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Specific Restrictions in the Progression of Venezuelan Equine Encephalitis Virus-Induced Disease Resulting from Single Amino Acid Changes in the Glycoproteins

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The pathogenesis of Venezuelan equine encephalitis virus (VEE) was examined in the mouse model using V3000, a virus derived from a molecular clone of the Trinidad donkey strain of VEE. These results were compared in parallel experiments with avirulent mutants of VEE derived by site-directed mutagenesis of the clone. Adult mice, inoculated subcutaneously in their left rear footpad with V3000, were followed in a time course study for 6 days in which 15 organs were tested for histopathological changes, for the presence of viral antigen by immunohistochemical staining, for the presence of viral nucleic acid by *in situ* hybridization analysis, and for content of viable virus. Virus was detected in the footpad inoculation site, but until 12 hr postinoculation (pi), the level of virus did not suggest early viral replication. By 4 hr pi, however, replication of V3000 was evident in the draining popliteal lymph node. At this early time point, no virus could be isolated from any other organ examined. At 12 hr, a significant serum viremia was observed, and virus was detected at a low level in a number of well vascularized organs, including spleen, heart, lung, liver, kidney, and adrenal gland. By 18 hr, high virus titers were present in serum and all the lymphoid organs examined, and these tissues appeared to be the major peripheral sites of V3000 replication. Virus in serum and peripheral organs was cleared by 3–4 days pi. In a second phase of the infection, V3000 invaded the central nervous system (CNS), replicated predominantly in neurons, and persisted in the brain until death by encephalitis. Pathologic findings as well as the results of immunocytochemical and *in situ* hybridization examination were generally coordinate with virus titration. A site-directed mutant of V3000, V3010, contained a mutation in the gene for the E2 glycoprotein at codon 76 (Glu to Lys) which rendered it avirulent after footpad inoculation. Detection of V3010 replication in the draining lymph node was sporadic and was sometimes delayed to as long as 3 days pi. Infrequent and/or delayed virus spread to other sites also was observed. Analogous experiments were performed with other mutants which were avirulent by the footpad inoculation route: V3014, a mutant differing from V3000 at three loci (E2 Lys 209, E1 Thr 272, and E2 Asn 239), as well as single-site mutants V3032 (E2 Lys 209) and V3034 (E1 Thr 272). The mutations in V3014 prevented spread beyond the draining lymph node. The single-site E2 Lys 209 mutation allowed spread to the draining lymph node and to other lymphoid organs without significant serum viremia or invasion of the CNS, and E1 Thr 272 was characterized by near normal peripheral replication, but only sporadic replication in the CNS. These results suggest that VEE-induced disease results from a sequence of events which can be defined by interdicting the pathogenic pathway with specific mutations in the VEE genome.

INTRODUCTION

Venezuelan equine encephalitis virus (VEE) is a member of the genus alphavirus in the family *Togaviridae*. The virus was originally isolated in Trinidad (Kubes and Rios, 1938; Gilyard, 1944) and is an insect-transmitted veterinary and human pathogen in the Americas (Peters and Dalrymple, 1990). A mouse model for VEE infection that approximates many elements of the human and equine disease, combined with a complete cDNA clone of the VEE genome, affords an important opportunity to apply the tools of molecular genetics to the analysis of VEE replication and disease induction *in vivo*.

The VEE virion, like that of other alphaviruses, is characterized by a lipoprotein envelope containing cell-derived lipids and two viral glycoproteins, E1 and E2 (Pederson and Eddy, 1974; Kääriäinen and Söderlund, 1978; reviewed in Strauss and Strauss, 1994). The alphavirus E1 and E2 glycoproteins associate as heterodimers, and three heterodimers intertwine to form each of the 80 spikes apparent on the surface of virions (Vogel *et al.*, 1986; Paredes *et al.*, 1993). Within the envelope is the nucleocapsid which consists of 240 copies of the capsid protein (Paredes *et al.*, 1992) and which encloses the 11,447-nt, single-stranded, positive sense RNA genome (Kinney *et al.*, 1992).

Several studies of VEE pathology and pathogenesis in

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the horse, other animal hosts, and humans have been published (Kissling *et al.,* 1956; Victor *et al.,* 1956; De la Monte *et al.,* 1985; Gleiser *et al.,* 1962; Gorelkin, 1973; Walker *et al.,* 1976; Jackson *et al.,* 1991). In these reports, the extent and severity of the pathologic changes induced by VEE in spleen, lymph node, pancreas, and the central nervous system (CNS) varied considerably with the infected host species, host age at the time of infection, inoculation dose, and inoculation route. A comparative pathology study in guinea pigs, mice, burros, and monkeys (Gleiser, 1962) identified mice as an experimental species which displays a two phase disease after a peripheral inoculation. In the initial peripheral phase, commencing within 1–2 days postinoculation (pi), the virus affected mainly lymphoid and myeloid tissues. By 4–6 days pi, the virus was cleared from the serum, and affected organs appeared to be regaining their normal morphology. The second, neurotropic phase of the disease ensued following invasion of the CNS 2 or 3 days pi and ended with the death of the animal at 6 or 7 days.

In vitro transcripts of full-length cDNA clones of alphavirus genomic sequences (Rice *et al.,* 1987; Davis *et al.,* 1989; Kuhn *et al.,* 1991; Liljeström *et al.,* 1991) are functional replicas of the genome and initiate a productive infection when introduced into cells by transfection. Several advantages for the study of pathogenesis are provided by this system. Genetic instability inherent in RNA viruses is minimized so that starting inocula are as homogeneous as possible. Individual mutations may be introduced at the cDNA level by site-directed mutagenesis, and the resulting virus mutants may be recovered following transcription and transfection. Infection of animals with cloned virus of defined genotype allows the unambiguous assignment of pathogenesis phenotypes to specific loci. Finally, starting with defined virus inocula permits one to evaluate genotypic and phenotypic alterations of the virus *in vivo.* In the studies reported here, virus was derived from a molecular clone of the Trinidad donkey strain of VEE, pV3000, and site-directed mutants generated from it (Davis *et al.,* 1989, 1991).

