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Attenuating Mutations in Glycoproteins E1 and E2 of Sindbis Virus Produce a Highly Attenuated Strain When Combined In Vitro

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Alterations in either the E1 or the E2 glycoprotein of Sindbis virus can affect pathogenesis in animals. Previously, we identified two distinct E1 glycoprotein gene sequences which differed in their effect on pathogenesis. One had an attenuation phenotype following subcutaneous inoculation of neonatal mice (E1 Ala-72, Gly-75, and Ser-237), while the other was virulent (E1 Val-72, Asp-75, and Ala-237). In this study, we examined the basis for this difference in pathogenesis by using a full-length cDNA clone of Sindbis virus from which infectious RNA could be transcribed in vitro. The relative contribution of each E1 residue to the pathogenesis phenotype was determined by using site-directed mutagenesis to alter each codon individually and in combination. Residues 75 and 237, in combination, appeared to be the major E1 determinants affecting pathogenesis. In addition, the effect of directly combining independently attenuating E1 and E2 mutations in the same virus was examined. The attenuating E1 sequences characterized in this study were coupled to a previously characterized attenuating mutation at E2 residue 114. The resulting recombinant virus, constructed in vitro, exhibited an increased attenuation of neurovirulence as compared with recombinant viruses containing either of the attenuating elements alone.

Sindbis virus is the type species of the *Alphavirus* genus, a group of mosquito-transmitted encephalitis viruses that include such human and veterinary pathogens as eastern, western, and Venezuelan equine encephalitis viruses (1). Sindbis virions contain a single-stranded, plus-sense RNA genome of 11,703 nucleotides (nt) (29) that is enclosed within an icosahedral nucleocapsid. The nucleocapsid is composed of a single repeating protein species, C, that complexes with the RNA, and is in turn surrounded by a host-derived lipid bilayer from which two virus-encoded glycoproteins, E1 and E2, protrude as heterodimers (reviewed in reference 27). These heterodimers appear to associate in a trimeric form and are the characteristic spikes on the virion surface (8, 30). The envelope glycoproteins are functionally involved in many biological activities of the virus, including early virus-cell interactions (6, 18) and pathogenesis in animals (7, 14, 20).

Sindbis virus infection of neonatal mice provides a model system for the study of alphavirus pathogenesis. Subcutaneous (s.c.) or intracerebral (i.c.) inoculation of as little as 1 PFU of wild-type Sindbis virus AR339 into 1-day-old mice results in the development of an acute encephalitis producing 100% mortality within 7 days (3, 10, 11, 23). Several determinants that affect Sindbis virus pathogenesis have been studied. To date, all characterized mutations that alter in vivo pathogenesis phenotypes have been localized to the E1 and E2 glycoprotein genes. One locus affecting pathogenesis involves residue 114 of E2. Substitution of arginine for serine at E2 residue 114 in the AR339-derived Sindbis virus mutant SB-RL (4) confers three coordinately linked phenotypes: attenuation in neonatal mice, accelerated penetration into cultured baby hamster kidney cells (BHK-21 cells), and increased sensitivity to neutralization by monoclonal antibodies directed to the E2c antigenic site (7, 17, 18). The pleiotropic effect of this single amino acid substitution was confirmed by using a full-length cDNA clone of Sindbis virus, Toto1101, from which infectious RNA could be transcribed in vitro (24). Two strains of virus differing only at E2 residue 114 were derived from recombinant clones, and these reproduced the pleiotropic phenotypes of the biological mutant (20).

Virus derived from the unsubstituted full-length Sindbis virus clone Toto1101 (24) was attenuated for neonatal mice when inoculated s.c. Replacement of the E1 and 6K genes of Toto1101 with the corresponding sequences from an AR339 cDNA clone resulted in a construct which produced virulent virus (20). Sequence analysis showed that these recombinant viruses differed at three amino acid positions, all in the E1 glycoprotein: residues 72, 75, and 237 (20).

In this report, we characterized the individual contributions of E1 amino acid residues 72, 75, and 237 to the attenuation phenotype of the recombinant Toto1101. Site-directed mutagenesis was used to generate virus mutants that differed by single amino acid substitutions at these positions. In addition, we constructed a model alphavirus vaccine by combining independently attenuating mutations from each of the glycoprotein genes to produce a highly attenuated Sindbis virus strain.

MATERIALS AND METHODS

Virus strains and cell culture. The recombinant Sindbis virus strain Toto1101 was generated from a full-length cDNA clone constructed by Charles Rice and co-workers (24). The recombinants TR2000, TR2100, and TR2200 were generated by replacing portions of Toto1101 with sequences from our prototype virulent and attenuated laboratory strains, SB and SB-RL, respectively, as described previously (20). For experiments described in this report, the specific clones TR2215 and TR2120 were used as representatives of the TR2200 and TR2100 classes, respectively (20).

