NOTE TO USERS

DISSERTATION

EARLY PATHOGENESIS

OF

VENEZUELAN EQUINE ENCEPHALITIS VIRUS

INFECTION IN HORSES

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MAX L TEEHEE ENTITLED EARLY PATHOGENESIS OF VENEZUEALEN EQUINE ENCEPHALITIS VIRUS IN HORSES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE FO DOCTOR OF PHILOSOPHY.

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ABSTRACT OF THESIS

EARLY PATHOGENESIS OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS INFECTION IN HORSES

In horses, it is not known which tissues support Venezuelan equine encephalitis virus (VEEV) replication during the early stages of disease, nor how the virus gains access to the CNS. My research focused on the early viral titers in tissues, the interferon response in horses and diagnosis of VEEV viremia by a quantitative RT-PCR.

Two horses each were challenged with the virulent Trinidad donkey strain of VEEV, and two sacrificed at 24, 48, 96 and 144 hours post challenge. Viremic titers peaked at $10^{3.8}$ to $10^{4.5}$ PFU/mL between 60 and 72 hours after challenge. VEEV titers peaked in the draining lymph nodes ($10^{3.9}$ to $10^{6.1}$ PFU/g tissue) at 24 to 48 hours. Highest tissue titers were noted in the bone marrow ($10^{6.2}$ to $10^{8.6}$ PFU/g) at 48 and 96 hours, the olfactory bulb ($10^{6.5}$ PFU/g) and tract ($10^{6.8}$ PFU/g) at 96 hours post challenge. Virus was detected in the dental pulp ($10^{2.8}$ to $10^{5.1}$ PFU/g), trigeminal nerve ($10^{2.9}$ to $10^{3.6}$ PFU/g), and olfactory tract and bulb, prior to detection of virus in the cerebrum. This data indicates the virus replicates in the lymph nodes and bone marrow, prior to entry into the CNS through the olfactory and trigeminal nerves.

The serum interferon (IFN) responses were assayed in horses challenged with four strains of VEEV, the TrD strain, a non-virulent IE strain, a virulent IE strain, and the V3526 vaccine strain. IFN response was greatest (320-1280 IU/mL) for the TrD strain challenge. The initiation, peak, and length of serum IFN titers in horses

challenged with TrD correlated with the initiation, peak and length of viremic titers. Virulent IE VEEV challenge of three horses resulted in no detectable serum IFN response, although clinical illness was severe in the horses. The avirulent IE and V3526 VEEV strains resulted in a low, transient but detectable IFN response. Results of the quantitative RT-PCR indicate viral antigen can be detected in horse sera at titers as low as 2.0 PFU/mL.

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CHAPTER I

LITERATURE REVIEW

INTRODUCTION

Venezuelan equine encephalitis is an important arthropod-borne encephalitic disease of equids in the Americas, with the ability to cause sickness and death in humans. Numerous epizootics during the past 90 years have caused widespread morbidity and mortality in humans and horses. There has been great economic impact on small rural communities and farms that rely upon horses and human labor. Additionally, the Venezuelan equine encephalitis virus (VEEV) easily causes infection in humans by the aerosol route, and thus has the potential for use as a biological weapon. While much information has been gleaned from the study of this disease in the past 80 years, many questions remain. Continued research is warranted to develop better vaccines, understand the source of epizootics and develop effective treatments.

The ultimate goal of research on Venezuelan equine encephalitis is to eliminate the disease from animals and humans. This can be accomplished only through the better understanding of the virus and the disease.

HISTORY OF VENEZUELAN EQUINE ENCEPHALITIS DISEASE AND RESEARCH

Since at least the early 1800s a severe, fatal encephalitic disease affected horses, and probably humans, in the Americas. The first known record of this disease in horses described an outbreak in Massachusetts in the summer of 1831, when over 75 pasture horses died. Speculation in 1831 was that a type of forage, possibly a plant toxin, caused the disease (Hanson, 1957). Infectious agents had not yet been discovered or described at this early time. Additional outbreaks of encephalitic disease were recorded between 1845 and the early 1900s, on the east and west coasts of North America and in Argentina.

By the late 1800s scientists had only begun to recognize the existence of viruses, which initially were called filterable disease agents. In 1898 the foot and mouth disease agent became the first animal virus isolated and described when it was isolated by filtration and then re-inoculated into animals (Loeffler & Frosch, 1898). Another breakthrough in understanding arboviruses occurred in 1900 when the yellow fever agent was shown to be transmitted by *Aedes* mosquitoes (Reed et al, 1901). In 1912, a widespread encephalitic epidemic in horses occurred in central and western North

America with an estimated death toll of 30,000 horses (Udall, 1913; Meyer, 1933; Sabattini et al, 1985). About this time, virus research was further advanced when white mice were found to be susceptible to many viruses by intracerebral inoculation, and the subsequent development of the virus neutralization test (Chamberlain, 1987). Since at least the 1920s, reports of widespread seasonal neurologic equine disease in the northern areas of South America suggest epizootics of equine encephalitis disease spread by arthropods (PAHO, 1972).

In the 1930s, scientists began to unravel the mystery of the equine encephalitic diseases. In 1930, an outbreak of encephalitic disease affecting at least 6000 horses, with a fatality rate of close to 50%, hit the San Joaquin Valley in California (Meyer et al, 1931). Brain tissues from dead horses were collected and a filterable infectious agent was isolated (Meyer et al, 1931). The next two years saw repeat outbreaks of this disease in horses in the western United States, and as far east as South Dakota, where more filterable agent was isolated (Giltner & Shahan, 1933). In the east coast states of New Jersey, Maryland and Virginia, an apparently similar disease struck, but this one had a nearly 90% equine death rate (Gillespie & Timoney, 1981). Again a filterable disease agent was isolated. These filterable agents were called separately, the California, South Dakota, and Maryland strains of equine encephalomyelitis. A cross challenge of guinea pigs, protected with antiserum from either the South Dakota, California or Maryland strains made it evident that two antigenically different strains were involved in the outbreaks (Giltner & Shahan, 1933). This initiated reference to the strains as the eastern and western strains. About the same time the eastern and western encephalomyelitis viruses were shown to be transmissible by *Aedes* species mosquitoes

in the laboratory (Kelser, 1933; Merrill, 1934). Unfortunately, this diverted investigators to looking at only *Aedes* species mosquitoes during epidemics, and virus could not be found in the mosquitoes. Then, to add to the confusion, encephalitic viruses were isolated from the kissing bug, *Triatoma sanguisuga* (Kitselman & Grundmann, 1940) and also found to be transmitted by the Rocky Mountain spotted fever tick, *Dermacentor andersoni* (Syverton & Berry, 1936). About this time, formalin-inactivated vaccines were made with the isolated eastern and western equine encephalitis viruses (Shahan & Giltner, 1934; Beard et al, 1938) and used in humans and horses, but these early vaccines lacked purity and could cause numerous reactions in the recipient.

Records from 1936 describe an epidemic of severe encephalitic disease in horses on the Guajira peninsula in Venezuela, South America. A filterable virus was isolated and Koch's postulates were soon satisfied when this isolated virus caused the same disease when inoculated into healthy horses, and the virus was again recovered from these newly challenged horses (Kubes & Rios, 1939). This filterable virus was shown to be antigenically different from both the eastern and western North American viruses although it caused similar disease in horses (Beck & Wyckoff, 1938; Kubes & Rios, 1939). Formalin-inactivated vaccines were made with the new virus as had been done with the eastern and western encephalitis viruses. These vaccines gave good protection in guinea pigs against challenge with the virus isolated from Venezuela, but the vaccine did not protect against eastern encephalomyelitis (Beck & Wyckoff, 1938). About this time the three diseases were initially referred to by their present names, western equine

encephalomyelitis (WEE), eastern equine encephalomyelitis (EEE) and Venezuelan equine encephalomyelitis (VEE).

In 1938, the eastern and western strains of equine encephalomyelitis viruses were recovered from brain tissues and sera of humans who died of encephalitic disease (Fothergill et al, 1938; Webster & Wright, 1938; Howitt, 1938A). This was the first reported isolation of an equine encephalitic virus from humans, although K.F. Meyer had considered the possibility in 1932 after two men had become ill, one fatally, both associated with encephalitic horses (Meyer, 1932). Thus, in the span of ten years, the eastern, western and Venezuelan encephalitic viruses were recognized and isolated, discovered to be transmitted by arthropods, found to be antigenically different, determined to infect humans, and researchers developed an effective vaccine.

In 1941, more evidence was obtained to suggest that only mosquitoes served as vectors for transmission of the equine encephalitic viruses in nature. From 1939 to 1941 a large epidemic of western equine encephalitis (WEE) covered much of the western states, with St Louis encephalitis (SLE) and WEE outbreaks occurring at the same time in the state of Washington. In the summer of 1941, during this epidemic in the Yakima Valley of Washington, over 15,000 biting arthropods were collected, and isolates of SLE and WEE viruses were obtained only from *Culex tarsalis* mosquitoes (Hammon et al, 1941B). This was made possible by use of dry ice as both an attractant and a cold preservative. This was the first reported isolation of virus from naturally infected mosquitoes, although additional isolations occurred on two other continents, nearly simultaneously, with two different viruses. These two viruses, yellow fever and Japanese encephalitis viruses, were isolated from naturally infected mosquitoes in

Africa and Japan, respectively (Mahaffy et al, 1942; Mitamura et al, 1950). Additionally, it was soon realized that the natural distribution of *Culex tarsalis* coincided with the known distribution of WEE and SLE (Jenkins, 1950). The same year, outbreaks of encephalitis in humans in Canada and Colorado were first diagnosed with the complement fixation test allowing for the diagnosis of recent exposure to the disease by a rapid test (Casals & Palacios, 1941). Also in 1941, a sero-survey of birds and mammals in the Yakima Valley area was able to detect antibodies in wild and domestic animals. This study provided evidence that WEE and SLE viruses were more wide-spread than generally suspected (Hammon et al, 1941).

Meanwhile, in the early 1940s, VEE epidemics repeatedly swept across South America. In 1942, the virus was recovered in Colombia (Soriano Lleras & Figueroa, 1942). One epizootic of VEE swept across Venezuela and eventually reached the island of Trinidad, in 1943, causing a large number of equine losses and death in humans (Randall et al, 1944; Kubes, 1944; Tigertt & Downs, 1962). This was the first time naturally-acquired VEEV infection in humans was recognized and described. The VEEV isolated from the brain of a donkey that died in Trinidad in 1943 became known as Trinidad donkey strain. A few years later, VEEV was recovered in Ecuador (Sotomayor, 1946). It became apparent that the range of VEE epidemics covered a wide area of northern South America. These different isolates of VEEV all appeared to cause the same disease in humans and horses, so all isolated agents were thought to be the same strain of virus.

Also in the 1940s, numerous investigators were accidentally infected with VEEV in the laboratory. The first known human case of VEE, due to laboratory

exposure, was described in 1943 (Casals et al, 1943). By 1947, at least twelve more human laboratory infections had been recorded. These laboratory infections usually caused a debilitating, but self-limiting illness (Lennette et al, 1943; Koprowski & Cox, 1947). Also in 1949, the first purified VEE vaccine grown in chicken embryo cells was prepared (Randall et al, 1949). This permitted the production of vaccines that were more efficient and resulted in less reaction in the recipient.

The 1950s saw continued outbreaks of VEE in South America, several scientific discoveries and the weaponization of the VEEV. In 1952 another major epidemic of VEE occurred in Colombia. During this epidemic a second naturally acquired human illness in Colombia was reported, which confirmed that humans were susceptible to natural infection (Sanmartin-Barberi et al, 1954). In 1954, the arboviruses were divided into three groups, depending on their cross-reactivity in the hemagglutination inhibition test (Casals & Brown, 1954). Group A arboviruses consisted of the EEE, WEE and VEE viruses. Group B viruses included dengue, St Louis encephalitis, and yellow fever viruses, which cross-reacted with each other. Remaining viruses that were non-reactive with other arboviruses were classified as Group C. As more became known about viruses, the group A arboviruses became the Alphavirus genus within the Togaviridae family. In 1954, another major epidemic of VEE involving horses and humans occurred in Colombia (Sanmartin-Barberi et al, 1954), and in 1956, birds were shown to be susceptible to VEE infection (Chamberlain et al, 1956). Military planners in the late 1950s realized the aerosol infection capabilities of VEEV, and the virus was developed for use as a biological weapon by both the Soviet Union and the United States.

Research on VEEV continued in the 1960s and 1970s. A live attenuated vaccine, TC-83, was developed in 1961 for use in laboratory workers (Berge et al, 1961; McKinney et al, 1963). This vaccine represents the 83rd passage of the Trinidad donkey strain of VEEV in guinea pig heart cells. Evidence suggested VEEV was endemic in Trinidad when VEEV was isolated from wild rodents and mosquitoes and 20% positive of a sample of humans less than 15 years old were found to be seropositive for VEEV. This also suggested that rodents and mosquitoes were involved in the endemic cycle in Trinidad (Downs et al, 1962).

Numerous experimental infections of domestic animals were conducted in the late 1950s through the early 1970s to determine the pathogenesis of VEEV infection and the ability of animals to serve as amplifiers of the virus. The comparative pathology of VEEV infection in various mammals (mice, Rhesus monkeys, burros, guinea pigs) was examined (Gleiser et al, 1962). Experimental VEEV infection of horses determined they could be infected by aerosol challenge, in addition to subcutaneous injection, and that horses could serve as amplifying hosts (Kissling et al, 1956). Additionally, the magnitude, duration and kinetics of viremia were determined in horses (Dietz et al, 1978; Kissling et al, 1956; Henderson et al, 1971; Walton et al, 1973). Experimental infections suggested that dogs were susceptible to VEEV infection and potentially could transmit the virus to mosquitoes (Davis et al, 1966; Bivin et al, 1967), but natural infections in dogs have not been found. Challenge of North American mammals with VEEV suggested that several species of rodents were highly susceptible and could potentially serve as reservoirs or amplifying hosts (Bowen, 1976).

