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A Single-Site Mutant and Revertants Arising *in Vivo* Define Early Steps in the Pathogenesis of Venezuelan Equine Encephalitis Virus

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The early stages of Venezuelan equine encephalitis virus (VEE) pathogenesis in the mouse model have been examined using a genetic approach. Disease progression of a molecularly cloned single-site mutant was compared with that of the parental virus to determine the step in the VEE pathogenetic sequence at which the mutant was blocked. Assuming that such a block constitutes a genetic screen, isolates from different tissues thought to be distal to the block in the VEE pathogenetic sequence were analyzed to determine the pathogenetic step at which revertants of the mutant were selected. Directed mutation and analysis of reversion *in vivo* provide two powerful genetic tools for the dissection of the wild-type VEE pathogenetic sequence. Virus from the parental virulent clone, V3000, first replicated in the draining lymph node after subcutaneous inoculation in the left rear footpad. Movement of a cloned avirulent mutant, V3010 (E2 76 Glu to Lys), to the draining lymph node was impaired, replication in the node was delayed, and spread beyond the draining lymph node was sporadic. Serum, contralateral lymph node, spleen, and brain isolates from V3010 inoculated animals were invariably revertant with respect to sequence at E2 76 and/or virulence in mice. Revertants isolated from serum and contralateral lymph node retained the V3010 E2 Lys 76 mutation but also contained a second-site mutation, Glu to Lys at E2 116. Modification of the V3010 clone by addition of the second-site mutation at E2 116 produced a virus that bypassed the V3010 block at the draining lymph node but that did not possess full wild-type capacity for replication in the central nervous system or for induction of mortality. A control construct containing only the E2 116 reverting mutation on the V3000 background was identical to V3000 in terms of early pathogenetic steps and virulence. Therefore, analysis of mutant replication and reversion *in vivo* suggested (1) that the earliest steps in VEE pathogenesis are transit to the draining lymph node and replication at that site, (2) that the mutation in V3010 impairs transit to the draining lymph node and blocks dissemination to other tissues, and (3) that reversion can overcome the block without restoring full virulence.

INTRODUCTION

Classical histopathogenesis studies have laid the groundwork for current understanding of several human and experimental viral infections, including those caused by alphaviruses (Fenner, 1949; Nathanson, 1980; Mims *et al.,* 1995; Roberts, 1962; Johnson, 1965; Jackson *et al.,* 1991). These descriptive approaches have outlined pathways of viral spread in the infected host. However, molecular methodologies, including reverse genetics, make it possible to elucidate specific viral genetic features that are associated with virulence and pathways of dissemination.

Molecular genetic studies of virus pathogenesis have their antecedents in experiments that examined the effects of strain differences and mutation on virus virulence and tissue tropism. Among these are early studies with reovirus (Spriggs *et al.,* 1983; Tyler *et al.,* 1986), lymphocytic choriomeningitis virus (Ahmed *et al.,* 1984; Ahmed and Oldstone, 1988), bunyaviruses (Endres *et al.,* 1991), and Sindbis virus (Davis *et al.,* 1986). With the advent of molecular clones of poliovirus (Racaniello and Baltimore, 1981) and alphaviruses (Rice *et al.,* 1987; Davis *et al.,* 1989; Liljeström *et al.,* 1991; Kuhn *et al.,* 1991), it became possible to unequivocally assign pathogenesis functions to particular nucleotide substitutions by comparing otherwise isogenic virus clones (e.g., Polo *et al.,* 1988; Lustig *et al.,* 1988; Westrop *et al.,* 1989; Tucker and Griffin, 1991; Davis *et al.,* 1991; Atkins *et al.,* 1994). We have continued the evolution of this genetic approach in these studies of Venezuelan equine encephalitis virus (VEE).

The pathogenesis of VEE in the mouse consists of a series of defined steps from infection of the animal until its death as a consequence of encephalitis (Davis *et al.,* 1994; Grieder *et al.,* 1995). This pathogenetic sequence may be viewed as analogous in concept to a biochemical

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pathway in which each step consists of a distinct enzymatic reaction that converts one intermediate into the next, culminating in the synthesis of the final product. Mutants that fail to synthesize the final product are defective in one of the enzymes of the pathway and accumulate the intermediate upon which that enzyme normally acts. The examination of multiple individual mutants illuminates both the specific enzymes and the intermediates comprising the pathway. In the context of viral pathogenesis, each step is a distinct interaction between the virus and specific cells and/or tissues that occurs in a milieu dynamically changing during the host response to infection. Each of these interactions governs the progression of the infection to its next stage, culminating in overt disease and/or death. A mutation that prevents a fatal outcome interferes with one of these specific virus–cell interactions and interrupts the pathogenetic sequence at the point where that interaction is required for progression of the disease. With respect to that particular mutation, the interaction point becomes an *in vivo* genetic screen for reversion, so that virus populations isolated downstream of the inhibited interaction will be highly enriched in revertants capable of overcoming or circumventing the block imposed by the original mutation. Therefore, the examination of multiple individual mutants, each of which fails to cause death of the animal, in conjunction with an analysis of their revertants, will identify those interactions necessary for disease progression. This information is of critical importance in the development of intelligent vaccine and/or therapeutic strategies, and it is this genetic approach to VEE pathogenesis that we have taken in the present experiments.

VEE causes equine enzootic and epizootic disease in tropical areas of the Western hemisphere. Human cases of VEE infection are associated with epizootics and may present as a mild flu-like febrile illness with pharyngitis, a fulminate febrile illness, or in approximately 1% of cases, encephalitis. Severe disease tends to occur preferentially in children (Ehrenkranz and Ventura, 1974). Recent outbreaks of VEE serve as reminders of the veterinary and medical threat of this disease (Rico-Hesse *et al.,* 1995; Rivas *et al.,* 1997; R. Tesh and S. Weaver, personal communication). In experimental infection of equines (burros), and in several other experimental models including the mouse, VEE progresses via distinct lymphotropic and neurotropic phases (Gleiser *et al.,* 1962). The clinical features and autopsy findings in human cases also suggest a biphasic pattern with sequential lymphoid and central nervous system involvement (Johnson *et al.,* 1968).

In this article, we describe the initial stages in VEE pathogenesis comparing molecularly cloned viruses: wild-type V3000, a single-site attenuated mutant, V3010, and V3533, a second-site revertant of V3010 based on a biological isolate from V3010-infected mice. The mutation in V3010 blocks progression of virus disease at an early step in the VEE pathogenetic sequence, and the second-site mutation in V3533 overcomes this early block.

RESULTS

Patterns of V3010 spread in the mouse

V3010 is a molecularly cloned mutant of VEE that is isogenic with the wild-type clone, V3000, except for a Lys for Glu substitution at codon 76 of the E2 glycoprotein gene (Davis *et al.,* 1991). While V3000 is uniformly fatal at a standard dose of 1000 PFU administered subcutaneously (sc) in the footpad, V3010 is avirulent in outbred CD-1 mice (Grieder *et al.,* 1995). Following footpad inoculation, V3000 rapidly spreads to the draining popliteal lymph node, where replication can be detected as an increase in plaque numbers within 4 h postinfection (p.i.). A viremia is well established by 12 h, virus is growing in all other lymphoid tissues by 18 h, and within 24–36 h, invasion of the central nervous system (CNS) is observed (Grieder *et al.,* 1995; Charles *et al.,* 1995; Fig. 1, row A). In contrast, the spread of V3010 to the draining lymph node and beyond was altered. In approximately 25% of the animals inoculated with V3010, the spread of the virus to the draining lymph node was delayed (Grieder *et al.,* 1995; Fig. 1, row B), while in the remainder of animals, this delay was either reduced or undetectable (Fig. 1, row C). However, in all cases dissemination of the virus beyond the draining lymph node to serum and other tissues was sporadic. The basis for this range of V3010 phenotypes has not yet been examined and could be explained, for example, as a function of host genotype differences in an outbred mouse population or as a stochastic effect. Regardless, however, these results clearly suggest that the V3010 lesion at E2 76 confers both a significant impairment in spread of the virus to the draining lymph node and a more substantial block to spread beyond the draining lymph node.

