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13. ABSTRACT *(Maximum 200 words)*

We have constructed a full-length cDNA clone of the virulent Trinidad Donkey strain of Venezuelan equine encephalitis virus, from which infectious RNA genomes can be transcribed *in vitro*, as the basis for development of a recombinant live attenuated vaccine strain for VEE. We are using recombinant DNA techniques to produce a low reversion live virus vaccine by combining multiple independently attenuating mutations in a single virus genome. Four triple mutants were avirulent in both CD-1 and C57Bl/6 mice. One of these mutants induced complete protection in C57Bl/6 mice against intraperitoneal (ip.) or aerosol challenge with the virulent parent virus, while all four mutants induced complete protection in CD-1 mice against ip. challenge. PE2 cleavage defective mutants were avirulent in C57Bl/6 mice inoculated subcutaneously (sc.) and in CD-1 mice inoculated sc., intracranially or intranasally (in.), and produced a solid immunity to ip. or in. challenge (CD-1 mice). The growth of these mutants is restricted in cultured mosquito cells. Collaborators at USAMRIID are testing the most promising attenuated mutants in monkeys.

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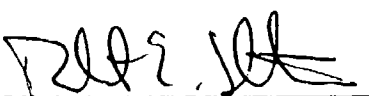

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INTRODUCTION

We have constructed a full-length cDNA clone of the virulent Trinidad Donkey strain (TRD) of Venezuelan equine encephalitis virus (VEE) as the basis for development of a recombinant live attenuated vaccine strain for VEE (Davis, et al., 1989). Infectious RNA genomes are transcribed from a T7 promoter positioned immediately upstream from the virus sequence, allowing changes in the DNA sequence to be expressed phenotypically in virus progeny. Our approach to vaccine development stems from previous studies showing that strains with multiple independently attenuating mutations are less likely to give clinical symptoms in vaccinees than strains with only one or two attenuating mutations (Almond, 1987). One possible explanation for this is that the probability of multiple reversion events at all of the mutated sites in a single virus genome is very low. The side effects associated with the current investigational vaccine for VEE, TC-83 (Edelman, 1986), may be the result of reversion of a small number of attenuating changes.

Previously, we analyzed biologically selected strains to identify a panel of single site mutations that significantly reduce virulence. These mutations were introduced into the full-length clone individually to prove that they were responsible for the attenuated phenotype (Davis et al., 1991). The work described in this report, completed between 7-1-93 and 6-30-94, was directed toward 1) increasing the number of candidate attenuating mutations by saturation mutagenesis of known attenuating loci, 2) the study of mutations that prevent proteolytic processing of PE2 and allow PE2 to be incorporated in place of mature E2 into viable, attenuated progeny virus, 3) construction of cDNA clones with various combinations of three attenuating mutations and testing for viability, attenuation and immunogenicity, and 4) preliminary studies of safety and efficacy of intranasal immunization with molecularly cloned multiple mutants.

I. Saturation mutagenesis of attenuating loci in the E2 glycoprotein

We are continuing saturation mutagenesis of the attenuating loci at E2 position 76 and E2 position 209, with the goal of comparing viability and level of attenuation afforded by all the possible amino acid substitutions at these two sites. These results will form the basis for choosing one site with a majority of lethal or attenuating substitutions, for which a double mutant attenuating codon can be designed. This strategy may become very important in light of the possible overattenuation of triple mutants (see below) by allowing changes at only two codons to give the theoretical reversion rate of a quadruple mutant. Eleven amino acid substitutions at E2 76 were made using the Kunkel mutagenesis procedure (Kunkel, 1985) in an M13 subclone of the VEE structural genes and, using the glycoprotein gene shuttle vector, eight of these mutations were placed into the full-length virulent VEE clone, V3000 (**Table 1**). Viability of RNA transcripts was assayed by transfecting BHK cell monolayers with ^{35}S-UTP-labeled RNA using Lipofectin (BRL), overlaying the monolayers with agarose and calculating specific infectivity [plaque forming units (pfu) per ^{35}S cpm incorporated] for mutant RNA relative to that of a viable control RNA. Three of the mutations tested were lethal. Six amino acid substitutions made at E2 position 209 and their effect on viability are also shown (**Table 1**).