BHK-21 cells were used for virus propagation and titration as well as RNA transfection. BHK-21 cells were obtained from the American Type Culture Collection (Rockville, Md.)

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in passage 53 and used between passages 55 and 65. The cells were maintained in Eagle minimal essential medium containing 10% donor calf serum, 10% tryptose phosphate broth, 50 U of penicillin per ml, and 50 μg of streptomycin per ml (MEM complete).

General recombinant DNA methods. DNA manipulations were performed essentially as described by Maniatis et al. (15) and Ausubel et al. (2). Plasmid DNA was prepared by the method of Holmes and Quigley (9), with a modification to eliminate the need for RNase treatment. Briefly, following removal of the protein-chromosomal DNA pellet, the RNA was precipitated by making the lysate 2 M in ammonium acetate and incubating it on ice for 2 h. After centrifugation and removal of the RNA pellet, plasmid DNA was precipitated by the addition of 95% ethanol. DNA fragments used in cloning procedures were purified from agarose gels with Geneclean (Bio101, La Jolla, Calif.). Nucleic acid-modifying enzymes were obtained commercially from Promega Biotec, New England BioLabs, Inc., Boehringer Mannheim Biochemicals, United States Biochemical Corp., or Amersham Corp. and used in accordance with supplier instructions.

In vitro mutagenesis of the Toto1101 E1 gene. Site-directed mutagenesis was performed by a modification of the procedure of Kunkel et al. (13). A 2,653-base-pair fragment of Toto1101 containing the E1 glycoprotein gene was subcloned into M13mp18 as follows. A *Dra*I (nt 9470)-*Dra*I (nt 12123) fragment of Toto1101 was blunt-end ligated into *Sma*I-digested, dephosphorylated M13mp18 replicative-form DNA to generate the mutagenesis cassette mp18TE1. A single-stranded uracil-containing DNA template was obtained by propagation of mp18TE1 in the *dut ung Escherichia coli* strain CJ236. Specific oligonucleotides to alter single E1 amino acid codons were synthesized on an Applied Biosystems model 380A DNA synthesizer: TE1-72 (5′-TA GCCTGCATGAACGGCCGGCTGA-3′), Ala-72 to Val; TE1-75 (5′-GCAGGTATAGTCTGCATGAGC-3′), Gly-75 to Asp; and TE1-237 (5′-AATCCTGATGCGGCCTGCGTGT-3′), Ser-237 to Ala. Phosphorylated oligonucleotide (4.8, 9.6, or 19.2 ng) was annealed to 400 ng of single-stranded uracil-containing mp18TE1 DNA and extended in the presence of deoxynucleotide triphosphates and T4 DNA ligase with either the Klenow fragment or Sequenase 2.0 (United States Biochemical Corp.). Double-stranded DNA was transformed into the *dut*$^+$ *ung*$^+$ *E. coli* strain JM101 and plated for plaque isolation. Individual phage clones were propagated, and single-stranded DNA was isolated (2). Clones containing the desired mutations were identified by dideoxynucleotide sequencing (see below) with oligonucleotide primers specific for the appropriate Sindbis virus sequences (7). DNA fragments (*Bss*HII [nt 9804]-*Xho*I [nt 11749]) from positive clones were subcloned into the full-length clone Toto1101 for the production of virus harboring the individual mutations. The mutations were again confirmed by sequence analysis directly from RNA transcripts synthesized in vitro or from purified virion RNA. For purposes of phenotypic testing, multiple clones of each type were generated (the virulence data were pooled for presentation).

RNA and DNA sequence analyses. Sequence data were obtained from RNA and single-stranded DNA templates by the dideoxynucleotide chain termination method (26). Purified virion RNA or genome-length RNA transcribed in vitro was sequenced with reverse transcriptase and oligonucleotide primers complementary to Sindbis virus E1 glycoprotein gene sequences (7, 32). Single-stranded M13 (mp18TE1) DNA was sequenced with the same Sindbis virus-specific oligonucleotide primers and the Klenow fragment as described by Ausubel et al. (2).