Meanwhile, VEE epizootics occurred in either northern South America or Central America every year between 1962 and 1973, with the exception of 1965. VEEV was isolated from sick human patients, wild birds and field rodents in Panama in 1961 and 1962 (Grayson & Galindo, 1968). In 1962, an outbreak of VEE in Venezuela and Colombia resulted in an estimated 32,000 human cases, of which 1199 exhibited neurological signs and 190 died (Sellers et al, 1965). VEEV was also isolated in Florida from mosquitoes and antibodies to the virus were found in cotton rats (Chamberlain et al, 1964), and Seminole Indians (Work, 1964). In 1967, another VEEV epizootic in Colombia extended, over the next few years, into Ecuador, Venezuela and Central America, until it reached the southern border of Texas in 1971 (PAHO, 1995). This outbreak resulted in an estimated 38,000-50,000 horse deaths, and over 320,000 human cases of VEEV infection.

In 1969, the VEE viruses were classified into four major categories (I-IV), and those in category I were further classified into subtypes IA through IE based on hemagglutination inhibition (HI) assays (Young & Johnson, 1969). In 1971, the Florida strain of VEEV was shown to be avirulent for horses, with little or no viremia present (Henderson et al, 1971), and in 1972, further evidence indicated that only the IA, IB, and IC strains caused encephalitis in experimentally challenged horses (Walton et al, 1972; Walton et al, 1973; Dietz et al, 1978). Shortly afterwards, the IA and IB strains of were found to be undistinguishable antigenically and combined into a single IAB strain (Johnson & Martin, 1974).

Research on VEEV during the last 25 years produced additional discoveries, and during that time period there was a long absence of VEE epizootics. The Cabassou

strain was isolated and became VEE subtype V (Digoutte & Girault, 1976). The minimum infectious dose of IE VEEV for *Culex taeniopus* was reported at less than 5 plaque forming units (Scherer et al, 1981). A new VEEV strain, isolated in Argentina, was indicated by hemagglutination-inhibition test to represent a new subtype of VEEV, subsequently classified as subtype VI (Calisher et al, 1985). The 1971 IAB epizootic strain in Texas was shown to be molecularly similar to the 1943 Trinidad donkey (TrD) isolate, which was used in the production of formalin-inactivated vaccines. This suggested the epizootic virus that reached Texas in 1971 may have originated from, or been sustained by, incomplete inactivation of TrD VEEV used in the vaccines (Kinney et al, 1992). This hypothesis was further supported by genetic similarities between the TrD strain and the 1971 Texas strain (Weaver et al, 1999).

In the late 1980s, it was suggested that epizootic VEE viruses had become extinct (Walton & Grayson, 1988), since the last report of epizootic VEEV activity occurred over 15 years previously on the Guajira Peninsula of Venezuela (Oberste et al, 1998). Then from 1992 to 1996, new outbreaks of epizootic VEE were reported in Venezuela, Colombia, and Mexico. The 1992 outbreak in Venezuela involved the IC subtype, caused 12 equine deaths and 39 human febrile cases (PAHO, 1995; Rico-Hesse et al, 1995). In 1993, there were 55 human cases and 66 equine cases of VEE in Venezuela. Also in 1993, an epizootic of VEE in Mexico affected 125 horses with a 50% case fatality rate. This small outbreak in Mexico represented the first confirmed equine outbreak attributed to VEEV subtype IE (Oberste et al, 1998; Gonzalez-Salazar et al, 2003). Then in 1995, over 25,000 human cases, with 42 deaths and over 504 equine cases with 474 deaths, were attributed to a IC VEEV epizootic in Venezuela and

Colombia (PAHO, 1995; Weaver et al, 1996). The same year, another small outbreak occurred in Mexico with the IE subtype (Oberste et al, 1998; Gonzalez-Salazar et al, 2003). In all four epizootics, the TC-83 vaccine was utilized to prevent the spread of the disease. An additional small outbreak of ID VEEV was reported in humans in Peru in 1994, with three confirmed clinical cases and eight cases of seroconversion (Watts et al, 1997; Oberste et al, 1998).

Currently, questions remain about the source of epizootic VEE viruses, IAB and IC, and mechanisms of persistence between epizootics. As mentioned earlier, molecular and genetic evidence suggested that the 1971 VEEV epizootic that reached Texas had originated from incomplete inactivation of the TrD virus used in vaccines. Additional phylogenetic and genetic analyses indicate the multiple IC epizootics in the 1990s probably arose from one lineage of enzootic ID subtype of VEEV (Kinney et al, 1992; Powers et al, 1997; Wang et al, 1999).

Recent research includes the search for better vaccines, the route of CNS invasion in mice infected with VEEV, and potential treatments for VEEV infections.

THE VIRUS

Classification

Venezuelan equine encephalitis virus is a member of the family *Togaviridae*. This name was derived from the Latin "toga" for cloak, because of the apparent cloak formed by the envelope around the virus, when viewed under an electron microscope.

Some other viruses with envelopes, such as flaviviruses were initially included in *Togaviridae*, but eventually reclassified based on differences in serological cross-reactivity and genome organization. *Togaviridae* is one of several virus families along with *Arteriviridae*, *Caliciviridae*, *Coronaviridae*, *Flaviviridae* and *Picornaviridae* that are single-stranded, positive sense, RNA viruses. The *Togaviridae* family includes the Alphavirus and Rubivirus genera. The members of the Alphavirus genus are all arthropod borne, although not all arthropod borne viruses are in the genus Alphavirus. Inclusion into the alphavirus genus is based upon serological cross-reactivity with one of the current alphavirus members (Calisher & Karabatsos, 1988; Porterfield, 1980). There are currently seven serogroups in the alphavirus genus (Calisher & Karabatsos, 1988). These are the Barmah Forest (BF) complex; the eastern equine encephalitis (EEE) complex; the Middelburg (MID) complex; the Ndumu (NDU) complex; the Semliki Forest (SFV) complex; the Venezuelan equine encephalitis (VEE) complex and the western equine encephalitis (WEE) complex. As of the 2000 ICTV meeting, twenty-six viruses were placed in the alphavirus genus.

Genome

The genomes of members in the family *Togaviridae* range in size from 9.7 kilobases (kb) for rubella virus to 11.8 kb for Sindbis virus. The genome of TrD VEEV is 11,447 nucleotides (nt) in length (Kinney et al, 1989; Meissner, 1999). The 5' end of the togavirus genome is capped by 7-methylguanosine and the 3' end is polyadenylated. All alphaviruses have untranslated regions (UTR) at both ends of the genome. In the TrD strain of VEEV, the 5' and 3' UTRs are 44 and 121 nucleotides in length,

respectively (Strauss & Strauss, 1994; Kinney et al, 1989). These UTRs are important in regulating the replication and translation of the virus genome. The genome can be divided into two general regions: the non-structural genes (nsP1 through nsP4) on the 5' two-thirds of the genome (Figure 1.1), and the structural genes on the 3' one-third of the genome (Trent et al, 1979; Rice & Strauss 1981; Strauss et al, 1984). The structural genes include the capsid (C), E1, E2, E3 genes and a small 6K gene. The order of the genome from the 5' end is: cap, UTR, nsP1, nsP2, nsP3, nsP4, junction, capsid (C), E3, E2, 6K, E1, UTR, polyA tail (Table 1.1).

Function of Gene Products

The functions of the alphavirus nonstructural gene products are an area of current investigation, some functions have been deduced, mainly from studies with Sindbis virus (SINV) and Semliki Forest virus. In SINV, essential viral replicase and transcriptase components appear to be synthesized initially as two polyproteins, P1234 and P123 (Strauss & Strauss, 1994). These two polyproteins synthesize genome-length plus and minus strand RNA (P1234) and a subgenomic 26S mRNA (P123). Additional functions of the individual proteins have been deduced. The protein expressed by nsP1 appears to initiate and/or continue synthesis of minus-strand RNA (Wang et al, 1991) and is also involved in capping the genomic and subgenomic RNAs during transcription (Ahola et al, 2000; Mi et al, 1989; Scheidel & Stollar, 1991). This protein also affects the cytoskeleton of virus infected cells (Laakkonen et al, 1998). The nsP2 protein functions as a helicase involved in unwinding the RNA duplex (Gomez de Cedron et al,

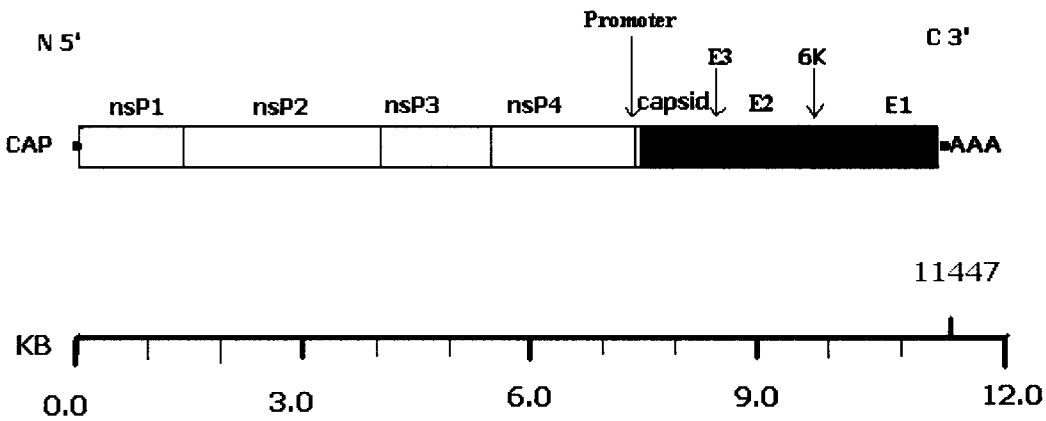

Figure 1.1. TrD VEEV Genome. Representation of the genome of TrD strain of VEEV drawn approximately to scale. CAP = 7 Methyl cap. AAA = polyA tail. nsP = non structural protein (Kinney et al, 1989; Meissner et al, 1999).

Table 1.1
VEEV TrD

	UTR	nsP1	nsP2	nsP3	nsP4	NT	Prom	C	E3	E2	6K	E1	UTR PolyA
Number of nts	44	1605	2382	1671	1821	8	30	825	177	1269	168	1326	121
Position													
Starting #	1	45	1650	4032	5703	7524	7532	7562	8387	8564	9833	10001	11327
Ending #	44	1649	4031	5702	7523	7531	7561	8386	8563	9832	10000	1326	11447

Table 1.1. VEEV TrD genome with gene position indicated in nucleotide number position. Nts = nucleotides; UTR = Untranslated region; nsP = non-structural protein; Prom = Promoter; C = Capsid; PolyA = Poly A tail (Kinney et al, 1989; Meissner et al, 1999).

1999) during transcription. It is also required for the synthesis of the 26S subgenomic mRNA, probably in the initiation of transcription (Suopanki et al, 1998). nsP2 is also the proteolytic enzyme that cleaves the non-structural polyprotein into the individual proteins during replication (Ding & Schlesinger, 1989; Hardy & Strauss, 1989). The functions of nsP3 are not completely understood, but it provides an essential function for viral RNA synthesis including minus-strand and subgenomic 26S mRNA synthesis (De et al, 2003; LaStarza et al, 1994). The nsP4 acts as the viral polymerase (Sawicki et al, 1990; Hahn et al, 1989), affects host cell-dependent replication (Fata et al, 2002) and is highly regulated in the infected cell (Strauss et al, 1983; de Groot et al, 1991). In Sindbis virus, synthesis of minus-strand RNA templates is regulated by the early formation of P123 and nsP4 protein complexes, which are important in replicating minus-strand RNA. Later in replication, accumulation of proteinase nsP2 processes the P123 protein into the separate nsP1, nsP2 and nsP3, which complex with nsP4 and can only synthesize positive stranded genomic RNA (Lemm & Rice, 1993; Lemm et al, 1994).

Structural proteins include the capsid (C) protein which is 275 amino acid residues in length in VEEV and can be divided into two distinct domains. The 96 residues on the N terminus are basic, and presumed to bind the viral genomic RNA (Rice and Strauss, 1981). The highly conserved C terminus acts as a proteinase, promotes capsid-capsid interactions during assembly and interacts with the cytoplasmic tail of the E2 glycoprotein. The E3 protein starts out as an E3/E2 complex (PE2) before cleavage into the separate E2 and E3 proteins. In Semliki Forest virus, the E3 protein is shed from the surface of the infected cell after cleavage in the trans-golgi. In SINV, the

E3 protein appears to be retained within the E1/E2 heterodimer complex, although the function is still unknown (Paredes et al, 1998). The E2 protein is a component of the trimer-dimer projections (discussed in the next section) on the surface of the virion. During assembly, the trans-membrane E2 protein acquires two or three N-linked glycosyl residues. The intracytoplasmic region has a second stretch of hydrophobic amino acids, which may serve to tether the protein to the inner surface of the membrane. The E1 protein is also a component of the trimer-dimer projections on the surface of the virion that are important in binding and fusion (Dubuisson & Rice, 1993). Fusion is accomplished by a conserved stretch of hydrophobic amino acids toward the N-terminal of E1. During translation, it acquires one or two N linked glycosyl residues with a short intracytoplasmic tail.

The small 6K protein is important in virus assembly (Ivanova et al, 1995) and in folding of the E1-E2 heterodimers (Yao et al, 1996). It also serves as a signal peptide for translocation of E1 (Melancon & Garoff, 1986), and is important for budding (Liljestrom et al, 1991). Additionally, it may interact with the E2 protein in the envelope (Strauss et al, 2002) although only small amounts of this protein are incorporated into the virion (Lusa, et al, 1991).