Previous studies with such molecularly cloned viruses have shown that certain mutations in the glycoprotein genes of alphaviruses can significantly alter their virulence in rodent model systems. A mutation in E2 codon 55 of Sindbis virus is a major determinant of neurovirulence in adult mice (Lustig *et al.,* 1988; Tucker *et al.,* 1993). Mutation at E2 114 reduces neurovirulence of Sindbis virus for neonatal mice (Davis *et al.,* 1986; Polo *et al.,* 1988), whereas a constellation of mutations in E1 (at E1 72, 75, and 237) predominantly acts to restrict invasion of the CNS in this model (Polo and Johnston, 1990). Mutations in the Sindbis E2c neutralizing antigenic site, composed of E2 residues 62, 96, and 159 (Pence *et al.,* 1990), and at E2 114 affect neurovirulence and/or neuroinvasion depending on the specific amino acid substitution (Polo and Johnston, 1991; Schoepp and Johnston, 1993). A mutation at E2 172 increases the survival time of neonatal mice infected with Sindbis and simultaneously decreases the efficiency with which the virus binds to unfractionated cultures of mouse brain cells (Tucker and Griffin, 1991), suggesting a role of the glycoproteins in cell tropism effected by their targeting the virus to specific cellular receptors (Wang *et al.,* 1991, 1992; Ubol and Griffin, 1991). However, reduction of virulence specified by other mutations in E2 did not correlate with their binding to mouse brain cell receptors (Schoepp and Johnston, 1993), suggesting that some glycoprotein mutations may influence cell tropism and virulence by affecting early virus–cell interactions other than binding or by altering the efficiency of virus maturation in a cell specific manner. Virus derived from a full-length cDNA clone of Semliki Forest virus (SFV4) reproduces the lethality of the L10 strain upon intranasal inoculation of adult BALB/c mice or intraperitoneal (ip) inoculation of pregnant animals (Glasgow *et al.,* 1991). A mutation at SFV4 E2 162 extended survival following intranasal administration (Balluz *et al.,* 1993) and allowed survival of pregnant mice inoculated ip, although most of the fetuses were killed (Glasgow *et al.,* 1994). In Ross River virus, a 21-nt deletion in the E2 gene was associated with reduced virulence (Vrati *et al.,* 1986). Mutations in the E2 precursor, which prevent its cleavage, uniformly lead to dramatically reduced virulence in adult mouse virulent strains of Sindbis (Russell *et al.,* 1989), in Sindbis strains pathogenic only for neonates (Heidner *et al.,* 1994; Heidner and Johnston, 1994), in SFV4 (Glasgow *et al.,* 1991), and in VEE (Davis *et al.,* unpublished observations).

The experiments reported here carefully examined the pathogenesis of V3000, the virus derived from pV3000, especially at early times after inoculation of adult mice. The objectives were to confirm that VEE derived from the molecular clone was representative of its biological progenitor and to determine the effect of specific glycoprotein mutations on the progression of VEE induced disease. We have examined the pathogenesis of virulent V3000 and four molecularly cloned mutants of the virus which are avirulent upon footpad inoculation. Each contains one or more mutations in the glycoprotein genes and each is otherwise isogenic with virus derived from pV3000. The results of this comparative pathogenesis study suggest that progression of VEE-induced disease can be interdicted by viral mutation at several points, including spread to the draining lymph node, spread to other lymphoid organs, establishment of viremia, and invasion of the CNS.

MATERIALS AND METHODS

Viruses and clones

A full-length cDNA clone of VEE (pV2000) was produced from the genomic RNA of TRD-E1, a clonal isolate

of the Trinidad Donkey strain of VEE (TRD; Kubes and Rios, 1938; Johnston and Smith, 1988; Davis *et al.,* 1989; 1991). A silent change in codon 170 of the E2 gene was introduced into pV2000 by site-directed mutagenesis to produce pV3000, removing a *Sma*I site as an aid in identification of V3000 sequences. A coding change from Asn to Ile at E2 codon 239 (nt 9279) in pV3000 was inadvertently made at the same time. Sequence analysis of the entire mutagenized region of V3000 (nts 8054 to 11,202) showed no other differences compared to TRD-E1. Virus was produced from pV3000 as follows. Plasmid DNA was prepared for run-off transcriptions by linearization at a unique *Not*I site downstream of the poly(A) tract, and *in vitro* transcription was carried out with T7 RNA polymerase. Transfection of baby hamster kidney (BHK) cells with *in vitro* RNA transcripts was performed with cationic liposomes (Lipofectin, BRL) using the manufacturer's recommended protocol. Virus-containing culture supernatants were harvested when cytopathic effect was significant in the cell monolayers (approximately 36 hr posttransfection), and virus stocks were frozen at −70°. Virus stocks derived from pV3000 were termed V3000.

VEE mutants were produced by site-directed mutagenesis using a modification of the Kunkel method (Kunkel, 1985; Kunkel *et al.,* 1987). In each case, mutagenesis was performed on a subclone of pV3000 containing the structural protein genes (see below). The relevant portion of the mutagenized subclone was used to replace the analogous region of pV3000, and the substituted region was sequenced in its entirety. Mutant V3010 was described previously (Davis *et al.,* 1991) and contains a single substitution (Lys for Glu) at E2 codon 76. V3014 also was described previously as containing a Lys for Glu substitution at E2 209. However, further sequencing of V3014 revealed two additional mutations relative to the V3000 sequence. One of these is a Thr for Ala substitution at E1 272; the other is at E2 239. At E2 239, V3014 has an Asn residue in common with the genomic sequence of TRD-E1, whereas V3000 has Ile. Therefore, V3014 differs from V3000 at 3 loci: E2 Lys 209, E2 Asn 239, and E1 Thr 272. Comparisons between viruses that differ in their coding sequences only at E2 239 (V3000: E2 Ile 239; V2000: E2 Asn 239) indicate no differences in their virulence (percentage mortality and average survival time) or their pathogenesis in CD1 mice (F. B. Grieder, J. F. Aronson, N. L. Davis, and R. E. Johnston, unpublished data). V3032 and V3034 contain only the individual E2 Lys 209 and E1 Thr 272 mutations, respectively. E2 Lys 76 and E2 Lys 209 were originally identified as attenuating mutations in viruses selected for rapid penetration in BHK cells (Johnston and Smith, 1988; Davis *et al.,* 1991). Changes at E2 239 and E1 272 occurred originally as adventitious mutations during site-directed mutagenesis.

V3032 and V3034 were constructed by standard methods (Sambrook *et al.,* 1989) using a pUC118-based shuttle vector containing the structural gene region of V3000 (from the *Hin*dIII site at nt 7290 to the *Not*I site downstream of the poly(A) tract). As a marker for shuttle vector sequences, the E2 gene was altered by replacement of the *Spe*I–*Apa*LI fragment (nt 8389–nt 9220) with the homologous region of pV2000 (Davis *et al.,* 1991) to reinstate the *Sma*I site at nt 9072. Another silent change, to inactivate the *Bsa*HI site in the E1 gene (nt 10,848), was made by site-directed mutagenesis. Sequence analysis of the mutagenized region confirmed that this was the only mutation, and replacement of the *Afl*II–*Sac*II fragment (nt 8,054–nt 11,199) of pV3000 with that from the modified shuttle vector resulted in a full-length clone whose RNA transcripts were fully infectious, yielding virus with a virulence phenotype identical to that of V3000. Therefore, the only sequence differences between the shuttle vector sequence and that of pV3000 were the silent *Sma*I and *Bsa*HI restriction site polymorphisms. Unique restriction sites flank the E2 (*Spe*I–*Sac*I) and E1 (*Sac*I–*Eag*I) genes in the shuttle vector, and a restriction site polymorphism (*Sma*I or *Bsa*HI) can be used to verify replacement of either gene by a mutagenized sequence. An E2 gene containing Lys at position 209 was produced in M13 phage using the Kunkel procedure and substituted for the homologous region in the shuttle vector. The glycoprotein gene region of pV3000 (nts 8,054 to 11,202) was replaced with that from the E2 Lys 209 shuttle vector to produce pV3032. pV3032 RNA transcripts were fully infectious, and the E2 gene contained only the change in E2 codon 209. V3034, containing only the E1 Thr 272 mutation, was produced by the same technique.

