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CONSTRUCTION AND EVALUATION OF A POLYVALENT GENETICALLY ENGINEERED VACCINE CANDIDATE FOR VEE

ANNUAL REPORT

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13. ABSTRACT *(Maximum 200 words)*

We have shown previously that combining multiple independently attenuating mutations in a single Venezuelan equine encephalitis virus (VEE) strain using recombinant DNA techniques is a feasible strategy for producing a stable, immunogenic live virus vaccine. To improve the initial vaccine candidate, two major areas have been pursued. First, the number of attenuating loci from which the constituent mutations will be selected has been expanded by further study of rapid penetration mutants, the investigational VEE vaccine strain, TC-83, and regions conserved among alphaviruses. Second, testing of alternate mutations at known attenuating loci has been initiated. Attenuating mutations have been compared in different strains of mice, and in a single strain infected by two different routes. These comparisons will aid in the selection of the two or three component mutations of the improved vaccine candidate. A shuttle vector has been constructed to facilitate the combination of different glycoprotein mutations with mutations in other regions of the genome.

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Table of Contents

Introduction

Live, attenuated virus vaccines have proved their effectiveness in controlling many virus diseases. They offer long-lived immunity and are relatively inexpensive to produce and administer. A well-known risk factor in the use of live virus vaccines is the possibility of reversion of attenuating mutations, which may give undesirable side effects. The present live virus vaccine for Venezuelan equine encephalitis virus (VEE), TC-83, produces frequent adverse reactions (Edelman, 1986), which may be caused by such reversion events.

We have proposed to overcome the disadvantage of reversion to virulence by using a molecular strategy to place multiple independently attenuating mutations into a single VEE vaccine strain. The very low probability of reversion at multiple sites in a single virus genome would give this vaccine strain a very stable attenuated phenotype. In addition, by introducing changes at a limited number of sites, whose major effect is to reduce virulence, the antigenic structure of the virus can be retained. Thus, the ability of the vaccine strain to induce a fully protective immune response in the vaccinee can be preserved.

Our molecular approach is based on 1), a full-length cDNA clone of the VEE genome from which infectious RNA genomes can be transcribed *in vitro* (Davis, et al., 1989), 2), the identification of attenuating mutations in both biologically selected attenuated virus strains (Davis, et al., 1991 - Appendix A) and attenuated mutants produced by targeted mutagenesis of conserved regions, and 3), introduction of single and multiple attenuating mutations into the full-length cDNA by site-directed

mutagenesis and recombinant DNA techniques.

This annual report describes results obtained during the period from July 1, 1991 to June 30, 1992 that pertain to the following technical objectives:

A. Expansion of the panel of mutations available for incorporation into the eventual vaccine candidate by identification of additional attenuating loci in the VEE genome.

B. Optimization for attenuation and immunogenicity at each locus by testing a catalog of amino acid substitutions, deletions and insertions, and by mutagenesis of flanking regions.

C. Identification of mutations which reduce transmission by mosquito vectors.

D. Evaluation of mutant recombinants in rodent models.

E. Construction of a shuttle vector to facilitate combination of mutant E2 and E1 glycoprotein genes.

Our studies of individual attenuating mutations, although they are not yet complete, have yielded sufficient information to allow us to begin construction of a new group of multiple mutants. The characteristics of these multiple mutants with respect to growth in tissue culture, attenuation in rodents, ability to induce a protective response, and mosquito transmission will determine which mutations we include in an improved VEE vaccine candidate.

A. Identification of Additional VEE Attenuating Loci

Further analysis of rapid penetration mutants

Selection for an increased rate of penetration of BHK cells coselects for attenuation of virulence in VEE (Johnston & Smith, 1988). Several attenuating loci in the E2 glycoprotein gene of VEE were identified in a panel of attenuated rapid penetration mutants (Davis, et al., 1991). In addition, three rapid penetration mutants shared a change from gln to arg at E2 position 81. However, one of these mutants was virulent, while the other two were attenuated. When the mutation at E2 81 was introduced into the full-length cDNA clone of the virulent Trinidad donkey strain of VEE, the resulting mutant virus was attenuated. Virus strains produced from four full-length clones shown to carry the E2 81 mutation were used to infect 6-week-old C57Bl/6 female mice (10 mice per strain, 5 x 10^3 pfu per mouse, ip.). One out of the 40 mice died on day 8 after inoculation; all the surviving mice were protected against challenge with the virulent parent virus. We assume from this result that the virulent rapid penetration mutant, which has the attenuating arg at E2 position 81, also carries a second mutation that reverts its attenuation phenotype, but not its penetration phenotype. Examples of this have been shown in the Sindbis virus-neonatal mouse model (Pence et al., 1990).

Although studies with the molecularly cloned mutants identified position 81 as an attenuating locus in E2, they also showed that this mutation was prone to reversion in tissue culture. Molecularly cloned virus from early times post-transfection was consistently attenuated, but later harvests often contained virulent revertants (data not

shown). This observation is consistent with the fact that a virulent second site revertant was isolated in the initial selection series. We conclude that the attenuating mutation E2 arg 81 is too unstable for use in a vaccine strain. However, another amino acid substitution at this locus, or a different arg codon, might be stable enough to be useful.

Analysis of mutations originally identified in TC-83

One of the sequence changes that distinguish the investigational VEE live virus vaccine, TC-83, from its virulent parent is an A rather than the parental G at nucleotide 3 of the 5'-noncoding region (Kinney, et al., 1989). This mutation has been shown to reduce virulence (Kinney, et al., 1991). We have produced clones with all three possible substitutions at this site and compared them with respect to their effect on virus viability and attenuation in two-week-old and adult CD-1 mice (Table 1).

TABLE 1
Attenuating mutations at nucleotide 3 of the 5'-untranslated region

virus strain	nucleotide at position 3	Infection of two-week-old CD-1 mice[a]		Infection of adult CD-1 mice[a]	
		% mortality	A.S.T.	% mortality	A.S.T.
V3000	G	100	3	100	8.25±1.28
U27E	U	91	3.91±1.38	87.5	8.67±1.21
U2811	C	82	7.78±1.39	0	NA
U4011	A[b]	64	7.57±1.72	12.5	13

[a]CD-1 Mice inoculated intraperitoneally with 10^3 pfu.
[b]Mutation originally found in investigational VEE vaccine strain TC-83.

All three mutations gave viable virus and reduced the mortality for mice; however, the substitution of either an A (as in TC-83) or a C at this locus was the most attenuating.

A predicted stem-loop structure in this region of the RNA that includes nucleotide 3 has been proposed to play a vital role in the replication cycle of the virus (Niesters and Strauss, 1990). Each of the three substitutions alters this predicted secondary structure. In addition, preliminary evidence suggests that a substitution at nucleotide 3 alters the level of viral RNA synthesis (K. McKnight and R. Johnston, unpublished results).

This attenuation locus is an important candidate for inclusion in a vaccine strain for two reasons. Mutations at this site are predicted to alter a different step in the virus life cycle and in the disease process than glycoprotein mutations. Therefore, combination of a change at this locus with a mutation in a surface protein gene may give a synergistic effect on virulence while avoiding the debilitating effect of multiple changes in a single virus structure or function. Secondly, mutation in the 5'-noncoding region reduces virulence without changing the antigenic structure of the virus particle.

Analysis of regions conserved among alphaviruses

Several regions of the VEE genome, both noncoding and coding, show extensive homology with other alphaviruses (Ou, et al., 1983; Kinney et al, 1986, 1989). The fact that these elements have been maintained throughout evolution of this genus suggests that they play important roles in the virus life cycle. A corollary of this hypothesis is that mutation of these regions may have specific effects on the ability of the virus to replicate, thereby altering its host range, tissue tropism, and/or virulence.

A region of the VEE E1 glycoprotein gene shows a high level of homology at the amino acid sequence level with other known alphavirus sequences (Kinney et al, 1986). It extends from position E1 74 to position E1 108, and is remarkable not only for

its homology but also for its lack of charged residues. By analogy with the paramyxoviruses, this region is proposed to be the fusogenic peptide involved in virus entry (Garoff et al., 1980). Previous results with Sindbis virus suggested that this region may contain attenuation loci (Polo and Johnston, 1990). Attenuating mutations were identified in the Sindbis E1 gene at position 72, just upstream of this region, and position 75, at its very N-terminal end.

We used saturation mutagenesis techniques to produce a panel of mutants in this conserved region of E1. Seventeen different amino acid substitutions were produced and placed individually in the full-length clone (Table 2).

TABLE 2
Mutations in the conserved region of the E1 glycoprotein gene

	E1 codon													
VEE	80	81	82	83	84	85	86	87	88	89	90	91	92	93
	GTC	TTC	ACA	GGG	GTT	TAC	CCG	TTC	ATG	TTG	GGT	GGT	GCA	TAT
	val	phe	thr	gly	val	tyr	pro	phe	met	trp	gly	gly	ala	tyr
mutants	ala	ile val tyr	ile pro	*trp* ala	phe	asn		val		arg gly	val asp	ala	ser	

Only one of these clones, which carried an ile in place of the parental phe at position 81, produced infectious RNA, as shown by specific infectivity measurements. Sixteen of the seventeen mutations were lethal, supporting the idea that the specific amino acid sequence of this region is functionally important in the virus life cycle. The substitution

of ile for phe at E1 81 produced a virus that was avirulent for adult C57Bl/6 mice. Ten out of ten mice survived inoculation with 10^3 pfu ip., and were immune to challenge with 10^4 pfu of the virulent parent virus. In addition, second site revertants were isolated following transfection with a clone carrying a lethal substitution of trp for gly at position 83. This second site mutation, which restored the viability in cell culture of the RNA genome, produced a virus that was avirulent in adult C57Bl/6 mice but gives solid immunity. The second site mutation has not yet been mapped. These E1 glycoprotein attenuating loci may be important constituent mutations of a new vaccine candidate. It may be possible to combine an E1 mutation with a 5'-noncoding change and an E2 mutation to produce a stably attenuated strain in which no single viral function is severely impaired.

