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Molecular Basis of Sindbis Virus Neurovirulence in Mice

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We examined a variety of strains of Sindbis virus for the genetic changes responsible for differences in neurovirulence in mice. SV1A (a low passage of the AR339 strain of Sindbis virus), a neuroadapted Sindbis virus (NSV), and two laboratory strains of Sindbis virus (HRSP and Toto1101) were examined. NSV causes severe encephalomyelitis with hind-limb paralysis and high mortality after intracerebral inoculation in weanling mice. In contrast, SV1A causes only mild, nonfatal disease in weanling mice; however, in suckling mice, SV1A causes a fatal encephalomyelitis after either intracerebral or subcutaneous inoculation. The two laboratory strains used have a greatly reduced neurovirulence for suckling mice and are avirulent for weanling mice. The nucleotide sequences and encoded amino acid sequences of the structural glycoproteins of these four strains were compared. Hybrid genomes were constructed by replacing restriction fragments in a full-length cDNA clone of Sindbis virus, from which infectious RNA can be transcribed in vitro, with fragments from cDNA clones of the various strains. These recombinant viruses allowed us to test the importance of each amino acid difference between the various strains for neurovirulence in weanling and suckling mice. Glycoproteins E2 and E1 were of paramount importance for neurovirulence in adult mice. Recombinant viruses containing the nonstructural protein region and the capsid protein region from an avirulent strain and the E1 and E2 glycoprotein regions from NSV were virulent, although they were less virulent than NSV. Furthermore, changes in either E2 (His-55 in NSV to Gln in SV1A) or E1 (Ala-72 in NSV to Val in SV1A and Asp-313 in NSV to Gly in SV1A) reduced virulence. For virulence in suckling mice, we found that a number of changes in E2 and E1 can lead to decreased virulence and that in fact, a gradient of virulence exists.

Sindbis virus is the type species of the genus *Alphavirus* in the family *Togaviridae*. It is among the least pathogenic of the alphaviruses, which include such important pathogens as eastern, western, and Venezuelan equine encephalitis viruses (10). In nature, Sindbis virus is transmitted by mosquitoes and its vertebrate hosts are usually birds or mammals (40). The viral genome is a plus-stranded RNA of 11,703 nucleotides. In the virion, it is complexed with capsid protein C to form an icosahedral nucleocapsid that is surrounded by a lipid bilayer in which two integral membrane glycoproteins, E2 and E1, are embedded (reviewed in reference 39). Both E1 and E2 are anchored in the membrane by a conventional C-terminal hydrophobic anchor, and they are closely associated with each other as a heterodimer that probably forms during synthesis of the glycoproteins (27, 30). This heterodimer is believed to be the functional subunit, and three heterodimers are associated to form a trimeric spike in the virus (8). The spike is the structure that binds to susceptible cells to initiate infection, and it also possesses neutralizing epitopes.

Sindbis virus infection of mice has been studied as an experimental model of acute viral encephalitis (9, 13). Wild-type Sindbis virus (AR339 strain) causes fatal encephalitis in suckling mice and nonfatal encephalitis in 4-week-old weanling mice (14). A strain of Sindbis virus that is highly lethal for weanling and adult mice was isolated after six intracerebral (i.c.) passages of an AR339 strain of Sindbis virus, alternating between suckling and weanling mice (11). This neuroadapted strain of Sindbis virus (NSV) has been genetically stable after many passages in cell culture. NSV replicates 10- to 50-fold more efficiently in the brains of mice than SV1A (a low-passage strain derived from wild-type Sindbis virus AR339), and its i.c. 50% lethal dose is 2 to 20 PFU in weanling mice. After i.c. inoculation with NSV, weanling mice become ruffled, develop kyphoscoliosis and hind-limb paralysis, and have high mortality (11, 12). There is poliomyelitis, particularly involving the ventral horns, in the thoracic and lumbar spinal cord (12). Stanley et al. (35) demonstrated that some anti-Sindbis virus E1 and E2 monoclonal antibodies discriminate between NSV and SV1A, suggesting that changes in the surface glycoproteins are associated with changes in virulence.

Olmsted et al. (20, 21) have selected Sindbis virus variants in vitro that are attenuated in suckling mice. Thus, strains of Sindbis virus can be essentially avirulent for mice of all ages, virulent for suckling mice but avirulent for weanling mice, or virulent for mice of all ages.

Recently, a complete cDNA clone of Sindbis virus has been constructed from which infectious RNA can be transcribed in vitro with SP6 RNA polymerase (28). This has made it possible to map changes among the various strains of Sindbis virus which lead to differences in virulence in mice, an approach similar to that used by Kohara et al. (15) to map virulence determinants in poliovirus.