Cells

Baby hamster kidney cells, obtained from the American Type Culture Collection in passage 53, were maintained in Eagle's minimum essential medium supplemented with 10% donor calf serum (DCS), 10% tryptose phosphate broth, 0.29 mg/ml L-glutamine, 100 units/ml penicillin, and 0.05 mg/ml streptomycin. For all experiments, cells were used between passages 55 and 65. Transfection procedures, preparation of virus stocks, and virus titration on BHK cells have been described previously (Davis *et al.,* 1991).

Assays for virulence and immunogenicity

Four- to 8-week-old, female CD1 mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were maintained on standard laboratory feed and water *ad libitum* and housed in the BL3 containment facility for at least 24 hr prior to the beginning of experiments. CD1 mice were inoculated subcutaneously (sc) into their left rear footpad or intracerebrally (ic) with 10^3 PFU. Percentage mortality and average survival time ± standard error (AST ± SE) were calculated following an observation

period of 14 days. All surviving mice were challenged with an ip dose of 10^4 PFU of virulent V3000.

Virus replication and histopathology

Adult, female CD1 mice were inoculated sc in the left rear footpad with 10^3 PFU of virulent V3000 or one of the VEE mutants in a volume of 25 μl. At 6, 12, 18, 24, 48, 72, 96, 120, and 144 hr pi, two mice were anesthetized and exsanguinated. Sera and the following tissues were collected for viral titration and for histopathological and immunocytochemical analysis: brain, eye, salivary gland, thymus, heart, lung, liver, spleen, pancreas, duodenum, kidney, adrenal gland, skeletal muscle (right quadriceps), left and right popliteal lymph nodes, and left footpad. Depending on the tissue, from 10 to 50% was used for titration and the remainder was processed for microscopic analysis. For brain, approximately one-fourth of the cerebral cortex from the right hemisphere was removed for virus titration. In the experiments shown in Figs. 1 and 2, samples for virus titration were homogenized in 4 vol phosphate-buffered saline containing 1% DCS using microtubes and pestles (Kontes). The final 20% (w/w) suspension was clarified by centrifugation, and the supernatants were frozen in aliquots at −70° prior to standard plaque titration on BHK cells. In other experiments, specifically with V3010, the tissue homogenates were frozen at −70° as 10% suspensions, thawed, and titered immediately after centrifugation. Tissue samples for histopathological and immunocytochemical analysis were fixed in 10% buffered formalin for at least 24 hr, embedded in paraffin, sectioned, and either stained with hematoxylin and eosin, or alternatively, stained for VEE antigen using the avidin–biotin and horseradish peroxidase system (Vector Laboratories, Burlingame, CA) with a polyclonal rabbit anti-VEE antibody (kindly provided by Dr. Jonathan Smith, USAMRIID, Frederick, MD) as primary reagent. Negative controls were tissues of control mice inoculated in parallel with PBS and sections from V3000-inoculated animals stained with normal rabbit serum.

In situ hybridization

A 993-bp *Pst*I–*Sac*I fragment (nt 9,493–nt 10,486) from the structural gene region of pV3000 was inserted into the polylinker of the transcription vector pGEM-3 (Promega). The plasmid was digested with *Sma*I (nt 9827) and used as template in an *in vitro* transcription reaction containing SP6 polymerase (Promega) and [α-^{35}S]UTP (Clabough *et al.,* 1991). The reaction product was a 678-nt RNA probe complementary to viral message sense RNA, containing the 6K gene and part of the E1 gene (nts 9,827–10,486). A second ^{35}S-labeled RNA probe of approximately 500 nt was transcribed from a pGEM-4 transcription vector containing the influenza hemagglutinin gene (a kind gift from Dr. Andrew Caton, Wistar Institute, Philadelphia, PA) and was used as a control probe for nonspecific hybridization. Incorporation of [α-^{35}S]UTP into each probe was typically 80 to 90% to give a sp act of approximately 10^9 counts per min (cpm)/μg. Probe activity was quantitated by counting 1-μl samples in an automated liquid scintillation counter (Pharmacia LKB).

Paraffin-embedded tissues were cut in 3-μm sections and mounted on aminopropyl triethoxysilane-coated slides. *In situ* hybridization was performed as previously described (Clabough *et al.,* 1991; Ausabel *et al.,* 1989; Gowans *et al.,* 1989) with some modification of washing procedures. Duplicate slides from each mouse were hybridized with 50,000 cpm of either the V3000 riboprobe or the influenza virus hemagglutinin probe in 5 μl of hybridization mix under a siliconized cover slip. As an additional control, tissue sections from mice inoculated with saline and treated as described above were hybridized with the V3000 probe. All slides were washed in 2× SSC (1× SSC contains 0.15 *M* NaCl and 0.015 *M* sodium citrate) containing 20 m*M* β-mercaptoethanol, but no formamide, at 55°. A final high stringency wash was performed in 0.1× SSC at 70° for 15 min. After dehydrating through graded ethanol, the slides were dipped in Kodak NTB-2 emulsion, allowed to dry, and stored in a light-tight slide box at 4° for 1 to 3 days. After development, the slides were stained routinely with hematoxylin and eosin. A positive VEE-specific signal by *in situ* hybridization was taken as evidence of virus replication in a given tissue.

RESULTS

The major advantages of examining pathogenesis using virus derived from molecular clones are that an inoculum can be employed that is as close as possible to a homogeneous population and that the effect of single nucleotide changes on pathogenesis phenotype can be assessed. However, the act of cloning a single genotype from a natural virus isolate raises the possibility that the clone may be derived from a minor variant not representative of the population as a whole. To ensure that V3000 reflects the pathogenic properties of the natural VEE isolate from which it was cloned and to supply baseline information for evaluation of the site-directed mutants, V3000 replication and spread in mice have been examined after sc inoculation of 10^3 PFU into the left rear footpad of adult CD1 mice. This route of inoculation was chosen to provide additional information about experimental VEE infection of mice by modelling introduction of virus by mosquito bite and by providing the opportunity to examine very early events in VEE pathogenesis, such as spread of the virus to a specific draining lymph node. Two animals were sacrificed at intervals pi, and tissues were removed for titration of infectious virus, histopathology, immunocytochemistry, and *in situ* hybridization.

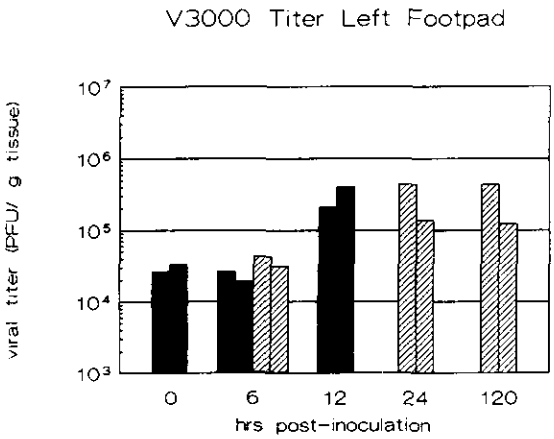


FIG. 1. Histogram of VEE titers (PFU/g tissue) in the left rear footpad. CD1 mice were inoculated into the left rear footpad with 10^3 PFU of V3000. Two mice were sacrificed at each time point, and the bars represent individual mice. Accumulated data from two independent experiments are shown: experiment one, solid bars; experiment two, hatched bars.