In vitro transcription and RNA transfection. Plasmid DNA containing a full-length cDNA copy of Sindbis virus RNA was linearized and used for runoff transcription reactions with SP6 RNA polymerase as described previously (20, 24). RNA transcripts were analyzed by electrophoresis in 0.7% agarose gels containing 0.1% sodium dodecyl sulfate and stained with ethidium bromide. Approximately half of the product RNA was diluted and introduced into BHK-21 cells by the DEAE-dextran transfection method described by Stollar et al. (28) or by cationic liposome-mediated transfection (Lipofectin; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). In the "lipofection" procedure, RNA transcripts were diluted to 100 μl with sterile H_2O and an equal volume of diluted Lipofectin reagent (1:1 in sterile H_2O) was added. The RNA-Lipofectin mixture was incubated at room temperature for 15 min. BHK-21 cells in 60-mm dishes were prepared for transfection by two consecutive 30-min incubations with Optimem (Bethesda Research Laboratories) at 37°C followed by the addition of 3 ml of fresh Optimem. The RNA-Lipofectin mixture was added to two 60-mm BHK-21 cultures (100 μl per plate). Transfected cells were incubated at 37°C for approximately 12 h, at which time the Optimem was replaced with MEM complete. Monolayers were incubated at 37°C until cytopathic effects were observed. Supernatants were harvested, clarified by low-speed centrifugation, and divided into aliquots to provide working stocks for phenotypic analysis.

Construction of virus recombinants. The construction of Toto1101, pTR2000, pTR2100, and pTR2200 has been described elsewhere (20, 24). Essentially, plasmid pTR2000 was generated from Toto1101 by replacement of the E1 and 6K genes (*Bss*HII [nt 9804]-*Sac*II [nt 11484]) with the corresponding cDNA fragment from an SB (AR339) clone. Plasmids pTR2100 and pTR2200 were generated from pTR2000 by replacement of the 5′ one-third of the E2 gene (*Stu*I [nt 8571]-*Pst*I [nt 9119]) with the corresponding cDNA fragments from the SB and SB-RL clones, respectively. Plasmids pTR2100 and pTR2200 were used as the basis for recombinants pTR1000 and pTR1050 constructed in this report. Plasmid pTR1050 was generated from pTR2100 by replacement of the SB-derived E1 and 6K gene sequences (*Bss*HII [nt 9804]-*Xho*I [nt 11749]) with the corresponding sequences from Toto1101. Plasmid pTR1000 was generated from pTR2200 in a similar way; the SB-derived E1 and 6K gene sequences of pTR2200 (*Bss*HII [nt 9804]-*Xho*I [nt 11749]) were replaced with the corresponding sequences from Toto1101. Replacements were confirmed initially at the DNA level by restriction endonuclease analysis. Toto1101 sequences contain a single *Stu*I site (nt 10770) in the region of replacement that is not present in SB-derived sequences (20). The fidelity of the replacements was further confirmed by sequence analysis of the resulting viruses.

Virulence testing in animals. Virus stocks were assayed for virulence in litters of 1-day-old CD-1 mice (Charles River Breeding Laboratories Inc., Wilmington, Mass.). With the exception of the 50% lethal dose (LD_{50}) determinations, multiple litters were inoculated s.c. with 100 PFU of virus in 50 μl of phosphate-buffered saline containing 1% donor calf serum (PBS-1%) for each virus stock. Animals were observed for a period of 14 days postinoculation to calculate percent mortality and average survival time of animals that died. For the LD_{50} determinations, litters (at least 10 animals per dose) were inoculated either s.c. (50 μl) or i.c. (10 μl) with 10-fold dilutions of virus in PBS-1%. Calculations were

TABLE 1. Virulence of Toto1101-derived recombinant Sindbis virus strains[a]

Recombinant	E1 amino acid residue			No. of mice dead/no. inoculated	% Mortality	Avg survival time (days) for animals that died
	72	75	237			
Toto1101	Ala	Gly	Ser	14/62	23	9.6
TR1101-72	Val	Gly	Ser	11/53	21	10.8
TR1101-75	Ala	Asp	Ser	91/139	65	8.7
TR1101-237	Ala	Gly	Ala	66/109	61	8.3
TR1101-75/237	Ala	Asp	Ala	45/46	98	6.8
TR2000	Val	Asp	Ala	21/21	100	6.4

[a] Virulence results were from s.c. inoculations of 100 PFU into 1-day-old mice, with a 14-day observation period.

performed by the method of Reed and Muench (22) and were based on a 14-day observation period. Approximately 3 to 4 weeks postinoculation, surviving animals, including PBS-1% controls, were challenged with S.A.AR86, a strain of Sindbis virus virulent for adult animals (25). Animals which survived Sindbis virus infection were immune to S.A.AR86 inoculated i.c. at a dose of 500 LD_{50}s in 50 μl of PBS-1%. Challenged animals were observed for an additional 14 days.

Virus growth in animals. Three litters of 1-day-old CD-1 mice per virus strain were inoculated s.c. with 100 PFU of virus in 50 μl of PBS-1%. On days 1 through 7, 9, 11, and 13 postinoculation, three mice (one from each litter) were sacrificed to recover serum and brains. Serum was diluted 1:100 in PBS-1%, divided into aliquots, and frozen at −70°C for later virus titration. Brains were homogenized as 20% suspensions (wt/wt) in PBS-1%, diluted 1:20, divided into aliquots, and frozen at −70°C. Virus titrations were performed by a standard plaque assay with BHK-21 cells.