V3010 reversion *in vivo*

Reversion analysis was utilized to determine more accurately the step in the VEE pathogenetic sequence at which the lesion in V3010 blocked progression of disease. If the block is at the level of the draining lymph node, then it should function *in vivo* as a genetic screen for the selection of revertants capable of proceeding through that pathogenetic step. This suggests that a high proportion of viruses isolated from sites downstream of the putative blocked step should be same-site or second-site revertants. The sporadic isolation of virus from tissues downstream of the draining lymph node is consistent with this hypothesis.

Table 1 shows the virulence and amino acid encoded

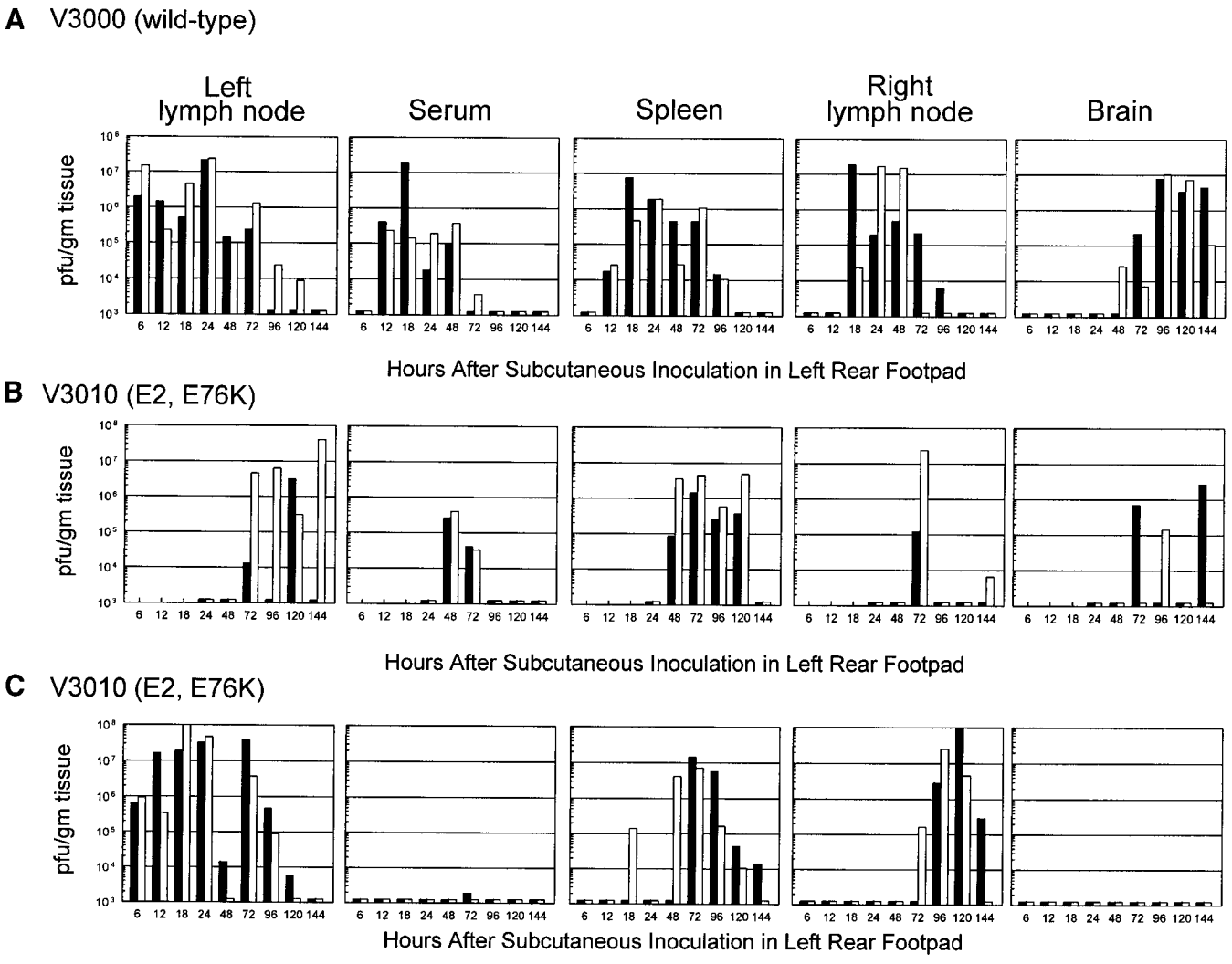

FIG. 1. *In vivo* replication of V3000 and an avirulent mutant, V3010. Six to 8-week-old CD-1 female mice were inoculated in the left rear footpad with 1000 PFU of V3000 (Row A, virulent wild type) or V3010 (Rows B and C, attenuated mutant: E2 Lys 76). Rows B and C show two experiments representative of the two different profiles of V3010 spread. At the indicated intervals p.i., two mice per group (solid and open bars) were sacrificed and selected tissues were harvested for viral titer determinations. Limit of detection was approximately 2×10^3, and bars drawn at the limit of detection indicate a titer at or below the limit.

at E2 76 of 17 isolates recovered from the draining lymph node, spleen, and brain at 120, 96, and 72 h, respectively, after inoculation with V3010. All the isolates from brain appeared to be same-site revertants characterized by the wild-type Glu residue at E2 76, 100% mortality, and an average survival time not significantly different from that characteristic of V3000. From spleen, the isolates had reverted to wild-type Glu at E2 76, but 4 of the 5 isolates displayed less than 100% mortality. This suggests a complex genotype that includes the wild-type residue at E2 76, which may allow progression beyond the draining lymph node, as well as one or more other mutations that block disease progression at an undetermined point downstream of the draining lymph node. Of similar complexity, 6 of 6 isolates from the draining lymph node retained the mutant E2 Lys 76, but 3 of these showed significant mortality compared to V3010, which is essentially avirulent. Overall, 11 of 11 isolates from spleen and brain had an altered phenotype and/or genotype compared with the inoculated V3010, and 3 of 6 isolates from the draining lymph node had an altered phenotype suggestive of a second-site reversion. This is consistent with a genetically specified block to V3010 movement beyond the draining lymph node.

In a second experiment, several plaque isolates were recovered from serum, draining lymph node, and contralateral popliteal lymph node from two individual mice 24 h after sc inoculation with V3010 (Table 2). Virus recovered from the draining lymph node formed uniformly small plaques on BHK cells, resembling those of parental V3010, whereas virus found in serum and contralateral lymph node of these two mice displayed an intermediate plaque morphology more like that of V3000.

