Sindbis Virus Mutations Which Coordinately Affect Glycoprotein Processing, Penetration, and Virulence in Mice†

DARCY L. RUSSELL,¹ JOEL M. DALRYMPLE,² AND ROBERT E. JOHNSTON^{1‡*}

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615,¹ and Division of Virology, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701²

Received 9 August 1989/Accepted 14 December 1989

Rapid penetration of baby hamster kidney cells was used as a selective pressure for the isolation of pathogenesis mutants of the S.A.AR86 strain of Sindbis virus. Unlike most Sindbis virus strains, S.A.AR86 is virulent in adult as well as neonatal mice. Two classes of mutants were defined. One class was attenuated in adult mice inoculated intracerebrally as well as in neonatal mice inoculated either intracerebrally or subcutaneously. Sequence analysis of the glycoprotein genes of the parent virus and three such mutant strains revealed a single point mutation which resulted in an amino acid change at position 1 in the E2 glycoprotein. The change from a serine in S.A.AR86 to an asparagine in the mutants created a new site for N-linked glycosylation which appeared to be utilized. This mutation did not retard release of infectious particles; however, mutant virions contained the E2 precursor protein (PE2) rather than the E2 glycoprotein itself. The mutants also lost the ability to bind two E2-specific monoclonal antibodies, R6 and R13. A second class of mutants was attenuated in neonatal mice upon subcutaneous inoculation but remained virulent in adults and in neonates when inoculated intracerebrally. Sequence analysis of three such strains revealed the substitution of an arginine residue for a serine at position 114 in the E2 glycoprotein. Reactivity with monoclonal antibodies R6 and R13 was reduced, yet members of this mutant class were more susceptible than S.A.AR86 to neutralization by these antibodies.

Sindbis virus is the prototype member of the *Alphavirus* genus of the family *Togaviridae* (3). Representatives of this group having veterinary and public health importance include eastern, western, and Venezuelan equine encephalitis (VEE) viruses. We have been examining the molecular basis of neurovirulence in this virus group with the ultimate goal of designing a new generation of vaccines for these agents.

Two mouse model systems have been used for the study of Sindbis virus neurovirulence. Most natural Sindbis virus isolates (e.g., AR339) and laboratory strains are lethal for neonatal mice at doses approaching 1 PFU administered by either an intracerebral (i.c.) or subcutaneous (s.c.) route (20, 34). In adult mice inoculated i.c., most of these isolates replicate to a significant titer in the brain, but the animals survive the infection without noticeable symptomatology. The transition from susceptible to resistant occurs between days 8 and 15 postpartum. However, some natural Sindbis virus isolates, such as S.A.AR86, are virulent in adult mice inoculated i.c. as well as in neonates (see below). All strains of Sindbis virus are avirulent in adult mice inoculated s.c. The objective of the experiments reported here was to isolate and characterize mutants of S.A.AR86 with altered neurovirulence for adult or neonatal mice or both.

Domains on the glycoprotein spikes of Sindbis virus are important determinants of neurovirulence (9, 28, 32, 33, 52). The spikes consist of heterodimers of viral glycoproteins E1 and E2, which are anchored in the lipid envelope of the virus by hydrophobic domains near their carboxy termini (37, 42, 50). Amino acid residues of E1 and E2 or interior to the lipid bilayer interact with the icosahedral nucleocapsid (36, 54).

The subunits of the nucleocapsid are composed of a single capsid protein species, C, that surrounds the single-stranded, positive-sense RNA genome (45, 50). A third glycoprotein, E3, is present in the virions of a related alphavirus, Semliki Forest virus (16).

The virion proteins are translated from a subgenomic 26S mRNA in the 5' to 3' order C, E3, E2, 6K, and E1 and are synthesized as part of a polyprotein precursor (14, 36, 41). The capsid protein has an inherent autoprotease activity which catalyzes the excision of C from the polyprotein (2, 18). The amino terminus of E3 serves as a signal sequence for the membrane insertion of PE2 (composed of E3 and E2), while the 6,000-molecular-weight peptide (6K peptide) serves a similar function in the insertion of E1 (54). In the latter case, the 6K peptide is proteolytically cleaved both at its amino terminus to release PE2 from the nascent polyprotein and at its carboxy terminus leaving E1 inserted into the ER membrane (53). PE2 and E1 form a heterodimeric unit (37) which is transported through the Golgi apparatus and ultimately to the plasma membrane (12). Capsid protein assembles with genomic RNA in the cytoplasm, nucleocapsids associate with cytoplasmic domains of the glycoproteins, and maturation occurs by budding through the plasma membrane (7). At some point in the progression of the PE2 and E1 glycoproteins from endoplasmic reticulum to plasma membrane, the E3 portion of PE2 is cleaved from E2 and released to the medium, while E2 becomes an integral part of the mature virion (53).

To identify elements which contribute to virulence, mutant strains with altered pathogenesis phenotypes have been isolated and their sequences have been compared with that of the parent strain. Starting with Sindbis virus strain AR339, which is virulent only in neonates, Griffin and Johnson (17) selected a strain of AR339 which is lethal for both adult and neonatal mice. This was accomplished by alternately passaging the virus in neonatal and adult mice by

* Corresponding author.

† Paper no. 31416 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC 27695-7601.

‡ Present address: Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC 27599-7290.