Structure

All Togaviridae viruses are 60-70nm in size, with an icosahedral T=4 symmetry (Paredes et al, 2003; Harrison et al, 1971; Paredes et al, 1993) and 80 trimeric spikes projecting out from the envelope surface (Paredes et al, 2001; Mancini et al, 2000; Rice & Strauss, 1982; Ziemiecki & Garoff, 1978). The icosahedral symmetry is based on

240 repeats of the capsid (C) protein that make up the nucleocapsid with the RNA genome inside (Pletnev et al, 2001; Mancini et al, 2000). The envelope of the virus consists of a lipid bi-layer derived from the host plasma membrane with embedded E1 and E2 viral glycoproteins. The E1 and E2 glycoproteins form into heterodimers, three heterodimers form a trimer group, or trimer-dimer, which project as trimeric spikes on the surface of the virion. There are 80 spikes, or 240 copies of the E1/E2 heterodimers, in the mature virus envelope (Mancini et al, 2000; Adrian et al, 1984; Harrison, 1986). Each of the 240 heterodimers interacts individually with a copy of capsid protein in the underlying nucleocapsid (Mancini et al, 2000; Owen & Kuhn, 1997; Skoging et al, 1996). The trimer-dimer group is held together by disulfide bonds between the three E1 glycoproteins (Ziemiecki et al, 1980). Multiple trimer-dimers form into 30 hexamers and 12 pentamers, which form the 20 triangular faces of the icosahedron (Paredes et al, 2001; Anthony & Brown, 1991). While the viruses in *Togaviridae* may have similar capsid protein components, these proteins in VEEV and SINV nucleocapsids differ in pentameric orientation (Paredes et al, 2001).

In SINV, the 80 E1/E2 spikes project out about 50 Å above the envelope membrane (Paredes et al, 2001). These spikes are important in attachment, fusion with and entry into the target cell, as will be explained later. The E1 and E2 glycoproteins that make up the spike appear to wrap around each other in an anti-clockwise direction (Paredes et al, 2001). At the tip of the spike the glycoproteins separate into a tripartite head. The most distal part of the tripartite head contains domains of the E2 glycoprotein. These domains of the E2 glycoprotein have epitopes recognized by monoclonal antibodies (Smith et al, 1995).

Attachment and Entry

Understanding the mechanisms for attachment and entry of the alphavirus virion to the host cell is an area of active investigation. Important features include the 80 trimer-dimer spikes, cell surface molecules, low pH of endosomes, and fusion of membrane lipids. Determining the details of attachment and entry of VEEV has been complicated since the virus can infect numerous mammallian, avian and mosquito cells.

Entry of alphaviruses into the target cell is accomplished by endocytosis, a low pH in the endosome and conformational change in the E1-E2 trimer. Alphaviruses take advantage of receptor-mediated endocytosis to gain entry into cells (DeTulleo & Kirchhausen, 1998; Marsh & Helenius, 1989). Conformational changes in the SINV trimer-dimer spike are evident after attachment and exposure to low pH (Flynn et al, 1990; Meyer et al, 1992). Low pH leads to a dissociation of the E1 and E2 heterodimer and subsequent trimerization of E1 proteins (Wahlberg & Garoff, 1992). The conformational changes in SINV trimer-dimer expose new epitopes (Wahlberg & Garoff, 1992), and allows E1 glycoprotein mediated fusion of the viral envelope with cellular membranes containing cholesterol and sphingolipids (Lu et al, 1999; Smit et al, 1999; Kielian et al, 1996). Fusion allows entry of the nucleocapsid into the cytoplasm, with subsequent uncoating of the RNA after binding to ribosomes (Singh & Helenius, 1992; Wengler & Gros, 1996).

Research with Semliki Forest virus suggests that the E1 glycoprotein in the envelope mediates virus fusion to the target membrane (Kielian et al, 1996). Other evidence suggests the E2 glycoprotein is important in attachment as mutations to the E2

glycoprotein of SINV prevented viral binding to vertebrate cells (Dubuisson & Rice, 1993). Monoclonal antibodies against the E2 glycoprotein neutralized infectivity and virulence in mice (Tucker & Griffin, 1991; Schoepp & Johnston, 1993). Monoclonal antibodies against residues 170-220 of the E2 spike glycoprotein neutralized the infection of SINV in chick cells (Strauss et al, 1991).

Several receptors on the host cells have been proposed for VEEV and other alphaviruses. Major histocompatibility complex (MHC) class I has been identified as a possible receptor for Semliki Forest virus (SFV) (Helenius et al, 1978). But SFV can enter cells without MHC class I, so this receptor is not necessary for all alphaviruses (Oldstone, 1980). SINV can bind to laminin receptors on baby hamster kidney cells (Wang et al, 1992), and it has been shown that VEEV and SINV can use these receptors on mosquito (C6/36) cells (Ludwig et al, 1996; Wang et al, 1992). Yet, SINV can also bind to avian cells, without the need for laminin receptors (Wang et al, 1992). Heparan sulfate, a ubiquitous glycosaminoglycan found on the outside of many cells in many species is under investigation in initial binding of alphaviruses (Byrnes & Griffin, 1998). Yet, heparan sulfate, which binds SINV, is not required for infection by Ross River virus (Byrnes & Griffin, 1998) and binding to heparan sulfate may be an *in vitro* adaptation (Klimstra et al, 1998). In addition to these receptors, fusion is promoted by the presence of cholesterol and sphingolipids for Semliki Forest virus (Kielian et al, 2000; Wilschut et al, 1995). It may be that alphaviruses are able to bind several different receptors on different species, or maybe co-receptors are required for attachment, or maybe a combination of both (Griffin, 2001).

Replication

In single-stranded, positive sense RNA viruses, the genome acts directly as the mRNA after uncoating in the cytoplasm (reviewed in Strauss & Strauss, 1994; Schlesinger & Schlesinger, 2001). In alphaviruses, the non-structural genes of the positive sense genome are translated into a polyprotein and the polyprotein processed into functional proteins. Translation starts near the 5' genomic terminus and continues through the non-structural genes to three stop codons just prior to the subgenomic RNA portion of the genome. The autoproteolytic activity of the nsP2 releases the nsP2 protein from the polyprotein (Ding & Schlesinger, 1989; Hardy & Strauss, 1989; Lemm et al, 1994). In alphaviruses, assembly of RNA replicase complexes occurs on the modified cytoplasmic surfaces of membrane vesicles (Barton et al, 1991; Froshauer et al, 1988). The non-structural proteins P123 and nsp4 replicate full-length complementary or negative strands of the genomic RNA (Lemm & Rice, 1993; Lemm et al, 1994). These negative strands serve as templates for the replication of both positive sense subgenomic 26S RNA strands and full length genomic RNA strands. The nsP2 protein is important in the initiation of subgenomic RNA replication with subsequent production of structural proteins (Suopanki et al, 1998). Early in replication, synthesis of negative sense RNA is favored; later in replication the P123 polyprotein is processed into separate proteins which favors the formation of the positive sense genomic strands and subgenomic mRNA (Lemm & Rice, 1993; Lemm et al, 1994) in preparation for assembly.

The 26S subgenomic strand is translated to structural polyproteins as early as 2-3 hours after infection. Host ribosomes initiate translation near the 5' end of the 26S

subgenomic strand. The first structural protein translated on the subgenomic polyprotein N-terminus, is the capsid protein which possesses serine protease activity and releases itself from the polyprotein (Strauss & Strauss, 1990). As the remainder of the polyprotein is translated and translocated into the lumen of the endoplasmic reticulum, it is cleaved by host-cell signalase (Liljestrom & Garoff, 1991). Four products are then present in the endoplasmic reticulum; the capsid, a PE2 protein, a 6K membrane-embedded protein and the E1 glycoprotein. The latter three products move together through the secretory vesicles from the endoplasmic reticulum to the cellular plasma membrane. The PE2 and E1 glycoproteins both have hydrophilic trans-membrane domains which remain in the lumen, hydrophobic membrane embedded domains, and short cytoplasmic portions. The majority of the 6K protein is hydrophobic and remains embedded in the ER membrane. These locations are important, since during transportation through the secretory vesicles, the host cell enzymes cause several post-translational modifications on the lumenal domains (Keegstra et al, 1975). One important modification is the cleavage of PE2 into E2 and E3 glycoproteins by a host cell furin-type enzyme (Watson et al, 1991).). This allows the E1 and E2 glycoproteins to form dimers and trimer-dimers, while still in the endoplasmic reticulum (Doms et al, 1993; Jones et al, 1977; Mulvey & Brown, 1994), before delivery to the plasma membrane (Jones et al, 1977). In Semliki Forest virus, the E3 glycoprotein does not remain with the virion but is shed from the plasma membrane, while in Sindbis the E3 glycoprotein remains with the virion (Gaedigk-Nitschko & Schlesinger, 1990; Lusa et al, 1991). Before assembly of virions begins in earnest, adequate quantities of capsid

proteins and genomic strands are needed in the cytosol, with equally adequate quantities of trimer-dimers in the plasma membrane.

Assembly

As the nucleocapsid forms in the cytosol, the N-terminus of the capsid protein is presumed to bind the genomic RNA and the C-terminus of the capsid interacts with other copies of capsid protein (Weiss et al, 1989). In Sindbis virus, a 132 bp-fragment in the nsP1 coding region of the genome interacts with the capsid protein, thus selecting for the encapsidation of genomic RNA, not subgenomic RNA (Frolova et al, 1997).

Early evidence suggested that nucleocapsids formed first, and then individually interacted with the E1-E2 trimer-dimers that were present in the host plasma membrane to form a lattice over the capsid and initiate budding. New research has brought this maturation and budding model into question as complete nucleocapsids are not required for maturation (Forsell et al, 1996) and mutated Semliki Forest virus, which cannot form nucleocapsids, still forms T=4 icosahedral virions at the plasma membrane (Forsell et al, 2000). Details on the old model are given here, as portions of the old model may still be relevant, and a new model has not been agreed upon by researchers.

In the earlier maturation and budding model, as the formed nucleocapsids in the cytoplasm near the host plasma membrane, the presence of the numerous trimer-dimers in the membrane initiate the envelopment process (Jones et al, 1977; Schlesinger & Schlesinger, 1986). This model suggests the attraction of the C-terminals of the nucleocapsids with the cytoplasmic tails of E2 proteins in the plasma membrane provides the energy to complete the envelopment and budding process (Anthony &

Brown, 1991). As adjacent capsid proteins in the nucleocapsid associate with more trimer-dimers in the plasma membrane, the trimer-dimers are drawn closer together, allowing formation of pentamers and hexamers. Disulfide bonds form between the E1 proteins in the trimers (Wahlberg et al, 1989; Ziemiecki et al, 1980) locking the subunits into the T=4 icosahedral lattice (Anthony & Brown, 1991; Caspar & Klug, 1962). Budding is achieved as the plasma membrane bends around the nucleocapsid, drawn by the attraction of additional C-terminals and E2 tails, until the plasma membrane layers meet, fuse and release the mature virus. The new evidence suggests that the nucleocapsid-E2 cytoplasmic tail interaction may not be required to initiate and drive the budding process. In the assembled virus particle, these interactions are still present.

PATHOGENESIS OF VEEV INFECTION

Pathogenesis in Horses

VEEV was determined to cause fatal encephalitis in horses and later in humans, in the late 1930s and 1940s. To better understand the pathogenesis of VEEV infection, numerous experimental infections of horses were carried out over the next few decades. Natural and experimental challenge of horses with virulent subtype IAB virus produced a spectrum of disease, from inapparent infection to death. This spectrum was divided into four clinical classifications: inapparent infection, moderate infection, severe non-fatal, and severe fatal (Walton, 1998). The spectrum of disease in horses infected with

various epizootic VEEV is evident from a comparison of clinical signs in three separate experimental studies (Kissling et al, 1956; Henderson et al, 1971; Walton et al, 1973), where clinical signs were evident in 73%, 100%, and 100% (respectively) of challenged horses. Signs consistent with encephalitis were evident in 36, 75, and 81% of the horses and 45, 50, and 81% of the horses died.

The typical progression of experimental epizootic IAB VEEV infection in horses can be determined from these studies and others. The incubation period or time after exposure until the febrile response, varied from 1-3 days, but was as short as 12 hours or as long as 5 days. A majority of horses developed fever between 24-48 hours post challenge (Walton et al, 1973; Kissling et al, 1956; Henderson et al, 1971). Viremia was detectable in most horses at the commencement of fever. The typical fever reached an upper range of 104-105°F (40-41°C) 24 hours later. Viremia also peaked at approximately 10^4 to 10^7 PFU/mL by the second or third day post challenge. By day 3 post challenge, evidence of anorexia, depression or excitability became apparent. Also on the second to third day post challenge, a variable degree of leukopenia developed in each horse that developed fever. The most severe leukopenia was reached between 3 and 5 days post challenge (Walton et al, 1973). In horses with mild or moderate manifestation of disease, fever, anorexia, and leukopenia lasted one to two days, and then the horse improved to normality by three or four days post challenge. Severe infections were normally characterized by a longer duration of fever, anorexia, and leukopenia, along with diarrhea and profound depression, but even some of these horses recovered 4-5 days after challenge. The length and severity of illness has not been a good predictor for the development of encephalitis. Signs of encephalitis typically

develop around day 4 to 6 post-challenge, or 3-5 days after first clinical signs. Viremia was cleared in most horses by the time signs of encephalitis became evident. Signs of encephalitis include behavioral changes such as circling, bumping into objects, stupor, and staggering. In fatal cases, neurological signs associated with encephalitis worsened until horses were unable to rise, and these horses often paddled constantly until death, typically around 7-10 days post challenge.