MATERIALS AND METHODS

Virus stocks and preparation of virus and viral RNA. SV1A (derived from a low-passage isolate of AR339 obtained from the American Type Culture Collection, Rockville, Md.) and

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NSV were grown and titers were determined on monolayers of primary and secondary chicken embryo fibroblasts (CEF), as previously described (35). Virus was precipitated from the culture fluid with polyethylene glycol and was purified by successive velocity sedimentation and equilibrium density centrifugation (23). The isolation of viral RNA was described previously (29).

cDNA cloning. cDNA synthesis followed essentially the procedure of Okayama and Berg (19) as modified by Lindqvist et al. (16). A plasmid vector referred to as proteus 1 was tailed with deoxyribosylthymine (40 to 60 residues) and used as a primer for first-strand cDNA synthesis. Second-strand synthesis then used *Escherichia coli* DNA polymerase I, *E. coli* RNase H, and *E. coli* DNA ligase. After second-strand synthesis, the double-stranded DNA was cut with *Hin*dIII and ligated intramolecularly. The resulting clones have an insert of 5,438 nucleotides [plus a variable length of poly(A)] and contain the entire structural protein coding region of Sindbis virus.

General recombinant DNA techniques. Restriction endonucleases and DNA-modifying enzymes were purchased from commercial sources and used essentially as recommended by the manufacturer. Plasmids were grown, purified, and analyzed by standard methods, with minor modifications (17).

Construction of hybrid genomes. Hybrid genomes were produced by replacing restriction fragments in Sindbis virus clone Toto1101 or Toto50 (28) with the corresponding regions from cDNA clones derived from NSV, with cDNA from strain SV1A, or with cDNA from a clone of Sindbis virus HRSP strain (for details of the restriction sites used, see Fig. 1 and 2). Virus was rescued from these recombinant clones and tested for its biological properties.

In vitro transcription and RNA transfection. RNA transcripts were synthesized as described elsewhere (28). Briefly, either supercoiled plasmid templates or plasmid DNAs which had been digested with an appropriate restriction endonuclease to produce a runoff transcript were transcribed in vitro by SP6 RNA polymerase in a solution containing 40 mM Tris chloride (pH 7.6); 6 mM MgCl; 2 mM spermidine; 1 mM each ATP, CTP, GTP, and UTP; 100 μg of nuclease-free bovine serum albumin per ml; 5 mM dithiothreitol; 1 mM $m^7G(5')ppp(5')G$ cap analog; 500 U of human placental RNase inhibitor per ml; 400 U of SP6 polymerase per ml; and 10 to 100 μg of template DNA per ml. Quantitation of the RNA transcript was effected by including a trace amount of [^{32}P]CTP in the reaction and counting a sample of the product after adsorption to DE81 paper (Whatman, Inc., Clifton, N.J.). Confluent monolayers of secondary CEF in 35-mm-diameter tissue culture plates (about 10^6 cells) were transfected with the resulting RNA. After they were washed once with Eagle minimal essential medium (7) containing Earle salts but not serum, the cells were incubated with 1.5 ml of Eagle medium containing 50 mM Tris chloride (pH 7.3) at 25°C and 200 μg of DEAE dextran per ml (500,000 average molecular weight; Sigma Chemical Co.) for 60 min at 37°C. This medium was removed, and 200 μl of in vitro-transcribed RNA diluted in phosphate-buffered saline was added to the cells and incubated at room temperature for 60 min with occasional rocking. PFU were quantitated by overlaying the monolayer with 3 ml of 1.2% agarose (SeaKem; FMC Corp., Marine Colloids Div., Rockland, Maine) in Eagle medium containing 2% fetal calf serum, followed by incubation at 30 or 40°C for 3 or 2 days, respectively. Plaques were visualized by staining with neutral red. Virus stocks were produced by removing the transfection mix and incubating the cells in 3 ml of Eagle medium containing 3% fetal calf serum for 48 h or longer at 30°C.

Sequence determination of SV1A and NSV cDNA. DNA sequencing was carried out on cloned cDNA by the method of Maxam and Gilbert (18) as modified by Smith and Calvo (34), using restriction fragments 3′ end labeled with the Klenow fragment of *E. coli* DNA polymerase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). NSV clones NSV5-37 and NSV5-44 and SV1A clones SV1A-3 and SV1A-21 were analyzed. Chain termination sequencing of RNA with reverse transcriptase as described by Ou et al. (22) and Zimmern and Kaesberg (41), using intracellular viral RNA as a template and synthetic oligonucleotides as primers, was also used to obtain part of the sequence. To test virus derived from hybrid cDNAs for the predicted sequence, certain regions of the RNA genome sequence were confirmed by chain termination sequencing in the same way. All of the sequencing was obtained on two clones or checked by the dideoxy sequencing of an RNA template (which gives the majority nucleotide at any position) to rule out cloning artifacts and sequencing of minor variants.