TABLE 1

Virulence and Sequence Characteristics of Viruses Recovered from V3010-Infected Mice

Recovered from draining lymph node				Recovered from spleen				Recovered from brain			
Isolate[a]	Mortality[b]	AST ± SE[c]	E2 76 Residue[d]	Isolate[a]	Mortality[b]	AST ± SE[c]	E2 76 Residue[d]	Isolate[a]	Mortality[b]	AST ± SE[c]	E2 76 Residue[d]
L41	16.6	9.0 ± 1.4	Lys	Sp1	0	—	Glu	B1	100	8.0 ± 0.6	Glu
L42	0	—	Lys	Sp4	100	7.0 ± 0.9	Glu	B2	100	6.8 ± 1.2	Glu
L43	0	—	Lys	Sp6	66.7	10.0 ± 0	Glu	B3	100	6.7 ± 0.8	Glu
L47	0	—	Lys	Sp7	0	—	Glu	B6	100	7.3 ± 0.8	Glu
L48	50	9.3 ± 0.6	Lys	Sp8	66.7	8.5 ± 0.7	Glu	B7	100	6.8 ± 0.8	Glu
L49	50	9.0 ± 1.0	Lys	V3000	100	6.7 ± 1.2	Glu	B8	100	6.8 ± 0.8	Glu
V3000	100	7.7 ± 0.6	Glu	V3010	0	—	Lys	V3000	100	6.7 ± 1.2	Glu
V3010	0	—	Lys					V3010	0	—	Lys

[a] Plaque isolates were recovered from three V3010-inoculated animals at 120, 96, and 72 h p.i. from the draining lymph node, spleen, or brain, respectively, in an experiment analogous to that shown in Fig. 1. The isolates were expanded by one passage on BHK cells.

[b] Six CD-1 mice per isolate were inoculated sc in the left rear footpad with 1000 PFU, and percentage mortality and average survival time were recorded.

[c] Average survival time ± standard error.

[d] Each isolate was sequenced across the E2 76 codon, from nt 8750 to 8900 (codons 63–112). Either no change or change only at the E2 76 codon was observed.

Dissemination of putative revertants

The *in vivo* phenotype of two of the putative revertants was examined. Seven-week-old female CD-1 mice were inoculated with 1000 PFU of expanded plaque isolates designated 4b2 or 4s2, isolated from the contralateral lymph node and serum, respectively, of the same animal. One of eight 4b2-inoculated mice died (12.5%), but all eight showed signs of illness by day 6 (Table 3). These signs ranged from hunching and ruffling to hind limb paresis. Three mice displayed transient hind limb paresis by day 7, recovered, and survived challenge with V3000. Isolate 4s2 had a similar virulence phenotype in mice: 25% mortality, 62.5% morbidity, with two of the survivors having experienced transient hind limb paresis. The nature of the illness in these mice indicated the presence of a systemic response to viral infection and, at least in some cases, neuroinvasion. However, the full virulence of the wild-type clone was not restored in these biologic isolates.

TABLE 2

Partial Sequence Analysis of Serum and Lymph Node Isolates

Virus	Source	Plaque size	E2 76	E2 116
V3000	Inoculum	Large	Glu	Lys
V3010	Inoculum	Small	Lys	Lys
6a1	dln	Small	Lys	Lys
6a2	dln	Small	Lys	Lys
6a3	dln	Small	Lys	Lys
6a4	dln	Small	Lys	Lys
6a5	dln	Small	Lys	Lys
4b2	cpln	Intermediate	Lys	Glu
4s1	Serum	Intermediate	Lys	Glu
4s2	Serum	Intermediate	Lys	Glu
4s3	Serum	Intermediate	Lys	Glu
4s4	Serum	Intermediate	Lys	Glu
4s5	Serum	Intermediate	Lys	Glu
6s1	Serum	Intermediate	Lys	Glu
6s2	Serum	Intermediate	Lys	Glu
6s3	Serum	Intermediate	Lys	Glu
6s4	Serum	Intermediate	Lys	Glu
6s5	Serum	Intermediate	Lys	Glu

Note. Plaque isolates were derived from two individual CD-1 mice (4 and 6) inoculated 24 h previously in the left rear footpad with 1000 PFU of the molecularly cloned attenuated mutant V3010. RT-PCR products corresponding to the complete E2 and E1 genes were sequenced. Predicted amino acid at E2 codon 76 or 116 is indicated in the last column. No additional mutations were identified in the region sequenced for any of these isolates. V3000, virulent wild-type VEE clone; dln, draining popliteal lymph node (left); cpln, contralateral popliteal lymph node (right).

The experiment illustrated in Fig. 2 was performed to confirm that dissemination beyond the draining lymph node was an inherent and reproducible feature of 4b2 and 4s2 isolates. Naive mice were inoculated sc in the left rear footpad with 4b2 or 4s2, and the spread of virus was traced to selected tissues during the first 48 h p.i. The pattern of viremia produced by 4b2 and 4s2 paralleled that of V3000. Like wild-type V3000, 4b2 and 4s2 produced maximal or close to maximal serum viral titers by 12 h p.i., the time point illustrated in Fig. 2. In contrast, at 12 h p.i. the serum titer in V3010-inoculated mice was below the limit of detection (33 PFU/ml). At 24–48 h p.i., V3010 displayed viremia only sporadically (data not shown). It is likely that this relatively late viremia reflected revertants in serum, although in this experiment larger plaque variants were not recovered from serum or other sites. Peak serum viral titers produced after inoc-

TABLE 3

Morbidity and Mortality of V3010 Revertants

Virus	Codon at E2 76/116	Percentage morbidity[a] (sick/total)	Percentage mortality (dead/total)	AST ± SE (days)
		Experiment 1		
V3010	Lys/Lys	0 (0/8)	0 (0/8)	—
4b2	Lys/Glu	100 (8/8)	12.5 (1/8)	8.0
4s2	Lys/Glu	62.5 (5/8)	25 (2/8)	8.0 ± 1.4
		Experiment 2		
V3000	Glu/Lys	100 (4/4)	100 (4/4)	7.0 ± 1.2
V3010	Lys/Lys	0 (0/4)	0 (0/4)	—
V3533	Lys/Glu	87.5 (7/8)	25 (2/8)	9.5 ± 0.7
V3048	Glu/Glu	100 (8/8)	100 (8/8)	6.4 ± 0.9

[a] Six to 8-week-old female CD-1 mice were inoculated sc with 1000 PFU of the indicated virus into the left rear footpad. The animals were observed for signs of illness ranging from hunching and ruffling to hind limb paresis.

ulation with 4b2 or 4s2 were about one order of magnitude lower than those for V3000. Thus, although revertants 4b2 and 4s2 were capable of overcoming the E2 Lys 76 specified block to spread beyond the draining lymph node, they were still impaired in terms of their quantitative ability to produce V3000 levels of viremia and virulence.