Replication and spread of V3000 in extraneural tissues

The highest amount of virus recovered from the footpad immediately following inoculation and at 6 hr pi was approximately 600 PFU total virus (Fig. 1). These values approximate the amount inoculated, although recovery of virus from this site may not be quantitative due to the difficulty of dispersing the tough collagenous tissue of the footpad. In contrast, VEE replication was detectable in the draining popliteal lymph node as early as 4 hr pi, when the amount of virus in the entire organ was approximately 5×10^4 PFU (data not shown) rising to 1×10^5 PFU at 6 hr (Fig. 2). Not until 12 hr did the levels of virus recovered from the footpad exceed the inoculum, suggesting that extensive replication of VEE at the site of inoculation was not a prerequisite for movement to the draining lymph node.

At 6 hr pi, virus was below the level of detection in serum (33 PFU/ml) and the other tissues examined (1.25×10^3 PFU/g) (Fig. 2). At 12 hr pi, V3000 was detectable in serum and in several other organs in addition to the draining popliteal lymph node. These included spleen, heart, lung, liver, kidney, and adrenal gland, where titers ranged from 2.5×10^3 to 2.2×10^7 PFU/g. At 18 hr pi, V3000 could be isolated from serum, from all the organs that contained virus at 12 hr, as well as from thymus, pancreas, salivary gland, and the contralateral popliteal lymph node. Therefore, the dissemination of VEE from the site of inoculation to tissues peripheral to the CNS was complete by 18–24 hr pi.

Each of the peripheral organs demonstrated one of three patterns of V3000 replication from 24 to 96 hr pi. One pattern, distinguished by high virus titers (1×10^5 to 2×10^7 PFU/g) through Day 4 pi, was observed in the serum, spleen, thymus, popliteal lymph nodes, and pancreas. The other pattern was characterized by somewhat lower virus titers (10^4 to 10^5 PFU/g) clearing by Day 2 or 3 pi (heart, lung, liver, kidney, adrenal gland, and salivary gland). The third pattern consisted of rare or no detection of VEE replication. For example, virus was detected in skeletal muscle (right quadriceps) of only three mice: two at 24 hr pi and one at 96 hr pi contained between 10^4 and 10^5 PFU/g. Virus was not detected in skeletal muscle at any other time. No virus could be isolated from duodenum (free of lymphoid tissue) at any time pi. As in previous studies of VEE infection of mice (see above), V3000 was essentially undetectable in all visceral organs examined and in the serum by Day 5 pi.

Microscopic evaluation of tissue sections for lesions, presence of VEE antigen and evidence of viral replication by *in situ* hybridization was consistent with the time course of replication and spread indicated by virus titration, considering the relative sensitivities of these methods. These observations also were generally consistent with previous studies of VEE pathogenesis using biological isolates (Kissling *et al.*, 1956; Victor *et al.*, 1956; Gleiser *et al.*, 1962; Tasker *et al.*, 1962; Kundin, 1966; Gorelkin, 1973; Jackson *et al.*, 1991). Selected examples of these data are shown in Fig. 3.

During the 24 hr following inoculation of V3000, histopathological changes were restricted to lymphoid tissues, consistent with the initial virus dissemination through the lymphatic system documented by virus isolation. In both draining and systemic lymph nodes, loss of follicular structure, disseminated single cell necrosis, subcapsular necrosis, and by 24 hr pi, an accompanying moderate infiltrate of acute inflammatory cells (Fig. 3A) were observed. Positive *in situ* hybridization signals were detected as early as 6 hr pi in the draining lymph node. Hybridization signal was most commonly superimposed over individual cells, rather than clusters of cells, even when the surrounding pathologic changes were diffuse, and the majority of the positive cells were found in the subcapsular region of the lymph nodes. Staining for VEE-specific antigen was associated with necrotic cells and debris (Fig. 3D). At 24 hr pi, the spleen showed an increased ratio of red to white pulp, lymphocyte necrosis in the center of splenic follicles, and disseminated single lymphocyte necrosis throughout the splenic parenchyma. Discrete, individual cells showed positive *in situ* hybridization signal between 12 and 72 hr pi. The thymus was characterized by a mild cortical atrophy with occasional disseminated single necrotic lymphocytes. *In situ* hybridization showed comparatively few infected cells in thymic tissue, and these were most abundant at Day 3 pi. In general, cell types positive for VEE antigen or *in situ* hybridization signal could not be determined definitively based on morphology and distribution. However, in some mice, strong positive hybridization signal was present in the spleen over cells which appeared to be megakaryocytes by morphological criteria (Fig. 3F). In a separate