It appears, from these partial results, that the E2 209 site may be more flexible, in that only two amino acid substitutions, *met* and *cys*, which are very similar chemically, gave a nonviable phenotype. At the E2 76 locus, however, three of the nine amino acids tested to date gave nonviable RNA genomes, and these residues represent very different chemical types. In addition, we showed previously that a single amino acid deletion at E2 codon 76 is also lethal. However, the final choice of site will rest not only on the proportion of lethal changes, but also on the proportion of attenuating changes, which remains to be determined.

Complete sequence analysis of the mutagenized regions and virulence testing of these mutants in rodents remains to be done, as well as production of the remaining amino acid substitutions at these sites. It will be of interest to determine the effect of different amino acid substitutions at E2 codon 209 on the neutralization epitope that includes this locus. Work by others (Kinney et al., 1988 ; Johnson et al., 1990) and unpublished results obtained with V3014 (a double mutant containing E2 lys 209 and E1 thr 272, J.T. Roehrig, personal communication) indicate that a phe (in place of the parental ile) at E2 position 207 or a lys (in place of the parental glu) at E2 209 eliminate binding of an $E2^b$-specific monoclonal antibody. Mutants containing the E2 lys 209 mutation are still able to elicit a protective immune response in mice and hamsters (Davis et al., 1991).

Table 1
Amino acid changes made at E2 positions 76 and 209

Viability of *in vitro* RNA transcripts from full-length cDNA clones containing single site mutants in the VEE E2 glycoprotein			
E2 amino acid 76[a]	Viability	E2 amino acid 209	Viability
glu (V3000)	+	thr (V3000)	+
ile	+	tyr	+
phe	-	pro	+
thr	-	thr	+
cys	ND[b]	cys	-
val	+	leu	+
leu	+	met	-
arg	+	lys(V3032)	+
pro	-		
lys(V3010)	+		

[a]ser, gln and tyr mutations were made in the M13 subclone and will be transferred into the full-length clone.
bNot determined.

II. Characterization of PE2 cleavage defective mutants

A. Construction of cDNA clones, determination of sequence, viability and PE2 cleavage phenotypes done previously

We identified two glycoprotein mutations that resuscitate lethal mutations at the PE2 cleavage site, E2 gln 243 and E1 ser 253. We tested the E1 mutation for its ability to resuscitate two types of cleavage site mutations. The change from *phe* to *ser* at E1 253 was placed into three different full-length cDNA clones, the virulent V3000 parent (V3040), a clone containing a deletion of the PE2 cleavage signal (E3 56-59, V3526), and a clone containing a change from *arg* to *asp* at E3 59, immediately upstream of the PE2 cleavage site (V3528) (Table 2). All sequences subjected to mutagenesis were confirmed by direct RNA sequencing of *in vitro* RNA transcripts. Specific infectivity measurements of RNA transcripts showed that although the two cleavage site mutations were lethal, clones containing E1 *ser* 253 in addition to these cleavage site mutations produced viable RNA transcripts. In addition, the E1 *ser* 253 mutation in the V3000 background (V3040) gave viable transcripts. SDS-polyacrylamide gel analysis of ^{35}S methionine-labeled virion proteins showed that the resuscitated viruses incorporated PE2 into their virus particles in place of mature E2. For V3040, the cleavage of PE2 appeared to be normal.

B. Growth in cultured cells

Two cell lines, BHK cells and C6/36 mosquito cells, were infected with the viruses shown in Table 2. The multiplicity of infection was 1-2, and the culture supernatants were sampled at 6 hr intervals. Virus titers represent plaque forming units as determined on BHK cell monolayers (Figure 1). V3526, V3528 and V3040 were indistinguishable from V3000 with

Table 2
PE2 cleavage defective mutants

PE2 cleavage site mutants - viability and virion protein composition				
virus strain	sequence at PE2 cleavage site	second-site mutation	viability of RNA transcript	E2/PE2 in the virion
V3000 parent	RKRR/S	none	+	E2
V3022	----/S	none	-	ND[a]
V3038	RKRD/S	none	-	ND[a]
V3526	----/S	E1 ser 253	+	PE2
V3528	RKRD/S	E1 ser 253	+	PE2
V3040	RKRR/S	E1 ser 253	+	E2

[a] Noninfectious virus particles are produced. Their polypeptide compositions have not been determined.