A second conserved region among alphaviruses is the cleavage site used in the processing of PE2 to give the mature E2 glycoprotein. Indeed, the four amino acid signal that precedes the N-terminal amino acid of mature E2, basic-X-basic-basic, and that directs the activity of a cellular protease, is a constant motif in the glycoprotein precursors of enveloped viruses (Schlesinger and Schlesinger, 1986; Hosaka et al., 1991). This cleavage event may play an important role in the production of functional virus glycoproteins. Previous studies with an adult neurovirulent strain of Sindbis virus, S.A.AR 86, showed that a mutation in the N-terminal codon of E2 that prevented this cleavage event led to the production of viable virus particles containing PE2 rather than E2 (Russell et al., 1989). Although this mutant grew as well as the parent in tissue culture, it was attenuated in mice.

TABLE 3
Mutations in the PE2 cleavage site and second-site resuscitating mutations

	virus strain	sequence at PE2 cleavage site	second-site mutation	% mortality in mice[a]
	V3000 parent	RKRR/S	NA	100
biological revertants	J2-8	----/S	E2 gln 243	0
	J9-1	----/S	E1 ser 253	0
molecularly cloned double mutants	BB	----/S	E2 gln 243	0 - 40
	Z	RKRD/S	E2 gln 243	9 - 27

[a]V3000 parent and biological revertants were tested by inoculating adult C57Bl/6 mice with 10^3 pfu ip. Molecularly cloned double mutants were tested by inoculating two-week-old CD-1 mice with 10^3 pfu ip. V3000 gives 100% mortality in both mouse models.

To determine whether the PE2 cleavage site was an attenuating locus in VEE, we used site-directed mutagenesis to introduce changes in this region. The four amino acid cleavage signal, E3 residues 56-59, was completely deleted in one group of mutant clones, while a second panel of mutant clones carried single amino substitutions for the arg residue just upstream of the cleavage site, E3 residue 59. The amino acid substitutions tested were leu, ile, thr, pro, asp, asn, phe, lys, met, ala, val, gln, tyr, and glu. Measurement of the specific infectivity of RNA genomes transcribed from these clones showed that all of these mutations are lethal in VEE. The prevention of PE2 cleavage is also lethal in the AR339 strain of Sindbis virus (H. Heidner and R. Johnston,

Ann. Meeting of the ASV, 1992). However, in both the VEE and Sindbis AR339 systems, it has been possible to isolate viable revertants from cells transfected with the mutant RNAs. Two independent revertants of the VEE mutant that contains a deletion of E3 codons 56-59 have been studied and shown to contain a second mutation not present in the transfected RNA. One mutation is at E2 243 (gln for the parental leu), the other is at E1 253 (ser for the parental phe). Molecularly cloned double mutants containing either the deletion or a lethal substitution at E3 position 59 coupled with the E2 243 mutation were viable. The putative resuscitating mutation at E1 253 is presently being tested in full-length clones containing either a deletion or a substitution at the PE2 cleavage site. Consistent with results obtained with the AR339 and S.A.AR 86 strains of Sindbis, both the biologically selected and molecularly cloned double mutants were attenuated in mice (Table 3). Therefore, the combination of a lethal mutation at the PE2 processing site with a resuscitating mutation elsewhere in the genome could be included in the design of a multiply attenuated vaccine strain.

B. Improvements of Known Attenuating Mutations

Alternative amino acid substitutions at known attenuating loci

Results obtained with the Sindbis virus-neonatal mouse system showed that the attenuation phenotype of single site mutants depended not only on the locus that was changed, but on the specific amino acid substitution (Polo and Johnston, 1991; Schoepp and Johnston, Virology, submitted). For example, at one codon in the E2 glycoprotein gene, any one of the 11 amino acid substitutions tested resulted in reduced virulence,

while at a second locus, only one of the five different residues tested gave an attenuated phenotype. In addition, specific amino acid substitutions at the same locus were shown to have different effects on neurovirulence and neuroinvasiveness. Three different amino acid substitutions at E2 position 96 produced virus that could no longer invade the brain, but was virulent upon intracerebral inoculation. Eight other substitutions at the same site reduced both neurovirulence and neuroinvasiveness. Past experience has shown that results with Sindbis virus are good predictors of the behavior of VEE. Therefore, these results have a direct bearing on the testing and eventual selection of attenuation loci for inclusion in a VEE vaccine candidate.

In light of the results with Sindbis virus, panels of different amino acid substitutions are being engineered at selected attenuating loci in the VEE genome to determine two properties of these sites, 1), the proportion of amino acid changes that reduce virulence, and 2), the availability of attenuating codons which would require two nucleotide changes to give a virulent amino acid. A catalogue of alternative amino acid substitutions has been made at position 81 of the E1 glycoprotein. As reported above, a change from phe to ile at this site produced a mutant that was avirulent for adult mice. An additional 14 amino acids have been placed at this locus, including tyr and val, which in preliminary experiments appeared to be lethal (Table 4). These 14 mutations will be placed individually into the full-length clone and tested for their effects on viability and virulence. Analogous mutant panels will be made for known attenuating loci at E2 position 76 and E2 position 209.

TABLE 4
Alternate Amino Acid Substitutions at Attenuating Locus E1 81

virus strain	E1 81 codon	E1 81 amino acid	virus strain	E1 81 codon	E1 81 amino acid
V3000 parent	TTC	phe	CC16	CAA	gln
S35	ATC	ile	CC21	TTA	leu
CC3	GGT	gly	CC24	AAG	lys
CC4	GTT	*val*	CC25	CGT	arg
CC5	TCT	ser	CC27	ATG	met
CC7	GCA	ala	CC29	AGT	ser
CC12	ACG	thr	CC32	ATT	ile
CC13	CAT	his	CC36	TAT	*tyr*

Small in-frame deletion at a known attenuating locus

Reversion of attenuation mutations to a more virulent phenotype is the most serious problem in the use of a live virus vaccine. Reasoning that attenuating deletion mutations will not be able to revert (although in theory they can still be suppressed by second-site mutations), we tested deletions in a known attenuating locus, E2 position 76. E2 76 was selected because the substitution of lys for the parental glu at this site was the most attenuating mutation identified among the fast-penetrating mutants (Davis, et al., 1991, Appendix A). A single codon deletion at position 76, or a three codon deletion (positions 75, 76 and 77) were independently introduced into the full-length clone using site-directed mutagenesis. Both of these deletions produced

noninfectious RNA. However, RNA containing the single codon deletion gave rise to viable progeny following transfection of BHK cells. Two of these isolates, which contained both the deletion at E2 76 and an unmapped second mutation, were tested for virulence in CD-1 adult mice. One mutant gave 30% mortality and an extended average survival time following inoculation of 10^3 pfu into the footpad, while the other mutant gave 0% mortality. If further studies show that only a limited number of second site mutations can restore viability to this deletion mutant, and if the double mutants are consistently attenuated, the inclusion of this type of double mutation in a vaccine strain may greatly reduce the possibility of reversion. However, it appears from these results that some loci will not accommodate a simple deletion mutation. Deletions, as well as small in-frame insertions, will be tested at other sites in the E2 and E1 glycoproteins.

Alternative amino acid substitutions at a "resuscitating" site

As described above, a change from leu to gln at position 243 of the E2 glycoprotein restores viability to genomes that carry a lethal mutation, either a four amino acid deletion or a single amino acid substitution, at the PE2 cleavage site. Preliminary results suggested that E2 243 gln is lethal when introduced alone into the full-length clone (data not shown). This raises the possibility of producing an attenuated double mutant for which same-site reversion of either mutation is lethal. (E2 243 gln may also be lethal in the case where a second-site mutation unmasks an alternative cleavage site in PE2.) To determine the best second site mutation for this purpose, six alternative amino acid substitutions at E2 position 243 were made using in vitro mutagenesis; they are lys, ala, thr, asn, arg and ser. These mutations are being tested, in

conjunction with the cleavage signal deletion mutation and with a single amino acid replacement at E3 position 59, for their ability to restore viability and to produce an attenuated double mutant. Results from these tests will indicate the pair of mutations that gives the best growth in tissue culture, the greatest reduction in virulence, and maintains restriction in the mosquito vector (See below) while presenting the lowest probability of reversion to a virulent virus.

C. Mutations Affecting Vector Infection and Transmission

Studies of virus growth in, and transmission by, Aedes taeniorhyncus mosquitoes have been done in collaboration with Dr. Mike Turrell at USAMRIID. All mosquito infections were done by intrathoracic inoculation. Transmission was measured by the ability of a mosquito known to be virus infected to transfer the virus to a susceptible hamster, which was then monitored for clinical signs and finally for ability to survive a challenge with virulent VEE.