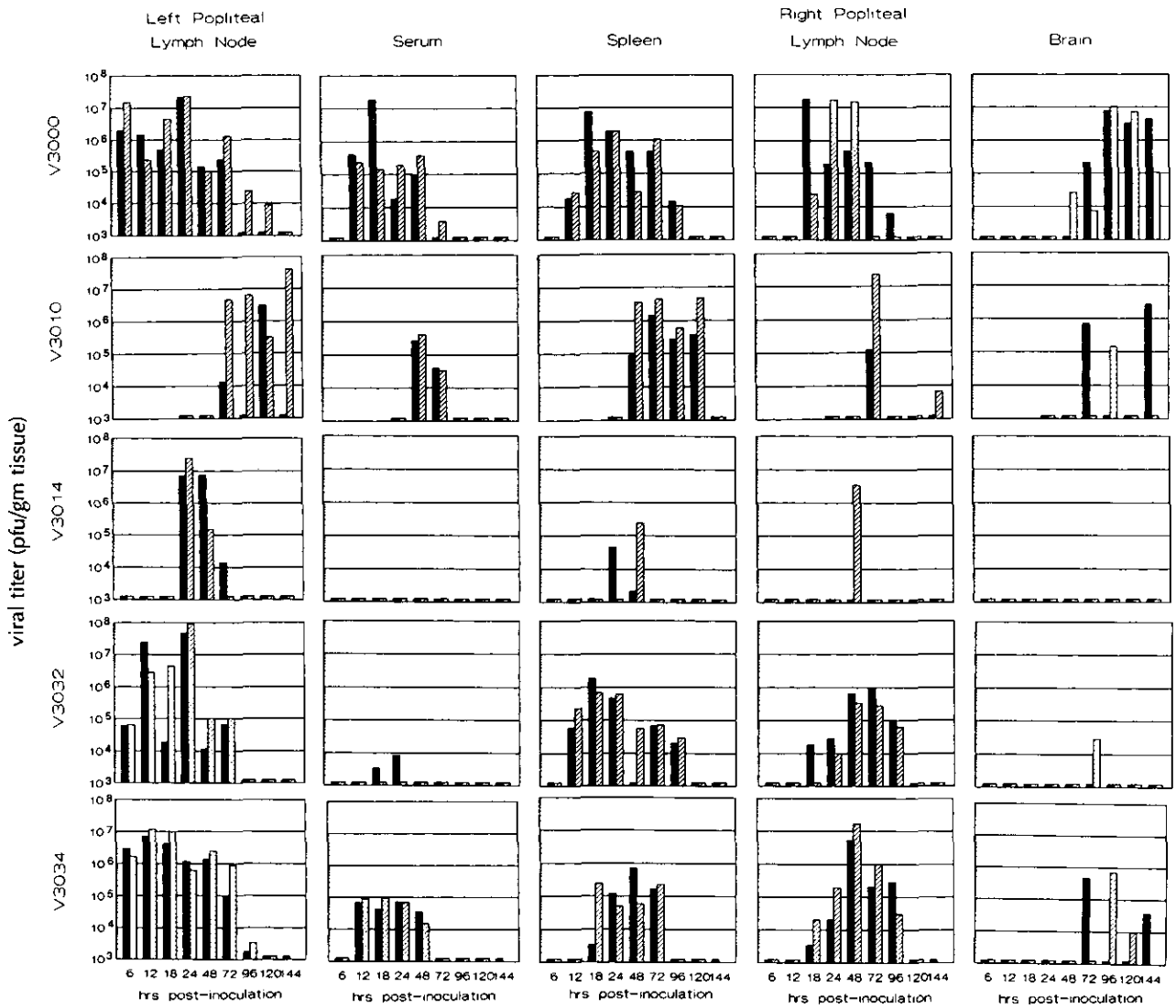
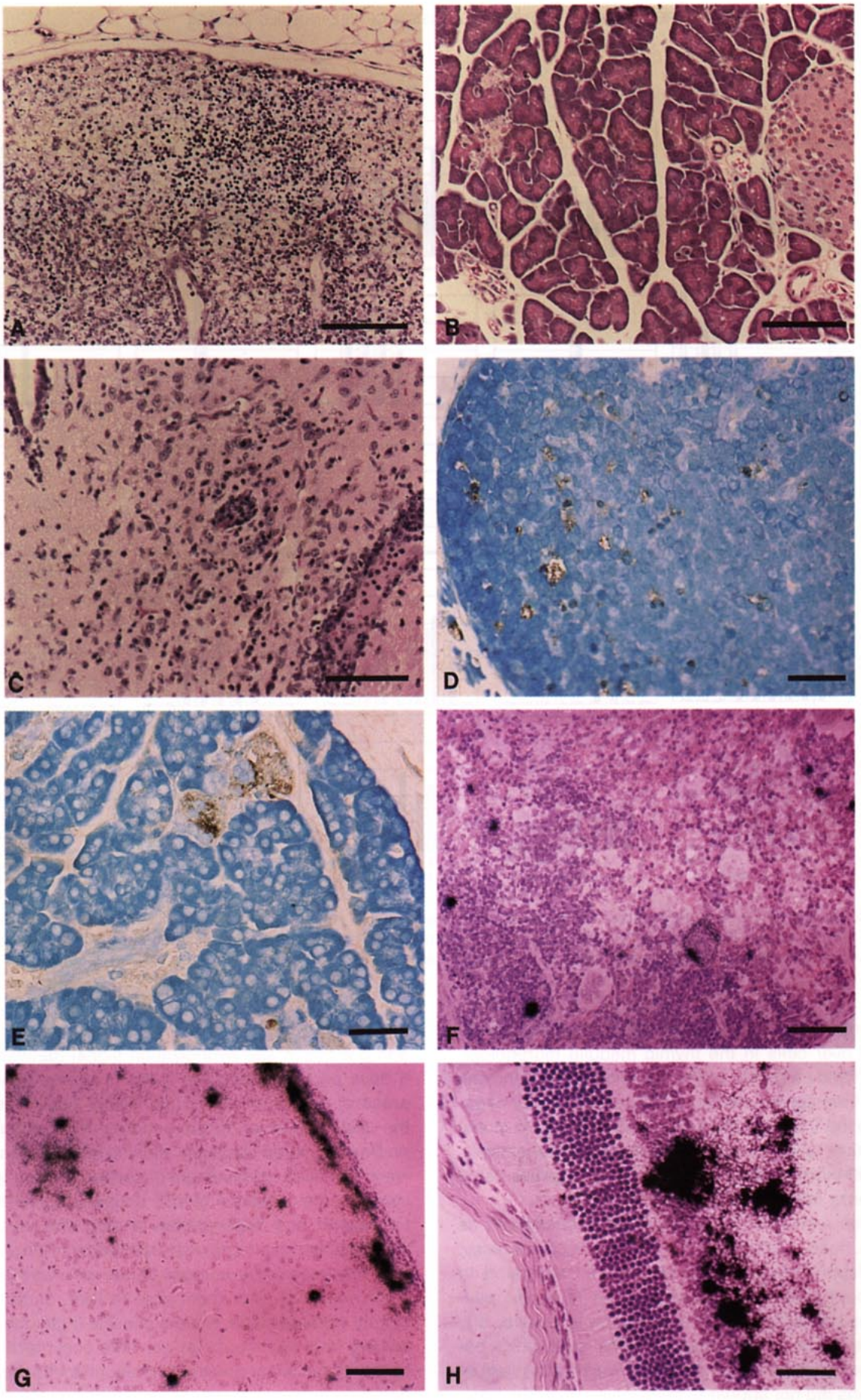


FIG. 2. Histograms of VEE titers (PFU/g tissue or PFU/ml serum) in representative organs. CD1 mice were inoculated into the left rear footpad with V3000, V3010, V3014, V3032, or V3034 (10^3 PFU/animal). Two mice (solid and hatched bars) were sacrificed at each time point, and tissues were removed for virus titration. Detection level was 1.25×10^3 PFU/g tissue or 33 PFU/ml serum.

experiment in CB17 mice, *in situ* hybridization signal was observed over cells in the thymic medulla morphologically identified as thymic epithelial cells (data not shown). Sections from saline-injected mice were uniformly negative using the V3000 probe or anti-VEE antibody in *in situ* hybridization and immunocytochemistry, respectively, and V3000-infected mice were uniformly negative using the influenza HA probe or normal rabbit serum.

VEE also replicated in nonlymphoid tissues such as pancreas, heart, and fat with differing levels of pathology. No significant histopathological changes appeared in nonlymphoid organs prior to 48 hr pi. At 72 hr, the pancreas was characterized by a moderate to severe, disseminated coagulative necrosis of acinar cells which became more pronounced at 96 hr. However, the islets of Langerhans did not appear to be involved (Fig. 3B). Positive staining for VEE antigen (72 and 96 hr pi; Fig. 3E) and positive *in situ* hybridization signal (between 3 and 6 days pi) were observed in the exocrine pancreas in areas of necrosis with no significant positive signal in the islets. However, in the pancreas of a single mouse on Day 5 pi, three positively staining islets of Langerhans were identified. Involvement of cardiac muscle was indicated by the presence of VEE antigen, *in situ* hybridization signal, and degeneration of myocytes. In those instances where an organ was positive by *in situ* hybridization, any associated adipose tissue also was positive.

No viral RNA was detectable in liver, salivary gland, kidney, adrenal gland, skeletal muscle (right quadriceps), or lung from any mouse at any time postinfection. Similarly, no consistent positive immunohistochemical signal could be detected in any of these organs, although liver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from one animal on Day 5 pi had positive staining in the centrolobular areas, and positive staining tubules were prominent in the kidney of one mouse on Day 3 pi. This suggests that these tissues are not usual sites of active virus replication, but are possibly areas of inconsistent antigen deposition.