respect to growth on BHK cells, reaching titers of 10^9 pfu/ml within 24 hours after infection. However, on C6/36 cells, the two resuscitated mutants grew to lower titers than V3000 and V3040, both of which reached 10^9 pfu/ml or greater by 34 hours post-infection. V3526, with a deleted PE2 cleavage signal, gave the lowest titer on C6/36 cells, only 10^6 pfu/ml by 34 hr pi., while V3528, with an *asp* at E3 59, reached a titer of 10^8 pfu/ml. This restricted growth in a mosquito cell line may correlate with inability to grow in or be transmitted by the insect vector, a phenotype which would reduce spread of a recombinant virus as well as decrease the opportunity for reversion during persistent infection of the mosquito.

C. Assay for virion production by PE2 cleavage defective mutants

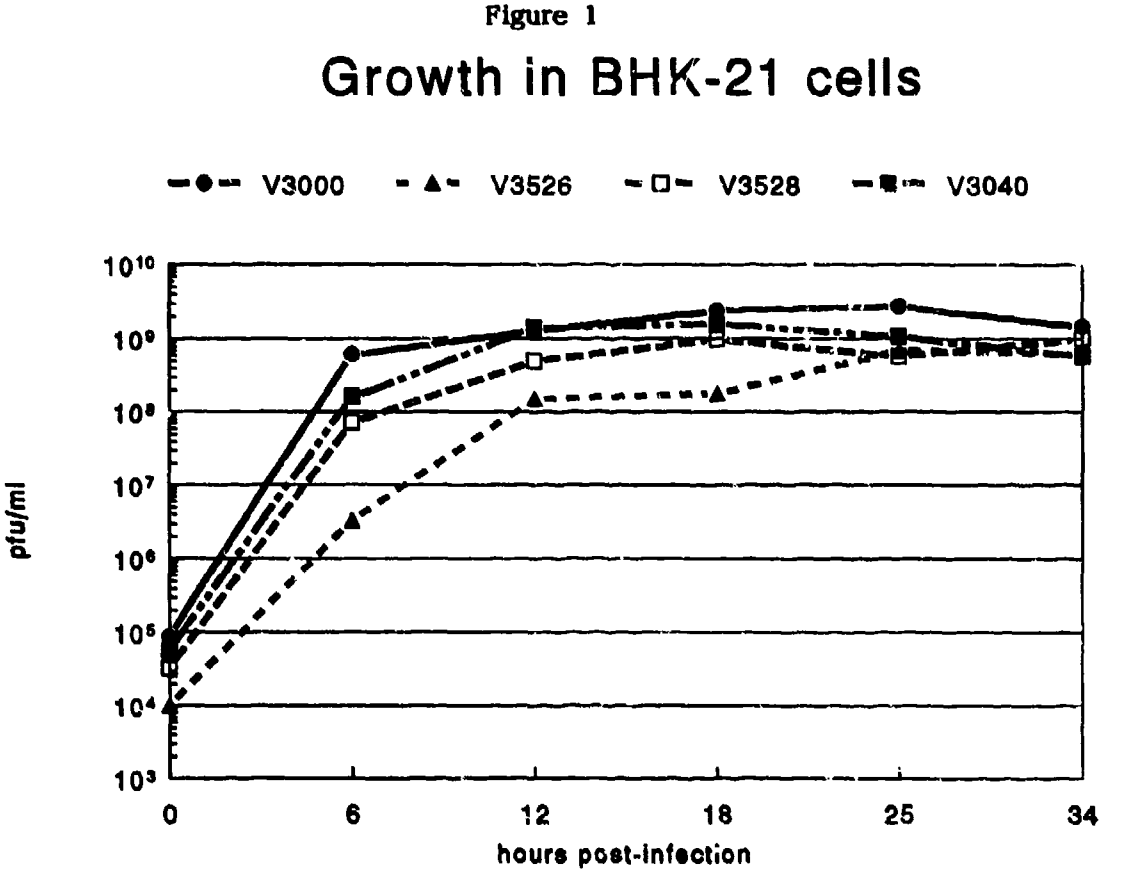
To determine whether noninfectious particles were produced by nonviable PE2 cleavage defective mutants carrying either a deletion of the cleavage signal (V3022) or E3 asp 59 (V3038), we transfected BHK cells with *in vitro* RNA transcripts by electroporation, added ^{35}S-methionine to the medium, and attempted to isolate virions by banding in potassium tartrate gradients. The distribution of ^{35}S methionine label in the gradients indicated that particles were