Animal tests. Viruses for animal inoculation were grown and assayed by plaque formation on BHK-21 cells. Three- to four-week-old BALB/cJ weanling mice of either sex (Jackson Laboratory, Bar Harbor, Maine) were inoculated i.c. with 1,000 PFU of virus in 0.03 ml of Hanks balanced salt solution containing 1% fetal calf serum. One- to three-day-old CD-1 (ICR) suckling mice (Charles River Breeding Laboratories, Wilmington, Mass.) were inoculated subcutaneously with 500 PFU of virus in each hind limb (footpad). Mice were observed for 25 days. The percent mortality was determined for weanling mice, and the percent mortality and mean day of death were determined for suckling mice. At least 10 weanling mice and two litters of suckling mice were inoculated with each virus strain. For recombinant strains, viruses grown from 2 to 4 different plaques were tested separately. These data have been pooled for presentation. For viruses which killed weanling mice at 1,000 PFU, the i.c. dose for 50% mortality was determined by the method of Reed and Muench (25), using groups of 10 mice at serial 10-fold dilutions.

RESULTS

Sindbis virus strains. Four strains of Sindbis virus and recombinants between various strains were used in these studies (Table 1). The passage history of these strains significantly affects the interpretation of the results. SV1A is a low-passage stock (7 to 9 passages in suckling mouse brain) received from the American Type Culture Collection and subsequently passaged five times in BHK cells (including three sequential plaque purifications) and twice in CEF. The AR339 strain that served as the parent for NSV had a more extensive passage history. It was obtained originally from H. Hineberg (Cleveland Metropolitan General Hospital) after an unknown passage history, and it was subsequently passed 10 times in mouse brain, once in BHK cells, and more than 10 times in CEF before neuroadaption (14). NSV was obtained from this AR339 strain by serial transmission six times by i.c. inoculation alternately in suckling and weanling mice (11). This virus was subsequently passed twice in CEF to isolate viral RNA for sequence analysis.

In addition, two laboratory strains of Sindbis virus derived from infectious RNA rescued from cDNA clones (28) were used; both were derived from the AR339 strain. The first of

TABLE 1. Sindbis virus strains

Virus strain[a]	Passage history[b]	Virulence in:	
		Suckling mice	Weanling mice
SV1A	9 SMB, 5 BHK, 2 CEF	+	−
NSV	Unknown passages in cell culture, 10 MB, 1 BHK, 10 CEF, 6(SMB + MB),[c] 2 CEF	+	+
Toto1101	Extensive passage in cell culture	±	−
HRSP	Extensive passage in cell culture	±	−

[a] All strains were derived by passage of the AR339 strain, isolated originally from a pool of *Culex univittatus* in August 1952, in Sindbis, Egypt, by inoculation into suckling mouse brain (40).

[b] Number and type of passage. SMB, Suckling mouse brain; MB, mouse brain; BHK, BHK-21 cells.

[c] Six i.c. inoculations alternately in suckling and weanling mouse brain.

these laboratory variants was rescued from cDNA clone Toto1101 and contains nucleotides 1 to 2713 and 9805 to 11703 from the HRSP strain described below and nucleotides 2714 to 9804 from a strain of uncertain passage history before cloning as Toto1101. The second strain, from clone Toto50, was derived from cDNA made to the HRSP strain of Sindbis virus (heat-resistant small-plaque strain). The HR strain was obtained by Burge and Pfefferkorn (5) from AR339 by selecting variants able to survive heating to 56°C. It had been subsequently passaged multiple times in both CEF and BHK cells before isolation of the small-plaque variant (38). HRSP was then passaged several times in CEF before being cloned as Toto50. The sequence of the HRSP strain has been published previously (28, 37).

Sequence analysis of the glycoproteins of NSV and of SV1A. Because monoclonal antibodies discriminate between NSV and SV1A (35) and attenuating mutations have been found in E2 (6, 21), it seemed likely that the glycoproteins would be important for neurovirulence in Sindbis virus. We therefore obtained the nucleotide sequence of the region encoding the glycoproteins, and from this we deduced the amino acid sequence of glycoproteins E3, E2, and E1 for both SV1A and NSV and compared this sequence with that previously published for HRSP (Fig. 1).