E3		1 Ser Ala Ala Pro Leu Val Thr Ala Met Cys Leu Leu Gly Asn Val Ser Phe Pro Cys Asn Arg Pro Pro Thr Cys Tyr Thr Arg Glu Pro Ser Arg																									
8439	UCU	GCU	GCA	CCA	CUG	GUC	ACG	GCC	AUG	UGC	UUG	CUU	GGA	AAC	GUG	AGC	UUC	CCA	UGC	AAU	CGC	CCG	CCC	ACA	UGC	UAC	
	C	A						A		U		C		U						G C					U		
33		Ala Leu Asp Ile Leu Glu Glu Asn Val Asn His Glu Ala Tyr Asp Thr Leu Leu Asn Ala Ile Leu Arg Cys Gly Ser Ser Gly Arg Ser Lys Arg																									
8535	GCU	CUC	GAC	AUC	CUC	GAA	GAG	AAC	GUG	AAC	CAC	GAG	GCC	UAC	GAC	ACC	CUG	CUC	AAC	GCC	AUA	UUG	CGG	UGC	GGA	UCG	
	C				U						U				U				U					U		C	
E2		1 Ser Val Thr Asp Asp Phe Thr Leu Thr Ser Pro Tyr Leu Gly Thr Cys Ser Tyr Cys His His Thr Glu Pro Cys Phe Ser Pro Ile Lys Ile Glu																									
8631	AGC	GUC	ACU	GAC	GAC	UUU	ACC	CCG	UAC	UUG	ACC	UAC	UUG	GGC	ACA	UGC	UCG	UAC	UGU	CAC	CAU	ACU	GAA	CCG	UGC	UUU	
	A						C		C			C							C					C		U	G
33		Gln Val Trp Asp Glu Ala Asp Asp Asn Thr Ile Arg Ile Gln Thr Ser Ala Gln Phe Gly Tyr Asp Gln Ser Gly Ala Ala Ser Ser Asn Lys Tyr																									
8727	CAG	GUC	UGG	GAU	GAA	GCG	GAC	GAC	AAC	ACC	AUA	CGC	AUA	CAG	ACU	UCC	GCC	CAG	UUU	GGA	UAC	GAC	CAA	AGC	GGA	GCA	
				C					U																	C	
65		Arg Tyr Met Ser Leu Glu Gln Asp His Thr Val Lys Glu Gly Thr Met Asp Asp Ile Lys Ile Ser Thr Ser Gly Pro Cys Arg Arg Leu Ser Tyr																									
8823	CGC	UAC	AUG	UCG	CUC	GAG	CAG	GAU	CAU	ACU	GUC	AAA	GAA	GCG	ACC	AUG	GAU	GAC	AUC	AAG	AUC	U					
				U					C						U												
97		Lys Gly Tyr Phe Leu Leu Ala Lys Cys Pro Pro Gly Asp Ser Val Thr Val Ser Ile Ala Ser Ser Asn Ser Ala Thr Ser Cys Thr Met Ala Arg																									
8919	AAA	GGA	UAC	UUU	CUC	CUC	GCG	AAG	UGU	CCU	CCA	GCG	GAC	AGC	GUA	ACG	GUU	AGC	AUA	GCG	AGU	AGC	AAC	UCA	GCA	ACG	
								A												U						C	
129		Lys Ile Lys Pro Lys Phe Val Gly Arg Glu Lys Tyr Asp Leu Pro Pro Val His Gly Lys Lys Ile Pro Cys Thr Val Tyr Asp Arg Leu Lys Glu																									
9015	AAG	AUA	AAA	CCA	AAA	UUC	GUG	GGA	CGG	GAA	AAA	UAU	U	CUA	CCU	CCC	GUU	CAC	GGU	AAG	AAG	AAU	CCU	UGC	ACA	GUG	
													U							A	A						
161		Thr Thr Ala Gly Tyr Ile Thr Met His Arg Pro Gly Pro His Ala Tyr Thr Ser Tyr Leu Glu Glu Ser Ser Gly Lys Val Tyr Ala Lys Pro Pro																									
9111	ACA	ACC	GCC	GCG	UAC	AUC	ACU	AUG	CAC	AGG	CCG	GGA	CCG	CAU	GCC	UAU	ACA	UCC	UAU	CUG	GAG	GAA	UCA	UCA	GGG	AAA	
		U		A										C		U			C		A					G	
193		Ser Gly Lys Asn Ile Thr Tyr Glu Cys Lys Cys Gly Asp Tyr Lys Thr Gly Thr Val Thr Thr Arg Thr Glu Ile Thr Gly Cys Thr Ala Ile Lys																									
9207	UCC	GGG	AAG	AAC	AUU	ACG	UAC	GAG	UGC	AAG	UGC	GCG	GAU	UAC	AAG	ACC	GGA	ACC	GUU	U	ACC	CGU	ACC	GAA	AUC	GGC	
	U							U					C							U			C		U	U	
225		Gln Cys Val Ala Tyr Lys Ser Asp Gln Thr Lys Trp Val Phe Asn Ser Pro Asp Ser Ile Arg His Ala Asp His Thr Ala Gln Gly Lys Leu His																									
9303	CAG	UGC	GUC	GCC	UAU	AAG	AGC	GAC	CAA	ACG	AAG	UGG	GUC	UUC	AAC	UCG	CCG	GAC	UCG	U	AUC	AGA	CAC	GCC	GAC	CAC	
																U						U	A				
257		Leu Pro Phe Lys Leu Ile Pro Ser Thr Cys Met Val Pro Val Ala His Ala Pro Asn Val Val His Gly Phe Lys His Ile Ser Leu Gln Leu Asp																									
9399	UUG	CCU	UUC	AAG	CUG	AUC	CCG	AGU	ACC	UGC	AUG	GUC	CCU	GUU	GCC	CAC	GCG	CCG	AAC	GUA	CAC	U	GUA	A	U		
				U															U							U	
289		Thr Asp His Leu Thr Leu Leu Thr Thr Arg Arg Leu Gly Ala Asn Pro Glu Pro Thr Thr Glu Trp Ile Ile Gly Lys Thr Val Arg Asn Phe Thr																									
9495	ACA	GAC	CAU	CUG	ACA	UUG	CUC	ACC	ACC	AGG	AGA	CUA	GCG	GCA	AAC	CCG	GAA	CCA	ACC	ACU	GAA	UGG	AUC	AUC	GGA	AAG	
			C																							C	
321		Val Asp Arg Asp Gly Leu Glu Tyr Ile Trp Gly Asn His Glu Glu Pro Val Arg Val Tyr Ala Gln Glu Ser Ala Pro Gly Asp Pro His Gly Trp Pro																									
9591	GUC	GAC	CGA	GAU	GCG	CUG	GAA	UAC	AUA	UGG	GCG	AAU	CAC	GAA	CCA	GUA	AGG	GUC	UAU	GCC	CAA	GAG	UCU	GCA	CCA	GGA	
												A			U	G							A				
353		His Glu Ile Val Gln His Tyr Tyr His Arg His Pro Val Tyr Thr Ile Leu Ala Val Ala Ser Ala Ala Val Ala Met Met Ile Gly Val Thr Val																									
9687	CAC	GAA	AUA	GUA	CAG	CAU	UAC	UAU	CAU	CGC	CAU	CCU	GUG	UAC	ACC	AUC	UUA	GCC	GUC	GCA	UCA	GCU	GCU	C			
																								A	C		
385		Ala Ala Leu Cys Ala Cys Lys Ala Arg Arg Glu Cys Leu Thr Pro Tyr Ala Leu Ala Pro Asn Val Ile Pro Thr Ser Leu Ala Leu Leu Cys																									
9783	GCA	GCA	UUA	UGU	GCC	UGU	AAA	GCG	CGC	CGU	GAG	UGC	CUG	ACG	CCA	UAU	GCC	CUG	GCC	CCA	AAU	GCC	GUG	A	C		
			U																						C		
417		Cys Val Arg Ser Ala Asn Ala Glu Thr Phe Thr Glu Thr Met Ser Tyr Leu Trp Ser Asn Ser Gln Pro Phe Phe Trp Val Gln Leu Cys Ile Pro																									
9879	UGU	GUU	AGG	UCG	GCU	AAU	GCU	GAA	ACA	UUC	ACC	GAG	ACC	AUG	AGU	UAC	UUA	UGG	UCG	AAC	AGC	CAG	CCG	UUC	UUC	UGG	
	C				C											G					U				U	C	

i.c. inoculation. The glycoprotein genes of the neuroadapted Sindbis virus strain (NSV) differ from AR339 by four coding changes, two in E1 (codons 72 and 313) and two in E2 (codons 55 and 209) (28). Neurovirulence in adult mice requires both the E1 and E2 genes from NSV, as shown by replacement of mutant and AR339 glycoprotein genes into full-length cDNA constructs of Sindbis virus. In another alphavirus, Ross River virus, a mutant deleted for E2 residues 55 to 61 displays an altered virulence phenotype in 1-week-old mice (52).

Our group has identified a domain on the E2 glycoprotein of Sindbis virus strain AR339 which controls virulence in neonatal mice. Substitution of arginine for the parental serine at E2 amino acid residue 114 significantly attenuates Sindbis virus as demonstrated with biologically selected mutants of AR339 (SB-RL and SB-FP [4, 5, 9, 31]) and with

full-length cDNA constructs of the Sindbis virus genome differing only at the E2 position 114 locus (33). This same codon influences two other Sindbis virus phenotypes as shown both in SB-RL (31, 32) and with the cDNA constructs (33). Arginine at E2 position 114, as compared with serine, accelerates viral penetration into BHK cells and allows efficient neutralization by E2-specific monoclonal antibodies (MAbs) reacting with the E2c antigenic site. Moreover, selection for accelerated penetration applied either to AR339 (32, 33) or to VEE (23) resulted in the isolation of rapidly penetrating, attenuated mutants. The covariant relationship between penetration and virulence suggests that a domain on the virion glycoprotein spike which influences early interaction with cultured cells overlaps a domain which influences pathogenesis. Mutations in such overlapping domains which lead to accelerated penetration into BHK cells may simulta-

