoprotein. Perhaps an E2 region of NSV enhances neuron tropism, binding, or entry. Neurovirulence of Semliki Forest alphavirus correlates best with such neuron tropism (1, 1a).

We conclude that neuroadaptation of SV selected for mutations in both the E1 and the E2 envelope glycoprotein gene products, but preponderantly in E2. These polypeptide changes substantially altered envelope topography, masking and unmasking biologically important NSV neutralization and hemagglutination sites. The conformational shifts coincided with the acquisition of increased neurovirulence and new lethality for adult mice. Selection of attenuated NSV mutants by these mabs may allow us to analyze fewer polypeptide changes between virulent and avirulent strains and to pinpoint critical neurovirulent epitopes at finer resolution.

ACKNOWLEDGMENTS

We thank Joseph J. Giffels, Querubin P. Mendoza, and Marcia Lyons for technical help, Linda Kelly for typing the manuscript, Fred Server, Neal Waxham, Dennis Brown, Mette Strand, Walter Gerhard, and Tom Moench for advice, Anne Bodner and Laurie Jackson for the gift of NS1 cells, and Norman Barker, Walter S. Massey, and Richard I. DeWitt for photography. J.S. thanks Theresa A. Pluth for her encouragement and Victor A. McKusick, John G. Bartlett, and Richard T. Johnson for support.

This work was supported by Public Health Service grants NS-18596 and NS-07000 from the National Institutes of Health.

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