In the nucleotide sequence obtained for the glycoprotein E3 region, there were no differences among strains SV1A, NSV, and HRSP. In glycoprotein E2, however, there were two nucleotide differences between SV1A and NSV, both of which led to amino acid substitutions. Nucleotide 8795 is A in SV1A and U in NSV, resulting in a Gln-to-His change at position 55 of E2. Nucleotide 9255 is G in SV1A and A in NSV, leading to the replacement of Arg in SV1A by Gly in NSV at position 209 of E2. Amino acid 55 is Gln in the HRSP strain (as it is in SV1A), whereas amino acid 209 is Gly in HRSP (as it is in NSV).

NSV and SV1A differ from HRSP in three additional amino acids of E2. Position 3 of NSV and SV1A is Thr, whereas HRSP has Ile at this position. Position 23 of SV1A and NSV is Glu; it is Val in HRSP (Glu-23 was previously shown to be the ancestral amino acid in HR [1]). Finally, position 172 of E2 is Gly in SV1A and NSV and Arg in HRSP. The changes at positions 3 and 172 may have arisen during selection of the HR strain.

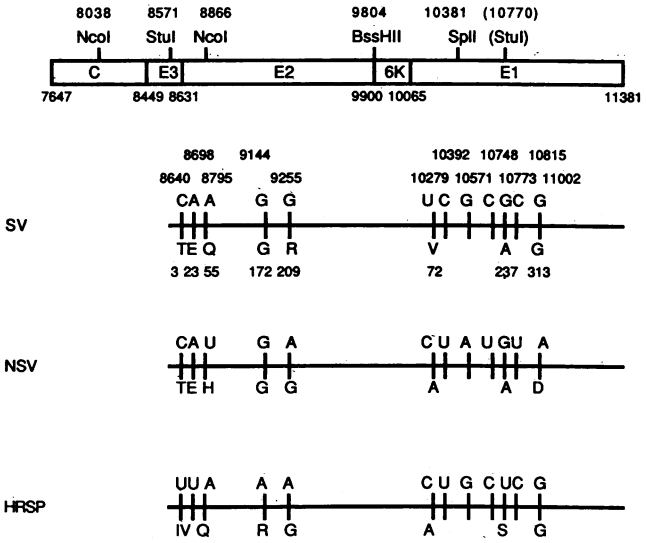

FIG. 1. Sequence differences between SV1A (SV), NSV, and HRSP. At the top is a schematic diagram of the structural region of the Sindbis virus genome. The positions of a number of restriction enzyme sites are shown, as well as the boundaries between the different proteins encoded, together with their coordinates numbered from the 5′ end of the RNA according to Strauss et al. (37). At the bottom are schematic sequence diagrams of three Sindbis strains. Nucleotides that differ between any of the strains are shown above the line (numbered from the 5′ end as described above), and encoded amino acids (if a change in coding is involved) are shown below the line, using the single-letter amino acid code. The amino acid numbers refer to the position within the glycoprotein; thus Q55 is residue 55 of glycoprotein E2, etc. The sequence from nucleotide 8571 in E3 to the end of the RNA was determined for SV1A and for NSV as described in Materials and Methods. The sequence for HRSP is from the work of Strauss et al. (37). C, Capsid protein; 6K, polypeptide 6K.

In glycoprotein E1, there were six nucleotide differences between SV1A and NSV which led to two amino acid changes. Silent changes were found at positions 10392 (C in SV1A → U in NSV), 10571 (A → G), 10748 (C → U), and 10815 (C → U). The coding changes were at position 10279 (U in SV1A → C in NSV), which led to the substitution of Val in SV1A by Ala in NSV at position 72 of E1, and at nucleotide 11002 (G → A), which led to the substitution of Gly by Asp at position 313. Position 72 is Ala in HRSP (as it is in NSV), whereas amino acid 313 is Gly in HRSP (as it is in SV1A). In addition to differing from each other at these positions, SV1A and NSV also differ in position 237 (Ala) from HRSP (Ser); this change may also have arisen during selection of the HR strain.

Of the eight nucleotide differences between SV1A and NSV, seven were transitions (three C → U, three G → A, and one U → C change). The transversion was the A → U change, which led to the substitution of Gln by His.

There were no changes between SV1A and NSV in the 3′ noncoding region.