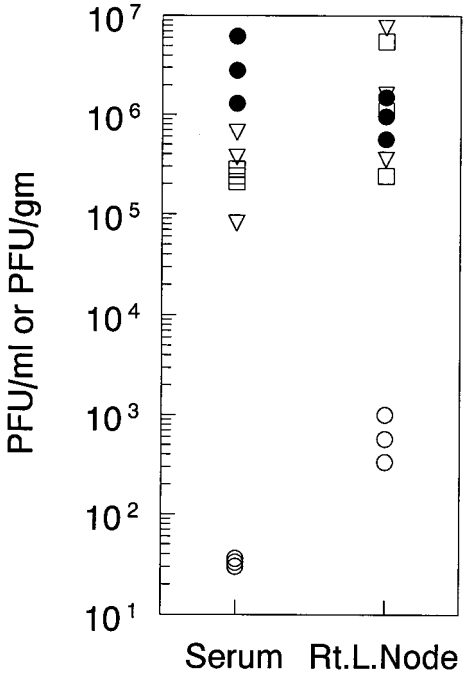

FIG. 2. Early dissemination of V3010 and its revertants. Six to 8-week-old CD-1 female mice were inoculated in the left rear footpad with 1000 PFU of V3000 (wild-type, filled circles), V3010 (cloned mutant, open circles), 4b2 (biological revertant, open squares), or 4s2 (biological revertant, open inverted triangles). At intervals through 48 h, three mice were sacrificed and tissues removed for titration. Shown are the serum samples from animals sacrificed at 12 h, the time at which V3000 had an established viremia. All three V3010-inoculated animals were below the limit of detection (33 PFU/ml), but are plotted at 30, 33, and 36 PFU for clarity. The samples depicted from the right (contralateral) popliteal lymph node are from animals sacrificed at 24 h, the time at which V3000 titers were at or near their peak in this tissue (limit of detection is 333 PFU/g).

The popliteal lymph node from the leg opposite the inoculated footpad, the contralateral lymph node, is a remote lymphoid site not directly linked via lymphatics with the draining lymph node of the inoculated leg. V3000 and revertants 4b2 and 4s2 spread to this site with nearly indistinguishable kinetics (Fig. 2 shows the titer in the contralateral popliteal lymph node at 24 h p.i.). As expected, the isolate derived from contralateral lymph node (4b2), as well as the serum isolated from the same mouse (4s2), reached and replicated in this site as efficiently as did V3000. However, of the three mice inoculated with V3010 and sampled at 24 h p.i., virus titer in the contralateral popliteal lymph node was at or near the limit of detection (333 PFU/g). These observations indicated that 4b2 and 4s2 resembled V3000 rather than parental V3010 in terms of their ability to produce viremia and spread to distant lymphoid sites. Hence, they were revertants with respect to the restricted early spread phenotype of V3010.

Identification of putative reverting mutations

Partial sequence analysis of the E2 glycoprotein gene of putative revertants (including 4b2 and 4s2) identified a second-site mutation that correlated with spread beyond the inoculated leg and its draining lymphatics. All 11 isolates from serum and the contralateral popliteal lymph node retained the attenuating residue at E2 76, but they also possessed a mutation at nucleotide 8909, which changed the amino acid at E2 116 from Lys to Glu (Table 2). The identical mutation at E2 116 was found in isolates from two separate mice. By contrast, 5 of 5 small plaque isolates from the draining (ipsilateral) lymph node were

genotypically identical (within the region sequenced) to the inoculated V3010 at E2 76 and E2 116. Thus, the second-site mutation at E2 116 was found only in plaque isolates recovered from sites distal to the draining lymph node in the pathogenetic sequence. This reinforces the suggestion that the E2 76 mutation in V3010 prevented systemic spread of V3010 and that an additional mutation(s) was required to enable V3010 to spread to serum and distant lymphoid sites.

Phenotypic analysis of a molecularly cloned E2 76/E2 116 revertant (V3533)

To confirm that the revertant phenotype demonstrated for 4b2 and 4s2 (i.e., viremia and early dissemination competence) was attributable to the E2 116 mutation, this mutation was placed into the genetic background of V3010, resulting in a double mutant (V3533: E2, E76K, and K116E). The E2 116 mutation also was placed into the V3000 background as a single mutant (V3048). Table 3 displays mortality data for mice inoculated with V3000, V3010, V3533, and V3048. The E2 116 mutation by itself (V3048) did not affect virulence relative to V3000. The cloned revertant (V3533) had virulence characteristics intermediate between those of V3000 and V3010, resembling the biologic revertants 4b2 and 4s2.

The pattern of early spread for V3533 showed striking fidelity to that for the biological revertants 4b2 and 4s2 (Figs. 2 and 3; additional data not shown). The pattern of peripheral replication of V3533 was similar, although not identical, to that of V3000. Relative to V3000, there were no discernable delays in reaching the draining lymph node or in development of viremia. However, the viremia generated by V3533 was of slightly lower titer and of shorter duration than that for V3000. Likewise, the first appearance and peak titers of V3533 in other lymphoid tissues (spleen, contralateral lymph node, and thymus) were similar to those characteristic of V3000. However, the highest titers were not sustained as long, and clearance from these tissues appeared to be somewhat more rapid than in V3000-inoculated animals. A similar pattern was apparent in the pancreas, where no replication of V3010 was detected; V3533 replication in the pancreas was to lower titers and was cleared more rapidly than V3000 (data not shown). Replication in muscle was rarely detected with any of these viruses and then only at titers just above the limit of detection. Although V3533 first appeared in the brain about the same time as V3000, CNS titers of V3533 were more variable, the peak titers were lower, and most importantly, the virus was cleared from the brain. A large proportion of V3533-infected animals survived. Therefore, the reversion conferred by the second-site suppressor mutation at E2 116 was with respect to the specific block in the pathogenetic sequence imposed by the E2 76 mutation in V3010 and was not sufficient to restore virulence to the level of V3000. This is consistent with the anatomical sites from which the original biological revertants were isolated (serum and contralateral lymph node; Table 2) and with the presence of same-site revertants in the brain (Table 1).

V3048, carrying the E2 116 mutation alone, was indistinguishable from V3000 in all tissues examined except the pancreas, where it replicated to lower titers (data not shown).

Replication at the site of inoculation

Many viruses are thought to replicate first at the site of inoculation followed by movement through the lymphatics to the draining lymph node, in concert with general models of virus infection (Fenner, 1949; Nathanson, 1980; Mims *et al.,* 1995). VEE, however, does not appear to follow this paradigm. As noted previously, V3000 titers at the footpad site of inoculation did not exceed levels consistent with recovery of the virus inoculum until 12 to 18 h p.i. (Grieder *et al.,* 1995; data not shown), whereas replication in the draining lymph node was evident within 4 h (Fig. 3). These data suggest that spread of VEE to the lymph node from the footpad is independent of replication at the site of inoculation. Replication evident in the footpad at 24 h or more p.i. could result from retrograde spread of the virus from the draining lymph node back down the inoculated limb or from the viremia. Viral titers in the footpad over the course of V3010 infection indicate that the mutant also is deficient in movement of virus back to the footpad and/or the ability to replicate in this tissue (data not shown). V3533, 4b2, and 4s2 were indistinguishable from V3000 in this phenotype.

Histopathological examination of footpads from V3000-inoculated mice 24 h p.i. showed focal coagulative necrosis involving skeletal muscle interstitium, rare myofibers, and areolar connective tissues of the foot. A sparse focal infiltrate of neutrophils accompanied this necrosis. V3010-inoculated footpads, observed at the same time p.i., showed scanty necrosis and inflammation, qualitatively similar to, but of considerably lesser degree than that seen in V3000-infected mice (data not shown).

Patterns of virus-specific *in situ* hybridization signal at 24 h p.i. corresponded with footpad viral titers (Fig. 4). Dense signal was seen in the footpads for V3000- and 4s2-infected mice (Figs. 4A and 4C), while very sparse signal was evident in some sections of V3010-inoculated footpads (Fig. 4B) after identical exposures and under identical hybridization conditions. V3000 replicated in the soft tissue and collagenous connective tissue of the footpad, particularly in the dermis, skeletal muscle, and tendon, with a striking predilection for periosteum and endosteum (Fig. 4D). V3000 tended to spread and replicate in a lamellar fashion, with signal focused in sheets of contiguous fibroblasts. Signal was seen in this pattern in the proximal leg, particularly involving the periosteum.