The reported morbidity rate of VEEV in horses during natural outbreaks (percent of horses exhibiting clinical signs during an outbreak) varies from 19-40%. (Groot, 1972). Morbidity rates are difficult to determine, since morbidity depends on the number of sick animals and healthy animals, after exposure to the virus. These parameters are especially difficult to determine in rural settings. Estimates are, therefore, based on data from a few farms in the affected area. For example, a questionnaire and serosurvey of 97 farms involved in the 1967 VEE epizootic in Colombia and Venezuela revealed an overall morbidity rate of 26% (451 out of 1755) in horses (Groot, 1972). The IE epizootic in Mexico involved a 30% (125 out of 417) morbidity rate in horses (Oberste et al, 1998A). The 1971 epizootic in Texas caused 3415 reported equine cases, which were estimated to represent a much higher morbidity rate (Groot, 1972). Variation in morbidity rates may reflect the vaccination status of the population and the history of previous outbreaks of epizootic or enzootic VEE, since prior exposure to VEEV probably provides long-lasting immunity (Hart et al, 2002; Pittman et al, 1996). The morbidity rate may also depend on the virulence of the VEEV strain, density of equids and mosquitoes in the region, and transmission rates in mosquitoes.

The fatality rate, or the percentage of horses that die after exhibiting clinical signs, has been reported to range from 38-83% (Groot, 1972). This rate probably reflects primarily the virulence of the epizootic strain, but also the density of infected mosquitoes and the susceptibility of the population. The 1971 epizootic that reached the United States resulted in a reported 3415 equine cases with 1426 equine deaths, a 42% fatality rate (PAHO, 1972). The 1995 epizootic in Colombia and Venezuela led to reporting of 474 equine deaths out of 979 cases, or a 48% fatality rate (PAHO, 1995).

Mice as Models of VEEV Pathogenesis

Horses and donkeys are the natural amplifying hosts for epizootic strains of VEEV, but experimental challenge of these animals is difficult and expensive, due to procurement costs and the necessity of conducting the work under BSL-3 containment. Use of primates is similarly problematic (Causey et al, 1961). For these reasons, VEE pathogenesis has been studied most frequently using mice as the animal model. Small rodents were found to be susceptible to infection with VEEV (Gleiser et al, 1962; Young et al, 1969; Bowen, 1976), suspected as reservoirs (Grayson and Galindo, 1968), and exhibited pathogenesis similar to horses (Gleiser et al, 1962; Kundin, 1966; Kundin et al, 1966; Jahrling & Scherer, 1973; Jackson et al, 1991). These similarities included early viremia, followed by depression and viral invasion of CNS leading to death.

Though similarities of VEEV pathogenesis in horses and rodents were noted, important differences exist. Rodents are much more sensitive to the fatal effects of VEEV infection. Epizootic strains of VEEV reportedly cause higher fatality rates in most laboratory rodents than in equids (Gleiser et al, 1962). Enzootic strains that are

avirulent for equids are reported to cause high morbidity and mortality in mice and hamsters (Austin & Scherer, 1971; Jahrling & Scherer, 1973; Ludwig et al, 2001). Currently, there are no rodent models that can correctly predict virulence of VEEV variants in horses. In mice, there is early destruction of lymphatic tissues (Jackson et al, 1991) and a severe prominent encephalomyelitis that may be responsible for the fatal outcome of VEEV infection (Gleiser et al, 1962; de la Monte et al, 1985). In hamsters, marked lymphoreticular and myeloid tissue destruction occurs (Austin & Scherer, 1971; Gorelkin & Jahrling, 1975; Walker et al, 1976). Lymphoreticular destruction leads to bacterial overgrowth and necrosis of intestinal lumen that can result in death, not from encephalitis, but from toxic shock (Gorelkin & Jahrling, 1975). While death in horses is postulated to be from encephalomyelitis based on the pathological changes noted in the CNS, the early lymphatic destruction is not well documented. Additionally, the peak and length of viremia in blood and tissues differ between mice and horses (Kissling et al, 1956; Walton et al, 1973; Gleiser et al, 1962).

Early Pathogenesis of VEEV infection in Mice

Pathological changes in horses due to VEEV infection have been studied at the point that encephalitic signs are evident, but little information is available on the early progression of disease. However, studies conducted during the last two decades have provided new information on the early immune response and pathogenesis of VEEV infections in small rodents. The following description of early VEEV pathogenesis is from mice models.

The first phase of infection is characterized by viral replication in the local lymphoid tissues and release of the virus into the circulatory system. After a mosquito injects the virus into the skin during a blood meal, evidence suggests that dermal Langerhans cells (resident dendritic) encounter and phagocytose the virions and migrate to the local draining lymph nodes (Steinman et al, 1997). By 30 minutes post infection, VEEV is detected in the local draining lymph node of mice challenged intradermally in the footpad (MacDonald and Johnson, 2000). Studies suggest that mosquitoes deliver virus extravascularly, yet virus moves out of the local tissue area within 10 minutes (Turell et al, 1999; Turell & Spielman, 1992). Within lymph nodes, Langerhans cells apparently present the degraded viral peptides to CD4 T-cells by antigen processing and presentation.

The majority of VEE virions appear to escape degradation in the Langerhans cells and instead replicate in the cytoplasm. Productive infection of Langerhans cells may even inhibit the Langerhans cells from further maturation (MacDonald and Johnson, 2000). This escape of degradation and presentation results in high viral titers in the draining lymph node as early as 6 hours post challenge.

Replication of virus in draining lymph nodes apparently leads to the release of virus into the blood and the initial release of IFN-α/β. Serum levels of IFN-α/β peak around 18-24 hours at concentrations of over 200,000 IU/ml of blood (Charles et al, 2001). Supplementation of IFN-α alone to mice prior to VEEV infection did not protect against encephalitis as would be expected, unless the IFN-α was conjugated to a polymer such as PEG (polyethylene glycol) that greatly increased the bioavailability and potency of IFN (Lukaszewski et al, 2000). IFN-α/β does prevent the spread of

VEEV into many tissues as studies have indicated in SCID (severe combined immunodeficient) mice and mice lacking IFN-α/β receptors with vesicular stomatitis virus (Muller et al, 1994). This first study indicated that VEEV was capable of infecting just about every tissue, while in immunocompetent mice the viral spread was limited by IFN-α/β (Lukaszewski et al, 2000).

At about 12 hours post challenge, VEEV was first detected in the circulatory system of mice. The viremic titers peaked at 10^6 -10^7 PFU/ml around 18-24 hours post challenge (Charles et al, 2001; Tasker et al, 1962). During this viremia, the virus apparently infects secondary tissues, primarily the lymphoid, pancreatic, and nervous tissue. By 24 hours, there are over 10^6 PFU of virus per gram of spleen tissue. More importantly, the virus probably gains entry into the peripheral nerves, as described later.

IgM antibody produced in response to VEEV infection is an important player in protection against development of fatal disease. IgM has been detectable in blood as early as 72 hours post challenge and, along with IFN, may be instrumental in clearance of virus from the blood. Around 72 hours post-challenge in mice, viremia titers decline, reaching undetectable levels by 96 hours post-challenge (Charles et al, 2001). IFN plays an indirect role in clearing viremia by reducing the number of newly infected cells.

Cytokine Expression during VEEV infection

Complex communications involving cytokines IL-1, IL-6, and both peripheral and central nervous systems, act on the hypothalamus to reset the thermal regulator, which increases body temperature (Reviewed in Roth & de Souza, 2001). The liver is also induced to synthesize acute phase proteins such as complement in the fight against

the pathogen. Also increased are ACTH, cortisol and the production of white blood cells needed to react against foreign pathogens (Goldsby, 2000). IL-6 gene expression levels in the draining lymph node increase over 100-fold in the mouse in response to VEE infection (Grieder et al, 1997).

While IL-12 secreted by macrophages is a potent inducer of Th1 CD4, CD8, and NK cells (the cell mediated immunity), studies suggest that IL-12 does not seem to affect the early immune response to VEEV. Although a 10-fold increased gene expression of IL-12 occurs in the draining lymph node within 24 hours of VEEV infection, elimination of IL-12 by anti-IL 12 antibodies or supplementation of IL-12 in deficient mice had no effect on the course of the disease (Grieder et al, 1997). Other studies reported additional IL-12 treatment may actually be deleterious during VEEV infection. (Lukaszewski et al, 2000). This may be due to the increased activation of the cell-mediated immunity that may result in the severe encephalitis noted later in the disease.

Expression of TNF-α and IL-2 was minimal early in VEEV infection (Grieder et al, 1997). In the locally infected lymph node, TNF-α levels increased about 6-fold while IL-2 increased only two-fold. TNF-α secreted by macrophages increases vascular adhesion and vascular permeability. IL-2, secreted by Th1 CD4 cells, enhances the cell mediated immune response. Supplementation or deletion of these cytokines did not alter VEEV pathogenesis in mice (Grieder et al, 1997). Thus, these two cytokines do not appear to be major players in VEEV infection.

Mechanisms for Neuroinvasion

The second phase of infection involves replication of the virus in the secondarily infected organs and viral invasion of the CNS. As mentioned earlier, viral titers increase in the spleen, lymphoid, and pancreatic tissue around 24 hours post challenge (Tasker et al, 1962). This second round of replication apparently sustains or increases, temporarily, the viremic titers. During this time period in mice, VEEV is first detected in the CNS (Charles et al, 1995; Tasker et al, 1962). There are two leading theories on how alphaviruses invade the CNS: virus breaching of the blood-brain barrier and retrograde viral movement from peripheral nerves. The majority of current evidence supports alphavirus invasion of the CNS through peripheral nerves. Invasion of the CNS by Trinidad donkey strain of VEEV (or its clone V3000), has been studied in mice. Virus could be detected in the olfactory neuroepithelium as early as 18-24 hours after initial infection, while other portions of the CNS were free of virus (Charles et al, 1995). Another study found 5.38 $\log_{10}$ LD_{50} of TrD virus in mice brains by 24 hours post challenge (Tasker et al, 1962). If VEEV gains access to the CNS through the peripheral nerves, the shortest and presumably, fastest route to the CNS would be through the olfactory sensory neurons. It has been theorized the virus invades the neurons through the fenestrated capillary endothelium, which are in close proximity to the olfactory sensory neurons. IL-1 and its action of increasing vascular permeability may actually increase the ability of the virus to invade secondary tissues, including spleen, liver, thymus, and peripheral nerves (Jackson et al, 1991). In one study, VEEV was first found in the olfactory bulb of the brain at 30 to 36 hours post infection (Charles et al, 1995). After 36 hours, the virus was then detected in other regions of the brain

connected to the olfactory bulb by efferent projections. At 48 hours post-infection, the virus had spread to the lateral olfactory tracts, pyriform cortex, hypothalamus, amygdala, hippocampus, and supraoptic nucleus (Charles et al, 1995).

VEEV may also gain entry into the CNS through dental nervous tissue (Charles et al, 1995). The virus apparently invades the unmyelinated dental neurons through the fenestrated capillary beds, which are in close proximity to the nerves. By 36 hours post challenge, the virus was detected throughout the tooth pulp, around the periodontal membranes, and in the branches of the trigeminal nerve in the tooth pulp. The virus, apparently through retrograde movement, gained entry into the parietal and temporal lobes of the brain by 48 hours post infection (Charles et al, 1995). This route of infection may not be as important as the olfactory route since the virus will already have gained entry into the CNS through the olfactory route. The importance of the dental nerve route of CNS invasion may be the existence of a secondary means of viral entry into the CNS, which could potentially overwhelm the CNS immune system.

Virus infection of CNS tissues may result in an over-reactive inflammatory immune response in the brain (de la Monte et al, 1985). The cells involved included microglial cells (the resident macrophages in the CNS), $CD8^+$ T cells, and neutrophils, which all infiltrate the CNS tissues (Charles et al, 2001). These infiltrates, along with the release of vascular permeability cytokines, IL-1, and possibly IL-8, cause pathogenic changes in the CNS, including edema, cerebral congestion, vasculitis and intracerebral hemorrhage, which characterize the encephalitis of VEE. The microglial and $CD8^+$ cells apparently kill VEEV infected CNS cells, causing cerebritis and meningitis (de la Monte et al, 1985). These pathological changes in mice are

presumably responsible for the majority of neurological signs, hind limb paralysis and possibly even death of the host.

Summary

In summary, the pathogenesis of VEEV in mice involves a complex interaction between the virus and the host immune system. Activation of CD4 T-cells by the dermal Langerhans cells in unvaccinated hosts results in release of interferons and cytokines, which cause increased vascular permeability, and activation of the cell mediated immunity. After VEEV invades the CNS, the acute inflammatory and cell mediated response results in CNS edema, cellular infiltrates, and potential destruction of vital CNS cells.

INTERFERON RESPONSE TO VEEV INFECTION

Interferons (IFNs) were discovered and named because of their ability to interfere with virus infections in eukaryotic cells (Nagano & Kojima, 1954; Isaacs & Lindemann, 1957; Wagner et al, 1963). Newcastle disease (Ruiz-Gomez & Isaac, 1963), vesicular stomatitis (Wagner et al, 1963), and foot-and-mouth disease (Sellers, 1963), are several diseases in which IFN appears to be a major factor in determining the outcome of virus infection and host determinant of virus virulence. The median lethal doses (LD_{50}) of VSV Indiana and Semliki Forest virus (SFV) are 10^7-fold and 10^6-fold lower in mice lacking type I IFN receptors relative to unaltered mice (Muller et al,

1994). The same experiments with mice lacking IFN-γ receptors (Type II IFN) did not show any significant difference for VSV and SFV, but there was 10^{3-5}-fold difference for vaccina virus (Muller et al, 1994). Similar results were obtained when anti-IFN serum was injected into mice prior to challenge with VSV (Gresser et al, 1976). *In vivo* virulence of other viruses, such as Sindbis (Vilcek, 1964) and influenza (Link et al, 1965) do not appear to be affected by IFNs. Interferons have been shown to be important players in the innate immune response to many viruses, but not all viruses.