The marked decline in the numbers of infected cells by Day 4 pi corresponded with the gradual return of peripheral tissues to normal histological appearance and the decrease in viral titers. Lymphoid tissues showed signs of repopulation with normal cells on Day 5 pi and were characterized by nearly normal histological appearance by Day 6. By Days 5 and 6 pi, the visceral organs, with the exception of the pancreas, were regaining normal morphology. Therefore, viral clearance from extraneural tissues was documented by the disappearance of detectable virus, return to normal structure, and absence of viral RNA in these organs.

Replication of V3000 in the brain

Invasion of the CNS was first detected by virus isolation from the brain on Day 2 or 3 pi, with titers ranging from 3.6×10^4 to 2.2×10^5 PFU/g fresh tissue (Fig. 2). Brain titers reached a plateau around Day 4 at 8×10^6 to 1×10^7 PFU/g, and these levels persisted throughout the subsequent observation period.

Coronal sections of the brain at 2-mm intervals were evaluated. Sections from V3000-inoculated mice showed *in situ* hybridization signal first appearing between 2 and 3 days pi, although no significant histopathological changes were observed through 3 days pi. The earliest detectable VEE-specific *in situ* hybridization signal was localized most often to olfactory tracts and associated nuclei (Fig. 3G).

Beginning on Day 4 pi, mild to moderate indications of neuronal degeneration were observed as well as hyperchromatic cells with vacuolization, mild meningitis, and perivascular cuffing with lymphocyte infiltrate. These increased in severity and magnitude through Day 6 pi at which time cerebral tissues showed severe, disseminated leptomeningitis and encephalitis. Characteristic changes at this final stage of disease included neuronal degeneration, severe perivascular cuffing with lymphocyte infiltrate, and gliosis with activation of microglia and astrocytes (Fig. 3C). Late in infection, viral RNA was disseminated throughout all areas of the cerebrum and brain stem. *In situ* hybridization signal was often seen overlying groups of cells, but was not associated directly with areas of perivascular cuffing. Signal was frequently observed over neurons. Identity of other positive cells in the brain could not be determined definitively based on morphology or distribution. In the cerebellum, isolated positive cells were observed in both the granular and molecular layers, but Purkinje cells were rarely positive.

Virus titers in the eyes, ranging from 1.1×10^4 to 7.8×10^5 PFU/g, were detected on Days 5 and 6. Neuronal cell layers of the retina contained large quantities of viral RNA on Day 6 pi (Fig. 3H). In general, *in situ* hybridization signal was highly localized and not present in all sections examined. These results are consistent with retrograde movement of the virus from the brain to the retina via axons of the optic nerve. Seeding of the retina from the circulation was unlikely, as virus titers in the serum at this time had fallen below the minimum level for detection of 33 PFU/ml.

Comparative virulence of V3000 and site-directed mutants

Defined mutations introduced into the V3000 genetic background had a profound effect on the ability of this virus to cause overt disease (Davis *et al.*, 1991). All the mutants were avirulent or highly attenuated when inoculated peripherally, and V3010 and V3034 were significantly attenuated when injected by the ic route (Table 1). V3032 and V3014 were considerably less attenuated when inoculated directly into the brain. All mice which survived infection by the mutants also survived subsequent ip challenge with 10^4 PFU V3000 without any clinical signs of infection, indicating that the mutants consistently grew *in vivo* to an extent sufficient for induction of a protective immune response.

Effect of mutation on progression of VEE-induced pathogenesis

The *in vivo* replication of the mutants was strikingly different from that of V3000 (Fig. 2). Each of the mutations (or clusters of mutations as in V3014) blocked progression of VEE-induced disease at a different step. The mutants were inoculated sc into the left rear footpad at a dose of 10^3 PFU, and, as in the V3000 experiment, two mice were sacrificed at the intervals indicated.

Movement from the site of inoculation to the draining lymph node. In the case of V3010 (Glu to Lys at E2 76),

FIG. 3. Tissue sections from VEE-inoculated CD-1 mice. Mice were inoculated with 10^3 PFU of V3000 into the left rear footpad, and sections of various tissues were subjected to staining with hematoxylin and eosin (A–C), immunostaining with an anti-VEE antibody, and counterstaining with methylene blue (D, E) or *in situ* hybridization with a VEE-specific riboprobe followed by staining with hematoxylin and eosin (F–H). Histological appearance of left popliteal lymph node at 24 hr is shown with disseminated single cell necrosis and some invading acute inflammatory cells (A). The pancreas at 72 hr shows necrosis of acinar cells but sparing of islets of Langerhans (B). The brain at 6 days pi exhibits neuronal degeneration, perivascular cuffing, and gliosis (C). Detection of VEE antigen in the lymph node at 24 hr pi (D) and the exocrine pancreas at 72 hr pi (E), and detection of VEE RNA in the spleen at 72 hr pi (F), the lateral olfactory tract of the brain at 48 hr pi (G), and the outer and inner ganglion layer of the retina at 144 hr pi (H). Bar, 100 μm.

TABLE 1

Virulence of Molecularly Cloned VEE

Virus	Intracerebral inoculation		Rear footpad inoculation	
	% Mortality	AST ± SE	% Mortality	AST ± SE
V3010 E2 Lys 76	22	7.5 ± 0.7	0	—
V3034 E1 Thr 272	11	6.0 ± 0	11	9.0 ± 0
V3032 E2 Lys 209	89	6.4 ± 0.9	0	—
V3014 E2 Lys 209 E1 Thr 272 E2 Asn 239	100	7.7 ± 0.5	0	—
V3000 (WT)	100	4.7 ± 0.7	100	5.4 ± 0.5

Note. Nine adult, female CD1 mice were inoculated with 10^3 PFU of the indicated viruses derived from molecular clones. Average survival time (AST) for those animals that died was calculated in days ± standard error (±SE) using an observation period of 14 days. All surviving mice were challenged ip with 10^4 PFU of V3000 at 3 weeks pi and showed no clinical signs of disease.

the mutation appeared to restrict VEE replication *in vivo* at a very early stage. In the experiment shown in Fig. 2, V3010 replication at the site of inoculation was not detected (data not shown), and no virus could be isolated from serum or the 15 organs examined until 48 hr pi. At this time, virus was detected only in serum and spleen of each of the two mice sacrificed. In experiments not shown here, an alternative pattern was observed. V3010 movement to the draining lymph node was delayed, but by 24 hr pi, significant levels of virus were recovered from the draining lymph node in approximately 75% of the animals examined (Aronson *et al.*, in preparation). In all experiments, however, progression to viremia and spread to other tissues was delayed and/or sporadic. At 72 hr pi (Fig. 2), virus was isolated from the serum and lymphatic organs (thymus, spleen, both popliteal lymph nodes) of both mice sampled and from pancreas and brain of one of the two animals sacrificed at this time point. During the remainder of the observation period, virus was detected sporadically in different organs, including left and right popliteal lymph nodes, thymus, spleen, pancreas, liver, duodenum, brain, and eye.