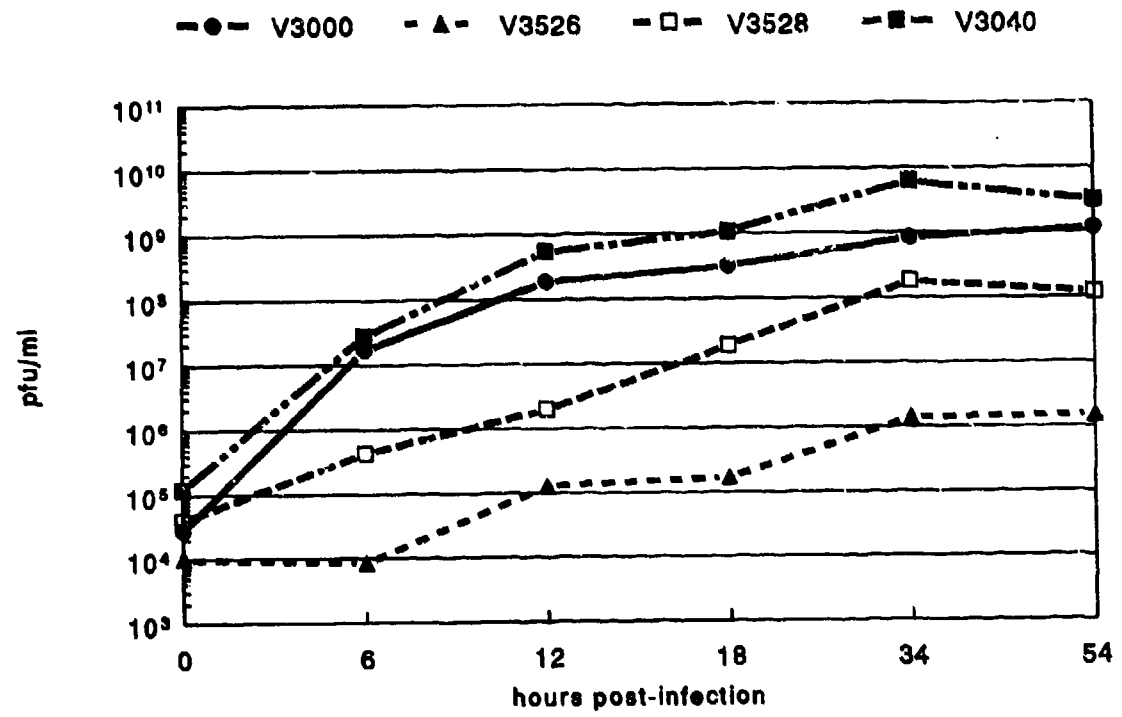
present, but that they were unstable (data not shown). Results obtained using sucrose gradients rather than potassium tartrate gradients suggested that approximately normal amounts of virions were made, that these particles were noninfectious on BHK cells, and that the proportion of viable revertants was very low for both mutants (data not shown). Therefore, these mutants are normal in their ability to assemble and release particles, like PE2 cleavage mutants of the prototype alphavirus, Sindbis virus. However, the mutant particles are not as stable as V3000 particles in high salt. Virus particles produced by the resuscitated mutants (V3526 and V3528), which also incorporate PE2 into their virions, will be tested for their stability in potassium tartrate and their specific infectivity (pfu on BHK cells per ^{35}S-met cpm) relative to V3000 virions. The low level of viable revertants arising from the PE2 cleavage mutants coupled with the fact that the resuscitating E1 ser 253 mutation by itself is attenuated in mice (see below) suggests that the rate of reversion to a virulent phenotype would be very low for these double mutants.

D. Genetic stability during growth in BHK cells

V3526, the resuscitated mutant with a deleted cleavage signal and E1 ser 253, made very small irregularly shaped plaques on BHK cells. By six hours after V3526 infection of BHK cells at a multiplicity of one, variants giving larger plaques began to appear. The proportion of larger plaques eventually reached about 30% of the total pfu. It is possible that these variants carry an additional mutation(s) which allow improved growth in BHK cells while maintaining the attenuated phenotype of the original mutant. We isolated large and small plaque variants from culture supernatant taken at 25 hours post-infection. Three of these isolates, one with the parental plaque type, one with a small, round plaque morphology and one with a large plaque type, were amplified by two passes on BHK cells during which their plaque phenotypes remained constant and homogeneous. They will be tested in mice by our collaborators at USAMRIID.