Mutation at the PE2 cleavage site

As described above, viable revertants were isolated from tissue culture cells transfected with RNA carrying a lethal deletion of the PE2 cleavage signal. One of these double mutants contained the deletion coupled with a change from the parental leu to gln at E2 position 243. Preliminary experiments indicated that this double mutant, J2-8, in addition to being attenuated in mice and hamsters, grew poorly in the mosquito vector. When the deletion mutation and the resuscitating E2 243 change were introduced into a full-length clone, the resulting recombinant double mutant, BB, gave

somewhat reduced titers in the mosquito. However, when the mutant-infected insects were allowed to feed on susceptible hamsters, the virus was not efficiently transmitted (data not shown). These results, although preliminary, suggest that this pair of mutations may contribute not only to the attenuation of a vaccine strain in vertebrates, but also may prevent the spread of the recombinant virus strain by its insect vector.

A rapid penetration mutation near a neutralizing epitope

An escape mutant selected for resistance to neutralization by monoclonal antibody 1A3B-7 was found to have a single amino acid substitution at E2 position 207 (Johnson et al., 1990). This mutant grew to lower titers in an Aedes albopictus cell line than its parent, and was restricted in its ability to infect and disseminate from the midgut of A.aegypti mosquitoes following oral infection (Woodward, et al., 1991). Since one of the attenuating mutations originally identified in a rapid penetration mutant mapped to E2 position 209, the molecularly cloned strain containing this mutation was tested for its ability to grow in mosquitoes following intrathoracic inoculation. Results so far indicate that this mutant is able to replicate and be transmitted by mosquitoes infected by this route. Further studies using oral infection of mosquitoes will show whether a mutation at E2 209 prevents dissemination in the mosquito and transmission to susceptible animals.

A deletion mutation in the C-terminus of NSP3

The nonstructural protein NSP3 has yet to be assigned a specific function in the alphavirus life cycle. Semliki forest virus and Sindbis virus NSP3 proteins are phosphorylated during infection, to give a population of differentially phosphorylated

molecules (Peranen et al., 1988; Li et al., 1990). NSP3 is the least conserved of the nonstructural proteins, at both the nucleotide and amino acid level (Kinney et al., 1989). One clue to its possible function was the finding that deletions in the C-terminal region of this protein, while having no effect on the viability of the mutant in cultured vertebrate cells, did decrease the efficiency of growth in cultured insect cells (La Starza et al., 1990). A viable deletion mutant of VEE has been mapped to the C-terminus of NSP3 (Davis et al., 1989). This molecularly cloned mutant, V1000, has comparable growth properties to the parental virus in three types of vertebrate cells, and is just as virulent in rodents. When V1000 was tested for its ability to grow in mosquitoes following intrathoracic inoculation, its growth pattern was indistinguishable from that of the parent virus.

D. Evaluation of Molecularly Cloned Mutants in Rodent Models

Comparison of attenuation in two strains of mice

Table 5 shows a comparison of per cent mortality following footpad inoculation of CD-1 mice, an outbred strain, and intraperitoneal inoculation of C57Bl/6 mice, an inbred strain, with the molecularly cloned rapid penetration mutants.

TABLE 5
Per Cent Mortality of Attenuated Mutants in Two Different Mouse Strains

	V3000 parent	E2 lys 76	E2 lys 120	E2 lys 209
CD-1 mice[a]	100	0	78	0
C57Bl/6[b]	100	0	0	0

[a]Adult mice injected into the footpad with 10^3 pfu.
[b]Adult mice injected intraperitoneally with 5×10^3 pfu.

Some of the virus mutants are avirulent in adults of both strains, eg. E2 lys 76 and E2 lys 209, while another, E2 lys 120, is avirulent in C57Bl\6 mice, but gives some mortality in CD-1 mice. Previously, testing in one-week-old and two-week-old C57Bl\6 mice showed that the changes at E2 76 and E2 209 gave lower mortality in young mice than the mutation at E2 120, although all were avirulent in adult mice (Davis et al., 1991, Appendix A). These results suggest that the use of different mouse models may reveal different levels, or different mechanisms, of attenuation among mutant viruses. The basis for the different behavior of a given mutant in mice of different ages or from different strains has not been determined.

Attenuation following different routes of inoculation

Measurements of mortality and average survival times were made following footpad and intracerebral inoculation to compare attenuated mutants with respect to neuroinvasiveness and neurovirulence (Table 6).

TABLE 6
Per Cent Mortality and Average Survival Time of Adult CD-1 Mice Infected by Two Different Routes

	V3000 parent		E2 lys 76		E2 lys 120		E2 lys 209	
	IC	FP	IC	FP	IC	FP	IC	FP
% mortality	100	100	22	0	100	78	100	0
AST (days)	4.67± 0.71	5.44± 0.53	7.5±0 .71	NA	6.0±0. 53	9.14±0 .69	7.67± 0.5	NA

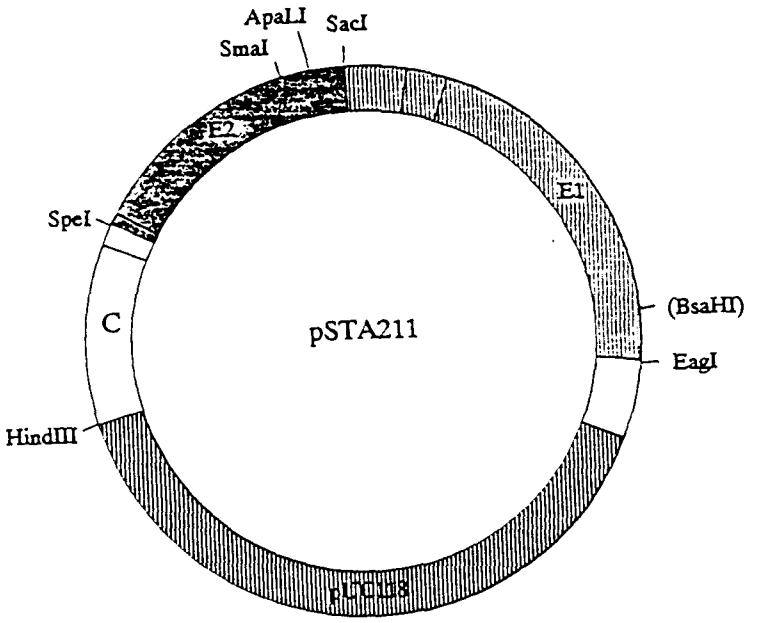
CD-1 mice were inoculated into the footpad (FP) or intracerebrally (IC) with 10^3 pfu and monitored over a 14-day period for % mortality and average survival time (AST)

In CD-1 mice, the E2 209 lys mutant was clearly blocked at invasion of the central nervous system, while the E2 76 lys mutant was inefficient at causing disease even when introduced directly into the brain. One strategy for producing a stable multiply attenuated vaccine strain is to combine mutations that block the disease process at different stages. The comparison of these two different routes of inoculation is a first step in distinguishing the blocks produced by individual attenuating mutations. More detailed studies of pathogenesis using selected mutants are being undertaken, funded from other sources, which will further characterize the defect in the disease process caused by these mutations.

E. Construction of a Shuttle Vector for Combining Mutant E2 and E1 Glycoprotein Genes

A new series of full-length cDNA clones will be engineered to contain combinations of the mutations described above. These clones will be mutated at three loci, a number that might not be expected to greatly reduce the ability of the virus to grow in tissue culture, but, if chosen properly, may block the virus's ability to cause disease at each of three independent steps. To facilitate the construction of these multiple mutants, a shuttle plasmid containing the structural genes of VEE was made such that unique restriction sites flank the E2 and E1 glycoprotein genes (Figure 1). First a DNA fragment of approximately 4 kbp, extending from the HindIII site (nt 7290 from the 5'-end of the VEE genome) to the NotI site downstream of the poly(A) tract, was removed from the full-length clone pV3000 (Davis, et al., 1991) and placed into the

FIGURE 1



multiple cloning site of pUC118 (Viera & Messing, 1987). Next, a BsaHI site in the subcloned E1 glycoprotein gene (nt 10,848) was removed by exchanging the SacI-EagI fragment (nt 9492-nt 10,890) for one in which BsaHI site had been altered by site-directed mutagenesis without altering the amino acid sequence. Finally, the SmaI site (nt 9072), originally made nonfunctional by a silent mutation in pV3000, was reinstated in the subclone by exchanging the SpeI-ApaLI fragment (nt 8389-nt 9220) of the subclone with the analogous fragment from the original cloned sequence of the Trinidad donkey strain of VEE. To test the authenticity of the resulting sequence, the DNA fragment flanked by the unique AflII (nt 8054) and SacII (nt 11,199) sites in the shuttle plasmid was used to replace the analogous region in the full-length pV3000 clone. In vitro RNA transcripts from this clone were shown to be infectious, and the sequence

between the SacI and EagI sites was determined, by direct RNA sequence analysis, to be identical to that in the original pV3000 clone. Finally, virus produced by transfection of BHK cells with these transcripts was tested for virulence in CD-1 mice (10^3 pfu, footpad). It gave 100% mortality with an average survival time identical to that measured in a parallel infection with V3000.

The shuttle plasmid will allow the combination of individual mutations in the E2 and E1 genes with mutations in the 5'-noncoding region. Each gene replacement will be monitored with a restriction enzyme, either SmaI or BsaHI. Also, replaced sequences that originated from mutagenized M13 subclones, which may contain unintentional changes, will be relatively small and can be easily checked by direct RNA sequencing.