After the initial discovery of interferons (IFNs), different types of interferons were realized, along with their antiviral mechanisms. Type I interferons include alpha and beta interferon (IFN-α; IFN-β), which are expressed by leukocytes and fibroblasts, respectively, in response to viral infections (Kimura et al, 1994; Siegal et al, 1999). Type II interferons include gamma interferon (IFN-γ) which is expressed by activated T lymphocytes and natural killer (NK) cells (Kimura et al, 1994). Additional research elucidated mechanisms by which type I IFNs assert their antiviral actions. The type I IFNs are produced by virally infected cells in response to dsRNA (Lampson et al, 1967). Macrophages, monocytes, and fibroblasts also produce type I IFNs but the process of induction is not understood (Biron, 1998; Gresser, 1984). Synthesized IFNs then bind to IFN-α and IFN-β receptors on other cells, activating the JAK-STAT pathway (Janus kinase group - signal transducers and activators of transcription), which induce several biochemical pathways that affect cell growth, metabolism, and degradation of mRNA (Biron, 1998). Two important genes induced by the JAK-STAT pathway are 2-5 oligoadenylate synthetase and protein kinase (Schindler & Darnell, 1995; Staeheli, 1990). The 2-5(A) synthetase enzyme activates RNAse L, a

ribonuclease, which degrades single-stranded RNA. The other enzyme, a protein kinase known as dsRNA-dependent protein kinase (PKR), induces phosphorylation of eIF-2. eIF-2 is a translation initiator factor required for protein synthesis within the cell. Phosphorylation inactivates eIF-2, leading to cessation of all protein synthesis in the virus infected cell (Biron, 1998; Gresser, 1984).

IFN-γ is secreted by activated T lymphocytes and natural killer (NK) cells. IFN-γ induces the expression of the cytokine interlukin-12 (IL-12) in nearby cells. IL-12 acts to enhance cytotoxic T cells, enhance the proliferation of activated T and NK cells, inhibit IgE production, and induce T helper cells (Th1) (Orange, 1994).

Different VEEV subtypes and strains have been studied for their sensitivity to and induction of IFNs. Sensitivity and resistance have been examined using IFN primed mouse fibroblast (L929) and chick embryo cell cultures. Viruses that are resistant to IFN cause cell death in IFN primed cells, while IFN protects cells from viruses sensitive to IFN. Virus-induced IFN production can be examined by challenge of small rodents with a virus, then assaying levels of serum IFN. These types of experiments have indicated that the TC-83 strain of VEEV is more sensitive to the antiviral effects of IFN than the virulent TrD strain (Jordan, 1973A), even though the TrD strain induces higher IFN production following *in vivo* challenge (Jahrling et al, 1976). The enzootic Pixuna strain (BeAr 35645) was more sensitive to IFN than TrD (Jahrling, 1976). When the IFN sensitivities of 25 isolates of enzootic and epizootic strains of VEEV were compared, seven of 10 epizootic strains (IAB and IC subtypes) were resistant to IFN, and the three remaining were intermediate in resistance. Likewise, eight of 14 enzootic (ID, IE, II, III, IV and VI subtypes) were sensitive to IFN, as evidenced by minimal

cytopathic effect in primed cell cultures exposed to the enzootic VEE viruses. Four were intermediate (all ID) and two (one ID and one IE) isolates were resistant (Spotts et al, 1998). Another study called into question the correlation of epizootic VEE viruses and resistance to IFNs. In these experiments, infectious clones of two epizootic IC and one enzootic ID strains were compared. One of the IC epizootic strains (IC – SH3) and the ID strain (ZPC738) differed by only 12 amino acids (Anishchenko et al, 2004). While this study found no appreciable difference in sensitivity to IFN between the two epizootic IC and the enzootic ID strains, the number of examined isolates was small. Additionally, of seven ID isolates tested in the Spotts study, one was resistant, four were intermediate in resistance, and only two were sensitive. Thus sensitivity to IFN for the ID and IE subtypes may not correlate to epizootic potential, but it appears that for subtypes IAB, IC, and II-VI, the correlation is appropriate.

IFNs have been effective as a treatment against some arboviral infections when treatment was started before or soon after challenge (Finter, 1966; Bradish & Titmuss, 1981). Use of the IFN-inducer poly I:CLC, a synthetic double-stranded polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine, has successfully treated mice, even after Banzi and Semliki Forest viruses produced viremia (Singh et al, 1989). Pre- and post-treatment with hyperimmune sera or poly I:CLC alone did not provide mice any protection against Semliki Forest virus, while combination treatments resulted in a 50% survival rate in mice injected intracranially with virus (Coppenhaver et al, 1995). *In vitro*, IFN-α pre-treatment reduced, by 100-fold, the replication of Sindbis virus (Depres et al, 1995) and Mayaro virus (Rebello et al, 1993), yet did not prevent cell death. IFN-α treatment of cells during infection did not affect virus

replication. However, Sindbis virus replication was decreased when a combination of IFN and a monoclonal antibody to the E2 protein were used to treat the cells (Despres et al, 1995).

For several attenuated VEEV strains, IFN appears to have the ability to prevent severe disease and fatalities in mice. The replication of TC-83 vaccine strain of VEEV was reduced in mouse cell cultures primed with mouse interferon as compared to primed cells infected with virulent TrD VEEV. The two virulent strains induced cell cultures to produce more IFN than the TC-83 strain (Jordan, 1973). The attenuated molecularly cloned VEEV strains, V3032 and V3043, are not fatal to genetically normal lab mice; yet, in mice with non-functional IFN α/β receptors, the attenuated V3032 and V3043 VEEV strains caused 100% mortality, with average survival times similar to virulent V3000 (Grieder & Vogel, 1999; White et al, 2001). In another study, administration of anti-IFN-α/β antibodies increased the severity of virulent V3000 infections (Grieder et al, 1997). IFN-β appeared to be more important than IFN-α in resistance to *in vitro* infections. When anti-IFN-β antibodies were titrated in cell cultures challenged with TC-83 and V3000 strains, higher CPE was noted than in cell cultures treated with titrated anti-IFN-α antibodies (Spotts et al, 1998).

Treatments for virulent VEEV infections have been explored using IFN. Treatment of mice infected with the virulent TrD strain of VEEV with type I interferons was unsuccessful (Spotts et al, 1998; Lukaszewski & Brooks, 2000). However, use of a PEGylated alpha interferon, to increase the serum half-life of IFN and thus its bioavailability and potency, protected mice from 25 LD_{50} of VEEV with over 75% of challenged mice surviving (Lukaszewski & Brooks, 2000).

In summary, interferons are important in resisting challenges with avirulent VEEV and may be a critical factor in the horses that survive challenge with virulent VEE viruses. Most avirulent and attenuated VEEV are sensitive to the antiviral effects of IFNs. Potential treatments of alphavirus infections may include a combination of drug therapies which include IFNs. Understanding the serum IFN responses in horses to virulent and avirulent VEEV challenge and vaccine administration may advance the development of viral treatments for horses.

DEVELOPMENT OF V3526 VEEV VACCINE STRAIN

VEEV has caused major outbreaks of influenza-like illness and encephalitis involving thousands of equines and humans in the last 80 years. Vaccines have been produced in an attempt to control epizootics and prevent laboratory infections. VEEV vaccines available for human use are the attenuated (TC-83) and killed virus (C-84) vaccines. TC-83 and TC-83-derived vaccines, such as C-84, have been the primary vaccines used in horses. Both of these vaccines have important deficiencies that necessitate development of an improved vaccine. TC-83 is reactogenic in 23% of human recipients and another 19% do not seroconvert following immunization (Pittman et al, 1996). Use of TC-83 in horses led to the infection of mosquitoes with the TC-83 strain, increasing the possibility of reversion to virulence (Pedersen et al, 1972; Kinney et al, 1992). The killed virus C-84 vaccine did not protect rodents against aerosol challenge with VEEV (Jahrling & Stephenson, 1984) and has only been used to boost

previous TC-83 recipients (Pittman et al, 1996). Additionally, live-attenuated vaccines historically are more advantageous than killed vaccines. The advantages include efficiency following a single immunization, longer duration of immunity and better protection against aerosol challenge.

The advent of new molecular tools in research provides the potential to design novel attenuated-live VEEV vaccines with less probability of reversion to virulence. One approach was to select for rapid penetration of cultured cells (Johnston & Smith, 1988). Another approach was to construct a molecular clone of VEEV TrD (Davis et al, 1989) and then introduce point and cleavage mutations to reduce virulence (Davis et al, 1991, 1995). Full length cDNA clones were made from attenuated VEE viruses (Table 1.2) and compared in virulence studies against the TrD strain (Davis et al, 1995). Several of these strains were then tested in mosquitoes for transmission rates (Turell et al, 1999), and in rodents and non-human primates for virulence and protection against TrD challenge (Pratt et al, 2003). Three attenuated candidates (V3524, V3526 and V3528) had low transmission rates in mosquitoes (Turell et al, 1999), were highly immunogenic, and protected against both aerosol and subcutaneous TrD or V3000 challenge in mice (Pratt et al, 2003). The V3526 and V3528 strains produced higher serum antibody levels in non-human primates than V3520. V3526 possessed a 12 nucleotide deletion, while V3528 only had a two nucleotide change, and these additional deletions in the genome were thought to provide additional safeguards against potential reversions. For these reasons the V3526 strain was selected for further

Table 1.2
VEEV laboratory mutations

Class of Mutant	Virus strain	Mutation loci	Types of mutation
Wild type	V3000	None	Derived from parent strain TrD
Single	V3010	E2 76	Single substitution:Glu→Lys
	V3032	E2 209	Single substitution:Glu→Lys
	V3034	E1 272	Single substitution:Ala→Thr
	V3040	E1 253	Single substitution:Phe→Ser
	V3042	E1 81	Single substitution:Phe→Ile
	V3043	nt 3A (5' UTR)	Single substitution:G→A
Triple mutants	V3519	E1 272, E2 76, E2 209	Three glycoprotein mutations
	V3520	E1 81, E2 76, E2 209	Three glycoprotein mutations
	V3522	nt3A, E2 76, E2 209	5' UTR & two glyco mutations
	V3524	nt3A, E1 272, E2 209	5' UTR & two glyco mutations
Cleavage site mutants	V3526	E3 (56-59), E1 253	Cleavage site deletion & Phe→Ser
	V3528	E3 59, E1 253	Cleavage site mutation & Phe→Ser
	V3531	E3 (56-59), E2 243	Cleavage site & Leu→Gln
	V3532	E3 59, E2 243	Cleavage site mutation & Leu→Gln

Table 1.2. V3000 served as template for single or multiple site mutations using the Kunkel method (Kunkel, 1985). The infectious VEEV RNA was transfected into baby hamster kidney cells (Davis et al, 1991). Virus strains above were obtained directly from baby hamster cell supernatant. The strains were then checked by direct sequence analysis (Davis et al, 1995).

research and development as a vaccine candidate. The V3526 VEE strain was found to be non-lethal after intracraniall injection of C3H/HeN mice, compared with lethality noted with both TC-83 and V3000 strains (Ludwig et al, 2001). Additionally, the avirulence of V3526 remained stable after multiple passages in mice and cell cultures. Additional evidence indicated the V3526 strain provided superior protection in mice against aerosol challenge (Hart et al, 2000), elicited antibodies more rapidly in mice than the C-84 and TC-83 VEEV vaccines, and the antibodies persisted for at least a year (Hart et al, 2002). In summary, the V3526 vaccine holds considerable promise as a safe and efficacious next-generation VEEV vaccine.

CHAPTER II

EARLY PATHOGENESIS OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS INFECTION IN HORSES

INTRODUCTION

Research on the early pathogenesis of Venezuelan equine encephalitis virus (VEEV) in mammals has been limited primarily to studies in rodents. Previous studies of VEEV infections in horses focused on the full-length disease course, not the early spread of virus throughout the horse. The research described here was designed to study the early pathogenesis of the virulent Trinidad donkey (TrD) strain of VEEV infection in horses. One group of three horses, controls from a VEEV vaccine study, was challenged with the TrD virus and allowed to progress for two weeks or until signs of clinical encephalitis were evident. The second group of six horses was challenged with

the TrD strain, and two animals sacrificed at 24 hours, 48 hours and 96 hours. Serum samples were obtained every eight hours. Virus isolation in cell culture, reverse-transcriptase polymerase chain reaction (RT-PCR), and immunohistochemistry (IHC) techniques were utilized to determine tissue viral loads and the presence of viral antigens.

As discussed in the literature review on VEEV pathogenesis in mice, VEEV initially infects dendritic cells and lymph nodes. Replication in lymph nodes leads to viremia and seeding of the reticuloendothelial system (spleen, liver, lymph nodes, and bone marrow) and other organs. Replication of virus in these secondary tissues increases the magnitude of the viremia, followed by detection of the virus into the CNS, presumably gaining entry through the olfactory sensory neurons or the blood-brain barrier. Invasion of the CNS leads to infection of neurons and, apparently in mice, a host immune response that has fatal consequences.

AIMS: Characterize the early pathogenesis of VEEV infection in horses. Determine which tissues produce high viral loads early in the course of disease, in an attempt to increase understanding of the replication patterns and target tissues in the horse. Provide information on the potential paths of invasion into the CNS in horses, by looking for early viral infection in neuronal paths leading into the CNS and in various regions of the CNS.

HYPOTHESES: Local and draining lymph nodes are expected to have the highest viral tissue loads early in infection. Tissue loads should increase in secondary organs such as spleen, pancreas, and dental pulp, prior to virus detection in the CNS. Before viral detection in the CNS, virus will be detectable in peripheral nerves such as

the trigeminal nerve from the dental pulp, the olfactory tract from the nose and the optic nerve from the eye. Evidence to support these hypotheses would support the model of VEEV invasion of the CNS through the peripheral nerves and not across the blood-brain barrier.

MATERIALS AND METHODS

Virus Strains

The VEEV used in this research was the Trinidad donkey strain (TrD) isolated originally in guinea pigs from brain tissue of a donkey that died in Trinidad during the 1943-44 epizootic (Kubes, 1944; Randall et al, 1944). It was classified as VEEV subtype IA by the short incubation hemagglutination-inhibition (HAI) test (Young and Johnson, 1969), which later became known as subtype IAB. The TrD virus and 64A99, a subtype IE virus described in Chapter 3, were verified as IAB and IE subtypes using a monoclonal antibody immunofluorescence assay (IFA) (Roehrig and Bolin, 1997; Roehrig et al, 1991). To conduct the IFA assay, infected Vero cells were fixed on glass slides, then exposed to a panel of VEEV subtype-specific monoclonal antibodies, followed by a FITC-conjugated secondary antibody. To determine the titer of viruses prior to inoculation, the viruses were passed in Vero cell cultures and the number of plaque-forming units (PFU) per mL determined by plaque assay. The TrD virus was diluted in sterile PBS for subcutaneous inoculation of horses.