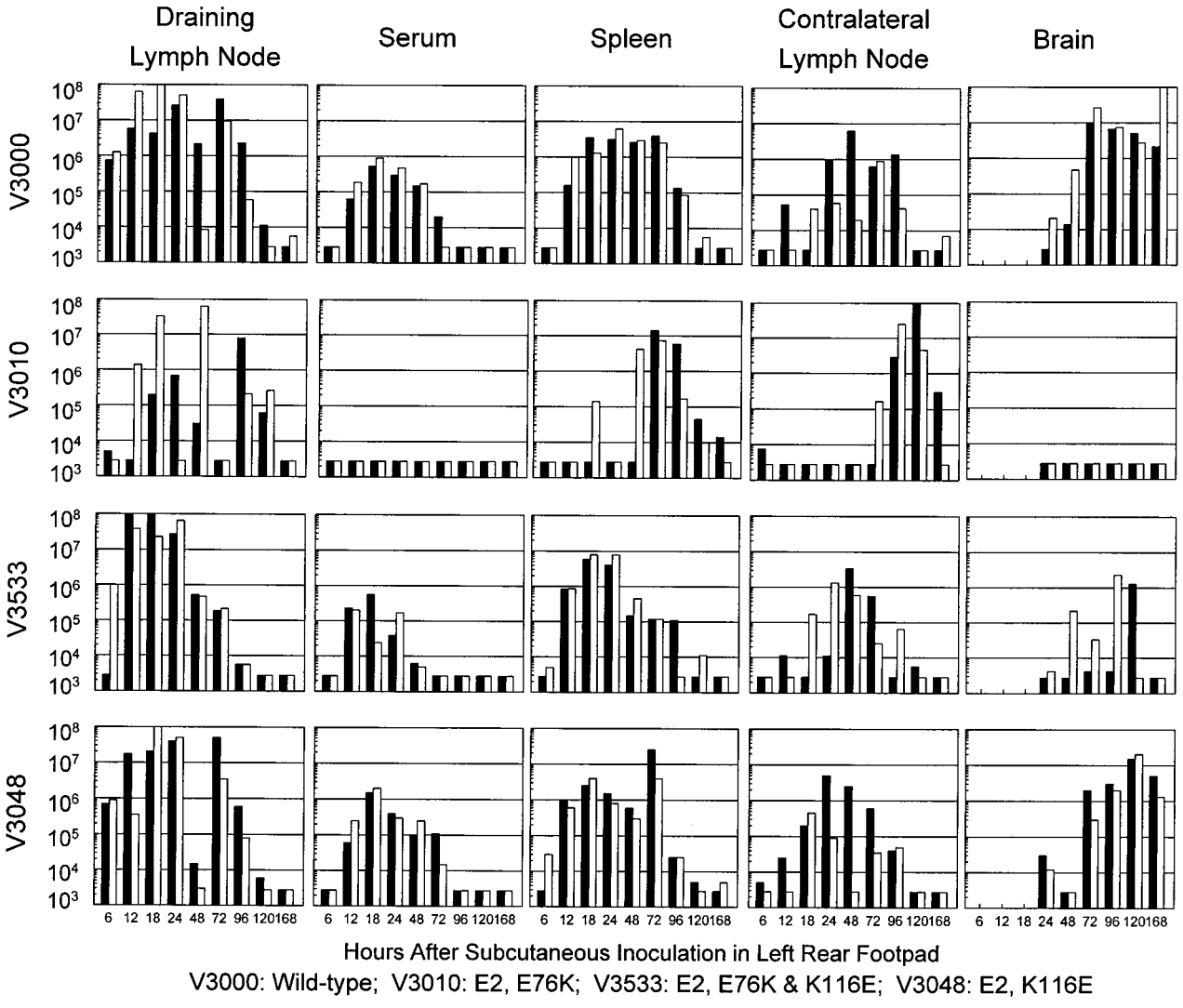

FIG. 3. *In vivo* replication of cloned VEE strains with mutations at E2 residues 76 and 116. Six to 8-week-old CD-1 female mice were inoculated in the left rear footpad with 1000 PFU of V3000 (wild-type; E2 Glu 76, Lys 116), V3010 (E2 Lys 76, Lys 116), V3533 (E2 Lys 76, Glu 116), or V3048 (E2 Glu 76, Glu 116). At the indicated intervals p.i., two mice per group (solid and open bars) were sacrificed and serum, footpad, ipsilateral (left) popliteal lymph node, contralateral (right) popliteal lymph node, spleen, thymus, pancreas, muscle, and brain were harvested for viral titer determinations. Limit of detection was approximately 2×10^3, and bars drawn at the limit of detection indicate a titer at or below the limit. A second experiment with a more limited subset of tissues using cloned viruses, and analogous experiments with V3010 and the biological revertants 4s2 and 4b2, gave similar results.

In addition, a concentration of silver grains was observed over spindled cells of the skeletal muscle interstitium, tendon, periosteum, and dermis (Fig. 4F). In contrast, V3010 replication was undetectable (even after extended exposures) or was observed rarely as a sparse, spotty signal (Fig. 4B). Replication in the spindled cells of the skeletal muscle was not observed in V3010-infected animals. The revertant 4s2 showed a pattern similar to that of V3000 (Figs. 4C and 4E). These results indicate a differential ability of V3000 and V3010 to replicate in fibroblasts and the unidentified spindled cells in the skeletal muscle interstitium, while the E2 116 second-site suppressor mutation appeared to restore this capacity. Alternatively, if viremia is required to seed these sites, then lack of signal may be secondary to the reduced viremia in V3010-infected animals.

DISCUSSION

Earliest stages of VEE infection and movement to the draining lymph node

The classical description of viral pathogenesis holds that viruses first replicate at the site of inoculation followed by spread to the draining lymph node (Fenner, 1949; Nathanson, 1980; Mims *et al.,* 1995). In the case of VEE, however, several lines of evidence suggest that the site of initial replication is the draining lymph node itself. VEE replication in the draining lymph node was demon-