Figure 1


Growth in BHK-21 cells
V3000
V3526
V3528
V3040
pfu/ml
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
0
6
12
18
25
34
hours post-infection


Growth in C6/36 cells
V3000
V3526
V3528
V3040
pfu/ml
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
0
6
12
18
34
54
hours post-infection

These results indicate that V3526 is genetically unstable and that only early harvest electroporation stocks with uniform plaque morphology should be used in experiments with this mutant. Further results will indicate whether a more stable attenuated mutant was isolated following growth in BHK cells. The putative causal mutation then could be identified by sequence analysis, added to the V3526 clone and tested for attenuation and stability.

E. Attenuation in C57Bl/6 mice

Finally, these mutants have been tested for virulence and protection in C57Bl/6 mice by our collaborators at Ft. Detrick. Both of the resuscitated PE2 cleavage mutants and V3040 were avirulent at a dose of 10^4 to 10^5 pfu, and gave complete protection from aerosol or intraperitoneal (ip.) challenge with virulent V3000 (**Table 4**).

III. Testing of multiple mutants for attenuation and protection in animal models

A. Glycoprotein gene mutations

Our strategy for construction of a low reversion live virus vaccine for VEE calls for combining multiple independently attenuating mutations in a single virus genome. Results to date suggest that three mutations will be sufficient. We previously reported the construction and preliminary testing of two triple mutants with two mutations in E2 and one in E1 (V3519: E2 76, E2 209, E1 272 and V3520: E2 76, E2 209, E1 81) Both triple glycoprotein mutants were avirulent when inoculated sc. (left rear footpad) into 5 week-old female CD-1 mice at a dose of 10^3 pfu and gave solid protection against an ip. challenge with 10^4 pfu of V3000 (**Table 3**). V3520 was also avirulent ic. at a dose of 10^3 pfu, while V3519 killed 1 mouse of 6. All of the survivors of ic. infection were also protected against V3000 challenge. V3519 and V3520 have now been tested for virulence and protection in C57Bl/6 mice and hamsters by our collaborators

at Ft. Detrick (**Table 4**). These animals were inoculated sc. (back of the neck) with a dose of 10^4 to 10^5 pfu and challenged with 10^5 pfu of V3000 either ip. or by aerosol. Both V3519 and V3520 were avirulent in C57Bl/6 mice, and V3520 killed only 1/20 hamsters with an extended survival time. However, in this experiment, neither strain gave complete protection of mice or hamsters against ip. or aerosol challenge with virulent V3000, consistent with the fact that neither elicited a serum neutralization titer over 1:36 (80% plaque reduction neutralization titer).

3. Mutations in the glycoprotein genes coupled with changes in the 5'-untranslated region

We constructed two additional triple mutants that contained an attenuating A at nucleotide position 3 of the 5'-untranslated region in combination with either E2 76 and E2 209 (V3522) or E2 209 and E1 272 (V3524). Initial experiments in 5 week-old female CD-1 mice showed that both of these mutants were avirulent when inoculated sc. (left rear footpad) at a dose of 10^3 pfu, and gave complete protection against an ip. challenge with 10^4 pfu of virulent V3000 (**Table 3**). V3522 was also avirulent and protective by the intracerebral route (ic.), while V3524 gave 100% mortality ic. Further studies with these mutants were conducted at Ft. Detrick in C57Bl/6 mice and hamsters (**Table 4**). Both mutants were avirulent in C57Bl/6 mice following sc. inoculation (back of the neck), but only V3524 gave complete protection against aerosol and ip. challenge with V3000. In hamsters, V3522 was avirulent, while V3524 gave 1 death out of 20 animals. Both protected against ip. but not aerosol challenge in hamsters.

It is not clear at this time why triple mutants that afforded complete protection against V3000 challenge in CD-1 mice did not reproducibly protect C57Bl/6 mice. It is possible that the mouse strain, the route of immunization (footpad vs. back of the neck) and/or the amount of V3000 challenge virus influenced the outcome. Nevertheless, these vaccine candidates were fully protective in CD-1 mice under the conditions used.