26	Leu	Ala	Ala	Val	Val	Val	Leu	Met	Arg	Cys	Cys	Ser	Cys	Cys	Leu	Pro	Phe	Leu	Val	Val	Ala	Gly	Ala	Tyr	Leu	Ala	Lys	Val	Asp	Ala	Tyr	Glu	Ala	21
9975	CUG	GCC	GCU	GUC	GUC	GUU	CUA	AUG	CGC	UGU	UGC	UCA	UGC	UGC	CUG	CCU	UUU	UUA	GUG	GUU	GCC	GGC	GCC	UAC	CUG	GCG	AAG	GUN	GAC	GCC	UAC	GAA		
	U		U	A						C		C														A								
3	His	Ala	Thr	Thr	Val	Pro	Asn	Val	Pro	Gln	Ile	Pro	Tyr	Lys	Ala	Leu	Val	Glu	Arg	Ala	Gly	Tyr	Ala	Pro	Leu	Asn	Leu	Glu	Ile	Thr	Val	Met		
10071	CAU	GCG	ACC	ACU	GUU	CCA	AAU	GUG	CCA	CAG	AUA	CCG	UAU	AAG	GCA	CUU	GUU	GAA	AGG	GCA	GGG	UAC	GCC	CCG	CUC	AAU	UUG	GAG	AAU	ACU	GUC	AUG		
																					U								C					
35	Ser	Ser	Glu	Val	Leu	Pro	Ser	Thr	Asn	Gln	Glu	Tyr	Ile	Thr	Cys	Lys	Phe	Thr	Thr	Val	Val	Pro	Ser	Pro	Lys	Val	Arg	Cys	Cys	Gly	Ser	Leu		
10167	UCC	UCG	GAG	GUU	UUG	CCU	UCC	ACC	AAC	CAA	GAG	UAC	AUU	ACC	UGC	AAA	UUC	ACC	ACU	GUG	GUC	CCC	UCC	CCU	AAA	GUC	AGA	UGC	UGC	GGC	UCC	UUG		
																							A		A	A								
67	Glu	Cys	Gln	Pro	Ala	Ala	His	Ala	Asp	Tyr	Thr	Cys	Lys	Val	Phe	Gly	Gly	Val	Tyr	Pro	Phe	Met	Trp	Gly	Gly	Ala	Gln	Cys	Phe	Cys	Asp	Ser		
10263	GAA	UGU	CAG	CCC	GCC	GCU	CAC	GCA	GAC	UAU	ACC	UGC	AAG	GUC	UUU	GGA	GGG	GUG	UAC	CCC	UUC	AUG	UGG	GGA	GGA	GCA	CAA	UGU	UUU	UGC	GAC	AGU		
			G		U	U								C			C			U					G									
99	Glu	Asn	Ser	Gln	Met	Ser	Glu	Ala	Tyr	Val	Glu	Leu	Ser	Val	Asp	Cys	Ala	Thr	Asp	His	Ala	Gln	Ala	Ile	Lys	Val	His	Thr	Ala	Ala	Met	Lys		
10359	GAG	AAC	AGC	CAG	AUG	AGU	GAG	GCG	UAC	GUC	GAA	UUG	UCA	GUA	UGC	GCG	ACU	GAC	CAC	GCG	CAG	GCG	AUU	AAG	GUG	CAU	ACU	GCC	GCG	AUG	AAA			
											C		C					U								C								
131	Val	Gly	Leu	Arg	Ile	Val	Tyr	Gly	Asn	Thr	Thr	Ser	Phe	Leu	Asp	Val	Tyr	Val	Asn	Gly	Val	Thr	Pro	Gly	Thr	Ser	Lys	Asp	Leu	Lys	Val	Ile		
10455	GUA	GGA	CUG	CGU	AUA	GUG	UAC	GGG	AAC	ACU	ACC	AGU	UUC	CUA	GAU	GUG	UAC	GUG	AAC	GGA	GUC	ACA	CCA	GGA	ACG	UCU	AAA	GAC	CUG	AAA	GUC	AUA		
																													U					
163	Ala	Gly	Pro	Ile	Ser	Ala	Leu	Phe	Thr	Pro	Phe	Asp	His	Lys	Val	Val	Ile	His	Arg	Gly	Leu	Val	Tyr	Asn	Tyr	Asp	Phe	Pro	Glu	Tyr	Gly	Ala		
10551	GCU	GGA	CCA	AUU	UCA	GCA	UUG	UUU	ACA	CCA	UUC	GAU	CAC	AAG	GUC	GUU	AUC	CAU	CGC	GGC	CUG	GUG	UAC	AAC	UAU	GAC	UUU	CCG	GAA	UAC	GGA	GCG		
							C		G			U															C			U				
195	Met	Lys	Pro	Gly	Ala	Phe	Gly	Asp	Ile	Gln	Ala	Thr	Ser	Leu	Thr	Ser	Lys	Asp	Leu	Ile	Ala	Ser	Thr	Asp	Ile	Arg	Leu	Leu	Lys	Pro	Ser	Ala		
10647	AUG	AAA	CCA	GGA	GCG	UUU	GGA	GAC	AUU	CAA	GCU	ACC	UCC	UUG	ACU	AGC	AAA	GAC	CUC	AUC	GCC	AGC	ACA	GAC	AUU	AGG	CUA	CUC	AAG	CCU	UCC	GCC		
																	G	U																
227	Lys	Asn	Val	His	Val	Pro	Tyr	Thr	Gln	Ala	Ala	Ser	Gly	Phe	Glu	Met	Trp	Lys	Asn	Asn	Ser	Gly	Arg	Pro	Leu	Gln	Glu	Thr	Ala	Pro	Phe	Gly		
10743	AAG	AAC	GUG	CAU	GUC	CCG	UAC	ACG	CAG	GCN	GCA	UCU	GGA	UUC	GAG	AUG	UGG	AAA	AAC	AAC	UCA	GGC	CGC	CCA	CUG	CAG	GAA	ACC	GCC	CCU	UUU	GGG		
										C		A		U															A		C			
259	Cys	Lys	Ile	Ala	Val	Asn	Pro	Tyr	Thr	Gln	Ala	Val	Asp	Cys	Ser	Tyr	Gly	Asn	Ile	Pro	Ile	Ser	Ile	Asp	Ile	Pro	Asn	Ala	Ala	Phe	Ile	Arg	Thr	
10839	UGC	AAG	AUU	GCA	GUC	AAU	CCG	CUU	CGA	GCG	GUG	GAC	UGC	UCA	UAC	GGG	AAC	AUU	CCC	AUU	UCU	AUU	GAC	AUC	CCG	AAC	GCU	GCC	UUU	AUC	AGG	ACA		
	U			A				C					U																					
291	Ser	Asp	Ala	Pro	Leu	Val	Ser	Thr	Val	Lys	Cys	Asp	Val	Ser	Glu	Cys	Thr	Tyr	Ser	Ala	Asp	Phe	Gly	Gly	Met	Ala	Thr	Leu	Gln	Tyr	Val	Ser		
10935	UCA	GAU	GCA	CCA	CUG	GUC	UCA	ACA	GUC	AAA	UGU	GAU	GUC	AGU	GAG	UGC	ACU	UAU	UCA	GCG	GAC	UUC	GGA	GGG	AUG	GCU	ACC	CUG	CAG	UAU	GUA	UCC		
												A								A			C			C								
323	Asp	Arg	Glu	Gly	Gln	Cys	Pro	Val	His	Ser	His	Ser	Ser	Thr	Ala	Thr	Leu	Gln	Glu	Ser	Thr	Val	His	Val	Leu	Glu	Lys	Gly	Ala	Val	Thr	Val		
11031	GAC	CGC	GAA	GGA	CAA	UGC	CCU	GUA	CAU	UCG	CAU	UCG	AGC	ACA	GCA	ACC	CUC	CAA	GAG	UCG	ACA	GUU	CAU	GUC	CUG	GAG	AAA	GGA	GCG	GUG	ACA	GUA		
				U			C									U						A												
355	His	Phe	Ser	Thr	Ala	Ser	Pro	Gln	Ala	Asn	Phe	Ile	Val	Ser	Leu	Cys	Gly	Lys	Lys	Thr	Thr	Cys	Asn	Ala	Glu	Cys	Lys	Pro	Pro	Ala	Asp	His		
11127	CAC	UUC	AGC	ACC	GCG	AGC	CCA	CAG	GCG	AAC	UUC	AUU	GUA	UCG	CUG	UGU	GGU	AAG	AAG	ACA	ACA	UGC	AAU	GCA	GAA	UGC	AAA	CCA	CCA	GCU	GAU	CAU		
		U				U					U	C					G									U					C			
387	Ile	Val	Ser	Thr	Pro	His	Lys	Asn	Asp	Gln	Glu	Phe	Gln	Ala	Ala	Ile	Ser	Lys	Thr	Ser	Trp	Ser	Trp	Leu	Phe	Ala	Leu	Phe	Gly	Gly	Ala	Ser		
11223	AUC	GUG	AGC	ACC	CCG	CAC	AAA	AAU	GAC	CAA	GAA	UUC	CAA	GCC	GCC	AUC	UCA	AAA	ACU	UCA	UGG	AGU	UGG	CUG	UUU	GCC	CUU	UUC	GGC	GGC	GCC	UCG		
												U								A														
419	Ser	Leu	Leu	Ile	Ile	Gly	Leu	Met	Ile	Phe	Ala	Cys	Ser	Met	Met	Leu	Thr	Ser	Thr	Arg	Arg	End												
11319	UCG	CUA	UUA	AUU	AUA	GGA	CUU	AUG	AUU	UUU	GCU	UGC	AGC	AUG	AUG	CUG	ACU	AGC	ACA	CGA	AGA	UGA												

FIG. 1. Nucleotide sequence and deduced amino acid sequence of the glycoprotein genes of S.A.AR86. Numbering is from the 5' end of the Sindbis virus genomic RNA (46). Nucleotide differences between S.A.AR86 and AR339 (9, 46) are given below the S.A.AR86 sequence.

neously reduce the efficiency with which virions complete requisite early interactions with target cells in the brain. We utilized the relationship between penetration and virulence of alphaviruses to isolate two classes of pathogenesis mutants from S.A.AR86.

MATERIALS AND METHODS

Virus strains and cell culture. The S.A.AR86 strain of Sindbis virus was obtained from Jordi Casals, Yale Arbovirus Research Unit. The virus was passaged three times in mouse brain at Yale and subsequently was passaged four times in BHK cells.

BHK cells were obtained from the American Type Culture Collection (Rockville, Md.) in passage 53 and used between

passages 55 and 65. The cells were maintained in Eagle minimal essential medium (MEM) supplemented with 10% tryptose phosphate broth, 10% donor bovine serum or 5% fetal bovine serum, 50 units of penicillin per ml, and 50 µg of streptomycin per ml (MEM complete).