Construction of recombinant viruses. To examine the effect of the different amino acid substitutions on the neurovirulence of Sindbis virus, a number of recombinant viruses were constructed. To accomplish this, restriction fragments from clones of SV1A, NSV, or HRSP cDNA were used to replace the corresponding restriction fragments in clone Toto1101 or, in one case, Toto50. These clones contain a complete cDNA copy of Sindbis virus inserted downstream from an

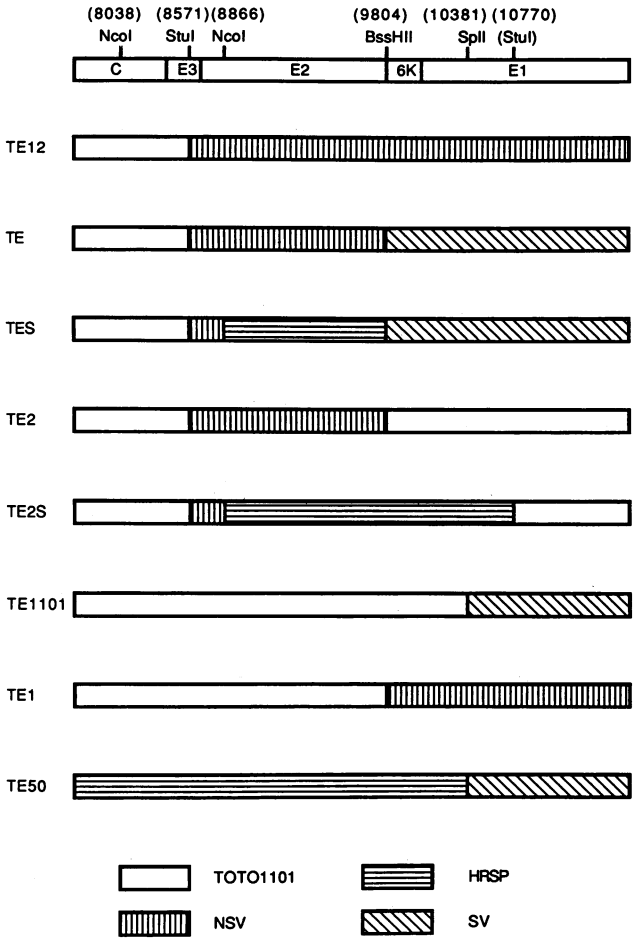

FIG. 2. Construction of recombinant Sindbis virus genomes in the glycoprotein region. Restriction fragments in clone Toto1101 (28) were replaced by the corresponding fragments from NSV, SV1A (SV), or HRSP (derived from Toto50), as indicated in the diagram. The restriction sites used and their coordinates in the viral genome are indicated.

SP6 RNA polymerase promoter from which infectious RNA can be transcribed in vitro (28). RNA was transcribed from the recombinant clones and transfected onto CEF cells. Monolayers were incubated at 30°C under liquid medium to rescue infectious virus or under agarose at 30 and 40°C for plaque assays. None of the recombinant viruses was temperature sensitive. The titers of the rescued viruses were between 10^8 and 10^9 PFU/ml. They were passaged once in BHK cells and then examined for their biological properties.

The constructs tested are illustrated schematically in Fig. 2. The restriction sites used to construct the hybrid genomes and their coordinates numbered from the 5′ end of the RNA (37) are also shown. The *Stu*I site at position 10770 is not present in all of the virus strains. Those with Ser at position 237 of E1 possess this site, whereas those that have Ala at this position do not. In all cases, the recombinants possess the nonstructural protein region and the capsid protein region from Toto1101 or from Toto50 and only the glycoprotein region or portions of the glycoprotein region are derived from other strains.

Neurovirulence of recombinant viruses for weanling mice. The neurovirulence of each of the recombinant viruses, the parental viruses (NSV, SV1A, HRSP), and viruses rescued from Toto50 and Toto1101 was tested in 3- to 4-week-old weanling mice inoculated i.c. with 1,000 PFU of each virus. In each case, two or more independent virus stocks were tested. The results were pooled, and the relevant data are summarized in Table 2.

TABLE 2. Neurovirulence of Sindbis strains in weanling mice[a]

Virus strain	No. dead/no. tested	% Mortality
NSV	21/21	100
TE12	15/35	44
TE	2/55	4
TES	0/20	0
TE2	0/20	0
TE2S	0/30	0
TE1	0/21	0
SV1A	0/20	0
Toto1101	0/10	0
Toto50	0/20	0

[a] Mice were challenged with 1,000 PFU of virus by i.c. inoculation. At least two independent stocks were tested, and the results were averaged.

Only NSV and the recombinant TE12 were virulent for weanling mice. TE12 has the nucleotide sequence from 8571 to 11552 of NSV and nucleotides 1 to 8570 and 11553 to 11703 from Toto1101, that is, glycoproteins E2 and E1 from NSV and the remainder of the genome from Toto1101 (Fig. 2). Toto1101 is avirulent for weanling mice, whereas TE12 is virulent, which demonstrates that the envelope proteins alone are sufficient to confer virulence to an otherwise avirulent strain. However, TE12 has a higher 50% lethal dose than NSV (50 versus 3, calculated by the method of Reed and Muench [25]), and the overall mortality is reduced (Table 2), which suggests that nucleotide sequences outside the envelope protein region are also important for neurovirulence. These sequences are presumably in the region encoding the replicase proteins, thereby affecting the efficiency of virus replication, although changes in the capsid protein or in noncoding regulatory sequences cannot be ruled out.