Conclusions

It has been shown previously that the combination of multiple independently attenuating mutations in a single virus strain using recombinant DNA techniques is a feasible strategy for producing a stable, immunogenic live virus vaccine for VEE (Davis, et al, 1991; Smith et al., 1991). This report describes progress toward an improved vaccine candidate. Two major areas of improvement have been pursued, 1) expansion of the number of attenuating loci from which the constituent mutations will be selected and 2), testing of alternate substitutions at known attenuating loci. The object of these studies is an improved multiple mutant that contains the most stable attenuating mutations available at a small number of loci, each of which affects a different stage of

the virus replication cycle. Thus, the debilitating effect of cumulative mutations in a single virus structure and/or function may be avoided, and changes to the antigenic structure of the virus will be minimized. The attenuating loci under consideration were identified in rapid penetration mutants, in the investigational VEE vaccine, TC-83, and among engineered mutations in regions conserved among alphaviruses. The further analysis of these loci will involve continued testing of alternate mutations. Virus strains that contain combinations of two and three mutations will be evaluated in sensitive rodent models for the degree of attenuation and ability to elicit a protective immune response.

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APPENDIX A

Attenuating Mutations in the E2 Glycoprotein Gene of Venezuelan Equine Encephalitis Virus: Construction of Single and Multiple Mutants in a Full-Length cDNA Clone

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Attenuated mutants of Venezuelan equine encephalitis virus (VEE) were isolated by selection for rapid penetration of cultured cells (R. E. Johnston and J. F. Smith, 1988, *Virology* 162, 437–443). Sequence analysis of these mutants identified candidate attenuating mutations at four loci in the VEE E2 glycoprotein gene: a double mutation at E2 codons 3 and 4, and single substitutions at E2 76, 120, and 209. Each candidate mutation was reproduced in an isogenic recombinant VEE strain using site-directed mutagenesis of a full-length cDNA clone of VEE. Characterization of these molecularly cloned mutant viruses showed that mutation at each of the four loci in the E2 gene was sufficient to confer both the accelerated penetration and attenuation phenotypes. Inoculation of the molecularly cloned viruses into rodent models that differ in their response to VEE suggested that individual mutations affected different aspects of VEE pathogenesis. Full-length clones containing multiple mutations were produced by combining independently attenuating mutations. Molecularly cloned viruses carrying two or three mutations were more attenuated in sensitive animal models than viruses which contained any single mutation alone. However, these highly attenuated strains still retained the ability to induce an immune response sufficient to protect against a high dose challenge with virulent VEE. These results indicate that production of a molecularly cloned live virus vaccine for VEE is feasible.

INTRODUCTION

The induction of viral disease in an animal requires the efficient execution of multiple viral functions. Due to the complexity of virulence as a genetic characteristic, mutations at numerous loci could interrupt the normal course of disease, resulting in an attenuated or avirulent phenotype. Mutations at a subset of loci affecting virulence may do so by adversely affecting replication in general. These mutants would replicate poorly in cell culture as well as in critical cells of the target organ(s). However, evidence from studies of several different virus systems has shown that other attenuating mutations disrupt specific aspects of disease progression *in vivo*. Such mutants have been selected in various ways, but they all share the property of dramatically reducing the virulence of the virus in a given animal host without affecting the ability of the virus to replicate in tissue culture. These mutants include those related to the Sabin vaccine strains of poliovirus (La Monica *et al.*, 1987; Westrop *et al.*, 1989), a point mutant in the S1 gene of reovirus (Kaye *et al.*, 1986), antibody escape mutants of rabies virus (Seif *et al.*, 1985), a neuroattenuated intertypic recombinant of herpes simplex virus (Javier *et al.*, 1987), a fusion-defective glycoprotein variant of La Crosse virus (Gonzales-Scarano *et al.*, 1985), and monoclonal antibody-resistant mutants of mouse hepatitis virus (Parker *et al.*, 1989).

We have investigated the genetics of virulence of two members of the alphavirus genus, Sindbis virus and Venezuelan equine encephalitis virus (VEE). Other members of this genus include Semliki Forest virus, eastern equine encephalitis virus, western equine encephalitis virus, Chikungunya virus, and Ross River virus. Attenuated mutants of Sindbis virus, isolated by direct selection for rapid penetration of baby hamster kidney (BHK) cells, or by selection for escape from neutralization by E2 glycoprotein-specific monoclonal antibodies, showed dramatically reduced virulence in mice, but replicated in cultured cells as well as, or better than wild-type virus (Olmsted *et al.*, 1984; 1986; Pence *et al.*, 1990). These mutations are localized in the E2 glycoprotein gene, and appear to exert their effect on virulence through changes in a structural feature(s) of the virion surface (Davis *et al.*, 1986; Pence *et al.*, 1990; R. Schoepp and R. Johnston, unpublished results).

Rapid penetration mutants of VEE also have been isolated (Johnston and Smith, 1988). Like the Sindbis

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mutants, the VEE mutants are significantly attenuated, implying that a structural domain that includes determinants of both penetration rate in cell culture and virulence *in vivo* is a general property of the alphavirus envelope spike. One possible explanation of this result is that mutations which optimize the early events in infection of BHK cells may simultaneously decrease the efficiency with which analogous early events are accomplished in critical target cells *in vivo*.

Four aspects of the VEE system make it well-suited for a genetic study of viral virulence and pathogenesis. First, like all alphaviruses, VEE is a relatively simple enveloped virus, whose 11,444-nucleotide-long, single-stranded, positive sense RNA genome encodes only seven genes (Kinney *et al.*, 1989). The three structural proteins include capsid protein, multiple copies of which form an icosahedron around the RNA, and two envelope glycoproteins, E1 and E2 (Pedersen and Eddy, 1974), which have been shown in other alphaviruses to associate in heterodimeric, transmembrane spikes (Rice and Strauss, 1982). Second, well-studied small animal models exist for VEE infection, the mouse and hamster, for which distinctive patterns of pathology have been described (Gleiser *et al.*, 1962). Third, a full-length cDNA clone of VEE has been constructed in conjunction with a T7 bacteriophage promoter. This clone can be transcribed *in vitro* with T7 RNA polymerase to yield infectious VEE RNA (Davis *et al.*, 1989), allowing the directed introduction of specific mutations into the VEE genome. Finally, the rapid penetration mutants described above are altered in their ability to cause disease, yet are sufficiently closely related to their virulent parent to allow identification of the causative mutations. A preliminary characterization of some of these mutants appeared previously (Davis *et al.*, 1990).

The experiments described in this report represent the starting point for two parallel studies: the investigation of the molecular genetics of VEE pathogenesis and the development of a molecularly defined and cloned, live, attenuated VEE vaccine. Candidate attenuating mutations were identified by sequencing the glycoprotein genes of the rapidly penetrating, attenuated VEE mutants. These mutations were then introduced individually into the full-length VEE clone to produce isogenic constructs differing only at the prescribed loci. Molecularly cloned viruses derived from these constructs were examined for their penetration phenotype in BHK cells and virulence in rodent models. Full length clones containing multiple mutations were constructed to analyze the interaction of independently attenuating mutations in animal models, and the suitability of such molecularly cloned viruses as live virus vaccines.

MATERIALS AND METHODS

Viruses and cells

Laboratory stocks of the Trinidad donkey strain of VEE (TRD) were obtained from the Centers for Disease Control (Ft. Collins, CO; FC) and P. Jahrling, U.S. Army Medical Research Institute of Infectious Diseases (Ft. Detrick, Frederick, MD; FD). These stocks were the parents for a panel of mutants isolated following one to four passages under a stringent selective pressure for accelerated penetration (Johnston and Smith, 1988). All experiments described in this report were carried out in compliance with the CDC–NIH guidelines described in "Biosafety in Microbiological and Biomedical Laboratories," 1988, and with guidelines established by the Recombinant DNA Advisory Committee of the NIH.

BHK cells were obtained from the American Type Culture Collection in passage 53, maintained in Eagle's MEM (EMEM) containing 10% donor calf serum and 10% tryptose phosphate, and used between passages 55 and 65. Vero cells (Vero-76, ATCC CRL 1587) were maintained in EMEM with 5% fetal bovine serum (FBS) and gentamicin (50 μg/ml), and were used between passages 140 and 150. Primary chicken embryo fibroblast (CEF) cultures were prepared by trypsinization of decapitated embryos and maintained in EMEM with 10% FBS and gentamicin.

Sequence analysis of viral RNA and DNA

Sequence data were obtained by the dideoxynucleotide chain termination procedure using both single-stranded DNA and RNA templates and oligonucleotide primers whose sequence is available upon request (Sanger *et al.*, 1977; Zimmern and Kaesberg, 1978). Single-stranded DNA templates were isolated from M13 phage particles using standard techniques (Ausubel *et al.*, 1987). VEE virion RNA was purified as described previously (Davis *et al.*, 1986). An alternative source of RNA templates was total cytoplasmic RNA isolated from virus-infected cells. RNA was isolated at 5 to 6 hr postinfection by incubation of cells in hypotonic buffer (0.01 *M* NaCl, 0.01 *M* Tris–HCl, pH 8.5, 0.0015 *M* $MgCl_2$), lysis with 0.45% (v/v) Nonidet P-40, removal of nuclei by centrifugation, extraction with phenol:chloroform:isoamyl alcohol (24:24:1) and with chloroform:isoamyl alcohol (24:1), and precipitation with ethanol. Reaction mixtures containing viral RNA synthesized *in vitro* from a full-length cDNA copy of the genome placed immediately downstream from a T7 promoter (Davis *et al.*, 1989) were used directly in the RNA sequencing protocol without purification.