Selection for rapid penetration. Rapidly penetrating mutants of S.A.AR86 were selected as described by Johnston and Smith (23) for VEE. Virus was allowed to adsorb to precooled cultures of BHK cells at 4°C for 1 h. After being washed with cold MEM complete, the cells were brought to 37°C by the addition of warm medium and 1 min was allowed for penetration to occur. The infected cell monolayers were treated with a trypsin-EDTA solution (GIBCO Laboratories, Grand Island, N.Y.) to remove the remaining extracellular virions and allow the separation of virions and cells by

TABLE 1. Amino acid comparison of Sindbis virus glycoproteins

Protein (amino acid no. ^a)	Amino acid	
	SB (AR339) ^b	S.A.AR86
E3 (20)	Asp	Asn
E2		
1	Arg	Ser
29	Val	Ile
61	Ala	Ser
116	Val	Ala
125	Leu	Met
212	Ser	Thr
243	Leu	Ser
247	Asp	Ala
277	Ile	Val
312	Val	Ile
375	Thr	Ala
386	Val	Ala
6K		
29	Phe	Val
30	Ile	Val
E1		
60	Ile	Val
61	Lys	Arg
72	Val	Ala
112	Ala	Val
116	Ser	Thr
169	Ser	Leu
302	Glu	Asp

^a Numbered from the N terminus of each protein.^b From Davis et al. (9).

differential centrifugation. The pelleted cells were washed twice with MEM complete, replated, and incubated at 37°C for 24 h, at which time the culture supernatants were harvested. Under these conditions, only those adsorbed virions penetrating during the 1-min incubation at 37°C could participate in successive rounds of virus replication. Virus from the fourth rapid penetration passage was used to infect cells at a multiplicity of infection (MOI) of 10^{-5} for 5 min at 37°C. At this time, the culture was trypsinized and the cells were washed twice by differential centrifugation and suspended in MEM complete. The cells were plated into 96-well tissue culture plates to give 3.5×10^4 uninfected cells per well and a maximum of 0.3 infected cells per well to obtain biological clones. Culture supernatants from wells showing cytopathic effect were harvested at 48 h postinfection.

Assay for animal virulence. Groups of three to four male C57BL/6 mice (6 to 8 weeks of age) were inoculated i.c. with 0.025 ml of virus (10^2 to 10^5 PFU) suspended in phosphate-buffered saline. The animals were observed daily for 21 days postinoculation; morbidity and mortality were recorded. Surviving animals were bled from the retro-orbital sinus to obtain serum for titration of anti-Sindbis virus neutralizing antibody. Litters of 8 to 15 CD1 mice (<24 h old) were inoculated s.c. with 0.05 ml of virus (50 to 1,000 PFU) or i.c. with 0.01 ml of virus (100 PFU) suspended in phosphate-buffered saline containing 1% donor calf serum. One or two animals per litter were injected with phosphate-buffered saline containing 1% donor calf serum to serve as controls. The animals were observed for 14 days postinoculation; morbidity and mortality were registered.

Penetration rate. BHK cells in 60-mm-diameter tissue culture dishes (four dishes per strain) were cooled to 4°C and

inoculated with 0.2 ml of virus suspension containing approximately 150 PFU. After 1 h at 4°C, the cultures were warmed to 37°C. Half of the cultures infected with each strain were incubated for 60 min and overlaid with MEM complete containing 1% agarose for plaque assay. The inoculum was removed from the remaining cultures after incubation at 37°C for 20 min. The monolayers were washed with 1 ml of trypsin and then incubated for 5 min at 37°C with fresh trypsin (1 ml). The action of the trypsin was inhibited by the addition of 5 ml of MEM complete. Two rounds of differential centrifugation in MEM complete separated both infected and uninfected cells from free virus. The number of trypsin-resistant infectious centers was determined as described previously (23). The fraction of virus penetrated within 20 min was derived by dividing the number of infectious centers by the amount of attached virus determined by plaque assay.

Sequence analysis of viral genomic RNA. Virions were purified on potassium tartrate gradients and concentrated by pelleting through a 20% sucrose cushion as described previously (32). RNA was extracted from sodium dodecyl sulfate (SDS)-dissociated virions with a mixture of phenol and chloroform (1:1, vol/vol) followed by chloroform. The RNA was concentrated by precipitation with ethanol and quantitated by determining the A_{260} . Oligodeoxynucleotide primers of 14 or 15 nucleotides as described previously (9) were used to sequence viral RNA genomes by the dideoxy-chain termination method adapted for RNA sequencing (1, 39, 55). Four additional primers were needed to complete the sequencing. These primers were synthesized automatically with a Pharmacia Gene Assembler. The primers were SE13 (5'-CATTCTATTGACAT-3'), SE16 (5'-ATCGCTCCGT ATTCC-3'), E21-c (5'-GCATTAGAACGATG-3'), and SE24 (5'-GTGTACTACGTTTCG-3'). cDNAs labeled internally with [α - 32 P]dATP were resolved on 6, 8, or 20% polyacrylamide gels containing 7 M urea. Sindbis virus strains were compared by the SEQUALIGN programs (22).

SDS-PAGE. Purified virions were concentrated by pelleting through 20% sucrose (wt/wt in TNE buffer [0.05 M Tris hydrochloride (pH 7.2), 0.1 M NaCl, 0.001 M EDTA] and quantitated by a protein microassay (Bio-Rad Laboratories, Richmond, Calif.). The pellets were suspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and boiled for 5 min immediately prior to electrophoresis. Virion proteins were resolved by electrophoresis through a 10% polyacrylamide gel containing SDS (26) and visualized either by a modified silver staining technique (29) or by autoradiography on Kodak XAR film.

Pulse-chase labeling of maturing virion proteins. BHK cells in 60-mm-diameter tissue culture dishes were infected with virus at an MOI of 50 for 1 h at 37°C. At this time, the inoculum was removed and either 2 ml of methionine-free MEM containing 10% tryptose phosphate and 10% dialyzed donor calf serum (MET-minus MEM) or 2 ml of MET-minus MEM plus 10 μ g of tunicamycin per ml was added to each plate. At 8 h postinfection, the medium was removed and the cells were pulse-labeled for 30 min in either MET-minus MEM or MET-minus MEM plus tunicamycin containing 10 μ Ci of [35 S]methionine per ml. The cells were harvested either immediately after the pulse or after a chase with MEM complete for 1 or 2 h. The infected cells were scraped from the dishes into 100 μ l of TNE buffer containing 1% Nonidet P-40 and 1 mM MgCl₂. This suspension was placed on ice for 5 min, and the nuclei were pelleted by centrifugation at $1,100 \times g$ for 5 min. The supernatants contained the cytoplasmic fraction of the cells. A 50- μ l sample of the cytoplasmic

fraction was treated with 50 μ l of twice-concentrated SDS-PAGE sample buffer, subjected to three cycles of freezing and thawing, and boiled for 5 min before electrophoresis. Virions were harvested from culture fluids by pelleting through 20% sucrose. These pellets were resuspended in SDS-PAGE sample buffer (26) and stored frozen at -20°C until electrophoresis.

Glucosamine/methionine ratio. BHK cells were infected with virus at an MOI of 0.1 for 1 h at 37°C and then incubated in MEM complete containing 5 μCi of [^3H]glucosamine per ml and 10 μCi of [^3S]methionine per ml for 18 h at 37°C . Virions were harvested from culture fluids and gradient purified. Proteins from purified virions were resolved by SDS-PAGE. After autoradiography, the protein bands were excised from the gel and passively eluted in 2% SDS overnight. Under these conditions, elution of radioactivity was 90%. Eluates were analyzed by liquid scintillation counting to determine [^3H]glucosamine and [^3S]methionine levels in the glycoproteins E1, E2, and PE2. The ratio of [^3H]glucosamine to [^3S]methionine was determined and normalized for the number of methionine residues in each protein. The unglycosylated capsid protein served as an internal standard.

Analysis of viral growth kinetics. BHK cells were infected with virus at an MOI of 1.0 for 1 h at 37°C . The inoculum was removed, and the cells were incubated in MEM complete. Samples for plaque assay were removed from two plates per strain at 2, 4, 8, 12, 16, and 20 h postinfection.

Specific infectivity. BHK cells were infected with virus at an MOI of 1.0 for 1 h at 37°C and incubated for 18 h postinfection in the presence of [^3S]methionine (10 $\mu\text{Ci}/\text{ml}$). Titers of gradient-purified virus preparations were determined by plaque assay, and the level of radioactivity in the undiluted virus was determined. From this information, the specific infectivity (PFU per counts per minute) was calculated for each strain.

Enzyme-linked immunosorbent assay. Sindbis virus strains were screened by standard enzyme-linked immunosorbent assay for reactivity with five anti-E2 MAbs as described by Olmsted et al. (32). These MAbs also were used to analyze the virions in a more native conformation by capture enzyme-linked immunosorbent assay, also described by Olmsted et al. (32).