Histopathological examination showed that the numbers of necrotic cells in lymph nodes, spleen, and pancreas from V3010-inoculated animals were greatly reduced when compared to tissues from animals inoculated with V3000. Coronal sections of brains from the three V3010-infected animals with detectable brain titers showed mild to moderate neuronal degeneration, gliosis, and perivascular cuffing with lymphocyte infiltrate (data not shown). These lesions were similar, but milder in intensity than those induced by V3000. No significant histopathological lesions were observed in sections of tissues with no detectable virus titers.

These observations suggest that the E2 Lys 76 mutation in V3010 interferes with the progression of VEE disease at a very early stage by disturbing normal movement of the virus from the site of inoculation to the draining lymph node and beyond. Virus recovered from serum and tissues other than the draining lymph node displayed variable plaque morphologies compared to the V3010 inoculum, suggesting that reversion may have allowed occasional dissemination of the virus beyond the draining lymph node.

The pattern of V3014 (E2 Glu 209 to Lys; E2 Ile 239 to Asn; E1 Ala 272 to Thr) was similar in some respects to V3010. After footpad inoculation, appearance of virus in the draining lymph node was delayed by approximately 18 hr. However, progression beyond the draining lymph node was limited to rare isolations from other lymphoid organs and was much less extensive than observed with V3010. If progression beyond the draining lymph node requires reversion, the multiple mutations in V3014 may have reduced the frequency with which reversion events occurred. Histopathological lesions were rare, and where found, were much less severe than V3000. These included a single small focus of necrotic cells in a Peyer's patch of one animal, mild necrosis of the exocrine pancreas, and mildly increased hematopoiesis in spleen.

Mutations blocking other steps in VEE pathogenesis. V3032 is a single mutant at E2 209 (Glu to Lys) which spread normally from the footpad to the draining lymph node and to other lymphoid organs, but did so while producing little or no viremia. This suggests that establishment of a detectable serum viremia is not a prerequisite for peripheral dissemination to lymphoid organs. At least in animals infected with V3032, virus associated with lymphocytes in the draining lymph node may move to other sites as these cells traffic normally to other lymphoid organs. Histopathological lesions following footpad inoculation of V3032 were minimal. Very mild myocardial degeneration, limited to individual muscle fibers, was noted in one animal at 24 hr. Also at this time, increased cell proliferation and inflammatory cells were observed in the draining lymph node and spleen, but there was no significant cell destruction.

Mutant V3034 (E1 Ala 272 to Thr) displayed peripheral replication very similar to that of V3000, although the maximum titers in serum and lymphoid tissues were somewhat lower than observed with virus from the parental clone. Nevertheless, isolation of virus from the brains of V3034-inoculated mice was sporadic, and the titers were lower than observed with V3000. As V3034 causes low mortality after ic inoculation, it is unclear whether infrequent isolation of this mutant from the brain reflects an impaired ability to invade the CNS or reduced competence for replication in the brain. As with the other mutants, pathological damage was very limited. At 72 hr pi,

interstitial edema in the pancreas was seen in one of the animals sacrificed, and a mild to moderate degree of necrosis in the exocrine pancreas was observed in the other animal at this time.

These experiments reinforce the notion that VEE pathogenesis may be interrupted at specific points in the pathogenic sequence by defined mutations in the genome of the infecting virus. In addition, they have delineated at least four critical steps in the sequence: movement from the site of inoculation to the draining lymph node, infection of other lymphoid organs, establishment of viremia, and invasion of the CNS.

DISCUSSION

The objectives of this study were (1) to determine the pathogenesis in the adult mouse model of virulent virus derived from the molecular clone of VEE, pV3000, with particular attention to the route(s) and time course of virus spread in infected animals, (2) to confirm that V3000 is representative of the Trinidad donkey strain of VEE from which it was cloned, and (3) to compare V3000 pathogenesis with that of site-directed avirulent or attenuated mutants derived from it. In addition, the experimental evaluation of VEE pathogenesis reported here differs from previous studies in two important respects. The inoculum virus was administered in a manner which mimicked the natural route of infection by mosquito bite, and inoculation into the footpad selected a defined draining lymph node and allowed examination of the earliest events in virus dissemination.

V3000 is representative of the Trinidad donkey strain of VEE

The VEE clones were derived from the genomic RNA of TRD-E1, which itself was derived from TRD by growth at limiting dilution (Johnston and Smith, 1988). Mortality and AST values for V3000 and TRD-E1 were similar (Johnston and Smith, 1988; Davis *et al.,* 1991), and the organ system pathology and course of V3000 infection *in vivo* was in general agreement with that observed in previous studies of VEE pathogenesis (Kissling *et al.,* 1956; Victor *et al.,* 1956; Gleiser *et al.,* 1962; Tasker *et al.,* 1962; Kundin, 1966; Gorelkin, 1973; Jackson *et al.,* 1991). Lymphoid and other peripheral organ systems were involved in an initial phase of disease, followed by clearance from the periphery, invasion of the CNS, and death due to encephalitis. This suggests that V3000 is generally representative of a majority population of TRD. However, our results differ somewhat from some previous studies (Tasker *et al.,* 1962; Jackson *et al.,* 1991). In the Jackson study, which used the TRD-E1 VEE isolate, virus antigen was demonstrated focally in the islets of Langerhans and in 40% of hepatocytes by Day 2 following ip inoculation of C57BL/6 mice with 1×10^4 PFU. Extensive involvement of these tissues was not detected in experiments with CD1 mice inoculated with either V3000 (this study) or TRD-E1 (Grieder and Johnston, unpublished data) into the rear footpad. Although involvement of the exocrine pancreas was consistently observed, positive immunocytochemical staining for VEE antigen was observed only in occasional islets in the pancreas of one V3000-infected animal on Day 5 pi and in hepatocytes of the other V3000-infected animal on Day 5 pi. Parallel tissue sections analyzed by *in situ* hybridization were negative throughout the infection. Also, no involvement of islet cells or hepatocytes was detected following ip inoculation of 5×10^4 PFU of V3000 into C57BL/6 mice (Grieder and Johnston, unpublished data). Similar inconsistencies may be found in earlier reports in which involvement of islet cells was (Kundin, 1966) or was not observed (Kissling, 1956). In the present case, the differences may be attributable to the properties of V3000 and possibly may be due to the Asn to Ile change at E2 position 239. However, V2000 and V3000, which differ in amino acid sequence only at this position, do not differ with respect to mortality and average survival time in either CD1 or C57/BL6 mice, nor do they differ in the extent of replication in the liver and islets of Langerhans cells of the pancreas in CD1 mice. Liver and pancreas pathology in C57/BL6 mice has not yet been examined.

Comparative pathogenesis of V3000 and site-directed mutants

Inoculation into the left rear footpad facilitated the examination of the earliest events in VEE pathogenesis. Immediately following inoculation, virus could be recovered from the footpad inoculation site in numbers consistent with recovery of inoculum virus. However, recovery of virus in excess of the inoculum level occurred only at 12 or more hr pi. The earliest viral replication was detected in the draining lymph node (at 4 hr pi). This suggests that early replication in the footpad was limited and that little VEE replication was required for movement of the virus to the draining lymph node. A mutation at E2 76 appeared to interfere with this event by preventing or delaying movement of the virus to the draining lymph node. Virus recovered from sites in the pathogenetic sequence "downstream" of the draining lymph node contained a very high proportion of revertants (Aronson *et al.,* manuscript in preparation). This suggests that reversion is required for movement beyond the draining lymph node and that the point of restriction imposed by the E2 76 mutation is at or before the draining lymph node.