Animals

Adult horses of varying ages and breeds were obtained from a local Colorado supplier. Prior to use, the horses were tested to verify they had no detectable antibodies to EEE, WEE and VEE viruses by the plaque reduction neutralization test (described later). Horses were tagged after gender, age and description were recorded. Age was determined by examination of dental wear patterns.

To prepare the horses, temperature recorders (iButtons, Dallas SemiConductor) were surgically implanted intramuscularly in the left flank of each horse, under xylazine sedation and local lidocaine block. The implanted recorders were programmed to record body temperatures hourly, starting seven days before challenge. After five to ten days, horses were entered into the BSL-3 facility and allowed to acclimate for at least four days. In BSL-3, the horses were fed pelleted horse feed twice daily, with an occasional cup of grain. All had free access to water. To humanely euthanize, horses were sedated with xylazine, then given an intravenous overdose of pentobarbital. Carcasses and debris were incinerated in the BSL-3 building. Animal usage was approved by the CSU Animal Use Committee and followed the Code of Federal Regulations #9 of the US Department of Agriculture.

Plaque Assays

Virus titers from inocula, sera and tissue homogenates were determined in duplicate in a double-overlay plaque assay. Briefly, ten-fold serial dilutions of each virus sample were prepared in BA-1 medium, and 0.1 mL of each dilution was added to confluent monolayers of Vero cells grown in six well plates. The plates were incubated

for one hour at 37C° in 5% CO_2 with intermittent rocking, then overlaid with 3mL per well of 0.5% agarose in minimal essential medium supplemented with 3% fetal bovine serum and antibiotics. After 24 hours of incubation, 2mL of a second 0.5% agarose overlay containing 0.005% neutral red was added. The plates were returned to the incubator, and the plaques were then counted on days 2-4 of incubation. The lower limit of detection using this method was 5 PFU/mL of serum (0.7 $\log_{10}$).

Plaque Reduction Neutralization Tests (PRNT)

Serum samples were tested for virus-neutralizing antibodies to EEE (NJ 60 strain), WEE (Fleming strain) and VEE (TrD strain) equine encephalitis viruses prior to challenge using a plaque reduction neutralization test (PRNT). Sera were heat-inactivated at 56°C for 30 minutes, then used to prepare serial two-fold dilutions in BA-1 medium. The diluted sera were mixed with an equal volume of virus diluted in BA-1 containing 10% FBS, to give approximately 100 PFU/0.1 mL in the final mixture. A volume of 0.1 mL of the serum-virus mixture was inoculated onto Vero cell monlayers in six-well plates. Plates were then processed as for viral plaque assays. Serum samples were screened in duplicate at 1:10 dilutions. Serum samples were considered positive if they induced a reduction in plaque count of >80% compared to controls at each particular dilution. The exception to this was in screening horses prior to use, where animals were excluded from study if a 1:10 dilution of their serum neutralized >50% of EEE, WEE, VEE virus.

Challenge

In the first study, a group of five horses were challenged by subcutaneous injection of 10^4 PFU of TrD virus and allowed to progress until definitive clinical signs of encephalitis were evident. The second group of six horses was challenged by subcutaneous inoculation of $10^{3.3}$ PFU of TrD VEEV. Two of these horses were euthanized at 24 hours, 48 hours and 96 hours after inoculation. In both studies, the remaining inoculum was back-titrated to verify the dose injected into the animals.

Collection of Specimens

Blood was collected by jugular veinipuncture in sterile serum separation vacuum tubes. To obtain serum, blood samples were allowed to clot for 8-12 hours at 4°C, centrifuged at 2000 x g for 10 minutes, separated, and the sera stored in screw top vials at -80°C until being assayed. Blood for complete blood counts (CBC) was collected in EDTA tubes, placed on a rocker within one hour of collection, and the CBC analysis completed within two hours of collection. Baseline CBCs were taken before challenge along with pre-challenge serum samples. After challenge, horses were observed at least twice daily for clinical signs.

At necropsy, multiple tissue samples were collected for virus isolation, reverse transcriptase PCR (RT-PCR), immunohistochemistry (IHC) and pathology (Table 2.1). Tissues for virus isolation and RT-PCR were collected sterilely in screw cap vials, placed immediately on dry ice and transferred to -80°C freezers. Tissues for IHC and pathology were placed in buffered formalin. Formalin was changed within 48 hours and the tissues allowed to fix for at least seven days. CNS tissues collected for virus and

Table 2.1
Tissues Collected at Necropsy

Tissue	VI	RT-PCR	IHC	Pathology
Olfactory bulb	√	√	√	√
Olfactory tract	√	√	√	√
Anterior cerebrum	√	√	√	√
Posterior cerebrum	√	√	√	√
Cerebellum	√	√	√	√
Brain stem	√	√	√	√
Cervical spinal cord	√	√	√	√
Lumbar spinal cord	√	√	√	√
Cranial nerve V at CNS	√	√		
Cranial nerve VII at CNS	√	√		
Optic chiasm	√	√		
Trigeminal ganglia	√	√		
Trigeminal nerve distal	√	√		
Retina	√	√		
Optic nerve distal	√	√		
Vitreous	√	√		
Bone Marrow	√	√		
Spleen	√	√	√	√
Liver	√	√	√	√
Pancreas	√	√		
Lacrimal gland	√	√	√	√
Salivary gland	√	√	√	√
Dental pulp	√	√		
Prescapular LN L & R	√	√	√	√
Superfical Cervical LN L & R	√	√	√	√
Deep cervical LN L & R	√	√	√	√
Superfical or Deep Inguinal LN Left	√	√	√	√
Mesentary lymph nodes	√	√	√	√

LN = Lymph Node
√ = Tissue collected
L & R = Left and Right

Table 2.1. List of tissues collected, from each horse at necropsy for either: virus isolation (VI), immunohistochemistry (IHC), reverse transcriptase-PCR (RT-PCR), or pathology. Check mark √ indicates tissue was collected. Additionally, tissues were collected from the heart, lung, adrenal gland, kidney and skeletal muscle for VI, RT-PCR and pathology from each horse.

pathology included olfactory bulb, olfactory tract, anterior cerebrum, posterior cerebrum, cerebellum, brain stem, cervical spinal cord, and lumbar spinal cord. The trigeminal (V) and facial (VII) cranial nerves were collected at the junction of the nerve with the CNS (Figure 2.1) for virus isolation and antigen retrieval. Samples from the peripheral nervous system collected for virus isolation and antigen detection included optic chiasm, trigeminal ganglia, trigeminal nerve, retina, and the optic nerve at the eye. Other tissue collected from the head for virus isolation and RT-PCR included salivary gland, lacrimal gland, dental pulp, and vitreous humor. Left and right lymph nodes were collected at the prescapular, superficial cervical, deep cervical, superficial or deep inguinal sites along with the mesentery lymph nodes, and used for virus isolation, RT-PCR, IHC and pathology. Other reticuloendothelial tissues collected for virus isolation and antigen assays included liver, bone marrow, spleen and pancreas. Other tissues collected for virus isolation and pathology included heart, lung, adrenal gland, kidney, and skeletal muscle.

Formalin fixed tissues for IHC and pathology were sectioned and sent to Colorado Histoprep, Inc to be embedded in paraffin sectioned and mounted on slides. Additional formalin fixed tissues for IHC were cut in 30μm sections by cryosection and mounted on gelatin-coated slides. Cryosectioned tissues included the junction of the CNS and trigeminal, facial, optic and olfactory cranial nerves (V, VII, II and I).

Tissue specimens were homogenized to 10% suspension of BA-1 supplemented with 7.5% FBS. Preparations were clarified by centrifugation at 5000 x g for five minutes. The supernatant was then processed for plaque assay. The lower limit of detection for tissue viral assay was 50 PFU/gram of tissue.

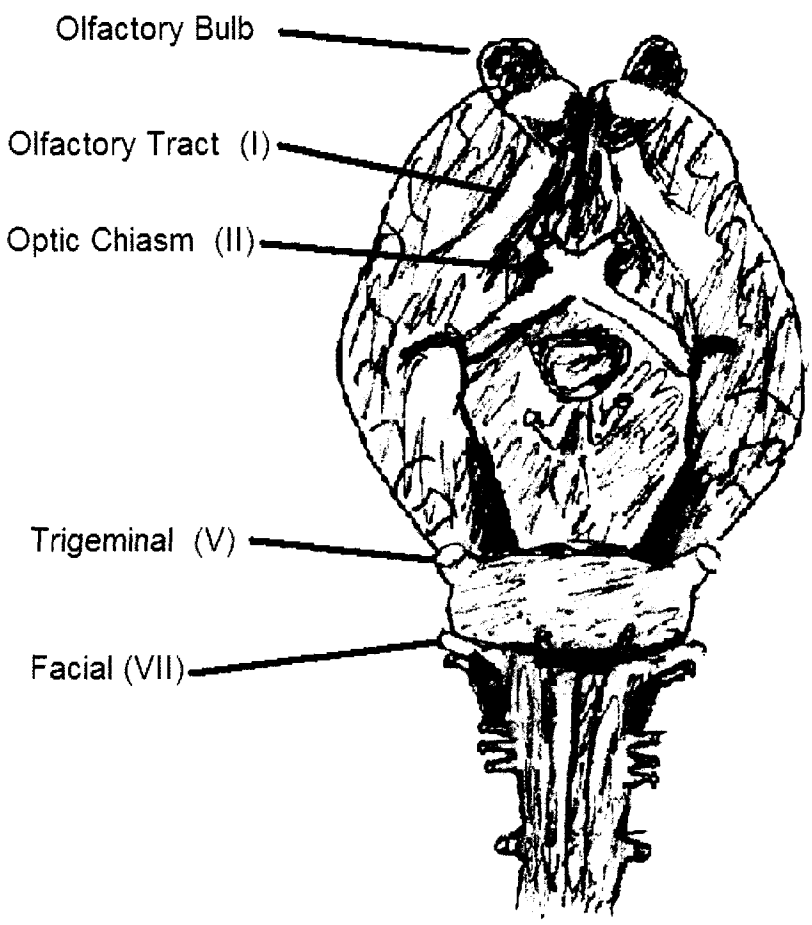

FIGURE 2.1

Cranial nerve sample collection sites

Figure 2.1. Yellow high-light on cranial nerves I, II, V and VII indicates the locations of sample collection from ventral horse brain for each nerve.

Immunohistochemistry

The paraffin mounted tissues were deparaffinized by heating to 60°C for 30 minutes and soaking in SafeClear (Fisher, Middletown, VA) for five minutes. Slides were re-hydrated in 100, 90 and 70 percent graded ethanol then held in de-ionized water. To break the disulfide bonds that mask antigenic sites, the tissue slides were soaked in Target Retrieval Solution (TRS) (DAKO, Carpineteria, CA), an antigen target retrieval system. Antigen retrieval times varied from 20 to 60 minutes in TRS, depending on the type of tissue and length of time in buffered formalin. CNS tissues were soaked from 30 to 60 minutes, while other tissue sections were soaked from 20 to 40 minutes. The slides were then processed, as described below, with either an avidin-biotin-alkaline phosphatase detection system or an indirect immunofluorescence assay (IFA).

The cryosectioned slides for IFA were soaked in a 25% sucrose solution for four days, frozen at -20°C and sectioned at 30 μm. Sections were mounted on gelatin-coated slides. Slides were bathed in TRS for 20 minutes at 95°C. The slides were then assayed with either the IFA or avidin-biotin-alkaline phosphatase system.

Both cryosectioned and paraffin mounted tissue sections were assayed with an avidin-biotin alkaline phosphatase detection system. Slides were blocked for 15 minutes with 20% goat serum in Tris-Saline-Tween 20 buffer at pH 7.4 (TST; 150mM NaCl, 25mM Tris- phosphate with Tween20). A 1:200 dilution of polyclonal mouse anti-VEEV ascites fluid (TC-83 strain; CDC, Atlanta, Georgia, courtesy of Dr. Ann Powers) was applied, as primary antibody, for 30 minutes at 37°C. The LSAB2 System (DAKO, Carpinteria, CA), an avidin-biotin-alkaline phosphatase kit, was followed according to

the manufacturer's instructions. Fast Red (DAKO, Carpinteria, CA) was used as the color indicator according to manufacturer's instructions. The slides were then counter-stained in Mayer's hematoxylin.

The remaining mounted and cryosectioned tissue slides were assayed by IFA. The slides were soaked in a fluorescent antibody wash buffer (FAWB; 0.0087M Na_2HPO_4; 0.0013M Na_2HPO_4;-H_2O; 0.145M NaCl). The slides were blocked for 15 minutes with 20% goat serum in FAWB and incubated with a 1:200 dilution of polyclonal mouse anti-VEEV ascites fluid for 30 minutes at 37°C. FITC-conjugated rabbit anti-mouse antibody diluted 1:100 in FAWB was applied. Three sections of each tissue were viewed under fluorescent microscopy and scored as either negative or positive.

Known negative and positive control slides were used for both avidin-biotin and IFA techniques. The negative control slides were paraffin mounted, unchallenged horse tissues. The positive control slides were paraffin mounted, TrD infected mouse brain slides from a previous study.