TABLE 2. Comparative penetration rates of Sindbis virus strains

Expt	% Penetration ^a					
	S.A.AR86	S3	S8	S11	S1	S12
1	4	31	ND ^b	ND	ND	ND
2	5	13	ND	ND	ND	ND
3	16	27	ND	ND	ND	ND
4	14	ND	58	37	100	49
5	15	ND	ND	34	76	85

^a The percentage of adsorbed virus which became resistant to removal by trypsin within 20 min at 37°C .

^b ND, Not determined.

RESULTS

Sequence comparison between Sindbis virus isolates S.A.AR86 and AR339. Sindbis virus isolates S.A.AR86 and AR339 are highly virulent for neonatal mice by either s.c. or i.c. routes but differ with respect to their virulence in adult mice. S.A.AR86 is lethal for adults inoculated i.c. at doses approaching 1 PFU, whereas AR339 induces no mortality in adults at doses of 10^6 PFU i.c. We compared the glycoprotein gene sequences of S.A.AR86 and AR339 (including the 6K and E3 regions) in an effort to identify putative coding changes which could account for the adult virulence phenotype. The comparison revealed 147 point mutations (Fig. 1) resulting in 22 amino acid coding differences (Table 1). It is clear that the extensive divergence between these two natural isolates of Sindbis virus makes it impossible to use a simple sequence comparison to evaluate the effect of these amino acid changes on the pathogenesis phenotype.

Selection of rapidly penetrating mutants of S.A.AR86. To select single-step mutants of S.A.AR86 which might be useful in identifying specific domains important for its pathogenesis phenotype, we passaged S.A.AR86 in BHK cells under a selective pressure for accelerated penetration as described by Johnston and Smith (23). This approach has been successful with both AR339 (9, 31, 33) and VEE (23) and is based on two considerations. First, selection for an altered penetration phenotype targets glycoprotein domains important in the early stages of virus-cell interaction, and second, mutation in these same domains or overlapping

FIG. 2. PE2 gene of S.A.AR86 and rapidly penetrating mutants. The point mutations and consequent amino acid differences from S.A.AR86 are indicated, as well as the potential glycosylation site in the E2 codon 1 mutants. The box at the right reviews the pathogenesis data presented in Table 3. A, Attenuated; V, virulent.

domains may influence pathogenesis by their effect on tissue tropism.

The S.A.AR86 population was enriched for rapidly penetrating mutants as described in Materials and Methods. Twenty-seven biologically cloned mutant strains were obtained. Six of these were examined in the work described here.

Penetration of BHK cells by S.A.AR86 and mutant strains. The rate at which the six mutant strains penetrated BHK cells was assessed as described in Materials and Methods. All six mutant strains under study penetrated BHK cells more rapidly than the S.A.AR86 parent did (Table 2).

Virulence in adult and neonatal mice. The six mutant strains segregated into two groups based on their virulence in mice (Table 3). S.A.AR86 and the first mutant group (S3, S8, and S11) displayed 100% mortality in adult mice following i.c. inoculation, although mice infected with the mutants had somewhat extended mean survival times. A similar relationship was observed in neonatal mice inoculated i.c. However, these mutants were attenuated in neonatal mice inoculated s.c. as compared with S.A.AR86. The attenuation was evident as reduced mortality and extended survival time.

Mutants constituting the second group (S1, S5, and S12) were essentially avirulent in adult mice inoculated i.c. and also were significantly attenuated in neonates inoculated by either i.c. or s.c. routes relative to their S.A.AR86 parent (Table 3).

Sequence analysis of S.A.AR86 and mutant glycoprotein genes. Because mutants isolated after selection for rapid penetration most probably contained alterations in structural proteins exposed on the virion surface, the sequence of representative mutant genomes was determined from the 3' end of the capsid gene through the E3, E2, 6K, and E1 genes. The S3 strain, representative of the mutant group virulent in adults but attenuated in neonates, differed from the parent S.A.AR86 strain at only two locations. One substitution at position 11282 (numbered from the 5' end of the Sindbis virus genome, [48]) in the E1 gene was a silent mutation from an adenosine in S.A.AR86 to a guanosine in S3. The other mutation was a coding change at position 8972. This nucleotide was an adenosine in S3 instead of a cytidine in S.A.AR86, resulting in the substitution of an arginine in S3 for a serine in S.A.AR86 at amino acid position 114 of the E2

glycoprotein (Fig. 2). Two other mutants from this phenotypic group (S8 and S11) were sequenced from nucleotide 8439 to nucleotide 9000 in the E2 gene. Like strain S3, these two mutants encoded arginine at E2 amino acid position 114. An identical mutation previously has been implicated in attenuation and penetration with both biologically selected mutants of strain AR339 and full-length cDNA clones (9, 33). It is likely that in the S.A.AR86 background, arginine substitution at E2 residue 114 also was responsible for attenuation in neonates inoculated s.c. and for the accelerated penetration characteristic of this mutant group. Yet this mutation appeared to have much less of an effect on the virulence of S.A.AR86 in adult and neonatal mice inoculated i.c.

The glycoprotein genes of representative mutants from the second group, characterized by attenuation in both adults and neonates, also were sequenced. S1, S5, and S12 differed from S.A.AR86 at a single base located at position 8632; this nucleotide was changed from a guanosine in the S.A.AR86 parent to an adenosine in the mutant strains. The resulting amino acid substitution was at E2 amino acid position 1 where a serine residue of S.A.AR86 was replaced with asparagine in the mutants (Fig. 2). This mutation converted the predicted amino acid sequence at the N terminus of E2 to Asn-Val-Thr, a potential site for N-linked glycosylation.

Effect of glycosylation site at E2 residue 1. Three experiments were performed to assess the effect of the putative glycosylation site at E2 position 1. First, proteins from purified preparations of extracellular virions were analysed by SDS-PAGE. Unlike virions of the S.A.AR86 parent and other Sindbis virus strains which contain E1, E2, and C, virions of the adult attenuated strains consisted of PE2, E1, and C (Fig. 3A). This result suggested that either the asparagine itself or the addition of carbohydrate to the putative glycosylation site at E2 position 1 prevented the processing of the E2 precursor protein, PE2. Furthermore, the isolation of a viable Sindbis virus mutant containing PE2 demonstrated that cleavage of the E2 precursor is not necessarily required for the maturation of infectious virions.

The second experiment was designed to determine whether the glycosylation site was, in fact, utilized by comparing the relative mobilities of the intracellular S.A.AR86 and mutant PE2 glycoproteins in SDS-PAGE. BHK monolayers were infected with either S.A.AR86 or

TABLE 3. Comparative pathogenicity of Sindbis virus strains

Virus strain	Pathogenicity in adults ^a injected i.c. with:				Pathogenicity in neonates ^b injected i.c. with 100 PFU		Pathogenicity in neonates ^b injected s.c. with 1,000 PFU	
	10 ⁵ PFU		50 PFU					
	% Mortality	AST ^c	% Mortality	AST	% Mortality	AST	% Mortality	AST
S.A.AR86	100 (3/3)	3	50 (3/6)	6.0	100 (7/7)	2.3	100 (27/27)	2.8
Group 1 mutants								
S3	100 (3/3)	5.5	83 (5/6)	5.8	100 (9/9)	3.6	83 (10/12)	7.1
S8	100 (3/3)	6.3			100 (6/6)	3.8	70 (7/10)	7.4
S11	100 (3/3)	4.5			100 (8/8)	5.6	80 (8/10)	6.8
Group 2 mutants								
S1	0 (0/7)				38 (3/8)	6.7	57 (4/7)	6.3
S5	12.5 (1/7)	6.0			86 (6/7)	9.5	77 (10/13)	5.4
S12	0 (0/7)				0 (0/7)		51 (7/13)	6.3

^a 6 to 8 weeks old.

^b Less than 24 h old.

^c AST, Average survival time. The average survival time of animals that died was calculated.

FIG. 3. SDS-PAGE of S.A.AR86 and rapidly penetrating E2 codon 1 mutants. (A) Proteins of purified S.A.AR86 virions and purified E2 codon 1 mutants S1, S5, and S12 were labeled metabolically with [35 S]methionine. (B) Intracellular proteins of S.A.AR86 and E2 codon 1 mutant S12 were labeled for 30 min with [35 S]methionine. C, Capsid protein.