Subcloning of the VEE structural gene region

Plasmid pV2000, containing pBR322-derived vector sequences and the complete VEE genome sequence downstream from a T7 phage promoter, was constructed using a modification of the procedure which produced the deleted plasmid, pV1000 (Davis *et al.*, 1989). Transcription of pV1000 *in vitro* yielded RNA which was infectious and produced high titers of virus following transfection of cultured cells. However, pV1000 contained a deletion of 102 nucleotides in the nsP3 gene. In the modified procedure, a preparation of plasmid pT7 5'V50 (Davis *et al.*, 1989) was partially digested with *Xho*I and then completely digested with *Xba*I to produce a heterogeneous population of DNA fragments, some of which contained the 102-nucleotide region deleted from pV1000. Following ligation with the appropriate *Xba*I–*Xho*I fragment from pTX 3'V50 (Davis *et al.*, 1989), constructs with the complete VEE sequence, including pV2000, were isolated. To prepare a substrate for *in vitro* mutagenesis, a *Hin*dIII–*Eco*RI fragment of pV2000 that contained the complete structural gene region (nucleotides 7291 to 11,444) was subcloned into M13mp19. The sequence of the glycoprotein genes in the subclone was determined and was found to be identical with that obtained directly from VEE virion RNA.

Site-directed mutagenesis

The VEE structural protein gene region subcloned into M13mp19 was the substrate for *in vitro* mutagenesis using the procedure of Kunkel (1985). Initially, a change in codon 170 of E2 that was silent with respect to amino acid coding, but that resulted in the elimination of a *Sma*I site was introduced into the subclone. Sequences derived from the subcloned genes could then be distinguished readily from analogous sequences in pV2000 by restriction analysis. In the background lacking the *Sma*I site, individual subclones were produced, each of which contained one of the candidate attenuating mutations. The unique *Afl*II restriction site in the capsid gene and the unique *Sac*II site near the 3' end of the E1 gene were used to substitute each mutated sequence separately for the corresponding sequence in pV2000. The presence of the desired mutation was verified by sequence analysis of the region containing the mutation, first in single-stranded DNA generated from the mutated subclone, and subsequently in full-length RNA transcripts produced *in vitro* from the substituted full-length clone. The specific infectivity (plaque forming units per cpm of ^{32}P-labeled UTP incorporated into *in vitro* RNA transcripts) of each construct was tested as described previously (Rice *et al.*, 1987), and was approximately the same for pV3000 (the full-length clone containing only the silent change in E2 codon 170) and for all of the mutant constructs derived from it. Therefore, RNA transcripts produced from these constructs were uniformly infectious. Virus derived from pV3000 was as virulent in rodent models as the Trinidad donkey strain of VEE, as measured by percentage mortality and mean day to death.

In vitro transcription and transfection

Preparation of plasmids for runoff transcription by digestion at the unique *Not*I site, transcription with T7 RNA polymerase, and DEAE-dextran-mediated transfection of CEF were performed as described previously (Rice *et al.*, 1987; Polo *et al.*, 1988; Davis *et al.*, 1989). Transfection of CEF and BHK cells also was performed with cationic liposomes (Lipofectin, BRL) using the manufacturer's recommended protocol. Virus-containing culture supernatants were harvested as soon as the monolayers showed significant cytopathic effect.

Assays for penetration rate, virulence, and immunogenicity of molecularly cloned mutant VEE strains

Viruses to be tested in these studies were obtained directly from transfected CEF culture supernatants and were used after appropriate dilution without further passage. Virus penetration into BHK cells was measured as the ability of attached virions to resist removal from the cell surface by trypsin and thereby to produce an infectious center, as described in Johnston and Smith (1988). Female C57BL/6 mice (6–8 weeks), female Syrian hamsters (7–8 weeks), and litters of ICR mice (1–3 days) were obtained from Harlan Sprague-Dawley (Indianapolis, IN), and were held in quarantine for at least one week prior to use. All animals were inoculated intraperitoneally, with adult animals and infant mice receiving 0.2 and 0.1 ml of the appropriate virus dilution, respectively. At 30 days after infection, adult animals were bled by cardiac puncture (hamsters) or from the retro-orbital sinus (mice) under ketamine hydrochloride (2 mg/100 g) and Rompun (xylazine, 1 mg/100 g) anesthesia. The animals were then challenged intraperitoneally with 5×10^4 PFU of the virulent V3000 strain as a direct test of immunity. In conducting the research described in this report, we adhered to the "Guide for Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

TABLE 1

MUTATIONS IN THE E2 GLYCOPROTEIN GENES OF RAPIDLY PENETRATING, ADULT MOUSE ATTENUATED MUTANTS OF VEE[a]

	E2 amino acid residue[b]								
Virus strain	3	4	76	120	209	245	278	308	323
FD-0-1	Glu	Glu	Glu	Thr	Glu	Lys	Phe	His	Gly
(virulent parent)	GAG	GAG	GAG	ACA	GAG	AAA	UUC	CAU	GGA
FD-3-6[c]	LYS	LYS							GLU
	AAG	AAG							GAA
FC-1-8	Glu	Glu	Glu	Thr	Glu	Lys	Phe	His	Glu
(virulent parent)	GAG	GAG	GAG	ACA	GAG	AAA	UUU	CAC	GAA
FC-4-2			LYS						
			AAG						
FC-4-7				LYS					
				AAA					
FC-1-2					LYS				
					AAG				
FC-4-6					LYS	ASN			
					AAG	AAU			

[a] Changes between the two parent stocks of Trinidad donkey strain VEE, FD-0-1 and FC-1-8, or between representative mutant virus strains and their parents are shown. Mutant FC-1-7 was identical with FC-4-7, and mutants FC-4-1 and FC-4-8 were identical with FC-1-2.

[b] E2 amino acids residues are numbered from the amino-terminal end of the mature protein.

[c] Mutant designations identify either the Fort Detrick stock (FD) or the Fort Collins stock (FC) of the Trinidad donkey strain of VEE as the parent and indicate the passage number followed by the number of the isolate.

ELISA titrations of sera from inoculated animals were carried out with either anti-mouse (Boehringer-Mannheim) or anti-hamster (Kirkegaard & Perry) peroxidase-labeled antibody conjugate, a single lot of each, using standard procedures after adsorption of VEE antigen to 96-well PVC plates (Dynatech Laboratories, Alexandria, VA). VEE antigen was prepared by solubilization and sonication of 1×10^8 infected BHK cells in 3 ml of 1% Triton X-100 in 0.01 *M* phosphate-buffered saline, pH 7.4, followed by clarification at 10,100 *g*. This lysate was utilized at a 1:2000 dilution. Optical densities obtained from similarly prepared, but uninfected, BHK cells (averaging 0.09 with anti-mouse, and 0.07 with anti-hamster) were subtracted from test results to correct for nonspecific reactions. Titers were recorded as the highest dilution of serum to give an optical density of at least 0.2.

RESULTS

Sequence analysis of two isolates of the Trinidad donkey strain of VEE

As described previously (Johnston and Smith, 1988), two laboratory stocks of the virulent Trinidad donkey strain of VEE, designated FC and FD, were the parents from which attenuated, rapid penetration mutants were selected. Sequence data were obtained from both of these parent strains as the basis for comparison with mutant sequences (Table 1). FC-1-8, a virulent, slow-penetrating isolate which was plaque purified following a single growth cycle under selective pressure for rapid penetration, represented the FC parent. The complete E2 gene sequence of this isolate was obtained from two sources. The majority of the sequence data was determined by direct sequencing of FC-1-8 virion RNA. Five nucleotides needed to complete the sequence were obtained from consensus nucleotides in the E2 gene sequences of seven mutants derived from FC-1-8. The sequence agreed exactly with that published previously (Kinney *et al.*, 1989). Complete sequence information was obtained for the E3, E2, and E1 genes of the FD parent, FD-0-1, using both virion RNA and a cDNA clone of FD-0-1 as template.

Within the region analyzed, FC-1-8 differed from FD-0-1 at three positions, all of which were in the E2 gene (Table 1). FC-1-8 contained a U rather than a C at position 9397, a C rather than a U at position 9487, and an A rather than a G at position 9531. The first two differences were noncoding changes in E2 codons 278 and 308. The third, however, encoded a Glu rather than a Gly at E2 position 323. In general, rapid penetration mutants derived from either parent strain retained the parental nucleotides at these positions. However, mutant FD-3-6, derived from FD-0-1, showed the FC-1-8 Glu codon at E2 position 323, while retaining the parental FD-0-1 nucleotides at the other two loci. Since both FC-1-8 and FD-0-1 are virulent and slow penetrating,

the FC-1-8 amino acid at E2 position 323 in the attenuated, rapid penetration mutant derived from FD-0-1 (FD-3-6) was not a candidate for the causal mutation in this isolate.

Sequence analysis of rapid penetration mutants

Experience with rapidly penetrating, attenuated mutants of Sindbis virus suggested that single nucleotide changes in the E2 gene might be responsible for the penetration and virulence phenotypes of the analogous VEE mutants (Davis *et al.*, 1986; Polo *et al.*, 1988; Pence *et al.*, 1990; R. Schoepp and R. Johnston, unpublished results). Therefore, the E2 genes of eight VEE rapid penetration mutants were analyzed by direct sequencing of viral RNA, and candidate attenuating mutations were, in fact, found in this gene (Table 1). The sequences of the entire E2 genes for mutants FC-1-2, FC-1-7, FC-4-1, FC-4-2, FC-4-6, FC-4-7, FC-4-8, and FD-3-6 were obtained. In five cases, FC-1-2, FC-4-2, FC-4-6, FC-4-7, and FC-4-8, sequence data were obtained for the entire E1 gene as well. No differences were seen between the E1 sequences of the mutants, the E1 sequence obtained from FD-0-1, and the VEE E1 sequence published previously (Kinney *et al.*, 1989).