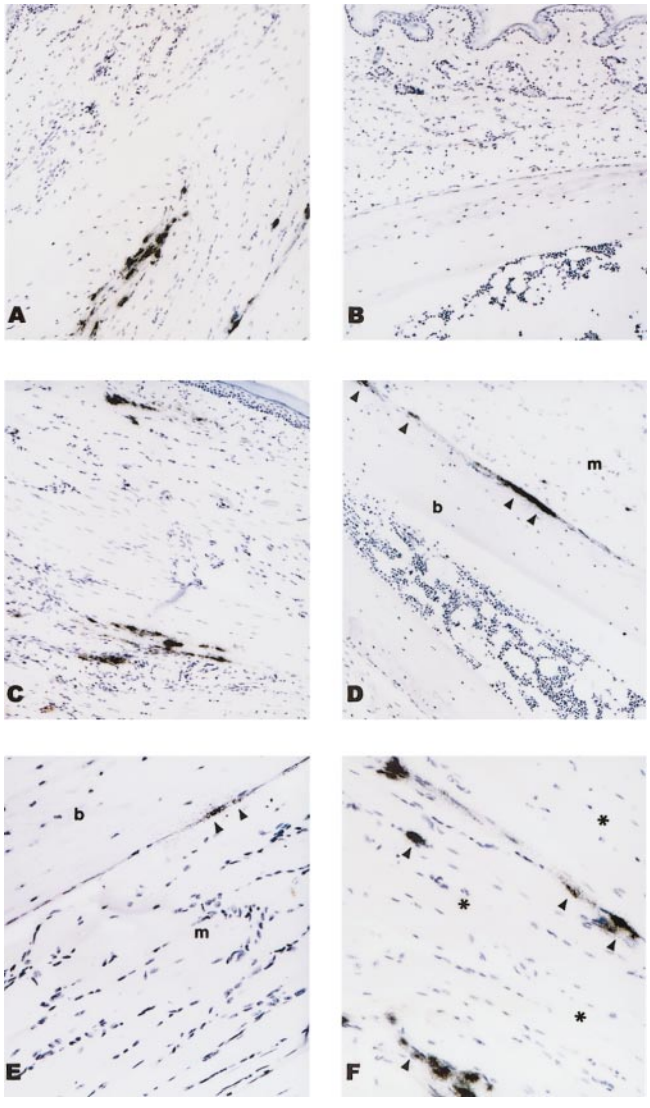

FIG. 4. *In situ* hybridization of inoculated legs. CD-1 mice were inoculated sc in the left rear footpad with 1000 PFU, sacrificed, and analyzed by *in situ* hybridization. (A) A histologic section of footpad from a V3000-inoculated mouse at 24 h p.i. shows specific signal in the subcutaneous muscle (original magnification 100×). (B) A comparable section from a V3010-inoculated mouse subjected to *in situ* hybridization lacked silver grains (original magnification 100×). (C) Footpad from a 4s2-inoculated mouse shows a pattern of virus localization similar to that for V3000 (original magnification 100×). (D) Silver grains were concentrated in the area of the periosteum of the foot in a V3000-inoculated mouse (arrows). b, bone; m, muscle (original magnification 200×). (E) A similar periosteal distribution of virus (arrows) was observed in a 4s2-inoculated mouse. b, bone; m, muscle (original magnification 200×). (F) In mice infected with V3000 or revertants, specific signal was distributed over spindled cells of the skeletal muscle interstitium (arrows) rather than over the muscle cells themselves (*). (V3000-infected mouse, 24 h p.i., original magnification 200×). Comparable results were obtained in separate experiments with V3000 and V3010.

strated within 4–6 h p.i., earlier than at any other anatomical site including the footpad where the virus was inoculated sc (Grieder *et al.,* 1995). A second line of evidence (MacDonald and Johnston, 2000) derives from studies that utilized VEE replicon vector particles (VRP; Pushko *et al.,* 1997) expressing the green fluorescent protein gene (gfp; Cormack *et al.,* 1996) in place of the VEE structural protein genes. The gfp–VRP unequivocally mark the first cell infected *in vivo* (because the replicon RNA lacks the structural protein genes), and the cell specificity of the VRP infection is dependent on the glycoproteins with which the VRP are assembled. The results of the VRP experiments are most consistent with infection of Langerhans cells in the footpad followed by migration of these cells to the draining lymph node where, in a V3000 infection, the earliest virus replication would be detected.

The E2 76 mutation in V3010 affects this process. In about 25% of animals infected with V3010, there was a delay in detection of replication in the draining lymph node, although the extent of eventual replication in these instances appeared close to wild-type levels. This indicates that movement of V3010 to the node was impaired to some extent. However, in the majority of animals, this impairment was much less apparent or undetectable, suggesting that V3010 spread to the lymph node occurred on a stochastic basis by a means different from that employed by V3000 and that this process was somewhat less efficient. Using gfp–VRP packaged in V3010 glycoproteins (MacDonald and Johnston, 2000), a small number of infected cells (relative to V3000 packaged gfp–VRP) were observed in the draining lymph node at 12 h p.i. These were unidentified small round cells in the medulla of the node, which also were observed as a minority population of infected cells with the V3000 packaged gfp–VRP. As V3010 fails to infect Langerhans/dendritic cells, its spread from the footpad could not take advantage of the rapid and efficient movement of these cells to the draining lymph node. Therefore, with limited replication of V3010 in fibroblasts (see Fig. 4), the mutant may rely on simple lymph drainage to move the inoculum virus to the node, a process that is unlikely to be as efficient as that employed by V3000. In the case of the pattern displayed in Fig. 1b, limited replication of V3010 at the inoculation site may be the source of revertants detected eventually in lymphoid tissues.

A second possibility is that the mutation in V3010 effectively blocks movement to the draining lymph node and that limited replication in the footpad allows the selection of revertants capable of movement. If this were true, then virus isolated from the node should always be revertant. This was not the case. Two sets of isolates from the draining lymph node were examined. In one set, all the isolates (five of five) retained the original E2 Lys 76 mutation and the characteristic small plaque phenotype of V3010. In the other set, six of six isolates retained E2 Lys 76, and three of these retained the avirulent phenotype of V3010. The other three isolates demonstrated sufficient mortality to distinguish them from the inoculated mutant. With the caveat that these isolates have not

been fully characterized as to phenotype and genotype, we conclude that reversion is not necessarily required for V3010 to reach the draining lymph node but that the altered cell tropism of this mutant results in less efficient movement to the node.

This notion is supported by studies of V3533, which consistently moves to the draining lymph node as efficiently as wild-type. gfp–VRP packaged in V3533 glycoproteins regain the ability to infect dendritic cells, although these do not appear to be precisely the same subset of dendritic cells targeted by V3000 (MacDonald and Johnston, 2000). These results suggest that the efficient movement of V3000 to the draining lymph node is genetically linked to its ability to infect dendritic cells but that dendritic cell infection is not absolutely required for the virus to eventually reach the draining lymph node.

Establishment of viremia

In contrast to movement of V3010 to the draining lymph node, spread beyond this tissue does require a reversion event. All virus isolates from serum (10/10), contralateral popliteal lymph node (1/1), spleen (5/5), and brain (6/6) from four different mice were genotypic and/or phenotypic revertants of the inoculated V3010. The molecularly cloned revertant V3533 induced a viremia with the same time course as V3000, although the peak titers were reduced approximately one order of magnitude.

A feature common to all neurotropic arboviruses is the ability to produce a prolonged high-titered viremia in susceptible hosts. This event appears to be required both for viral invasion of the CNS and for transmission to other mammalian hosts by insect vectors (Janssen *et al.,* 1984). In several alphavirus (Grimley and Friedman, 1970; Liu *et al.,* 1970; Murphy *et al.,* 1973) and bunyavirus (Janssen *et al.,* 1984; Griot *et al.,* 1993) infections, virus replicates in skeletal muscle both at the site of inoculation and at locations remote from it. Viral replication in muscle is thought to fuel the viremia. VEE infection of immunocompetent adult mice lacks this striking skeletal muscle tropism (Grieder *et al.,* 1995; Charles *et al.,* manuscript submitted for publication). Instead, VEE disseminates early to lymphoid tissue and replicates to high titer there. While it is presumed that this lymphotropic phase sustains the viremia, the mutant data indicate that the question is more complex. V3010 (as well as another mutant, V3014; Grieder *et al.,* 1995) can replicate in the draining lymph node without the induction of a viremia, suggesting that replication alone at this site is insufficient to fuel the viremia. The mutants may be incapable of escaping from the lymph system, they may be unstable in lymph or serum, or they may be cleared much more rapidly from the serum. Alternatively, replication at an as yet unidentified site may be the source of viremia in VEE infection.