RESULTS

Analysis of attenuating loci in glycoprotein E1. Previous work in this laboratory (20) and others (14) showed that virus derived from a full-length cDNA clone of Sindbis virus, Toto1101, developed by Rice and co-workers (24), was attenuated for neonatal mice inoculated s.c. A recombinant, TR2000, generated by replacement of the Toto1101 E1 and 6K gene sequences with those from an SB (AR339) cDNA clone, differed from the attenuated Toto1101 in that virus produced from this construct was virulent for neonatal mice following s.c. inoculation (20; Table 1). Sequence analysis of these two recombinant viruses revealed only three nucleotide differences, each of which resulted in amino acid differences at residues 72, 75, and 237 in glycoprotein E1 (20). Thus, one or more of these residues, individually or in combination, were responsible for the different pathogenesis phenotypes.

To assess the relative contribution of each residue to the pathogenesis phenotype, we performed site-directed mutagenesis to create a panel of recombinant strains isogenic with Toto1101 except for single amino acid substitutions. The three amino acid differences in the virulent construct TR2000 were reproduced as individual substitutions in Toto1101 (see Materials and Methods) for the production of virus harboring each mutation. The pathogenesis phenotype of each virus recombinant in neonatal mice was determined and compared with that of attenuated Toto1101 and virulent TR2000 (Table 1). At a dose of 100 PFU administered s.c., Toto1101 produced 23% mortality, with an average survival time of 9.6 days, while TR2000 produced 100% mortality, with an average survival time of 6.4 days. Substitution of the TR2000 valine for alanine at E1 residue 72 of Toto1101, (recombinant TR1101-72) did not significantly alter virulence from that of Toto1101, producing 21% mortality, with an average survival time of 10.8 days. However, single amino acid substitutions of aspartate for glycine at residue 75 (recombinant TR1101-75) and of alanine for serine at residue 237 (recombinant TR1101-237) increased the mortality induced by Toto1101 from 23% to 65 and 61%, respectively.

As residues 75 and 237 both appeared to be involved in the pathogenesis phenotype, site-directed mutagenesis again was used to incorporate the two amino acid changes into Toto1101 (Table 1). Substitution of both aspartate at residue 75 and alanine at residue 237 (recombinant TR1101-75/237) produced a level of virulence very close to that of TR2000, with 98% mortality and an average survival time of 6.8 days. Of 46 animals inoculated, 1 survived the 14-day observation period but eventually died on day 20 postinoculation. On the basis of these data, it appeared that E1 residues 75 and 237 in combination were the major glycoprotein determinants affecting the pathogenesis phenotype of Toto1101, while residue 72 may have played a minor role.

Construction of virus recombinants containing multiple attenuating mutations. The effects of combining independently attenuating mutations in the same virus were examined as a model for the informed construction of live alphavirus vaccines. Two well-characterized attenuating elements, one from each of the two glycoproteins, were chosen for inclusion. The first was the attenuating E1 configuration of Toto1101: E1 alanine 72, glycine 75, and serine 237. The second was an arginine-for-serine substitution at E2 residue 114 that was characterized previously (7, 20). A panel of recombinant viruses which included a virulent parental strain, strains which differed from the virulent parental strain by only the attenuating E2 or E1 element, and a strain which contained both the E2 and E1 attenuating elements was constructed. The virus recombinants were made by replacing specific gene sequences of one full-length clone with the corresponding cDNA sequences from another as described in Materials and Methods. A schematic of the recombinants is shown in Fig. 1 and includes the restriction sites used in the constructions, the origins of the substituted sequences, and all amino acid differences among the strains. TR2200 differed from the virulent TR2100 by only the arginine-for-serine substitution at E2 residue 114 (Fig. 1). TR1050 retained the wild-type serine residue at E2 residue 114 but differed from TR2100 by the substitution of the attenuating E1 sequences derived from Toto1101. TR1000 contained both the arginine-for-serine substitution at E2 residue 114 and the attenuating E1 sequences derived from Toto1101.

Comparative attenuation of E1 and E2 mutations. The virulence of each recombinant was initially assayed by s.c. inoculation of a standard 100-PFU dose into neonates. TR2100 consistently produced 100% mortality, with average survival times of less than 6 days. Although attenuated in comparison to TR2100, the recombinants TR2200 and TR1050 were essentially indistinguishable from each other at this dose, producing mortality rates of less than 30%, with extended survival times for the animals that died (data not shown).