All of the other strains are avirulent in weanling mice. Thus, strains containing only glycoprotein E1 or only E2 from NSV are avirulent, which demonstrates that changes in both E1 and E2 were necessary for the transition in neurovirulence from SV1A to NSV.

Neurovirulence of recombinant viruses for suckling mice. Our major interest in these studies was to define the changes that led to virus attenuation in weanling mice. However, Sindbis virus has also been used as a model system for the study of neurovirulence in suckling mice (6, 20, 21, 24), and it seemed of interest to compare the virulence of these constructs in suckling mice as well (Table 3). Strains NSV and TE12 were essentially indistinguishable in their neurovirulence (although the mean day of death may be slightly higher in studies with TE12). Thus, although Toto1101 is much less virulent for suckling mice than NSV, possession of the glycoproteins from NSV is sufficient to render Toto1101 fully virulent for suckling mice. Strains that contain E2 from NSV and E1 from NSV, SV1A, or Toto1101 were also neurovirulent in suckling mice, causing 100% mortality after subcutaneous inoculation, as did a construct containing only the N-terminal domain of E2 from NSV with the remainder of E2 from HRSP and E1 from SV1A (construct TES). The survival time of the animals varied from 3 to 5 days, depending on the construct tested. When E2 was derived from Toto1101 (construct TE1 or virus from

TABLE 3. Neurovirulence of Sindbis strains in suckling mice[a]

Virus strain[b]	No. dead/ no. tested	% Mortality	MDOD[c]
NSV	31/31	100	3.3
TE	77/77	100	3.6
TE12	20/20	100	3.9
SV1A	20/20	100	4.5
TES	47/47	100	4.8
TE2	20/20	100	5.2
TE2S	31/36	86	8.3
TE1101[d]	31/44	(70)	(9.4)
TE1	27/40	68	9.9
Toto1101	33/66	50	8.1
Toto50 (HRSP)	21/49	43	10.8
TE50[d]	4/21	(19)	(12.8)

[a] Mice were challenged with 1,000 PFU of virus by subcutaneous inoculation at 1 to 3 days of age.

[b] Fig. 2. At least two independent stocks were tested, and the results were averaged.

[c] MDOD, Mean day of death after subcutaneous injection of 1,000 PFU.

[d] The neurovirulence of these constructs is dependent on the exact age of the mice and drops from 100% mortality in 1- to 2-day-old mice to 20% in 3- to 4-day-old mice (see text).

Toto1101) or Toto50 (HRSP strain) the resultant virus was less virulent. With these strains, 30 to 60% of the inoculated mice survived and those that died had an extended survival time. The construct TE50 is discussed below. Thus, there is a gradient of neurovirulence in suckling mice extending from a mortality of 100% with a survival time of 3 days to a mortality of 20 to 40% with a 12-day survival time.

Correlation of amino acid changes with neurovirulence. The amino acid changes among the various strains of Sindbis virus in the E2 and E1 glycoproteins that have been assayed for neurovirulence have been summarized in Table 4, with the strains listed in descending order of neurovirulence. Sequence data for NSV and SV1A and for constructs containing sequences from these viruses are from Fig. 1; the Toto50 sequence is the HRSP sequence determined by Strauss et al. (37), and the data for Toto1101 are from the work of Polo et al. (24) and R. E. Johnston (personal communication).

For weanling mice, as noted above, changes in both E2 and E1 contribute to the differences seen between SV1A and NSV. In E2 the change at position 55 from His (in NSV) to Gln (in SV1A) appears to be primarily responsible for attenuation (compare NSV and construct TE12 with SV1A and construct TE1), although the changes from Thr to Ile at position 3 and Gly to Arg at position 209 cannot be evaluated independently from these constructs. In glycoprotein E1, either the change of Ala to Val at position 72 or the change of Asp to Gly at 313 or both appear to attenuate the virus (compare NSV and construct TE12 with SV1A and construct TE).

TABLE 4. Amino acid differences among Sindbis virus strains

Virus strain[a]	Amino acid in glycoprotein at position indicated:									
	E2						E1			
	3	23	55	172	209	251	72	75	237	313
NSV	T	E	H	G	G	A	A	D	A	D
TE12	T	E	H	G	G	A	A	D	A	D
TE	T	E	H	G	G	A	V	D	A	G
SV1A	T	E	Q	G	R	A	V	D	A	G
TES	T	E	H	R	G	A	V	D	A	G
TE2	T	E	H	G	G	A	A	G	S	G
TE2S	T	E	H	R	G	A	A	D	S	G
TE1101	I	E	Q	G	G	V	A	G	A	G
TE1	I	E	Q	G	G	V	A	D	A	D
Toto1101	I	E	Q	G	G	V	A	G	S	G
Toto50	I	V	Q	R	G	A	A	D	S	G
TE50	I	V	Q	R	G	A	A	D	A	G

[a] Strains are listed in order of decreasing virulence. The source of the sequence data for each strain is described in the text.