Spread to other peripheral tissues and brain

The second-site suppressor mutation in V3533 overcame the failure of V3010 to establish a viremia but did not restore wild-type virulence. Replication of V3533 in peripheral lymphoid tissues reached the approximate levels characteristic of V3000, but these peak levels were not sustained for the same length of time prior to clearance. A more dramatic difference was observed in the brain where the variation of virus titers between animals was more pronounced, the peak titers were not as high, and clearance of virus resulted in the survival of a high proportion of V3533-infected animals. The V3533 suppressor mutation was selected in response to a genetic screen at the level of viremia induction; therefore, there was no selection for a wild-type phenotype. Rather, the selective pressure was local and specific to the block in the pathogenetic sequence imposed by the original mutation. In support of this notion was the finding that brain isolates from V3010-infected animals appeared to be same-site revertants that restored wild-type virulence.

Structure/function correlates within the E2 gene

Regions of the E2 glycoprotein near codon 116 have been examined in several contexts. E2-120 was found to be an important attenuating locus in the TRD-derived vaccine strain TC-83 (Kinney *et al.,* 1993). An attenuated variant of Ross River virus possessed five E2 mutations compared to wild-type; these included mutations at E2-3, E2-67, and E2-119 among others (Vrati *et al.,* 1988). In Sindbis virus, a mutation in the E2-114 codon is associated with attenuation of virulence in mice, rapid penetration in BHK cells, and altered reactivity with E2c monoclonal antibodies (Davis *et al.,* 1986). This amino acid residue aligns very closely with E2 116 in VEE (Kinney *et al.,* 1986). Amino acid changes at positions 62, 96, or 159 in the E2 gene of Sindbis are associated with escape from neutralization by E2c monoclonal antibodies; some mutations in these sites were also able to suppress the attenuation imposed by the E2-114 mutation (Pence *et al.,* 1990; Schoepp and Johnston, 1993). These disparate loci in the Sindbis E2 glycoprotein may form a "pathogenesis domain," a folded structure composed of noncontiguous amino acid residues that determine virulence properties of the virus. In VEE, E2 76 and E2 116 may participate in an analogous pathogenesis domain. Until detailed three-dimensional structural characterization of the virion surface is completed, however, these models remain highly speculative.

Implications for live virus vaccines

The *in vivo* fate of the VEE attenuated mutants and their *in vivo* reversion can serve as a model for the performance of live virus vaccines in inoculated individuals and provides some rather straightforward sugges-

tions regarding vaccine design. First, an attenuating mutation early in the pathogenetic sequence would be preferred from a safety perspective. Slowing the progression of disease near the beginning of the sequence will allow an effective innate response to control the infection as the adaptive immune system is stimulated. Selective pressure for reversion at an early blocked step is not a selection for reversion to wild-type, whereas a mutation that blocks a late event is likely to be subject to pressure for reversion to full wild-type virulence in order for the virus to complete the pathogenetic sequence. A potential drawback in selecting a mutation blocking an early pathogenetic event is the possibility of suppressing replication at such an early stage that a protective immune response is not induced. Thus, it is theoretically possible to "overattenuate" a vaccine with a single mutation. A second vaccine implication is that multiple mutations are better than single mutations if each of the multiple mutations blocks at a different step in the sequence. If reversion pressure is specific to the block, then double mutants are likely to require at least two reversion events to revert to full virulence. However, a single reversion event could conceivably circumvent a double mutant in which each of the constituent mutations blocked the same step. Third, in contrast to the notion that a live virus vaccine should be largely disabled in its general replicative capacity, a successful vaccine will replicate very strongly *in vivo* but in a way that does not cause overt disease. Given careful characterization of candidate attenuating mutations, safe and effective vaccines can be designed with a minimal number of mutations. Such vaccines will be characterized by near wild-type growth through some relatively early step in the pathogenetic sequence but virtually no progression beyond that stage. This will lead to induction of a strong immune response without risk of disease.

The pathogenetic sequence at the molecular level

It is clear that progression through the VEE pathogenetic sequence requires multiple but different interactions between virus and specific cells *in vivo.* The mutations we have used to date in defining these different interactions are in the glycoprotein genes, suggesting that at least some of these steps are governed by the ability of the virus to attach to and infect different cell types or by the ability of the glycoproteins to envelop sufficient quantities of infectious virus in these cells. The subtlety of these differences is underscored by the fact that these are single-site mutants, predominantly in the E2 gene. In the context of receptor interactions, for example, this means that a single amino acid substitution that substantially blocks interaction with the receptor on one differentiated cell type may have little or no effect on the analogous interaction in a different cell. This suggests a wide variety of viral glycoprotein–cell receptor interactions, each of which differs from the others in perhaps small but significant ways. Examination of these interactions using the approach suggested here will allow the genetic linkage of subtle changes in virus–cell interactions to specific alterations in the VEE pathogenetic sequence leading to fatal disease.

MATERIALS AND METHODS

Viruses and clones

Virus phenotypically indistinguishable from the Trinidad donkey strain of VEE (TRD-E1) was derived from the full-length cDNA clone pV3000 as previously described (Davis *et al.,* 1989; Grieder *et al.,* 1995). The clone pV3010 was derived by site-directed mutagenesis of pV3000 and contains a single point mutation resulting in a Lys for Glu substitution at E2 codon 76 (Davis *et al.,* 1991). Infectious transcripts were prepared by *in vitro* transcription of linearized plasmid with T7 RNA polymerase. Transcripts were transfected into baby hamster kidney (BHK) cells using cationic liposomes (Lipofectin, BRL) according to the manufacturer's instructions or by electroporation. Infectious progeny virus was harvested from transfected culture supernatants as soon as a significant cytopathic effect was observed (approximately 36 h posttransfection). Infectious virus derived from pV3000 or pV3010 is termed V3000 or V3010, respectively. All viral stocks were stored in aliquots at −70°C and were tested in mice to verify virulence phenotypes (percentage mortality and average survival time) after intracranial and sc inoculation. Virus stocks used in these experiments were the transfected culture supernatants without further cell culture passage.

The E2 116 mutation (A to G at nt 8909) was introduced into the structural genes of pV3000 (cloned in M13) using the site-directed mutagenesis procedure of Kunkel (1985; Davis *et al.,* 1991). An 800-bp *Bst*XI restriction fragment comprising the mutagenized region was removed from M13 RF and cloned into a pUC118-based shuttle vector containing either pV3000 or pV3010 structural genes from which the analogous *Bst*XI fragment had been excised (Grieder *et al.,* 1995). The E2 glycoprotein gene was excised from each shuttle vector as an *Spe*I–*Sac*I restriction fragment containing the E2 116 mutation alone or both E2 116 and E2 76 mutations. This fragment was then substituted for the analogous fragment in the full-length pV3000 clone. The entire *Spe*I–*Sac*I replacement was sequenced to confirm that the desired mutations were the only ones present. The full-length clone containing the E2 116 mutation alone was designated pV3048; the clone containing mutations at codons 76 and 116 of E2 was designated pV3533. Virus stocks were generated from these clones as described above.

In experiments of this type, it is possible to introduce lethal mutations into the clone and still retrieve infectious virus due to reversion after transfection of the RNA tran-

scripts into cells. To ensure that this was not the case with the mutations used here, specific infectivities of the transcripts were determined by transfection of radioactively labeled transcripts into BHK cells followed by agarose overlay and development of plaques (Rice *et al.,* 1987). All of the mutant transcripts had specific infectivities similar to transcripts derived from pV3000, indicating that the introduced mutations were not lethal with respect to replication in BHK cells.