Movement to the draining lymph node also was delayed in animals inoculated with V3014, which harbors mutations at E2 209, E2 239, and E1 272. However, the basis for the delay in V3014 movement may not be the same as the basis for the E2 76 phenotype, since V3014 differed from the E2 76 mutant as follows. Recovery of virus at sites beyond the draining lymph node was much

less frequent with V3014, perhaps because it is composed of three mutations rather than one. Also, V3014 gave 100% mortality when inoculated ic, while the E2 76 mutant was significantly attenuated by this route. This suggests that replication of E2 76 may be restricted in numerous cell types within the host.

Within 12 hr of V3000 inoculation, a significant serum viremia was evident, and by 18 to 24 hr, virus replication was detected in all of the lymphoid organs examined. It was assumed previously that the source of the primary viremia was virus replication in the draining lymph node. However, serum viremia in V3032 (E2 209)-infected mice was below the limits of detection, while spread to and replication in other lymphoid organs appeared to be largely unimpeded. This indicates that a serum viremia is not required for seeding of VEE in other lymphoid organs and that replication in these tissues may not be a major source of VEE viremia. The possibility of cell-associated hematogenous spread from the draining lymph node is currently under examination.

V3032 is avirulent from a footpad inoculation yet causes high mortality when inoculated ic. In a preliminary experiment, administration of V3032 by intravenous inoculation increased mortality to about 30% from 0% mortality associated with footpad inoculation. Therefore, the ability of the V3032 mutation to restrict serum viremia may be the basis for its lack of virulence from a footpad site of inoculation.

Clearance of the virus from the serum and peripheral tissues was essentially complete by 4 days pi, and at this time point the lymphoid organs had begun to reassume their normal morphological appearance. The basis for peripheral clearance is not known, but is assumed to be mediated by the immune system through an early classical cellular and/or humoral response, or through cytokine responses in the infected animal. We hope to clarify this issue in ongoing studies of VEE infection of scid and reconstituted scid mice.

Invasion of the CNS

Invasion of the CNS constitutes a second phase of VEE pathogenesis. Virus replication in the brain was detected first at 48 hr pi and rose to high levels by the day of death. Viral replication and pathology within the brain were predominantly associated with cells identified morphologically as neurons. Extensive lymphocyte infiltration and perivascular cuffing suggested that at least a portion of the observed pathology was mediated by a specific cellular immune response.

A possible mechanism of VEE invasion of the CNS was suggested by the initial sites of V3000 replication in the brain. Consistent with the titration data, *in situ* hybridization signal was first detectable in the brain at 48 hr pi. At the earliest times, signal almost always was found in the lateral olfactory tracts and the hippocampus. Often, signal was observed in these areas exclusively. These observations led to the hypothesis that a major route by which VEE gains access to the brain from the circulation is via the olfactory system. The olfactory system has been suggested as the route by which St. Louis encephalitis and eastern equine encephalitis viruses invade the CNS from the circulation (Monath *et al.*, 1983; Sabin and Olitsky, 1937). The anatomical arrangement of the olfactory system makes it particularly vulnerable to viral invasion from the bloodstream. The axons of olfactory receptor neurons lie in close proximity to fenestrated capillaries and project directly back to the olfactory bulb, providing a short, direct path from the periphery to the CNS in a viremic host. At the point where they are most closely apposed to the capillary bed, these axons are unmyelinated. Thus, during the viremic, lymphoid phase of the disease, virus may escape from the capillaries in the nasal neuroepithelium and infect the olfactory sensory neurons, effectively avoiding the blood brain barrier. The possibility of VEE transport to the brain via olfactory nerves is supported by the high susceptibility of the olfactory bulb and tracts to intranasal VEE administration in the *Macaca rhesus* model (Danes *et al.*, 1973a,b) or aerosol administration to mice (Ryzhikov *et al.*, 1991).

At later times after VEE infection, virtually all areas of the brain become involved. Dissemination from neurons of the olfactory system to other areas of the brain can be attributed in part to spread across synaptic connections. It also is likely that the virus invades other nerve tracts in the periphery, such as the trigeminal nerve, which leads to somewhat delayed involvement of other regions of the brain.

The characteristics of the peripheral infection initiated by V3034 (mutation at E1 272) were similar to those of V3000 in that movement to the draining lymph node, establishment of viremia and replication in other lymphoid organs occurred with the same time course. However, virus was recovered infrequently from the brain. Because V3034 displayed very low virulence upon ic inoculation, it is not clear whether V3034 administered in the footpad entered the CNS inefficiently or was simply less capable of replication in the brain.

Intracerebral inoculation of V3014 induced 100% mortality, like V3000, but with an extended survival time. Interestingly, two of the three constituent mutations in V3014, when present as single mutations in the V3000 background, caused less than 100% mortality when inoculated ic (E2 209, 89%; E1 272, 11%). In a genetic sense, this result suggests that the third change, at E2 239, is a dominant neurovirulence mutation. This is consistent with the fact that the Asn at this position in V3014 is the same amino acid as in the virulent TRD strain. At a structural level, it is likely that virulence phenotype is determined by the overall conformation of the virion spikes as well as by specific domains on the two individual glycoproteins which comprise them. Therefore, the

phenotype of a mutant containing three glycoprotein mutations is not necessarily the summation of the individual phenotypes but could represent a conformation of the glycoprotein spikes, and hence a phenotype, not evident in any of the individual mutants.

Implications for vaccine design

The experiments reported here have implications for the design of live virus vaccines for VEE. Clearly, the choice of mutations for inclusion in such vaccines would be limited to those which block progression of the infection prior to the point where disease symptoms would be evident. Also, the level of viremia in vaccinees is of particular importance in the design of vaccines for viruses transmitted by mosquito vectors. Mutations which result in a very low level viremia are much less likely to be transmitted.

Mutational interdiction of VEE pathogenesis

The pathogenetic events following experimental infection of mice with VEE follow a specific sequential program ending in encephalitis and death. We have examined the pathogenesis of VEE in genetic terms, an approach pioneered in investigations of reovirus, lymphocytic choriomeningitis virus, and poliovirus pathogenesis (Spriggs *et al.,* 1983; Ahmed and Oldstone, 1988; Evans *et al.,* 1985). In such an approach, VEE pathogenesis is considered an interactive genetic system composed of the virus on one hand and on the other, the cells of each organ system in which the virus replicates. Much as one might expect of a biochemical pathway, specific mutations in the VEE genome, which result in a highly attenuated or avirulent phenotype, block the progression of disease at specific points in the pathogenetic sequence. These include the movement of virus from the site of inoculation to the draining lymph node, movement from the draining lymph node to other organs, establishment of viremia, and invasion of, or replication within, the CNS. By determining the basis for restriction in each case, the process of disease induction by VEE can be understood more clearly at an organismal, cellular, and molecular level.

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