Table 3
Testing of Attenuated Triple Mutants in CD-1 Mice

Attenuation and Induction of Protective Immunity in Outbred Female CD-1 Mice				
virus strain	attenuating mutations	route of immunization	survival (survivors/total)	challenge[b] (survivors/total)
V3000		ic.	0/3	
		fp.[a]	0/3	
V3519	E2 lys 76 E2 lys 209 E1 thr 272	ic.	5/6	5/5
		fp.	6/6	6/6
V3520	E2 lys 76 E2 lys 209 E1 ile 81	ic.	7/7	6/6
		fp.	6/6	6/6
V3522	E2 lys 76 E2 lys 209 nt3 A	ic.	4/4	4/4
		fp.	4/4	4/4
V3524	E2 lys 209 E1 thr 272 nt3 A	ic.	0/4	
		fp.	4/4	4/4

[a]Inoculations were sc. into the left rear footpad at a dose of 10^3 pfu.
[b]Challenge was ip. at a dose of 10^4 pfu of V3000.

C. PE2 cleavage signal deletion with E1 resuscitator and an additional attenuating mutation

We constructed full-length cDNA clones containing a PE2 cleavage site mutation and E1 ser 253 resuscitator combined with one additional attenuating mutation. Our first attempt was a multiple mutant containing the PE2 cleavage site deletion, the resuscitator at E1 253 and the attenuating *lys* at E2 209. Multiple independent isolates of this clone failed to give infectious RNA transcripts, indicating that this combination is lethal. In subsequent constructs we added an attenuating change from the parental G to A at nucleotide 3 of the 5'-untranslated region, or the

Table 4
Testing of Molecularly Cloned Mutants in C57Bl/6 Mice and Hamsters

Attenuation and Induction of Protective Immunity in Rodents[a]							
virus strain	attenuating mutations	C57Bl/6 mice			Hamsters		
		immunization[b]	challenge		immunization[b]	challenge	
			ip.	aerosol		ip.	aerosol
V3000		2/10		2/2	0/10		
V3010	E2 lys 76	10/10		7/10	7/10		6/7
V3032	E2 lys 209	10/10		10/10	2/10		1/1
V3034	E2 thr 272	10/10		10/10	2/10		2/2
V3042	E1 ile 81	10/10		10/10	2/10		1/1
V3040	E1 ser 253	10/10		10/10	0/10		
V3519	E2 lys 76 E2 lys 209 E1 thr 272	20/20	6/10	2/10	20/20	4/10	0/10
V3520	E2 lys 76 E2 lys 209 E1 ile 81	20/20	3/9	9/10	19/20	4/7	3/10
V3522	E2 lys 76 E2 lys 209 nt3 A	20/20	5/10	3/10	20/20	9/9	7/10
V3524	E2 lys 209 E1 thr 272 nt3 A	20/20	10/10	10/10	19/20	9/9	9/10
V3526	RKRR del E1 ser 253	20/20	10/10	10/10			
V3528	E3 asp 59 E1 ser 253	20/20	10/10	10/10			
saline		20/20	0/10	0/10	20/20	0/10	0/10

[a]Survivors/total
[b]Inoculations were sc. into the back of the neck at a dose of 10^4 pfu to 10^5 pfu.

attenuating change E1 ile 81 to the PE2 cleavage site deletion and E1 ser 253 resuscitator. *In vitro* RNA transcripts from these clones were viable. The mutant containing the nt3 A mutation produced nearly normal sized plaques on BHK cells, while the mutant with the E1 ile 81 change produced extremely small plaques. The sequence of the region of E1 that was mutagenized to

insert the E1 ile 81 mutation will be checked by direct RNA sequencing techniques, and progeny virus will be tested for growth in BHK cells, attenuation in mice and ability to induce a protective immune response.