RESULTS

Cells infected with VEEV strains TrD and 64A99 were confirmed as subtype IAB and I subtype viruses by immunostaining of infected cells using subtype-specific monoclonal antibodies (Table 2.2). As expected, the TrD strain was positively stained

TABLE 2.2

IFA Monoclonal Antibody VEEV Assay

	Vero	IE	TrD
PBS	-	-	-
1A3A-5	-	-	+
1A3B-7	-	+	+
1A1B-9	-	+	-

\+ positive IFA staining
– negative IFA staining

Table 2.2. Two VEEV subtypes (IE-64A99; IAB – TrD) and negative control (Vero cells only) in an indirect immunofluorescence antibody assay (IFA) using monoclonal antibodies (MAbs) to the E2 genome.

with the 1A3A-5 and 1A3B-7 antibodies, while the 64A99 strain was positively stained with the 1A3B-7 and 1A1B-9 antibodies (Figure 2.2, FIGURE 2.2 A, B, C, D, E, F).

Clinical Signs and Virus Isolation

Two horses (MT31 and MT32) were sacrificed 24 hours after they were challenged with the Trinidad Donkey (TrD) strain of VEEV. Neither horse demonstrated any abnormal clinical signs during the 24 hours. Changes were not detected in hematologic parameters or body temperatures. Viremia was detected in both horses at 24 hours post infection (pi). The viremic titer of the two horses was 70 (MT31) and 800 (MT32) PFU/mL at 24 hours post challenge. Attempts to isolate virus by Vero cell culture from MT31 tissues were fruitless. Several tissues from MT32 produced virus titers. The spleen and mesentery lymph node (150 PFU/gram of tissue), the right deep cervical lymph node (50 PFU/gram), and the left superficial lymph node ($10^{6.1}$ PFU/g tissue) all yielded virus (Figure 2.3 and Table 2.3). Virus was not found in any other tissues.

Horses number MT21 and MT33 were sacrificed 48 hours after challenge. Neither horse demonstrated abnormal clinical signs, except increased body temperatures noted in both horses. MT21's temperature initially rose around 28 hours post challenge with a peak temperature of 41°C (105.8°F) from 35-38 hours post challenge. Horse MT33's initial rise in body temperature was around 26 hours, with the highest temperature of 39.5°C (103.1°F) reached several times between 26 and 48 hours. Viremia was detected in MT33 with $10^{2.2}$ PFU/mL at 16 hours post challenge and in

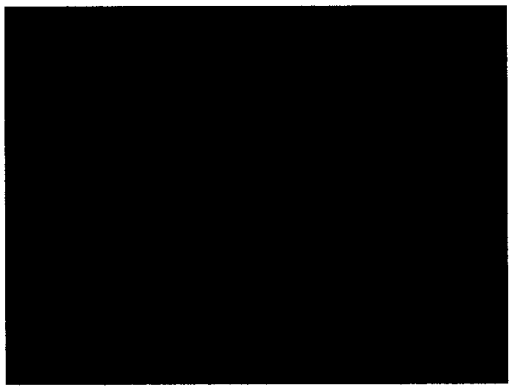

Figure 2.2 A. Control PBS

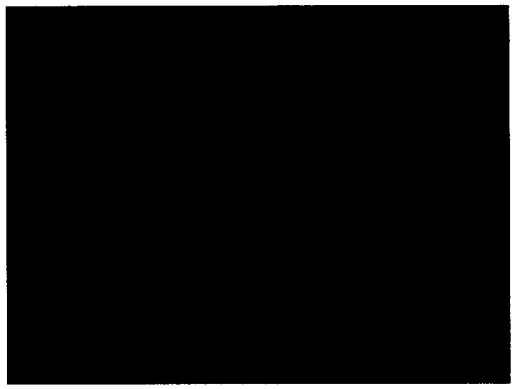

Figure 2.2 B. TrD with 1A1B-9

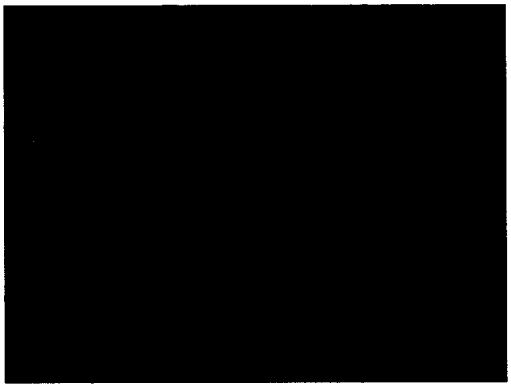

Figure 2.2 C. IE with 1A3B-7

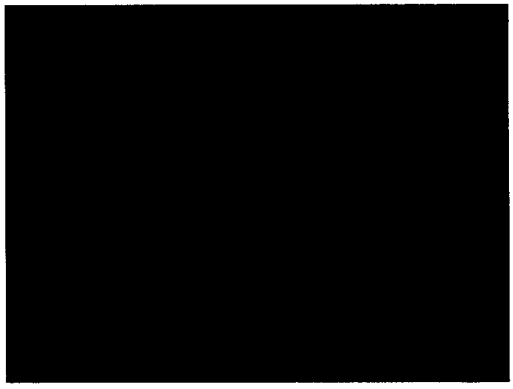

Figure 2.2 D. TrD with 1A3B-5

Figure 2.2 E. IE with 1A3B-9

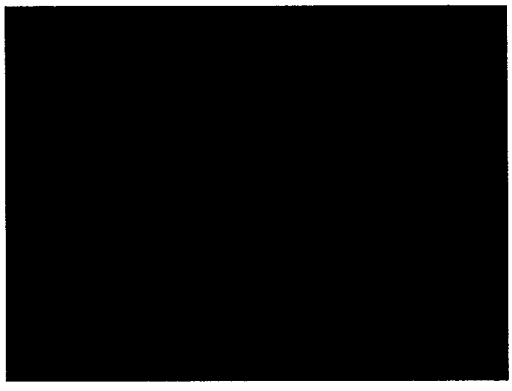

Figure 2.2 F. TrD with 1A3B-7

Figure 2.2. Monoclonal antibody stain of Vero cells infected with IE and TrD VEEV.

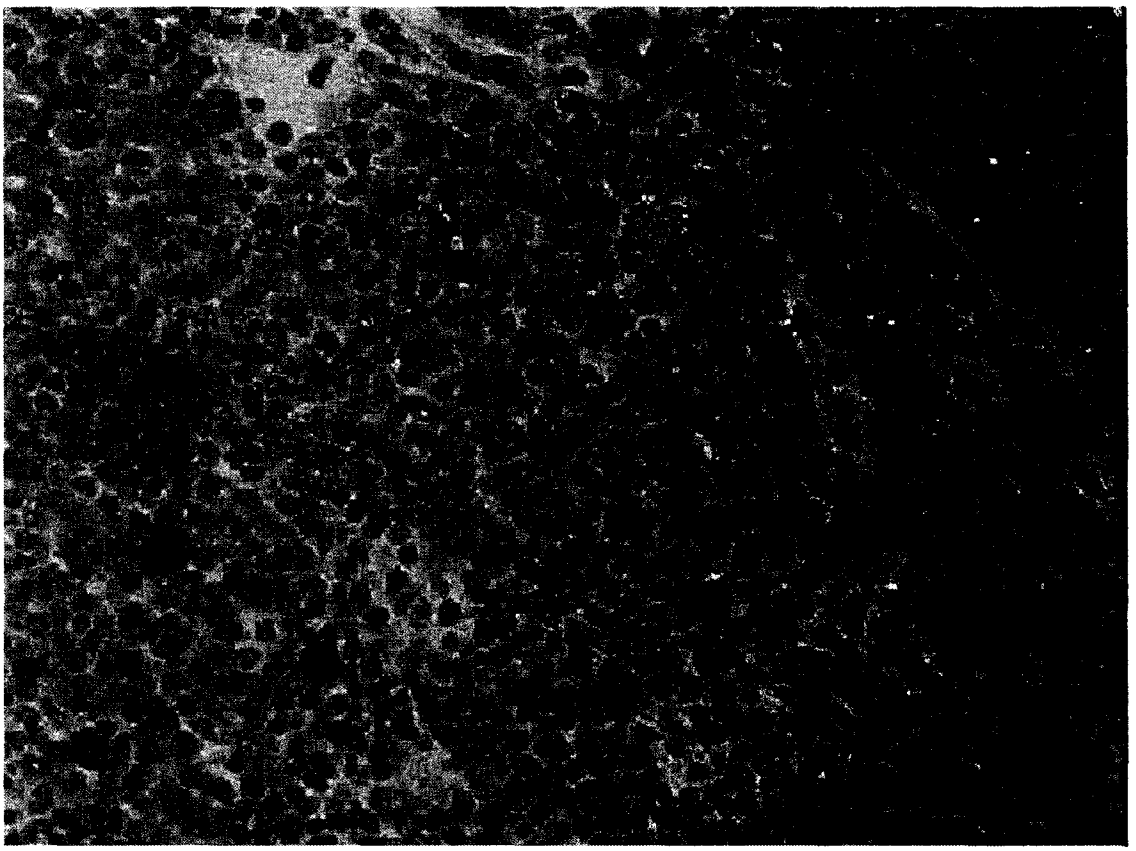

FIGURE 2.3. MT32 Left Superficial cervical lymph node, Fast Red ICH, x400.

TABLE 2.3 Tissue Viral Loads

Days post infection	1	2	2	4	4	6	6
Animal Number	MT32	MT21	MT33	MT34	MT22	DP16	DP18
Olfactory Bulb	<	<	1.8	6.5	IHC	IHC	IHC
Olfactory Tract	<	<	<	6.8	IHC	IHC	IHC
Anterior Cerebrum	<	1.6	2.1	<	4.7	2.7	2.5
Posterior Cerebrum	<	1.6	2.1	<	4.4	IHC	IHC
Cerebellum	<	1.9	2.1	<	4.8	4.4	2
Brain Stem	<	1.6	2.2	1.9	4.7	3.8	1.7
Optic Chiasm	<	1.9	<	2.6	4.2	3.7	4.2
Trigeminal Ganglia	<	<	<	<	<	ND	ND
Trigeminal N Face	<	2.9		3	3.6	ND	ND
Dental Pulp	<	5.1	3.1	2.8	4	3.6	2.7
Retina	<	1.9		1.6	4.3	ND	ND
Optic N at Eye	<	<	<	<	<	ND	ND
Lacrimal Gland	<	<	0.7	1.7	4	<	<
Salivary Gland	<	<	0.7	1.7	3.3	<	<
L Superficial Cervical LN	6.1	2.5	3.9	2.3	4.6	3	4
R Superficial Cervical LN	<	2.4	4.1	3.5	4.4	IHC	ND
L Deep Cervical LN	<	ND	6.5	5.6	5.9	2.7	3.3
R Deep Cervical LN	1.7	ND	ND	5.4	5.2	ND	ND
L Inginal LN	<	6	4.7	ND	4.6	ND	ND
R Inginal LN	<	3	3.1	ND	4.3	ND	ND
Mesentary LN	2.2	4.6	4.8	2.9	4.5	<	<
Bone Marrow	2.5	8	6.2	6.2	8.6	3.6	3.4
Cervical Spinal Cord	<	1.6	1.8	1.8	4.5	<	<
Lumbar Spinal Cord	<	<	1.9	<	4.9	<	<
Cerebral Spinal Fluid	<	<	<	<	<	<	<
Heart	<	<	2.5	<	2.8	<	<
Lung	<	2.2	1.6	1.8	5.2	<	<
Pancrea	<	4.7	ND	3.8	ND	<	<
Liver	<	<	2.6	<	ND	<	<
Spleen	2.2	2.9	3.4	3.1	6.4	2.4	2.5
Kidney	2	2.7	2.5	ND	5.3	<	<
Adrenal Gland	<	<	2.4	1.6	5.4	<	<

LN = Lymph Node
– immunohistochemistry assay positive

< not detected IHC
ND not done

TABLE 2.3: Tissue Viral Load. Virus titers in log 10 PFU/g tissue after infection with the Trinidad Donkey strain of VEEV. Horse MT01 data are not included since virus was not detectable in any tissues at 24 hours post challenge. The viremia in horse MT22 did not peak, but continued to increase through day 4 post challenge.

MT21 with $10^{2.2}$ PFU/mL at 24 hours post challenge. Viremia was highest at time of euthanasia, $10^{4.5}$ PFU/mL for MT21, and $10^{3.8}$ PFU/mL for MT33 (Figure 2.4).

Virus was isolated from multiple tissues, with highest titers in bone marrow (MT21 10^{8} and MT33 $10^{6.2}$), left inguinal lymph node (10^{6} and $10^{4.7}$), and left deep cervical lymph node ($10^{3.9}$ and $10^{6.5}$), mesenteric lymph node ($10^{4.6}$ and $10^{4.8}$), pancreas ($10^{4.7}$ MT21 only), and dental pulp ($10^{5.1}$ and $10^{3.1}$). Virus was not detected in either horse in the olfactory tract, trigeminal ganglia, facial portion of trigeminal nerve, retina, optic nerve at the eye, or cerebral spinal fluid. White blood cells decreased 45% and 24% for MT21 and MT33, respectively, by two days post challenge versus the day of challenge.