The situation for neurovirulence in suckling mice is more complex. As noted above, the different strains do not exhibit an all-or-none effect. Also, it is difficult to exclude cooperative interactions between the different changes found. With these provisos we can draw a number of conclusions. Firstly, as indicated above, only changes in glycoproteins E1 and E2 lead to virus attenuation in the constructs tested. Secondly, in glycoprotein E2, Gln-55 appeared to be required, but not sufficient, for attenuation. Attenuated E2s also had Ile-3, Arg-172, or Gly-209. The Gly-to-Arg change at position 172 did lead to a slight increase in survival time when combined with His-55 (compare TE with TES and TE2 with TE2S) and could have been involved in attenuation. The Thr-to-Ile change at position 3 could also have been attenuating, according to the data presented here, although Polo et al. (24) have argued that it is not involved in attenuation. The change at position 209 (as well as the changes at positions 23 and 251) did not appear to be involved in attenuation. Thirdly, in glycoprotein E1, two changes appear to be important in this group of constructs, i.e. the Ala-to-Val change at position 72 and the Ala-to-Ser change at position 237. When TE is compared with TE2 or TES is compared with TE2S, it is clear that the two changes together lead to decreased mortality and extended survival.

To separate the two changes in E1, the constructs TE1101 and TE50 were made. In initial experiments, TE1101 demonstrated an intermediate virulence, with 70% of the suckling mice dying and the mean day of death at 9.4 days (Table 3). However, the results were variable from litter to litter, in contrast to the results obtained with other constructs, and it appeared that the effects of this virus might be more age dependent. Further experiments to test this possibility revealed that results with TE1101 were, in fact, strongly age dependent. In 1- to 2-day-old mice, the mortality was 100% (10 of 10 mice; mean day of death, 5.7). In slightly older mice (3 to 4 days old), mortality was 20% (2 of 10 mice died, with a mean day of death of 14.5). Similarly, results with construct TE50 were age dependent in the same way. In 1- to 2-day-old mice, 10 of 10 mice died (mean day of death, 10.2); in 3- to 4-day-old mice, 2 of 10 died (mean day of death, 10). Toto50 and Toto1101 exhibited no age dependence in similar experiments with 1- to 2-day-old and 3- to 4-day-old mice. These results taken together suggest that the Ala-to-Ser change at position 237 is somewhat attenuating in suckling mice but that the Ala-to-Val change at position 72 may also contribute. The Asp-to-Gly change at position 75 does not appear to make a significant contribution to attenuation in this system, and the effect of the Asp-to-Gly change at position 313 is unclear.

DISCUSSION

The AR339 strain of Sindbis virus demonstrates age-dependent virulence in mice. Mice up to 8 days old develop acute encephalitis that is fatal; older mice also develop encephalitis, but the infection is not fatal (14, 26). More virus

can be isolated from the brain and other tissues of suckling mice than from older mice. The AR339 strain was originally isolated by the inoculation of suckling mice and passaged several times in this host (40), which may have been partially responsible for the neurovirulent characteristics of the strain. Starting from AR339, more virulent strains which are neurovirulent in weanling mice have been isolated by alternate i.c. passage in suckling and weanling mice (11); presumably the passage in weanling mice selected for virus that was able to replicate better in the central nervous system of older mice. More attenuated strains of Sindbis virus have also been obtained from the AR339 strain. Olmsted et al. (20, 21) have used several selection procedures, including selection for rapid penetration and growth in vitro, to isolate variants that were attenuated in suckling mice (see also reference 24). Barrett and Atkins (2) have shown that temperature-sensitive mutants are often attenuated. The present report and Polo et al. (24) have also shown that laboratory strains represented by HRSP (and Toto50) and Toto1101 are attenuated for suckling mice. These strains have been maintained by extensive passage in cell culture, usually in chicken cells or BHK cells (and the HR strains were selected for the ability to tolerate high temperatures). The passage of virus in cell culture is a standard method for selecting attenuated virus for vaccines and appears in this case also to have selected variants which are reduced in virulence for mice. We should note, however, that the virulent properties of NSV and of SV1A have been maintained upon passage in cell culture, although an extensive series of passages has not been tested for its effects.