Each of the rapid penetration mutants had a change at one of four E2 loci: at codon 76, 120, or 209 or a double change at codons 3 and 4. (In these experiments codons 3 and 4 were considered a single locus.) Two of the mutants had additional coding changes within the regions sequenced. Mutant FD-3-6 had the change from the FD parent amino acid to the FC parent amino acid at codon 323 described above, and mutant FC-4-6 showed a change in E2 codon 245 in addition to the change at position 209. As mutants FC-1-2 and FC-4-6 both carried an identical change at position 209, but mutant FC-1-2 showed the parental amino acid at 245, it seemed unlikely that the change at 245 in mutant FC-4-6 was related to the attenuation phenotype. Therefore, this mutation was not examined further in the present study.

Single nucleotide changes in E2 determine penetration and virulence phenotypes

The following experiments were performed to determine whether the nucleotide changes listed in Table 1 were sufficient to cause the altered penetration and virulence phenotypes of the mutants. As outlined under Materials and Methods, we used a full-length clone of the Trinidad donkey strain of VEE (parent FD-0-1) as a substrate for *in vitro* mutagenesis to reproduce each of the candidate mutations listed in Table 1 in the E2 gene of a full-length VEE clone. We then measured the penetration and virulence phenotypes of the viruses produced from these full-length clones. The wild-type control, V3000, was virus produced from plasmid pV3000, the parent full-length clone from which all of the mutant clones were derived (Materials and Methods).

Penetration rates for V3000 and for the molecularly cloned mutant virus strains are shown in Table 2. The penetration rates measured for V3000 as well as for some of the mutants varied from experiment to experiment. However, within each of three experiments, the mutants invariably gave higher penetration rates than the parent. Some of the mutants (e.g., V3026 and V3014) showed higher penetration rates that other mutants (e.g., V3010 and V3012). A range of accelerated penetration rates was also reported previously for the biologically selected rapid penetration mutants (Johnston and Smith, 1988). The penetration rate measured for V3000 was comparable to that reported previously for the Trinidad donkey strain of VEE in BHK cells (Johnston and Smith, 1988). These results demonstrated that the single nucleotide differences between the molecularly cloned mutants and their parent produced a rapid penetration phenotype in each case.

The original rapid penetration mutants whose E2 genes are represented in Table 1 were attenuated in adult C57BL/6 mice (Johnston and Smith, 1988). In the original experiments, a dose of 10^3–10^4 PFU injected intraperitoneally resulted in no clinical symptoms and generated complete resistance to later challenge with virulent VEE. The same protocol was followed with the molecularly cloned viruses, and the results are shown in Table 2. The single nucleotide change in the E2 gene of each of the molecularly cloned mutants produced an attenuated phenotype equivalent to that seen with the biologically selected rapid penetration mutants. The molecularly cloned mutant-infected mice also survived a challenge with 5×10^4 PFU of virulent V3000. Combined with the data from the penetration rate assay, these results identify the missense mutations found in E2 as determinants of both BHK cell penetration rate and adult mouse virulence phenotypes.

The molecularly cloned mutant viruses gave distinctive plaque sizes on both vero cells and BHK cells. The mutation at E2 position 76 and the double change at E2 positions 3 and 4 each produced a small plaque phenotype on both cell types, while the mutations at E2 120 and E2 209 reduced the plaque size on Vero cells but gave significantly larger than wild type plaque size on BHK cells. All of the molecularly cloned mutants produced yields similar to the wild-type parent following transfection of CEF or BHK cell monolayers with comparable amounts of RNA. Therefore, these

TABLE 2

PENETRATION AND ADULT MOUSE VIRULENCE PHENOTYPES OF MOLECULARLY CLONED MUTANT VEE VIRUSES

Recombinant virus strain	E2 mutant locus	Percentage penetrated in 15 min[a]			Percentage mortality in adult mice[b]
		Exp 1	Exp 2	Exp 3	
V3000	None	14	17	26	100
V3026	3 (Lys) 4 (Lys)	96	ND[c]	77	0
V3010	76 (Lys)	27	ND	50	0
V3012	120 (Lys)	ND	39	36	0
V3014	209 (Lys)	ND	51	82	0

[a] Penetration rate assayed by escape from inactivation by trypsin as described in Johnston and Smith (1988).

[b] Ten C57BL/6 mice per group, inoculated with 1×10^4 PFU ip., observed for 60 days.
Mean day to death, 10.8.

[c] ND, not determined.

mutants exhibited no significant growth restriction in two types of cultured cells.

E2 mutations differ in their effect on virulence

Changes at four different loci in E2 independently produced a dramatic reduction in virulence for adult mice. Although a self-limiting, immunizing infection was established by all of the mutants, it is possible that different aspects of pathogenesis might be affected by these mutations. Two approaches were taken to compare the relative attenuation achieved by these mutations. In the first approach, three of the molecularly cloned viruses and their parent were used to infect infant mice, because virulence of alphaviruses for young mice has been suggested to be qualitatively different from adult virulence (Russell *et al.*, 1989). In the second approach, the virulence of these constructs was compared in adult mice and hamsters at different doses.

Infant mice in the age groups used invariably undergo a lethal infection when inoculated intraperitoneally with 5 PFU of the unaltered TRD strain of VEE. The results from inoculation of 10^4 PFU of molecularly cloned viruses carrying the E2 Lys 76, E2 Lys 120, or E2 Lys 209 mutations into mice aged 8 or 15 days are shown in Figs. 1A and 1B, respectively.

In the 8-day-old animals, the E2 Lys 120 and E2 Lys 209 mutations were not appreciably attenuating, resulting in survival times only 24 to 48 hr longer than those obtained with the V3000 (parent) or V2000 viruses. However, a 4-day extension in survival was observed with the E2 76 Lys mutation. The relative attenuating effect of these mutations was more evident in the 15-day-old mice, in which infection with the molecularly cloned E2 120 Lys virus resulted in a 2-day increase in survival time relative to the unmutated parent virus, but infection with the molecularly cloned E2 Lys 209 and E2 Lys 76 mutants produced extended survival times and 60 and 90% survivors, respectively. Acquisition of resistance to these mutant viruses, even at high doses, appears to be relatively rapid in mice. In light of these results, the mutations could be ranked in order of increasing attenuation, from E2 Lys 120 to E2 Lys 209 to E2 Lys 76, indicating that the individual mutations result in functionally different viruses with distinct *in vivo* phenotypes.

The differential *in vivo* behavior of these constructs was also apparent from comparative virulence studies carried out in adult C57BL/6 mice and Syrian hamsters. In these studies, animals were inoculated intraperitoneally with 5×10^3 or 5×10^5 PFU and monitored for 30 days. Surviving animals were bled to determine their antibody response and challenged with 5×10^4 PFU of the virulent V3000 parent to assess their level of immunity. The results of these studies are summarized in Table 3.

Infection of either adult mice or hamsters with the Trinidad donkey strain of VEE, even with moderate doses given peripherally, consistently results in lethal infection. However, mean survival times are significantly different as hamsters succumb to the early lymphotropic stage of the disease, whereas mice survive this phase but die 5–7 days later of encephalitis (Table 3; Gleiser *et al.*, 1962). Infection of adult mice with moderate doses (5×10^3 PFU) of molecularly cloned viruses carrying the E2 Lys 76, E2 Lys 120, or E2 Lys 209 mutations resulted in 100% survival. Infection with the molecularly cloned mutant carrying the double change at E2 3 and 4 resulted in 90% survival. These findings are consistent with the results of the previous experiment (Table 2), and show the significant attenuating effect of these mutations. Increasing the challenge dose 100-fold (to 5×10^5) produced an occa-

TABLE 3

VIRULENCE AND IMMUNOGENICITY OF MOLECULARLY CLONED MUTANT VEE VIRUSES CONTAINING SINGLE MUTATIONS

Virus strain	Dose[a]	Adult mice					Hamsters				
		S/T[b]	MDD[c]	AB[d]	GMT[e]	Immune[f]	S/T[b]	MDD[c]	AB[d]	GMT[e]	Immune[f]
V3000	5×10^5	0/10	8.1 (0.99)		-		0/10	2.1 (0.56)			
(wild-type)	5×10^4	0/10	9.2 (0.63)				0/10	2.4 (0.51)			
V3026	5×10^5	9/10	[9]	9/9	10,159	9/9	4/10	3.8 (0.98)	4/4	2,690	4/4
(E2 3, 4)	5×10^4	9/10	[10]	9/9	7,465	9/9	6/10	4.3 (0.95)	6/6	1,425	6/6
V3010	5×10^5	10/10		10/10	4,222	10/10	7/10	4.4 (0.57)	7/7	1,766	7/7
(E2 76)	5×10^4	10/10		10/10	7,879	9/9	7/10	7.3 (4.9)	6/7	2,539	6/7
V3012	5×10^5	9/10	[10]	8/8	11,737	9/9	0/10	3.5 (1.3)			
(E2 120)	5×10^4	10/10		10/10	11,143	9/9	0/10	3.4 (0.51)	-		
V3014	5×10^5	8/10	10.5 [10, 11]	8/8	6,979	7/7	4/10	4.2 (0.40)	4/4	21,526	4/4
(E2 209)	5×10^4	10/10		8/10	3,200	8/10	5/10	4.6 (0.54)	5/5	6,400	5/5
None		10/10		0/10		0/10	10/10		0/10		0/10

[a] Dose, PFU inoculated intraperitoneally.
[b] S/T, number of survivors/number inoculated.
[c] MDD, mean day to death within observation period of 30 days (standard deviation) 3 or more deaths, [days to death] 1–2 deaths.
[d] AB, number of animals whose serum at 1:100 dilution gave an optical density of more than 0.2/number inoculated.
[e] GMT, geometric mean titer in ELISA, where titer was the reciprocal of the highest dilution to give an optical density of 0.2, not including animals with titers of less than 100.
[f] Immune, number of animals surviving for at least 30 days after challenge with 5×10^4 PFU of V3000 inoculated intraperitoneally.

sional death but nonetheless resulted in 80 to 100% survival.