However, differences in attenuation between the recombinants were evident when LD_{50}s were compared. TR2200 differed from TR2100 only by the arginine-for-serine substitution at E2 residue 114. This single amino acid change

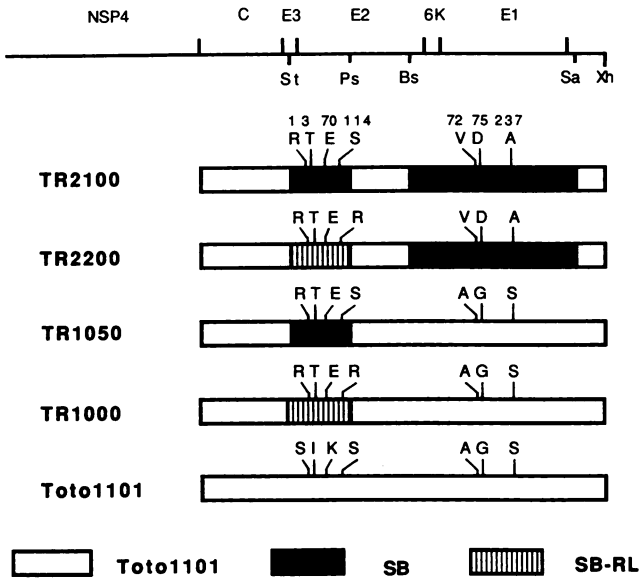

FIG. 1. Construction of recombinant Sindbis virus strains. The schematic shows the derivation of glycoprotein gene sequences of the recombinants and restriction endonuclease sites used in their construction. Amino acid coding differences among the strains are shown. All remaining nonstructural gene and noncoding region sequences not depicted are identical to those of Toto1101. St, *Stu*I (nt 8571); Ps, *Pst*I (nt 9119); Bs, *Bss*HII (nt 9804); Sa, *Sac*II (nt 11484); Xh, *Xho*I (nt 11749).

produced an increase in the LD_{50} following s.c. inoculation of less than 1 PFU for the virulent TR2100 to more than 10^7 PFU for TR2200 (Table 2). In addition, a comparable high level of attenuation was observed following direct inoculation into the brain. By this route of inoculation, the LD_{50} for TR2200 was 1.4×10^6 PFU (Table 3).

TR1050 differed from TR2100 only by the presence of the attenuating E1 configuration derived from Toto1101. This strain also showed a high degree of attenuation following s.c. inoculation into mice, with an LD_{50} of 1.5×10^4 PFU (Table 2). However, TR1050 showed very little difference from its virulent parental strain TR2100 following i.c. inoculation, producing 55% mortality with a dose of only 2 PFU per animal (Table 3). Clearly, the level of attenuation specified by the E2 residue 114 mutation in TR2200 was much greater than that characteristic of the combination of E1 mutations in TR1050 for either route of inoculation. In addition, the attenuating effects produced by the E2 and E1 loci appeared to differ. The predominant effect of the attenuating E2 mutation appeared to be reduced neurovirulence, while the effect of the attenuating E1 configuration appeared to be primarily on a property related to peripheral replication and/or invasion of the brain.

TABLE 2. Virulence of Sindbis virus strains inoculated s.c.[a]

Recombinant	% Mortality with dose (PFU) of:					LD_{50} (PFU)[b]
	10^3	10^4	10^5	10^6	10^7	
Toto1101	0	4	18	82	100	3×10^5
TR1050	16	55	50	100	ND[c]	1.5×10^4
TR2200	0	18	9	14	17	$>1 \times 10^7$
TR1000	0	5	31	13	0	$>1 \times 10^7$

[a] Virulence results from inoculations of 1-day-old mice are shown as percent mortality after a 14-day observation period.
[b] Calculated by the method of Reed and Muench (22).
[c] ND, Not determined.

TABLE 3. Virulence of Sindbis virus strains inoculated i.c.[a]

Recombinant	% Mortality with dose (PFU) of:								LD_{50} (PFU)[b]
	10^0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	
Toto1101	22	38	34	100	100	100	ND[c]	ND	56
TR1050	55[d]	90[e]	100	100	100	95[f]	ND	ND	<2
TR2200	ND	ND	ND	0	21	44	18	58	1.4×10^6
TR1000	ND	ND	ND	10	10	0	0	5	$>1 \times 10^7$

[a] Virulence results from inoculations of 1-day-old mice are shown as percent mortality after a 14-day observation period.
[b] Calculated by the method of Reed and Muench (22).
[c] ND, Not determined.
[d] The actual dose was 2 PFU, as measured by titration of the virus inoculum.
[e] The actual dose was 16 PFU, as measured by titration of the virus inoculum.
[f] The lone surviving animal died on day 23, prior to challenge with S.A.AR86.