S12. The cultures were labeled with [35 S]methionine for 30 min beginning at 8 h postinfection (Fig. 3B); PE2, E1, and C were labeled in both S.A.AR86 and S12 infected cells. The E1 glycoproteins and the C proteins of the two viruses comigrated in the gel. However, the PE2 of S12 migrated more slowly than the PE2 of S.A.AR86, as would be predicted if the protein contained an additional carbohydrate chain. Identical 35 S-labeled infected cultures were incubated in fresh medium containing excess unlabeled methionine (chase) for 1 h. In contrast to the S.A.AR86-infected cells in which PE2 disappeared after the chase period, the relative migration rate of PE2 of the S12 strain was unchanged (data not shown). Addition of tunicamycin altered the mobility of PE2 and E1 in cells infected with either S.A.AR86 or S12 (Fig. 4). However, in this case the S.A.AR86 and S12 PE2 proteins, lacking N-linked carbohydrates, comigrated.

In a third experiment, cells were infected with S.A.AR86 or S12 in the presence of [35 S]methionine and [3 H]glucosamine. Purified extracellular virions were subjected to SDS-PAGE, and the excised protein bands were analyzed by liquid scintillation spectroscopy after elution from the gel. The capsid protein served as an unglycosylated internal standard. The glucosamine/methionine ratio of the mutant and S.A.AR86 proteins was determined and adjusted for the number of methionine residues in each protein. This value reflected the relative number of utilized glycosylation sites on the E1 glycoproteins of both S.A.AR86 and S12, the E2 glycoprotein of S.A.AR86, and the PE2 glycoprotein of S12 (Table 4). The E1 proteins of S.A.AR86 and S12 had equivalent glucosamine/methionine ratios, indicating equivalent degrees of glycosylation. However, the PE2 protein of S12 contained approximately twice as much labeled glucosamine per labeled methionine residue as did the E2 protein of S.A.AR86. This result was consistent with the E2 protein of S.A.AR86 having two conserved and utilized glycosylation sites (predicted by the sequence comparison with AR339) and PE2 of S12 having four utilized glycosylation sites: the conserved site in E3, the two conserved sites in E2, and the newly created site at E2 position 1.

The results of these three experiments suggested (i) that the putative glycosylation site created by the asparagine substitution at E2 position 1 was utilized, (ii) that the asparagine substitution itself or the addition of carbohydrate at this position prevented the proteolytic processing of PE2,

FIG. 4. Protein composition of cytoplasmic extracts from BHK cells infected with S.A.AR86 or S12 in the presence or absence of tunicamycin. The viral and cellular proteins were pulse-labeled for 30 min with [35 S]methionine after an 8-h methionine deprivation. Lane 1, 14 C-labeled molecular weight standards (Sigma Chemical Co., St. Louis, Mo.): bovine serum albumin, 66,000; chicken egg albumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000. Lane 2, S.A.AR86; lane 3, S12; lane 4, S.A.AR86 in the presence of tunicamycin; lane 5, S12 in the presence of tunicamycin; lane 6, uninfected cells. PE2* and E1* indicate the positions of unglycosylated PE2 and E1. The relative migration of these unglycosylated proteins relative to their glycosylated forms has been determined previously (27, 40). C, Capsid protein.

and (iii) that the unprocessed PE2 quantitatively replaced E2 in mature mutant virions.

Growth and specific infectivity of S.A.AR86 and E2 asparagine 1 mutants. Previous work in several laboratories (5, 6, 19, 24, 46) indicated that virion maturation and production of infectious virus was prevented when proteolytic processing of PE2 into E2 and E3 was blocked by monensin, by addition of extracellular antibodies directed against either of the glycoproteins, or by mutation (e.g., *ts20*). However, when growth kinetics of S.A.AR86 and S12 were compared (Fig. 5), both strains grew to high titer (10^8 to 10^9 PFU/ml) with no significant differences in latent period or logarithmic phase. The specific infectivities of purified virions labeled with [35 S]methionine were determined as described in Materials and Methods. The specific infectivity of mutant S12 virions containing PE2 (6.7×10^3 PFU/cpm) was no less than that of S.A.AR86 virions (2.6×10^3 PFU/cpm). Equivalent results were obtained when specific infectivities were based on protein content of purified virus preparations as determined by colorimetric assay (Bio-Rad) (data not shown). These results indicate that cleavage of PE2 is not necessarily required for efficient maturation of infectious Sindbis virions and that the mutant strain which contained unprocessed PE2 grew at least as well in BHK cells as its S.A.AR86 parent.

Antigenic profile of S.A.AR86 and derived mutants. The virion structures of S.A.AR86 and representatives of each mutant class were probed with neutralizing MAbs specific

TABLE 4. Analysis of the number of utilized glycosylation sites

Virus	Protein	[³⁵ S]Met cpm	cpm of [³⁵ S]Met/no. of Met residues	[³ H]glucosamine cpm	cpm of [³ H]glucosamine/ cpm of [³⁵ S]Met per residue	Normalized to S.A.AR8	No. of predicted CHO sites
S.A.AR86	E1	2,186	219	7,234	33	1.0	2
S12	E1	884	88	2,756	31	0.9	2
S.A.AR86	E2	1,593	228	5,056	22	1.0	2
S12	PE2	698	87	4,269	49	2.2	4

for E2 antigenic sites situated on the surface of native virions. There are three defined neutralizing antigenic sites on E2 designated E2a, E2b, and E2c (8, 32, 38, 43, 47). Sequencing of neutralization escape mutants has identified amino acid residue 190 as a constituent of the E2a site (49), residue 216 as a major determinant of the E2b site (10), and residues 62, 96, and 159 as elements of the conformational E2c site (21; D. F. Pence and R. E. Johnston, unpublished observations). Several mutations in Sindbis virus AR339 which affect the binding or biological activity or both of E2c MAbs also affect pathogenesis in vivo (21). S.A.AR86 and representatives of the two mutant groups (E2 asparagine 1 mutants S1, S5, and S12; E2 arginine 114 mutant S3) were screened for reactivity with MAbs to antigenic sites E2a (MAb 49), E2b (MAbs R8 and R10), and E2c (MAbs R6 and R13) (Table 5). Binding of E2c MAbs to all four mutant

strains was decreased in comparison with that of the S.A.AR86 parent. The three adult attenuated strains containing PE2 showed a particularly marked reduction.

Binding of E2c MAbs to the mutant having E2 arginine 114 was detectable but reduced compared with E2c MAb binding to S.A.AR86. However, the mutant was neutralized more efficiently by E2c antibodies than was S.A.AR86 (Table 5). A similar phenotype was observed previously in E2 arginine 114 mutants of AR339 (31, 32) and in virus derived from full-length clones containing E2 arginine 114 (33).

DISCUSSION

The isolation of S.A.AR86 pathogenesis mutants and their preliminary characterization relate to several important issues of Sindbis virus biology. The first of these is the relationship between virulence in neonatal mice and virulence in adults. Various natural Sindbis virus isolates, laboratory strains, and mutants display one of three virulence phenotypes: (i) avirulent in neonates and adults, (ii) virulent in neonates but avirulent in adults, or (iii) virulent in both neonates and adults. Within each of the three categories, strain differences in mortality and mean survival times are evident. Moreover, Sindbis virus strains which are virulent in both neonates and adults generally show reduced survival times in neonates (by both i.c. and s.c. routes) compared with strains which are virulent in neonates only. These observations suggested a quantitative progression of increasing virulence from strains avirulent in neonates to strongly virulent strains lethal for adults. The S.A.AR86 mutant class typified by S3 was not consistent with this pattern. Compared with S.A.AR86, S3 was attenuated in neonates by s.c. inoculation, yet it remained as virulent as S.A.AR86 upon i.c. inoculation of neonates or adults. Therefore, as illustrated by S3, adult and neonatal virulence phenotypes differ qualitatively.

FIG. 5. Growth kinetics of S.A.AR86 and S12 in BHK cells. Symbols: ■, S.A.AR86; ●, S12.

TABLE 5. Analysis of MAb binding and neutralization

MAb	Optical density at 450 nm				
	S.A.AR86	S1	S5	S12	S3
Binding					
R6	1.12	0.09	0.19	0.17	0.46
R13	1.23	0.10	0.20	0.19	0.72
R8	1.39	0.97	1.24	1.23	1.17
R10	1.79	1.14	1.63	1.66	1.06
49	1.86	1.56	1.92	1.99	1.62
Neutralization (R6)					
	<100 ^a	ND ^b	ND	ND	400

^a The plaque reduction neutralization endpoint was expressed as the reciprocal of the highest dilution of MAb R6 that resulted in plaque reduction greater than or equal to 70% of a control sample incubated with nonimmune ascites fluid.

^b ND, Not determined.