These mutations, however, were not as attenuating in hamsters as they were in mice, as was noted with the original biological mutants (Johnston and Smith, 1988). This differential was most clearly evident with the molecularly cloned E2 Lys 120 virus which showed no attenuation for hamsters, producing survival times essentially equivalent to those of the parent. The remaining three mutants were clearly attenuated in hamsters relative to the unmutated parent virus, giving 40 to 70% survival. Also, as was seen in the mice, increasing the virus dose 100-fold did not appreciably alter the outcome of infection.

In general, surviving animals seroconverted with significant antibody titers and were completely immune to challenge with the virulent parent virus. The only exception was the low dose inoculation of mice with the E2 Lys 209 mutant, which immunized only 80% of the animals.

Increased attenuation results from combining individual mutations

The study of Sindbis virus neurovirulence has shown that a strain engineered to carry multiple attenuating mutations is less virulent than strains which contain any one of the constituent attenuating mutations alone (Polo and Johnston, 1990). These findings and the well-documented experience with revertants of the poliovirus vaccine strains (Almond, 1987), form the rationale for engineering a VEE vaccine strain with multiple, independently attenuating mutations. The possibility of additive or synergistic attenuating phenotypes in VEE was explored. Multiple rounds of *in vitro* mutagenesis were performed on a single template to produce E2 genes which contained two or three of the mutations described in Table 1. These multiply mutated E2 genes were then substituted into the full-length VEE clone and the molecularly cloned virus progeny were tested for virulence in infant mice, adult mice, and hamsters as described above. As the multiple mutants were tested in parallel with the single-site mutants, the same sets of animals infected with V2000 or V3000 that were used as virulent controls in experiments described in Figs. 1A and 1B, and Table 3 served as controls for these experiments.

As was seen with Sindbis virus, the multiple mutants were significantly more attenuated in these models than strains carrying any one of the single mutations (Figs. 1C and 1D, and Table 4). In the infant mice, for example, the addition of E2 Lys 120 to E2 Lys 76 resulted in a molecularly cloned virus producing approximately 60% survivors in the 8-day-old animals, whereas each mutation resulted in 100% mortality when tested individually. The triple mutant (E2 Lys 76, E2 Lys 120, and E2 Lys 209) was completely atten

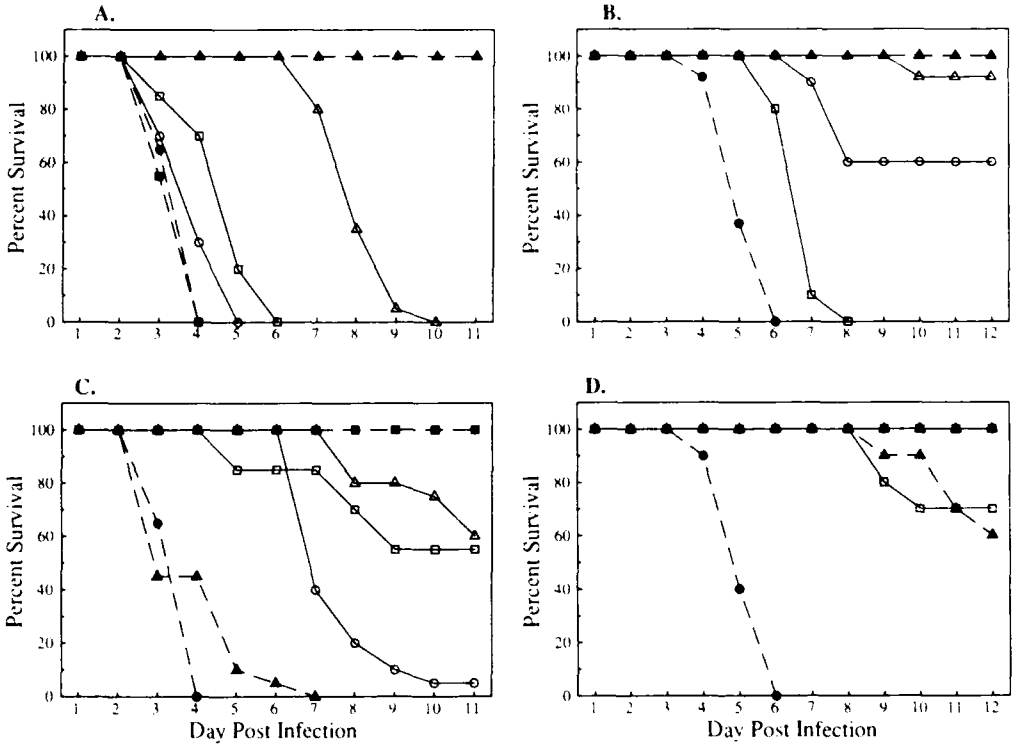

FIG. 1. Survival of infant mice infected with molecularly cloned mutant VEE viruses. Eight day old mice (A and C) or 15-day-old mice (B and D) were inoculated intraperitoneally with VEE mutants (1×10^4 PFU) carrying single (A and B) or multiple mutations (C and D). Symbols for A and B: ●, V3000 (virulent parent); ■, V2000 (virulent wild-type molecularly cloned virus); ▲, negative control (untranscribed plasmid DNA); ○, V3014 (E2 Lys 209); □, V3012 (E2 Lys 120); △, V3010 (E2 Lys76). Symbols for C and D: ●, V3000; ■, negative control; ▲, 120/209; ○, 76/209; □, 76/120; △, 76/120/209. In D, 76/209, 76/120/209, and the negative control all produced 100% survival.

uated in the 15-day-old mice, and produced approximately 60% survivors in the 8-day-old mice. Similarly, the double mutant containing E2 Lys 76 and E2 Lys 209 yielded 100% survival in the 15-day-old mice.

The additive effect in attenuation was also clearly apparent from the data obtained from infection of adult hamsters, where the double mutants containing either E2 Lys 76 plus E2 Lys 209, or E2 Lys 120 plus E2 Lys 209, and the triple mutant were essentially avirulent, inducing self-limited, immunizing infections. The additive effect cannot, however, be predicted *a priori*. The addition of E2 Lys 120 to E2 Lys 209, which individually resulted in 100 and 50% mortality, respectively, produced a virus avirulent for hamsters. The addition of E2 Lys 120 to E2 Lys 76, however, did not increase the attenuation observed over E2 Lys 76 alone.

As would be expected from the results obtained from infections with the individual mutants, the multiple mutants were attenuated in adult mice, resulting in inapparent, immunizing infections. However, the triple mutant, while protecting all of the mice inoculated with the higher dose, failed to immunize 20% of the animals at the lower dose. In hamsters, the lower dose of the triple mutant induced a protective immune response in all the animals inoculated.

Our results demonstrate that these molecularly cloned multiple mutant viruses compare favorably with the existing investigational live, attenuated vaccine, TC-83, which, although clearly attenuated in these animal models, produced 20% mortality in hamsters at a dose of 2×10^4 PFU. In preliminary experiments, the molecularly cloned triple mutant (E2 Lys 76, E2 Lys 120, and E2 Lys 209) proved to be attenuated, immunizing, and protective in guinea pigs and horses (data not shown).

DISCUSSION

In this study, we have used a series of isogenic full-length clones of VEE to show that single nucleotide changes found in the E2 genes of VEE rapid penetration mutants are sufficient to produce both an accelerated penetration rate in BHK cells and attenuation in adult mice. As was first described for Sindbis virus, these mutations presumably define a domain of the VEE glycoprotein spike which controls early interac-

TABLE 4

VIRULENCE AND IMMUNOGENICITY OF MOLECULARLY CLONED MUTANT VEE VIRUSES CONTAINING MULTIPLE MUTATIONS

Virus strain	Dose[a]	Adult mice				Hamsters				
		S/T[b]	AB[d]	GMT[e]	Immune[f]	S/T[b]	MDD[c]	AB[d]	GMT[e]	Immune[f]
76/209	5×10^7					9/10	[6]	9/9	2,962	9/9
	5×10^5	10/10	9/10	8013	10/10	10/10		10/10	3,200	10/10
120/209	5×10^7					10/10		10/10	5,198	10/10
	5×10^5	10/10	10/10	9523	10/10	10/10		9/10	10,159	8/9
76/120	5×10^7					7/10	6.0 (0.0)	7/7	8,613	7/7
	5×10^5	10/10	10/10	7607	10/10	8/10	6.0 [6, 6]	7/8	4,755	7/8
76/120/209	5×10^7	10/10	10/10	1714	10/10	10/10		10/10	1,600	10/10
	5×10^5	10/10	7/10	883	8/10	10/10		10/10	1,392	10/10
TC 83[g]	2×10^4	20/20	18/20	3200	19/20	8/10	5.0 [4, 6]	8/8	4,935	8/8

[a] Dose, PFU inoculated intraperitoneally.

[b] S/T, number of survivors/number inoculated.

[c] MDD, mean day to death within observation period of 30 days (standard deviation) 3 or more deaths, [days to death] 1–2 deaths.