By constructing recombinant viruses among strains that differ in virulence, we have mapped a number of virulence determinants. The situation in weanling mice seems clear-cut. There are determinants in both glycoproteins E2 and E1 that lead to attenuation, and there are determinants in the nonglycoprotein regions as well. In glycoprotein E2, the change of His to Gln at position 55 is attenuating, and in glycoprotein E1, the change of Ala-72 to Val and the change of Asp-313 to Gly lead to attenuation. The results with suckling mice were more complex. As noted above, it appears that a spectrum of neurovirulence exists with different virus strains, and in at least some cases the virulence is sharply age dependent. In addition, changes at multiple sites appear to have unpredictable effects, and thus the genetic background of the virus is important. Finally, when our results are compared with those of Johnston and colleagues (6, 20, 21, 24), it also appears that there may be differences that depend upon the strain of mouse. Although we have tested a large number of constructs, it would require testing many more and comparing the changes in different combinations to completely resolve the ambiguities. However, our results indicate that His-55 and Gly-172 (and possibly Thr-3) in E2 and Ala-72 and Ala-237 (and possibly Asp-313) and E1 are involved in neurovirulence in suckling mice (the situation with E1 is peculiar; one interpretation of the results is that forms of E1 virulent for weanling mice are less virulent for suckling mice and vice versa). Davis et al. (6) have shown the change from Ser to Arg at position 114 of E2 is attenuating. The fact that changes in the glycoproteins can attenuate the virus is consistent with studies of several other virus systems in which structural proteins have been implicated as determinants of virulence (3, 4, 31, 32).

The concept of neurovirulence is complex. The efficiency of replication of the virus in peripheral tissues, the efficiency of crossing the blood-brain barrier, and the efficiency of replication in the central nervous system once the virus invades are all important. There appear to be many ways to change a virulent virus so that it becomes less virulent. Changes in the replicase so that the virus replicates less rapidly may do so. Changes in the glycoproteins may affect the speed of virus penetration or maturation, thus affecting the growth rate, or these changes may affect tissue tropism and thus the ability of the virus to invade certain tissues, such as the central nervous system.

Our simple hypothesis to explain the results of this study is that the changes in the surface glycoproteins that lead to attenuation result in changes in the affinity of the virus for receptors on uninfected cells, thus altering its cell tropism. The effect need not be all or none. If altered virus bound to receptors in certain cells or tissues with reduced affinity or if the alteration produced virus that bound to a different class of receptors with variable expression in different tissues, the alteration in tissue tropism or cell type preferences could be relative, affecting the kinetics of virus replication in different organs and allowing host defenses to clear the infection and prevent death.

It is not known whether the changes in the Sindbis virus glycoproteins leading to differences in neurovirulence for mice lead to differences in attachment to cellular receptors. Smith and Tignor (33) compared the binding of two different field isolates of Sindbis virus differing in virulence and found that neuroblastoma cells (N-18) as well as nonneural cells (CER) had increased numbers of receptors for the virulent AR86 strain compared with the avirulent AR339 strain. Distinct receptors for the two strains were postulated on the basis of sensitivity to enzymatic degradation. Whether the same would be true for the more closely related strains used in this study (both derived from AR339) is not known. Comparative studies of the replication of NSV and SV1A in the brains and spinal cords of i.c. inoculated weanling mice have shown that both viruses infect the central nervous system and that the target cells for replication are the same, primarily neurons and ependymal cells (A. C. Jackson, T. R. Moench, B. D. Trapp, and D. E. Griffin, Lab. Invest., in press). However, more virus is produced and neurons show greater injury after infection with NSV than SV1A. Further study is required to determine whether this is due to a decrease in the effective concentration of receptors for SV1A, leading to slower spread of the virus, or whether both SV1A and NSV recognize the same receptors on the same cells but subsequent steps in NSV replication are more efficient. The surface glycoproteins of alphaviruses are also important in other steps of virus replication, such as penetration and fusion with lysosome or phagosome membranes to release the RNA, and for interaction with the capsid protein during virion maturation. Many of these steps involve conformational changes in the E1-E2 heterodimer which might be affected by changes in the amino acid structure of these glycoproteins.

The fact that a variety of changes in E1 and E2 affect neurovirulence and the fact that the effects of each change are different have obvious implications for vaccine development. Historically, one of the problems encountered during virus passage to develop attenuated strains has been overattenuation, so that the vaccine strain is no longer efficacious. On the other hand, multiple attenuating mutations are desirable so that the frequency of reversion to virulence is negligible. The ability to test the effect of individual attenuating changes and to mix them at will in recombinant strains in the approach used here could be of great value when applied to other viruses for which a vaccine is desired.

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