Similar LD_{50} experiments were performed for Toto1101. Following inoculation either s.c. or i.c., Toto1101 produced a pattern of attenuation similar to that of TR1050, although with significantly higher LD_{50}s. This greater degree of attenuation for Toto1101 than for TR1050 was therefore due to one or more sequence differences outside the E1 gene. In addition to the attenuating E1 configuration, Toto1101 harbored three amino acid differences in glycoprotein E2 that were not found in TR1050 (residues 1, 3, and 70). Recent work by S.-C. Lin, J. M. Polo, and R. E. Johnston (unpublished data) has suggested that the lysine residue at E2 residue 70 may contribute to the greater attenuation evident in virus derived from Toto1101.

Surviving animals from the LD_{50} experiments were challenged i.c. with 500 LD_{50}s of S.A.AR86, a strain of Sindbis virus virulent for adult animals (25), to determine whether they had received a virus inoculum sufficient to induce a protective immune response. All surviving animals inoculated s.c. in the LD_{50} experiments were completely refractory to challenge with S.A.AR86, demonstrating replication of the original virus inoculum and stimulation of a protective immune response. Similarly, surviving animals inoculated i.c. in the LD_{50} experiments were refractory to a subsequent challenge with S.A.AR86, with the exception of the lowest dose of TR1050 and Toto1101. In these instances, only 50 and 22% of the surviving animals were protected, respectively. The lack of a protective immune response at a 1- to 2-PFU dose in some animals inoculated i.c. was anticipated, considering the statistical probability of receiving no virus in the original inoculum.

Replication of attenuated recombinants in animals. Following peripheral inoculation of Sindbis virus into mice, the ultimate target site for virus replication and induction of encephalitic disease is the brain. Differences in the level of attenuation attributable to the E2 of E1 mutation may therefore be due in part to an altered ability of the virus to gain entry into the brain or to replicate within brain tissue. We examined virus replication in neonatal mice following s.c. inoculation of the recombinants. By quantitating the amount of infectious virus found in the brain and serum after various intervals, we observed several differences (Fig. 2).

The virulent parental strain TR2100 showed a rapid accumulation of virus in both the serum and brain within 24 h after s.c. inoculation of 100 PFU. The titers in the brain continued to increase through day 4 to $>10^{10.3}$ PFU/g, after which time all animals had died from the infection. Previ-

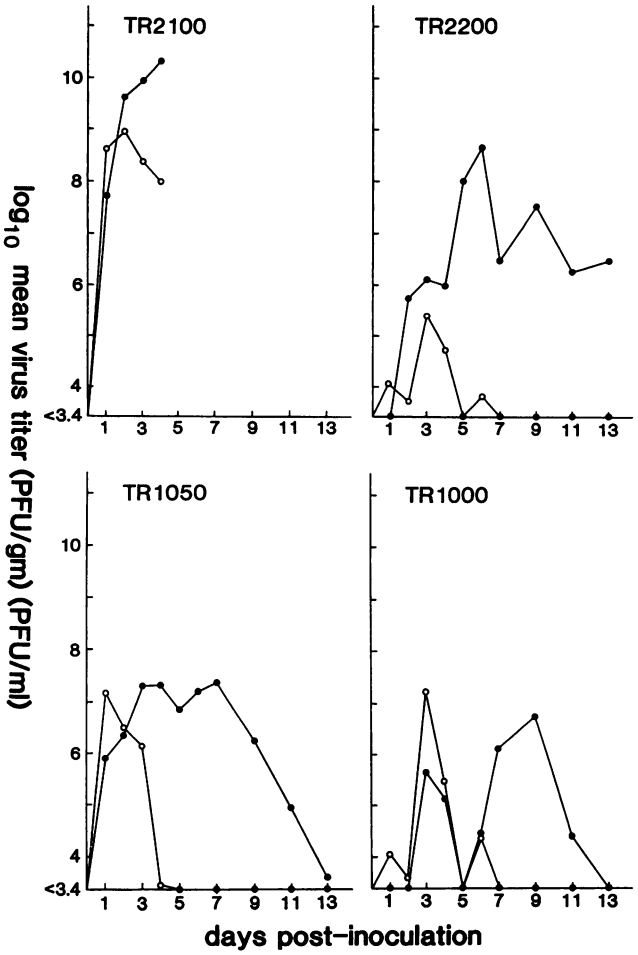

FIG. 2. Replication of recombinant Sindbis virus strains in neonatal mice. One-day-old animals were inoculated s.c. with 100 PFU of virus and sacrificed at intervals postinoculation to recover serum and brains. Virus titers were determined by a standard plaque assay. Datum points represent the geometric mean of three samples for serum (○) and brains (●), except for the day-13 sample for TR2200, which represents one sample. Sample ranges for titers in brains in each of the four virus infections on the day when the highest titer was reached were as follows: TR2100, day 4, 10.25 to 10.40; TR2200, day 6, 8.58 to 8.82; TR1050, day 7, 7.14 to 7.67; and TR1000, day 9, 6.62 to 6.80.