Cells

BHK cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% donor calf serum, 10% tryptose phosphate broth, 0.29 mg/ml L-glutamine, 100 U/ml penicillin, and 0.5 mg/ml streptomycin. Virus was quantitated by plaque assay on subconfluent monolayers of BHK cells.

Mice

Adult female CD-1 mice (6–8 weeks old), specific pathogen- and orphan parvovirus-free (Charles River), were maintained on standard lab chow and water *ad libitum.* Animals were acclimatized for 1 week in the BSL-3 facility before experimental manipulation. In all experiments, mice were inoculated sc in the left rear footpad with 1000 PFU of virus in 10 μl of phosphate-buffered saline (PBS) (low endotoxin) with 1% DCS (PBS/DCS) or 10 μl of diluent alone. All inocula were retitered at the time of the experiment to ensure that mice had received the appropriate dose.

Isolation of revertants

Candidate revertants were recovered as plaque isolates from tissues of mice inoculated sc in the left rear footpad with 1000 PFU of V3010. Isolates from brain (B1–3; B6–8) were derived at 72 h p.i. from an animal that had detectable virus in both the left (draining) and the right popliteal lymph nodes, spleen, serum, thymus, pancreas, and brain. Isolates from the left popliteal lymph node (L41–L43; L47–L49) were derived at 120 h p.i. from a mouse with detectable virus in the left popliteal lymph node and spleen. Isolates from the spleen (Sp1; Sp4; Sp6–8) were derived at 96 h p.i. from a mouse with detectable virus titers in the left popliteal lymph node, spleen, thymus, and pancreas.

A second set of candidate revertants was isolated at 24 h p.i. from two individual mice inoculated as described above with V3010. Serum and tissue homogenates from spleen and right popliteal lymph node from these two animals yielded predominantly larger plaques than V3010 on BHK cells; V3010 produced small, angular plaques. Virus from the left popliteal lymph node yielded uniformly small plaques.

RNA isolation and sequence analysis of biologic isolates

Intracellular RNA was isolated from one 60-mm dish corresponding to each expanded plaque isolate as follows. At 4 to 6 h p.i. dishes were rinsed with warmed PBS. Total cellular RNA was isolated using the RNA-zol B method (Cinna Biotecx, Houston, TX). The quantity and quality of RNA were assessed initially by SDS–agarose gel electrophoresis. E1 and E2 genes were amplified separately by RT-PCR using primer pairs JFA-1 (virus sense, 5′-GACAGCCCTTTCAGTCGTCATGTG-3′) and Vc9918 (virus complement, 5′-AAGGCGGCCAGAGGGATCAGC-3′) for E2, and Vs1276 (virus sense, 5′-ACCTGGGAGTCCTTGGATCACC-3′) and Vc11356 (virus complement, 5′-TGCCAATCGCCGCGAGTTCTATGT-3′) for E1. Reverse transcription was carried out at 42°C for 30 min in 20-μl reaction volumes containing 1 μl of total intracellular RNA (approximately 200 ng), 1 pmol each forward and reverse primer, 50 U MuLV-RT (Boehringer Mannheim), 0.01 M DTT, 20 U RNAsin, 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 2.5 mM $MgCl_2$, and 1 mM each dATP, dCTP, dGTP, and dTTP. Reverse transcriptase was heat-inactivated by boiling of the samples for 2 min. *Taq* polymerase (2.5 U, Boehringer Mannheim) was added in buffer yielding final concentrations of 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 2.0 mM $MgCl_2$ in a final volume of 100 μl. PCR was performed in a Perkin–Elmer 4600 thermocycler with the following cycling parameters: 93°C for 1 min; 42°C for 1 min; 72°C for 2 min; for 25 cycles. PCR mixtures then were treated with DNase-free RNase to inactivate infectious RNA, extracted with phenol/chloroform, and electrophoresed on 0.7% agarose gels. The anticipated 1.6-kb E2 PCR product and 1.5-kb E1 PCR product were visualized on ethidium bromide-stained gels and purified from the gel using the GeneClean kit (Bio101). Gel-purified PCR products were sequenced using the Δ *Taq* cycle sequencing kit (USB). ^{35}S-labeled products were separated on a denaturing, 6.5% acrylamide wedge gel and visualized by autoradiography after exposure of Kodak XAR5 film.

Assay for virulence

Six or eight CD1 mice for each group were inoculated in the left rear footpad with 1000 PFU of the indicated transfected culture supernatant (V3010, V3000, V3048, V3533) or 1000 PFU of an expanded biologic isolate (4b2 or 4s2). Mice were observed daily, and the numbers of dead and visibly ill mice (hunched, ruffled, depressed, and/or paralyzed) were recorded. Results are reported as the average survival time in days with standard error. All mice surviving the 14-day observation period were challenged with 10^4 PFU V3000 intraperitoneally to ensure that survival was due to attenuation of virulence rather than failure of the initial inoculation.

Examination of wild-type and mutant pathogenesis

To compare the ability of virus to disseminate beyond the site of inoculation, groups of CD-1 mice were inoculated with 1000 PFU of virus in the left rear footpad as described above. At intervals through 168 h p.i., two mice from each group were anesthetized with metofane and exsanguinated via the axillary artery into serum separator tubes. Additional samples for viral titration that were collected from each animal included the following: footpad (all soft tissue on the plantar aspect of the left foot, removed to the bone with sterile scalpel blade), ipsilateral (left) popliteal lymph node, contralateral (right) popliteal lymph node, spleen, thymus, pancreas, muscle, and brain. Tough tissues of the footpad were quickly minced on ice with sterile scalpels, transferred to a tared sterile microtube with pestle (Kontes), and triturated in 9 vol of PBS/DCS. Soft tissue samples were placed directly into tared microtubes with pestles, triturated in 9 vol of PBS/DCS, and stored frozen at −70°C. Samples were thawed and centrifuged at 15,000 *g* for 15 min to remove tissue debris, and the clarified supernatants were plaque titered on BHK cells.

Histology and *in situ* hybridization

Two mice were inoculated in the left rear footpad with V3000, V3010, 4s2, or diluent as described above and sacrificed at 24 h p.i. The left popliteal lymph node was dissected and fixed in neutral buffered formalin. The remainder of the left leg was disarticulated at the hip, soft tissues were sectioned to the bone, and the entire leg was fixed in a decalcification solution of 4% paraformaldehyde, 8% EDTA in PBS, pH 6.6. Fixation/decalcification was carried out at 4°C with one change of fixative over 10 days. Tissues were subsequently processed through graded alcohols, embedded in paraffin, and sectioned at 3 μm onto Probe-on+ slides (Fisher Scientific). Hematoxylin- and eosin-stained sections were examined for lesions. Serial sections were used for *in situ* hybridization as described (Grieder *et al.,* 1995). ^{35}S-labeled riboprobe was transcribed from a pGEM-3 plasmid containing the subcloned *Pst*I–*Sac*I fragment from the structural gene region of pV3000. The resulting 678-nucleotide riboprobe was complementary to viral message sense RNA and hybridized with full-length or subgenomic viral mRNA across portions of the 6K and E1 genes. Control irrelevant probe was transcribed from a pGEM-4 transcription vector containing influenza hemagglutinin (HA) gene sequences (kindly provided by Dr. Andrew Caton, Wistar Institute). *In situ* hybridization was performed exactly as described (Grieder *et al.,* 1995). Controls for probe specificity included serial sections hybridized with HA probe, and tissues from PBS-inoculated control mice hybridized with VEE probe.

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