The mutation responsible for the S3 phenotype was an arginine-for-serine substitution at E2 codon 114. This is the same coding change previously identified in mutants of strain AR339 (SB-RL and SB-FP) which also are attenuated for neonatal mice. SB-RL is attenuated in neonatal mice inoculated s.c., and compared with that of its parent, AR339, SB-RL invasion of the brain is delayed (unpublished observation). S3 also was attenuated in neonates after s.c. inoculation. Both S3 and SB-RL caused 100% mortality in neonates i.c., although S3, derived from S.A.AR86, was more rapidly lethal (2 to 4 days) than SB-RL (14 to 21 days). Two inferences may be drawn from these results. First, the arginine substitution at E2 position 114 exerts a similar effect in both AR339 and S.A.AR86 mutants despite the significant differences in genetic background. Second, in neonatal mice, one attenuating effect of the E2 114 mutation in either background may be to retard invasion of the target organ.

A second biological issue inherent in this work is the relationship between glycoprotein domains important for penetration of cultured cells and domains important for virulence *in vivo*. The mutants of S.A.AR86 discussed here represent the third instance in which selection for accelerated penetration coselected for attenuation. Coselection was demonstrated previously with Sindbis virus strain AR339 (31, 33) and with VEE (23). We have suggested that an alphavirus glycoprotein domain(s) influencing penetration overlaps a domain(s) which affects pathogenesis and that mutation of the shared region could affect both phenotypes (23). With Sindbis virus strain AR339, we have demonstrated that such mutations occur at the E2 position 114 locus (33). Mutations resulting in increased efficiency of interaction between virions and BHK cell membrane components may simultaneously decrease the efficiency with which virions interact with analogous elements on the plasma membranes of target cells *in vivo*.

In addition to its effects on penetration and virulence, the E2 arginine 114 substitution in AR339 modulates the biological activity of neutralizing MAbs reactive with the E2c antigenic site (31). These antibodies bind equally well to native virions having either serine or arginine at E2 114 (32); however, the antibodies neutralize the arginine-containing strains much more efficiently. The E2 arginine 114 mutation in S.A.AR86 reduced the ability of E2c-specific MAbs to bind to native virions or to virions disrupted by nonspecific attachment to an enzyme-linked immunosorbent assay plate. Nevertheless, these arginine-containing mutants, like those of AR339, were more efficiently neutralized by the E2c antibodies than was S.A.AR86.

The third point is the pleiotropic effect of the asparagine substitution at E2 position 1. E2 asparagine 1 was the only coding change in the glycoprotein genes between the adult attenuated mutant class and the S.A.AR86 parent. This suggested that the E2 position 1 mutation was responsible for several mutant phenotypes: avirulence in adult mice inoculated i.c., attenuation in neonatal mice inoculated i.c. and s.c., rapid penetration into BHK cells, loss of reactivity with E2c-specific MAbs, hyperglycosylation of PE2, failure to cleave PE2 to E3 plus E2, and consequent incorporation of PE2 into mature virions in place of E2.

In contrast to the predominant effect of the E2 position 114 mutation on neuroinvasiveness, the E2 asparagine 1 mutation reduced the neurovirulence of S.A.AR86 for both adults and neonates inoculated i.c. Accelerated penetration into BHK cells and altered reactivity with E2c-specific MAbs were phenotypes shared by the E2 asparagine 1 and the E2 arginine 114 mutations. E2c MAb neutralization escape

mutations mapped to E2 residues 62, 96, and 159. Mutations at each of these loci affected pathogenesis (10; Pence and Johnston, unpublished observations). Reduction in, or loss of, E2c MAb-binding activity is consistent with residues at E2 position 1 and E2 position 114 also being structurally related to the E2c antigenic site. However, the structural basis for the multiple effects of E2 asparagine 1 may reside in the substitution of this residue itself, in the addition of an N-linked carbohydrate chain to the asparagine, and/or in the effect of the additional 64 N-terminal amino acids not present on glycoprotein E2 of S.A.AR86.

The most dramatic biochemical effect of the E2 asparagine 1 mutation was the inhibition of PE2 cleavage and the quantitative incorporation of the precursor into infectious virions. The basis for the cleavage inhibition may be either a direct effect of the asparagine at position +1 relative to the cleavage site or a steric effect of the carbohydrate addition. Previous experiments in which PE2 cleavage was inhibited by antibodies to the glycoproteins, temperature-sensitive mutations, or monensin indicated that PE2 cleavage is required for the maturation of infectious virus (5, 6, 19, 24, 46). This was clearly not the case with the S.A.AR86 mutants. The mutant virions had the same specific infectivity as S.A.AR86 and displayed equivalent growth in BHK cells. In addition, Presley and Brown (submitted for publication) have demonstrated that infectious virions of the HR Sindbis virus strain containing substantial amounts of PE2 were produced in cells treated with relatively low concentrations of monensin. Both the S.A.AR86 mutants at E2 position 1 and the observations of Presley and Brown (submitted) suggest that PE2 cleavage is not a prerequisite for virion maturation or infectivity in cell culture. However, it is of interest that this cleavage may be required for Sindbis virus neurovirulence *in vivo*. The amino acid sequence immediately upstream from the S.A.AR86 PE2 cleavage site is Arg-Ser-Lys-Arg-, similar in its arrangement of basic amino acids to glycoprotein cleavage sites in other alphaviruses (13, 15, 25), flaviviruses (35), retroviruses (44), myxoviruses (11), and paramyxoviruses (51). Especially relevant are the paramyxovirus and myxovirus systems. Surveys of Newcastle disease virus strains have shown that the inability to cleave the fusion protein is correlated with loss of virulence (30, 51). In addition, a mutation creating a new glycosylation site in the avian influenza virus hemagglutinin prevents cleavage and renders the virus avirulent (11). Oligonucleotide-directed mutagenesis of a full-length cDNA clone containing the S.A.AR86 glycoprotein genes is in progress. Critical amino acid residues near the PE2 cleavage site will be altered to determine the sequence requirements for this important processing event and to ascertain the structural basis for the effects of the E2 asparagine 1 substitution on Sindbis virus penetration, MAb reactivity, and neurovirulence in adult and neonatal mice.

ACKNOWLEDGMENTS

We thank David F. Pence and Loretta Willis for excellent technical assistance.

This work was supported by the U.S. Army Summer Faculty Research Program and Public Health Service grants A122186 and NS26681 from the National Institutes of Health.

LITERATURE CITED

1. Ahlquist, P., R. Dasgupta, and P. Kaesberg. 1981. Near identity of 3' RNA secondary structure in bromoviruses and cucumber mosaic virus. *Cell* 23:183-189.
2. Aliperti, G., and M. J. Schlesinger. 1978. Evidence for an