IV. Protection against intranasal challenge with virulent V3000

We showed previously that subcutaneous inoculation with 10^3 pfu of the molecularly cloned attenuated double mutant V3014 (E2 lys 209, E1 thr 272) afforded complete protection against intranasal (in.) challenge with 10^4 pfu of V3000 (Annual report, Sept. 1993). In a subsequent experiment we followed the spread of the intranasal V3000 challenge virus in the V3014-immunized mouse. Mice were inoculated in the footpad with 10^3 pfu of V3014 and three weeks later were challenged with either 10^3 pfu of V3000 sc. or 2×10^3 pfu of V3000 in. Animals were sacrificed at various times after challenge for virus titration of the footpad, draining popliteal lymph node, spleen, olfactory neuroepithelium, olfactory bulb, brain and of the serum. No infectious virus was recovered from any of these sites in immunized mice at any time from 6 hr to 24 hr post-challenge. (Level of detection was 6 pfu/gm in brain and serum, and 33 pfu/gm in other tissues.) Since these animals had high ELISA titers of VEE-specific serum antibody, it is possible that low titers of progeny virus were masked in this experiment. Therefore, we will repeat this experiment using *in situ* hybridization analysis of the head as a more sensitive assay for viral replication. Based on the virus titration results, however, it appears possible that the immunity induced by infection with the attenuated mutation V3014 greatly decreases replication of virulent challenge virus introduced either in. or sc., even at primary sites.

We extended this investigation to examine protection induced against in. challenge by intranasal immunization (**Table 5**). It appeared likely that if an attenuated virus could be introduced safely onto a mucosal surface, it could very efficiently induce a mucosal immune

response. The resuscitated PE2 cleavage signal deletion mutant, V3526, was avirulent in CD-1 mice inoculated in. with either 10^4 or 10^5 pfu. Subcutaneous and ic. inoculations were done as controls, and these routes also gave 0% mortality, except for the high dose ic. which gave one death in six mice. When these mice were challenged in. with 10^4 pfu of virulent V3000, they all survived with no signs of VEE-induced disease. Unimmunized control mice all succumbed to V3000 challenge with an average survival time of 7.25 days. Another attenuated mutant, V3014, gave 33% mortality (2/6) following in. inoculation with 10^3 pfu, and 2/4 immunized mice succumbed to in. challenge with V3000 on day 8 post-challenge. Therefore, V3526 appears to be highly attenuated by the in. route, but is still able to establish an immunizing infection, while V3014 does not efficiently infect by the in. route, and when it does infect is likely to produce disease. Comparisons of the in. and sc. routes with additional multiple mutants followed by in. and/or aerosol challenge will give information concerning the preferred virus genotype and route for induction of mucosal immunity.

Serum and saliva samples were collected from some of the mice inoculated in. with V3526 three weeks post-inoculation and tested for VEE-specific IgG (sera) or IgA (sera and saliva). Serum IgG ELISA titers ranged from 1000 to 4000 (reciprocal of dilution required to give $O.D._{450} > 0.2$, measured against gradient purified V3000). All but one of the mice tested had high serum IgA titers (2000-4000), but no VEE-specific IgA could be detected in the saliva. Therefore, although these mice were all resistant to a high dose in. challenge with V3000, and most had high levels of circulatory IgA prior to challenge, the levels of secretory IgA in saliva were below our level of detection.

Table 5
Immunization by Intranasal Inoculation

Inoculation with V3526 (RKRR del + E1 ser 253) and in. challenge with V3000			
immunization route	immunization dose (pfu)	deaths/immunization	deaths/challenge
intranasal	10^4	0/6	0/6
	10^5	0/6	0/6
intracranial	10^3	0/6	0/6
	10^5	1/6	0/5
subcutaneous (footpad)	10^5	0/6	0/6

CONCLUSIONS

Over the past two years, we have demonstrated the feasibility of combining three attenuating mutations in different regions of the genome to produce stable, highly attenuated vaccine candidates. Four triple mutant cDNA clones, each containing a distinct constellation of mutations, were constructed and used to produce virus stocks with usable titers following electroporation of BHK cells. The constituent mutations were chosen such that more than one step in the virus life cycle would be affected to lower the probability that a single reversion event could produce a virulent virus. In CD-1 and C57Bl/6 mice, these triple mutants were avirulent. One triple mutant induced complete protection in C57Bl/6 mice. In hamsters, all of the triple mutants were significantly more attenuated than TC-83 (two mutants were avirulent and two gave 5% mortality), but none gave complete protection against virulent virus challenge. However, all of the triple mutants were completely protective in the CD-1 mouse/V3000 challenge model. Further direct comparisons are needed to determine the factors (such as route of immunization, mouse strain and challenge dose) which contributed to the inconsistency of the results in mice.