ously, indirect fluorescent-antibody staining of wild-type Sindbis virus AR339-infected brains showed specific cellular staining of viral antigen on day 1 postinfection, suggesting that the presence of virus in the brain was not necessarily associated with blood contamination (J. E. Humphreys and R. E. Johnston, unpublished results). The time course of replication for the virulent TR2100 was essentially indistinguishable from that for Sindbis virus strain AR339.

The attenuating E1 sequence in the recombinant TR1050 produced a very different pattern of virus replication in mice following s.c. inoculation. Although virus replication in the brain prior to day 2 was obscured by the elevated virus titers in the blood, virus clearly was detected in the brains of TR1050-infected animals by 48 h postinoculation. In addition, the maximum titer in the brain was reduced ($10^{7.4}$ PFU/g) as compared with that of TR2100 and was reduced to almost undetectable levels by day 13. Finally, titers in the serum of TR1050-infected animals were reduced in comparison to those produced by the virulent TR2100. Toto1101 produced a pattern of replication in mice similar to that produced by TR1050, with the exception of a slightly decreased titer in serum (data not shown).

The presence of the attenuating arginine substitution at E2 residue 114 of TR2200 also produced a marked decrease in the maximum titer in the brain ($10^{8.7}$ PFU/g) as compared with that of the virulent TR2100. However, the replication of TR2200 differed from that of the attenuated TR1050 in that infected mice showed a definite delay in the appearance of detectable virus in the brain until 2 days postinoculation. Also, the level of TR2200 remaining in the brain on day 13 was still relatively high in comparison to that of TR1050. A repeat of these experiments, which included day-21 and -35 observations, showed that TR2200-infected mice had no detectable virus in their brains at these later times (data not shown).

Combining the attenuating E1 and E2 elements. To directly examine the effects of combined attenuating loci from each of the two glycoproteins, we constructed the recombinant TR1000. TR1000 differs from the virulent TR2100 by the presence of both E2 residue 114 arginine substitution and the attenuating E1 configuration derived from Toto1101. Following s.c. inoculation, both TR1000 and TR2200 (E2 residue 114 Arg) produced an LD_{50} of greater than 10^7 PFU (Table 2). However, direct inoculation of TR1000 into the brain showed it to be more attenuated than either TR1050 or TR2200, the strains containing single attenuating determinants. TR1000 produced lower mortality rates across a range of doses and still failed to produce 50% mortality with doses of up to 10^7 PFU inoculated i.c. (Table 3). In addition, surviving animals were completely refractory to a subsequent challenge with 500 LD_{50}s of S.A.AR86. The data obtained from these LD_{50} experiments thus demonstrate an increased level of attenuation associated with the combination of attenuating E1 and E2 mutations in TR1000.

Examination of TR1000 replication in mice showed a different growth pattern (Fig. 2). Most notable was a delay in the appearance of detectable virus in the brain until at least 3 days postinoculation. This initial peak was followed by an abrupt decline to undetectable levels of virus on day 5 and then substantial amounts of virus through day 13. Because the level of virus in serum at day 3 was almost 100 times that found in brain, the day-3 to -4 peak in the titer in the brain may well have reflected the elevated viremic stage and consequent contamination of brain homogenates with blood-borne virus. If this were the case, then TR1000 replication in the brain would have been delayed until day 6 postinoculation. In addition to an extended delay in the appearance of virus in the brains of TR1000-infected mice, maximum titers in the brain were greater than 3 orders of magnitude lower than with the virulent TR2100 and were at or below the titers of either of the recombinants containing single attenuating elements. These data, in conjunction with the LD_{50} determinations, strongly suggest an additive attenuating effect due to the combined attenuating loci in TR1000.

DISCUSSION

The development of full-length cDNA clones which are infectious in themselves or from which infectious RNA can be transcribed in vitro has aided dramatically the study of pathogenesis determinants for RNA viruses. In the Sindbis virus system, clones such as Toto1101 (24) have been instrumental in identifying several virulence determinants within the envelope glycoproteins E1 and E2. In particular,

amino acid changes at E2 residue 114 (12, 20) or a combination of changes at E2 residues 55 and 209 and E1 residues 72 and 313 (14) greatly influence the ability of the virus to cause disease in animals. Further, in the course of these previous experiments, it was found that Toto1101 itself contains at least one attenuating mutation in the E1 gene (20).