- autoprotease activity of Sindbis virus capsid protein. *Virology* **90**:366–369.
3. Andrews, C. H., and H. G. Pareira. 1972. *Viruses of vertebrates*, 3rd ed. Bailliere Tindall, London.
 4. Baric, R. S., D. W. Trent, and R. E. Johnston. 1981. A Sindbis virus variant with a cell-determined latent period. *Virology* **110**:237–242.
 5. Bracha, M., and M. J. Schlesinger. 1976. Inhibition of Sindbis virus replication by zinc ions. *Virology* **72**:272–277.
 6. Bracha, M., and M. J. Schlesinger. 1976. Defects in RNA+ temperature-sensitive mutants of Sindbis virus and evidence for a complex of PE2-E1 viral glycoproteins. *Virology* **74**:441–449.
 7. Brown, D. T., and J. F. Smith. 1975. Morphology of BHK-21 cells infected with Sindbis virus temperature-sensitive mutants in complementation groups D and E. *J. Virol.* **15**:1263–1266.
 8. Chanas, A. C., E. A. Gould, J. C. S. Clegg, and M. G. R. Varma. 1982. Monoclonal antibodies to Sindbis virus glycoprotein E1 can neutralize, enhance infectivity and independently inhibit haemagglutination or haemolysis. *J. Gen. Virol.* **58**:37–46.
 9. Davis, N. L., F. J. Fuller, W. G. Dougherty, R. A. Olmsted, and R. E. Johnston. 1986. A single nucleotide change in the E2 glycoprotein gene of Sindbis virus affects penetration rate in cell culture and virulence in neonatal mice. *Proc. Natl. Acad. Sci. USA* **83**:6771–6775.
 10. Davis, N. L., D. F. Pence, W. J. Meyer, A. L. Schmaljohn, and R. E. Johnston. 1987. Alternative forms of a strain-specific neutralizing antigenic site on the Sindbis virus E2 glycoprotein. *Virology* **161**:101–108.
 11. Deshpande, K. L., V. A. Fried, M. Ando, and R. G. Webster. 1987. Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. *Proc. Natl. Acad. Sci. USA* **84**:36–40.
 12. Erwin, C., and D. T. Brown. 1980. Intracellular distribution of Sindbis virus membrane proteins in BHK-21 cells infected with wild-type virus and maturation-defective mutants. *J. Virol.* **36**:775–786.
 13. Faragher, S. G., A. D. J. Meek, C. M. Rice, and L. Dalgarno. 1988. Genomic sequences of a mouse-avirulent and a mouse-virulent strain of Ross River virus. *Virology* **163**:509–526.
 14. Fuller, F. J., and P. I. Marcus. 1980. Sindbis virus. I. Gene order of translation *in vivo*. *Virology* **107**:441–451.
 15. Garoff, H., A.-M. Frischauf, K. Simons, H. Lehrach, and H. Delius. 1980. Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. *Nature (London)* **228**:236–241.
 16. Garoff, H., K. Simons, and O. Renkonen. 1974. Isolation and characterization of the membrane proteins of Semliki Forest virus. *Virology* **61**:493–504.
 17. Griffin, D. E., and R. T. Johnson. 1977. Role of the immune response in recovery from Sindbis virus encephalitis in mice. *J. Immunol.* **118**:1070–1075.
 18. Hahn, C. S., E. G. Strauss, and J. H. Strauss. 1985. Sequence analysis of three Sindbis virus mutants temperature-sensitive in the capsid protein autoprotease. *Proc. Natl. Acad. Sci. USA* **82**:4648–4652.
 19. Johnson, D. C., and M. J. Schlesinger. 1980. Vesicular stomatitis virus and Sindbis virus glycoprotein transport to the cell surface is inhibited by ionophores. *Virology* **103**:407–424.
 20. Johnson, R. T., H. F. McFarland, and S. E. Levy. 1972. Age-dependent resistance to viral encephalitis: studies of infections due to Sindbis virus in mice. *J. Infect. Dis.* **125**:257–262.
 21. Johnston, R. E., N. L. Davis, D. F. Pence, S. Gidwitz, and F. J. Fuller. 1987. Nucleic acid sequence analysis of Sindbis pathogenesis and penetration mutants, p. 467–476. *In* M. A. Brinton and R. L. Rueckert (ed.), *Positive strand RNA viruses*. Alan R. Liss, Inc., New York.
 22. Johnston, R. E., J. M. Mackenzie, and W. G. Dougherty. 1986. Assembly of overlapping DNA sequences by a program written in BASIC for 64K CP/M and MS-DOS IBM-compatible microcomputers. *Nucleic Acids Res.* **14**:517–527.
 23. Johnston, R. E., and J. F. Smith. 1988. Selection for accelerated penetration in cell culture coselects for attenuated mutants of Venezuelan equine encephalitis virus. *Virology* **162**:437–443.
 24. Jones, K. J., R. K. Scupham, J. A. Pfeil, K. Wan, B. P. Sagik, and H. R. Bose. 1977. Interaction of Sindbis virus glycoproteins during morphogenesis. *J. Virol.* **21**:778–787.
 25. Kinney, R. M., B. J. B. Johnson, V. L. Brown, and D. W. Trent. 1986. Nucleotide sequence of the 26S mRNA of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and deduced sequence of the encoded structural proteins. *Virology* **152**:400–413.
 26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
 27. Leavitt, R., S. Schlesinger, and S. Kornfeld. 1977. Tunicamycin inhibits glycosylation and multiplication of Sindbis and vesicular stomatitis viruses. *J. Virol.* **21**:375–385.
 28. Lustig, S., A. C. Jackson, C. S. Hahn, D. E. Griffin, E. G. Strauss, and J. H. Strauss. 1988. Molecular basis of Sindbis virus neurovirulence in mice. *J. Virol.* **62**:2329–2336.
 29. Morrissey, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* **117**:307–310.
 30. Nagai, Y., H.-D. Klenk, and R. Rott. 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virology* **72**:494–508.
 31. Olmsted, R. A., R. S. Baric, B. A. Sawyer, and R. E. Johnston. 1984. Sindbis virus mutants selected for rapid growth in cell culture display attenuated virulence in animals. *Science* **225**:424–427.
 32. Olmsted, R. A., W. J. Meyer, and R. E. Johnston. 1986. Characterization of Sindbis virus epitopes important for penetration in cell culture and pathogenesis in animals. *Virology* **148**:245–254.
 33. Polo, J. M., N. L. Davis, C. M. Rice, H. V. Huang, and R. E. Johnston. 1988. Molecular analysis of Sindbis virus pathogenesis in neonatal mice by using virus recombinants constructed *in vitro*. *J. Virol.* **62**:2124–2133.
 34. Reinarz, A. B. G., M. G. Broome, and B. P. Sagik. 1971. Age-dependent resistance of mice to Sindbis virus infection: viral replication as a function of host age. *Infect. Immunol.* **3**:268–273.
 35. Rice, C. M., E. M. Lenches, S. R. Eddy, S. J. Shin, R. L. Sheets, and J. H. Strauss. 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science* **229**:726–733.
 36. Rice, C. M., and J. H. Strauss. 1981. Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins. *Proc. Natl. Acad. Sci. USA* **28**:2062–2066.
 37. Rice, C. M., and J. H. Strauss. 1982. Association of Sindbis virion glycoproteins and their precursors. *J. Mol. Biol.* **154**:325–348.
 38. Roehrig, J. T., D. Gorski, and M. J. Schlesinger. 1982. Properties of monoclonal antibodies directed against the glycoproteins of Sindbis virus. *J. Gen. Virol.* **59**:421–425.
 39. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 40. Scheefers, H., U. Scheefers-Borchel, J. Edwards, and D. T. Brown. 1980. Distribution of virus structural proteins and protein-protein interactions in plasma membrane of baby hamster kidney cells infected with Sindbis or vesicular stomatitis virus. *Proc. Natl. Acad. Sci. USA* **77**:7277–7281.
 41. Schlesinger, M. J., and S. Schlesinger. 1973. Large molecular-weight precursors of Sindbis virus proteins. *J. Virol.* **11**:1013–1016.
 42. Schlesinger, M. J., S. Schlesinger, and B. W. Burge. 1972. Identification of a second glycoprotein in Sindbis virus. *Virology* **47**:539–541.
 43. Schmaljohn, A. L., K. M. Kokubun, and G. A. Cole. 1983. Protective monoclonal antibodies define maturational and pH-dependent changes in Sindbis virus E1 glycoprotein. *Virology* **130**:144–154.
 44. Schwartz, D. E., R. Tizard, and W. Gilbert. 1983. Nucleotide sequence of Rous sarcoma virus. *Cell* **32**:853–869.

45. **Simmons, D. T., and J. H. Strauss.** 1972. Replication of Sindbis virus. I. Relative size and genetic content of 26S and 49S RNA. *J. Mol. Biol.* **71**:599-613.
46. **Smith, J. F., and D. T. Brown.** 1977. Envelopment of Sindbis virus: synthesis and organization of proteins in cells infected with wild-type and maturation-defective mutants. *J. Virol.* **22**: 662-678.
47. **Stanley, J., S. J. Cooper, and D. E. Griffin.** 1985. Alphavirus neurovirulence: monoclonal antibodies discriminating wild-type from neuroadapted Sindbis virus. *J. Virol.* **56**:110-119.
48. **Strauss, E. G., C. M. Rice, and J. H. Strauss.** 1984. Complete nucleotide sequence of the genomic RNA of Sindbis virus. *Virology* **133**:92-110.
49. **Strauss, E. G., A. L. Schmaljohn, D. E. Griffin, and J. H. Strauss.** 1987. Structure-function relationships in the glycoproteins of alphaviruses, p. 365-378. *In* M. A. Brinton and R. L. Rueckert (ed.), *Positive strand RNA viruses*. Alan R. Liss, Inc., New York.
50. **Strauss, J. H., B. W. Burge, E. R. Pfefferkorn, and J. E. Darnell.** 1969. Identification of the membrane protein and "core" protein of Sindbis virus. *Proc. Natl. Acad. Sci. USA* **59**:533-537.
51. **Toyoda, T., T. Sakaguchi, K. Imai, N. M. Inocencio, B. Gotoh, M. Hamaguchi, and Y. Nagai.** 1987. Structural comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of Newcastle disease virus. *Virology* **158**:242-247.
52. **Vrati, S., S. G. Faragher, R. C. Weir, and L. Dalgarno.** 1986. Ross River virus mutant with a deletion in the E2 gene: properties of the virion, virus-specific macromolecule synthesis, and attenuation of virulence for mice. *Virology* **151**:222-232.
53. **Welch, W. J., and B. M. Sefton.** 1979. Two small virus-specific polypeptides are produced during infection with Sindbis virus. *J. Virol.* **29**:1186-1195.
54. **Wirth, D. F., F. Katz, B. Small, and H. F. Lodish.** 1977. How a single Sindbis virus mRNA directs the synthesis of one soluble protein and two integral membrane glycoproteins. *Cell* **10**: 253-263.
55. **Zimmern, D., and P. Kaesberg.** 1978. 3'-Terminal nucleotide sequence of encephalomyocarditis virus RNA determined by reverse transcriptase and chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **75**:4257-4261.