The most promising of these triple mutants is being tested in monkeys by our collaborators at USAMRIID.

Alteration of the PE2 cleavage site leads to attenuation of virulence in Semliki forest virus, two strains of Sindbis virus and in VEE (Russell et al., 1989; Glasgow et al., 1991; Heidner et al., 1994; and Davis et al., 1994). The VEE PE2 cleavage defective mutants behave similarly to the Sindbis mutants with respect to their growth in BHK cells and restricted growth in C6/36 mosquito cells. Like the Sindbis mutants, the VEE cleavage defective mutants produce noninfectious particles, but unlike the prototype, these particles appear to be unstable in high salt. Two resuscitated cleavage site mutants, one with a 4 amino acid deletion of the cleavage signal (RKRR) and one with a lethal amino acid substitution in the cleavage signal that requires two nucleotide changes to give either a lys or arg, are avirulent in both CD-1 and C57Bl/6 mice by the subcutaneous route. The resuscitated deletion mutant is also avirulent following intranasal and intracranial inoculation of CD-1 mice. Protective immunity was demonstrable in all of these cases. These results mark these mutations as excellent candidates for inclusion in a vaccine, and they will be tested further in combination with an additional attenuating change to further reduce the theoretical probability of reversion.

Finally, protection against challenge across a mucosal surface has been tested both by aerosol and by intranasal administration of virulent V3000. This is an important attribute of a VEE vaccine, since the virus is infectious by this route. The results were mixed for the triple mutants, but a strong protective response was raised with both a double mutant (V3014) and the cleavage defective mutants in mice. It remains to be seen whether this result can be extended to other animal models.

LITERATURE CITED

Almond, J.W. 1987. The attenuation of poliovirus neurovirulence. Ann. Rev. Microbiol. 41: 153-180.

Davis, N.L., L.V. Willis, J.F. Smith and R.E. Johnston. 1989. *In vitro* synthesis of infectious Venezuelan equine encephalitis virus RNA from a cDNA clone: Analysis of a viable deletion mutant. Virology 171: 189-204.

Davis, N.L., N. Powell, G.F. Greenwald, L.V. Willis, B.J.B. Johnson, J.F. Smith and R.E. Johnston. 1991. Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: Construction of single and multiple mutants in a full-length cDNA clone. Virology 183: 20-31.

Davis, N.L., F.B. Grieder, J.F. Smith, G.F. Greenwald, M.L. Valenski, D.C. Sellon, P.C. Charles and R.E. Johnston. 1994. A molecular genetic approach to the study of Venezuelan equine encephalitis virus pathogenesis. Arch. Virol. [Suppl.] 9: 99-109.

Edelman, G. 1986. Early Ft. Detrick experience with TC-83. Conference on "Venezuelan Equine Encephalitis Vaccines in Man," at U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Frederick, MD, 12/16/86 - 12/17/86.

Glasgow, G.M., B.J. Sheahan, G.J. Atkins, J.M. Wahlberg, A. Salminen and P. Liljeström. 1991. Two mutations in the envelope glycoprotein E2 of Semliki Forest virus affecting the maturation and entry patterns of the virus alter pathogenicity for mice. Virology 185: 741-748.

Heidner, H.W., K.L. McKnight, N.L. Davis and R.E. Johnston. 1994. Lethality of PE2 incorporation in Sindbis virus can be suppressed by second-site mutations in E3 and E2. J. Virol.. 68: 2683-2692.

Johnson, B.J.B., J.R. Brubaker, J.T. Roehrig and D.W. Trent. 1990. Variants of Venezuelan equine encephalitis virus that resist neutralization define a domain of the E2 glycoprotein. Virology 177: 676-683.

Kinney, R.M., J.J. Esposito, B.J.B. Johnson, J.T. Roehrig, J.H. Mathews, A.D.T. Barrett and D.W. Trent. 1988. Recombinant vaccinia-Venezuelan equine encephalitis (VEE) virus expresses VEE structural proteins. J. Gen. Virol. 69: 3005-3013.

Kunkel, T.A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82: 488-492.

Russell, D.L., J.M. Dalrymple and R.E. Johnston. 1989. Sindbis virus mutations which coordinately affect glycoprotein processing, penetration, and virulence in mice. J. Virol. 63: 1619-1629.