Attenuating mutations in the Toto1101 E1 glycoprotein gene. We have characterized the E1 glycoprotein gene sequences which attenuate Sindbis virus clone Toto1101 for neonatal mice. Toto1101 was attenuated following s.c. inoculation of neonates, while TR2000 (Toto1101 containing an E1 gene from Sindbis virus strain AR339) was virulent (20). The two recombinants differed only at E1 residues 72, 75, and 237 (Ala, Gly, and Ser and Val, Asp, and Ala, respectively).

In this report, the contribution of each of the E1 residues towards the pathogenesis phenotype was examined by site-directed mutagenesis of Toto1101. Individual amino acid substitutions at these positions failed to produce a virulence phenotype, although single changes at E1 residue 75 or 237 did result in a significant increase in virulence. A similar observation for E1 residue 237 was made by Lustig et al. (14). The combined substitution of aspartate 75 and alanine 237 produced a level of virulence nearly equal to that of TR2000. Thus, these two loci together appear to represent the major attenuating determinants in the Toto1101 E1 sequences, while the mutation at E1 codon 72 may have only a modulatory effect. Previous work had suggested a potential role in virulence for E1 residue 72, in conjunction with other changes in E2 and E1 (14), and E1 residues 72 and 313 each were linked to a change in the optimum pH for pH-dependent virus fusion (5).

Assembly of a model alphavirus vaccine. The ability to identify multiple attenuating loci, coupled with the availability of full-length cDNA clones, provides an opportunity for the development of live, recombinant vaccine strains containing multiple, well-defined attenuating mutations. This approach first was suggested by Racaniello and Baltimore (21), following the construction of the first infectious cDNA clone of poliovirus.

Retrospective support for this approach has been obtained in genetic studies of the Sabin vaccine strains of poliovirus. With full-length cDNA clones of poliovirus, it was determined that the Sabin type 1 vaccine strain and an avirulent type 2 strain, P712, each contained multiple attenuating loci (16, 19), while attenuation of the Sabin type 3 vaccine strain appeared to be controlled by only two point mutations (31). The predilection of the type 3 vaccine strain for reversion and the frequency of vaccine-related poliomyelitis associated with type 3 relative to type 1 are consistent with the small number of attenuating loci in the type 3 vaccine. It is clear that engineering multiple unrelated attenuating mutations in a single strain would produce a vaccine candidate with a lower reversion frequency.

Using the Sindbis virus model system and attenuating loci from each of the two glycoproteins, we have begun to examine this approach. The attenuating E1 sequences characterized in this report were coupled to a previously identified attenuating locus at E2 residue 114 to generate the recombinant TR1000. TR1000 was highly attenuated for neonatal mice, an experimental model in which 1 PFU of wild-type virus can initiate a lethal infection. With TR1000, we were unable to determine an LD_{50} with doses as high as 10^7 PFU inoculated s.c. or i.c. However, the level of virus replication was sufficient to confer immunity to challenge with the highly neurovirulent S.A.AR86 strain.

This high level of attenuation may be due to multiple independent defects in the ability of TR1000 to induce encephalitis and death. The contribution of the E1 mutations to the attenuation of TR1000 appeared to result, at least in part, from a defect at or prior to invasion of the central nervous system. For TR1050, which contained only the attenuating E1 mutations, LD_{50}s for s.c. and i.c. routes of inoculation were 1.5×10^4 and <2 PFU, respectively, suggesting a decreased efficiency of virus entry into the central nervous system following peripheral inoculation. Inoculated directly into the brain, TR1050 produced mortality rates approaching that of its virulent parental strain, TR2100.

The mechanism of attenuation produced by the E2 residue 114 mutation appeared to differ from that of the attenuating E1 sequences. On the basis of LD_{50} comparisons between s.c. and i.c. routes of inoculation, the E2 residue 114 mutation in TR2200 appeared to affect primarily neurovirulence, as significant attenuation was still observed following direct inoculation into the brain (LD_{50}, 1.4×10^6 PFU). Examination of TR2200 replication in mice suggested that the E2 residue 114 mutation also specified decreased efficiency of neuroinvasion, as virus was not detected in the brain until 48 h postinoculation. However, similar results might have been observed if TR2200 had accessed the central nervous system quickly but was deficient in establishing replication there.

By combining attenuating mutations which appear to exert their predominant individual effects on different aspects of the pathogenic process, we have produced a highly attenuated model alphavirus vaccine. Further characterization of these and other combinations of attenuating mutations as to their degree of attenuation, induction of a protective immune response, and frequency of reversion to a virulence phenotype is in progress. However, we feel that the results obtained with the Sindbis virus model to date demonstrate that this approach is a feasible one for the informed derivation of live alphavirus vaccines of veterinary and public health significance.

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