[d] AB, number of animals whose serum diluted 1:100 gave an optical density of more than 0.2/number inoculated.

[e] GMT, geometric mean titer in ELISA, where titer was the reciprocal of the highest dilution to give an optical density of 0.2, not including animals with titers of less than 100.

[f] *Immune, number of animals surviving for at least 30 days after challenge with* 5×10^4 *PFU of V3000 inoculated intraperitoneally.*

[g] TC 83, the existing experimental vaccine strain of VEE.

tions between the virus and cultured cells as well as virulence in animals. Furthermore, we have combined three of these mutations in a single virus to produce a prototype molecularly cloned vaccine for VEE.

The attenuating E2 mutations do not impair the ability of the virus to induce a protective immune response.

A major concern in selection of attenuating mutations to be included in a molecularly cloned vaccine strain is the effect such mutations might have on the immunogenicity of the vaccine. Two properties of the virus must be maintained: its ability to replicate to some extent in the host, and its antigenic structure. With respect to replication, the attenuating mutations characterized in this report allow undiminished growth in tissue culture, and sufficient growth in the animal host to produce an immune response which renders the animal refractory to a high level challenge with virulent virus. Therefore, these mutations do not reduce virulence by severely restricting the ability of the virus to replicate, but act specifically to attenuate the ability of the virus to produce disease. This characteristic is important not only for mutations to be included in a molecularly cloned virus vaccine, but also for mutants to be used in the study of VEE pathogenesis.

With respect to antigenic structure, the locus at E2 209 falls within a region of the glycoprotein predicted to be an antigenic site (Jameson and Wolf, 1988). This prediction was verified by the observation of Kinney *et al.* (1988) that expression of a recombinant vaccinia virus containing a VEE E2 gene with a substitution of Lys for Glu at E2 209 greatly reduced detection of the $E2^h$ epitope in cells infected with the recombinant virus. Cells infected with the molecularly cloned mutant V3014, described here, which contains a Lys at E2 209, also did not react with an $E2^h$-specific monoclonal antibody (J. Roehrig, personal communication). In addition, a neutralization escape mutant selected with an $E2^h$-specific monoclonal antibody contained a mutation at E2 residue 207 (Johnson *et al.*, 1990). These observations, taken together, indicate that the change from a Glu to a Lys at E2 209 significantly altered an epitope recognized by a neutralizing monoclonal antibody.

In this report, we show that viruses which carry the E2 Lys 209 mutation are nonetheless able to elicit a highly protective immune response. Two factors may be at work in this case. The presence of neutralizing antibodies specific for other, unaltered epitopes on this mutant virion may be sufficient to provide the immunity which we observed. In addition, antigenic sites other than those identified as neutralization targets on mature VEE virions, including possible targets of cell-mediated immunity, may be important participants in the development of a protective immune response. Previous work with Sindbis virus demonstrated that passive immunization of mice with nonneutralizing monoclonal antibodies was protective (Schmaljohn *et al.*, 1983). For these reasons, attenuating mutations which alter a single neutralization site may neverthe-

less be considered for inclusion in a molecularly cloned vaccine.

Changes in the antigenic structure of these attenuated mutants may also affect the development of cross-protective immunity against VEE strains of subtypes other than IA, the subtype of the Trinidad donkey strain. Mice inoculated with molecularly cloned single mutants carrying the E2 Lys 76, E2 Lys 120, or the E2 Lys 209 mutation, or with the molecularly cloned triple mutant carrying all three changes, were challenged with the 71-180 strain of VEE, subtype IB, and were completely protected against disease (J. Smith, N. Powell, L. Willis, G. Greenwald, N. Davis, and R. Johnston, unpublished results). Further testing will show whether cross-protective immunity is produced against other VEE subtypes.

Different attenuating E2 mutations lead to different effects on pathogenesis

We intend to use the isogenic molecularly cloned strains described above to study VEE pathogenesis. Ultimately, viral pathogenesis is a result of successive infections of various target cells that progress from the site of entry to permissive cells of specific organ systems. In this way, the characteristic pattern of disease is produced. Distinct stages have been defined in VEE pathogenesis, including an early involvement of lymphoid and myeloid cells, and, in animal models which survive this phase, eventual invasion of the central nervous system. In theory, the defects caused by different attenuating mutations could interrupt the disease progression at different stages. Alternatively, different attenuating mutations could result in qualitatively distinct patterns of disease, by causing, for example, a significant change in tissue tropism.

We have presented two lines of evidence suggesting that the attenuated mutants of VEE described here can be distinguished on the basis of their pathogenesis. The first line of evidence is that infant mice develop resistance to different mutants at different ages. Age-related resistance in Sindbis infection of the mouse has been well documented (Reinarz *et al.*, 1971; Johnson *et al.*, 1972), although the basis for the reduced susceptibility of older animals is not clearly understood. However, studies with attenuated mutants of the adult neurovirulent S.A.AR 86 strain of Sindbis indicate that neonatal and adult virulence are qualitatively different (Russell *et al.*, 1989). Mice of different ages, in a sense, are distinct models for the study of viral pathogenesis, and mutants which are phenotypically indistinguishable in the adult mouse may show different degrees of virulence in younger mice. In the 1-week-old mouse model the mutation from Glu to Lys at E2 76 was more attenuating than any other mutation tested. Experiments in 2-week-old mice showed that the mutation from Glu to Lys at E2 209 was more attenuating than the change from Thr to Lys at E2 120. These results suggest that these mutations produce different *in vivo* phenotypes.

We have also examined virulence in different adult animal models. Hamsters die during the initial lymphotropic/myelotropic stage of VEE infection, prior to the point where central nervous system involvement is clinically apparent. Virulence for these animals, therefore, is a sensitive test of the ability to carry out this first stage of pathogenesis. The individual mutations differ in their virulence for the hamster, with the mutation at E2 position 76 being the most attenuated. This observation is consistent with the hypothesis that the defect caused by the mutation at E2 residue 76 may be expressed during the early lymphotropic stage of the disease.

A third type of analysis also indicates that different attenuating mutations block infection at different stages. These experiments involve comparisons of virulence following intracerebral and subcutaneous inoculations. Preliminary results in adult mice infected with molecularly cloned VEE mutants indicate that V3010 and V3014, which contain mutations at E2 positions 76 and 209, respectively, are much more virulent when inoculated intracerebrally than when inoculated into the footpad (F. Grieder and R. Johnston, unpublished data). This pattern is reminiscent of other alphavirus mutations which block disease progression at or before invasion of the brain (Polo and Johnston, 1990).

The mutation at E2 position 120 appears to be the least attenuating mutation in all of the animal models studied, although it did show an additive attenuating effect in hamsters when combined with the mutation at E2 209. E2 position 120 is the site of one of the seven amino acid changes between the Trinidad donkey strain of VEE and the live, attenuated vaccine strain derived from it, TC-83 (Kinney *et al.*, 1989). TC-83 has an arginine at E2 120, while the causal mutation in the rapid penetration, attenuated mutant described here is a lysine. The biochemical similarity of the Lys and the TC-83 Arg substitutions at E2 120 suggests that E2 Arg 120 contributes to the attenuated phenotype of TC-83.

All of the attenuating mutations characterized in this report result in the substitution of a Lys for the wild-type amino acid. In addition, we have preliminary evidence for an Arg substitution at a fifth attenuating locus, and, as described above, TC-83 E2 Arg 120 may be an attenuating mutation. Both Arg and Lys, large, highly charged residues, are nonconservative replacements for any other amino acid and might be expected

to result in a phenotypic change because of this characteristic alone. Conceivably, the use of trypsin in the original selection for rapid penetration led to this result. However, in the Sindbis system, the rapid penetration phenotype is not linked to Lys or Arg substitution. A third possibility is that an increase in the net positive charge of the virion surface may facilitate early interactions with the negatively charged cell surface, and thereby contribute to a rapid penetration phenotype.

Strains with multiple attenuating mutations are more attenuated than single mutants

Experience with live virus vaccines has shown that virus strains with multiple attenuating mutations are unlikely to be involved in cases of vaccine related illness, while those with only a few mutations appear to revert readily toward wild-type virulence (Almond, 1987). Data presented here demonstrate an additive or synergistic effect of combining individual attenuating mutations in the E2 gene of VEE. Infection with double mutants and the triple mutant resulted in significantly lower mortality and longer survival times, both in 1-week-old mice and in the hamster, than did infection with the single mutants. The attenuation observed with the multiple mutants as well as with the single mutants was comparable at doses of 5×10^3 and 5×10^5 PFU, suggesting that a wide range of doses can be explored for effective immunization. The multiple mutants generally gave smaller plaque sizes and often replicated to lower titers following transfection than the single mutants. However, they grew to sufficient levels in the animal to produce a fully protective immune response. Further study is required to show whether the increased attenuation of the multiple mutants stems from a lower rate of reversion, since more than one locus must revert to give a virulent phenotype, or from the additive effect of the multiple attenuating mutations.

The results presented here are important for three reasons. First, combining three of these mutations in a single genotype produced a highly attenuated virus which induced a protective immune response in sensitive animal models. An improved live, attenuated vaccine for VEE based on this approach would contain defined mutations that could be assayed at each step of production and also serve as genetic markers to identify it in the field. Second, the isogenic full-length clones of VEE with specifically attenuating mutations in E2 will be invaluable tools in further investigations of VEE pathogenesis. Finally, the approach that has been used to identify and combine attenuating, rapid penetration mutations of VEE and Sindbis could be applied to other alphaviruses for which a live, attenuated vaccine strain is needed.

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