Horses MT22 and MT34 were sacrificed four days (96 hours) after challenge with the TrD strain of VEEV. Both horses exhibited mild depression by day three and moderate depression on day four. Body temperatures initially began to rise at around 31 hours for both horses. MT22's peak temperature of 40.5°C (104.9°F) was reached at 44-46 hours post challenge, and remained in the 39.5-40°C (103.1-104°F) range until euthanasia. MT33's peak temperature of 40.0°C (104.0°F) was reached at 38 hours post challenge, dipped to 38.5°C (101.3°F) at 65 hours, then rose again to the 40.0°C (104.0°F) by time of euthanasia. Viremia was detected first at 24 hours in MT22 and 32 hours in MT34. Viremic titers in both horses rose at similar rates until to 56 hours post challenge when viremic titers in MT22 and MT34 were $10^{4.8}$ & $10^{4.6}$ PFU/mL, respectively. Viremia titers in MT34 peaked at 56 hours pi and then declined until reaching $10^{2.6}$ PFU/mL by 96 hours post challenge, except for a short biphasic rise to $10^{4.4}$ PFU/mL at 88 hours. In contrast, the viremia titers of MT22 increased throughout

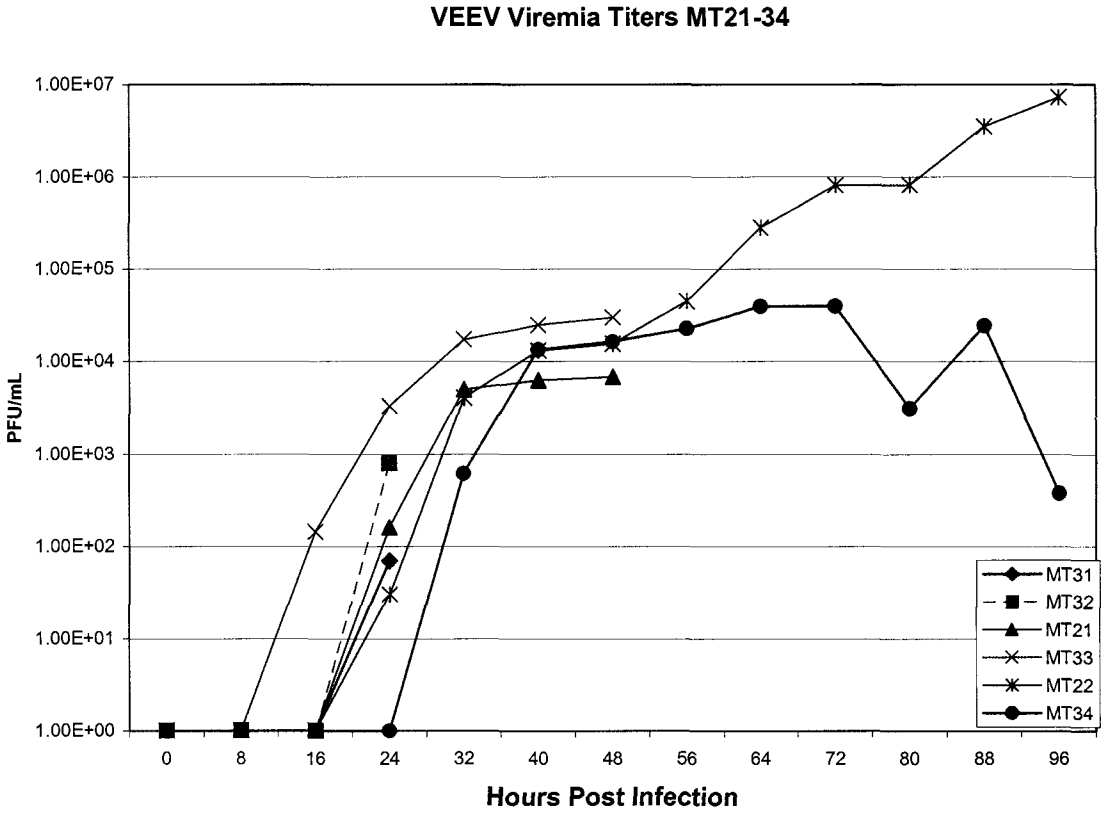

Figure 2.4 VEEV Viremia titers in MT21-34. Six horses were infected with TrD VEEV and two horses each sacrificed at 1, 2 and 4 days post inoculation. Serum samples were taken every eight hours and assayed for viral titers in Vero cells.

the study, with a peak of $10^{6.9}$ PFU/mL at euthanasia. White blood cell (WBC) count for MT22 decreased from 12,900 cells/μl, prior to challenge, to 1,600 cells/μl at euthanasia. WBC for MT34 decreased around 50%, with the lowest levels at euthanasia. MT22 had detectable virus titers in every tissue tested. Highest virus titers, for both MT22 and MT34, were found in the bone marrow ($10^{8.6}$ & $10^{6.2}$, respectively), spleen ($10^{6.4}$ & $10^{3.1}$), and deep cervical lymph nodes ($10^{5.9}$ & $10^{5.6}$). Only MT34 had detectable viral titers in the olfactory bulb ($10^{6.5}$) and olfactory tract ($10^{6.8}$). The posterior cerebrum, anterior cerebrum and bone marrow were positive for viral antigen by IFA. Cerebrum and cerebellum were positive by alkaline phosphatase IHC. Two cell types in the olfactory tract were positive for viral antigen by fluorescent antibody and alkaline phosphatase IHC, neuronal like cells with dendritic like processes, and pyramid shaped cells. (Figures 2.5 & 2.6)

Two horses (DP16 and DP18) were sacrificed at six days post infection, after developing neurologic signs of encephalitis due to TrD VEEV. Clinical signs consisted of depression, head pressing, and teeth grinding starting on day four post challenge. Increased body temperature was evident by 48 hours (DP16), and 52 hours (DP18) (Figures 2.7 and 2.8). DP16's temperature increased to 40.5°C (104.5°F) by 60 hours post challenge, and peaked at 41.0°C (105.8°F) at 4.5 days post challenge. DP18's temperature peaked at 39.5°C (103.1°F) by 2.5 days, returned to normal for 3-5 hours at day 4, then increased again to 39.0°C (102.2°F), in a biphasic response (Figure 2.8).

FIGURE 2.5. MT 22 Olfactory tract infected pyramid shaped cell x400, indirect immunofluorescence assay.

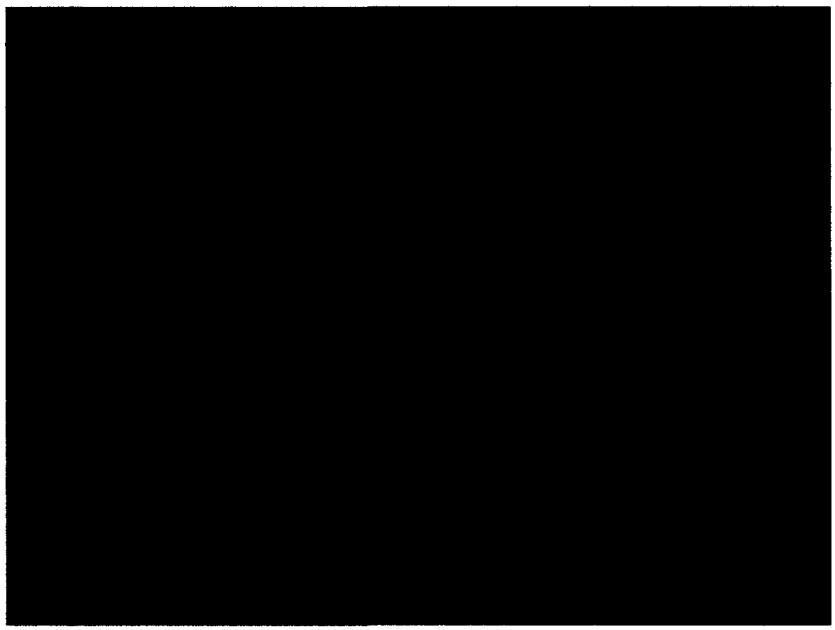

FIGURE 2.6 MT22 Aggregate of viral antigen positive cells by indirect immunofluorescence assay, in olfactory tract x400.

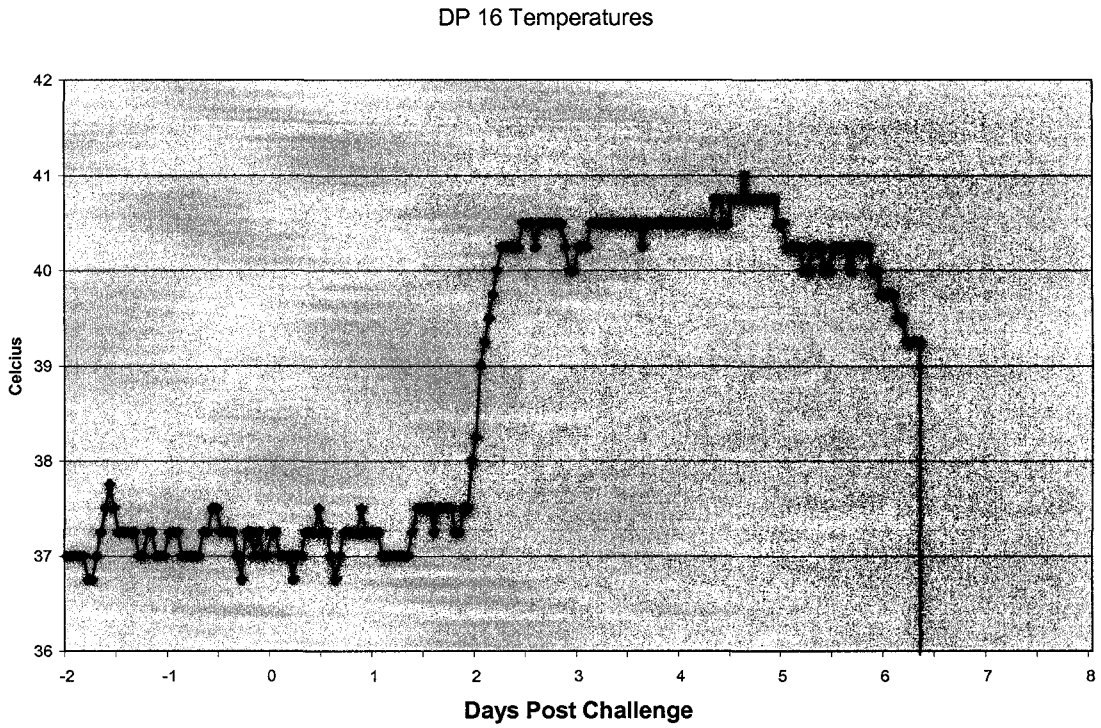

FIGURE 2.7. Body temperature of DP16, in degrees Celsius, after inoculation with TrD VEEV.

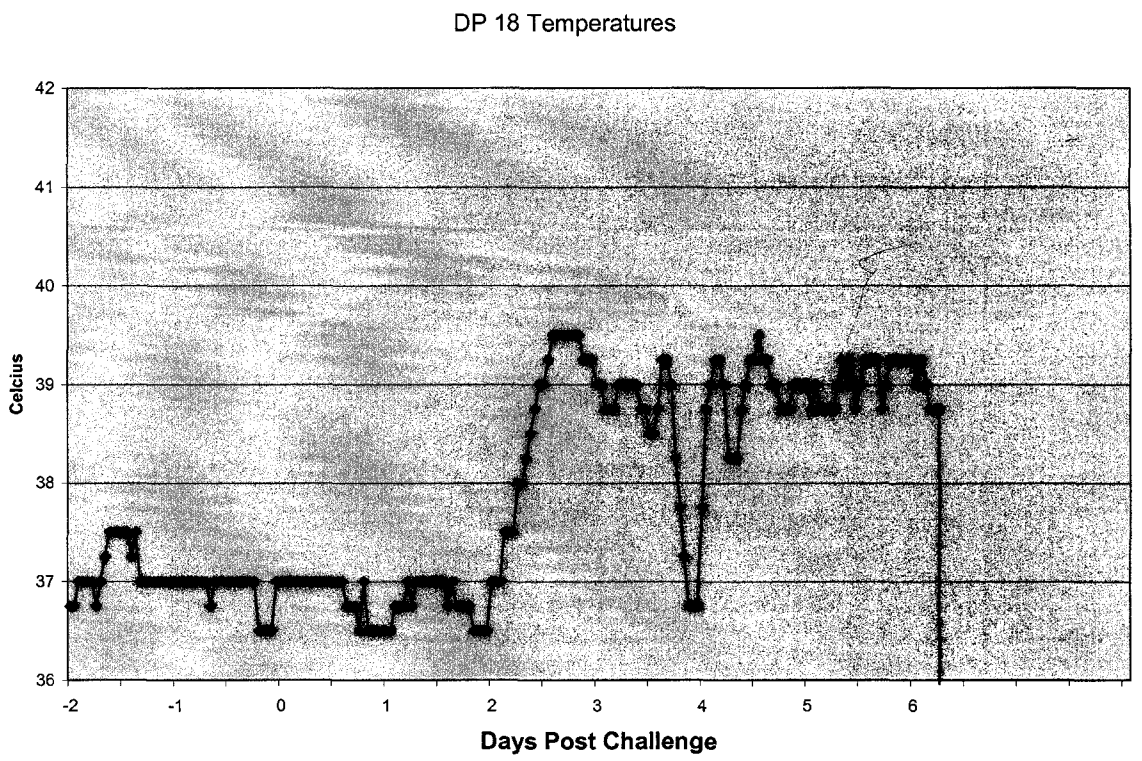

FIGURE 2.8. Body temperature of DP18, in degrees Celsius, showing biphasic response after inoculation with the TrD strain of VEEV.

Viremia was first detectable at 24 hours for both horses. DP16 had a peak viremic titer of $10^{4.2}$ PFU/mL at 3.5 days, and then viremic titers decreased until they were no longer detectable by five days post challenge. DP18 had peak viremia titer of $10^{4.6}$ PFU/mL at 3.5 days, which also decreased until viremia was undetectable at five days post challenge. DP16's WBC count fell from 10,000 cells/μl at challenge to 4,000 cells/μl by day five post challenge, then increased to 8500 cells/μl by day six. DP18 WBC count decreased from 7000 cells/μl at challenge to 2000 cells/μl at day five post challenge (Figure 2.9).

The highest tissue virus titers were from the bone marrow ($10^{4.2}$ and 10^{2} PFU/g; DP16 and DP18, respectively), the cerebellum ($10^{5.2}$ and 10^{2}), and from optic chiasm ($10^{3.7}$ and $10^{4.2}$). Dental pulp at six days post challenge, yielded $10^{3.7}$ (DP16) and $10^{2.6}$ (DP18) PFU/g of tissue (Table 2.3). Virus titers were not obtained from the olfactory tracts of DP16 or DP18, but both tissues along with the cerebrum, were positive for viral antigen by immunohisto-chemistry (Figure 2.10 & 2.11).

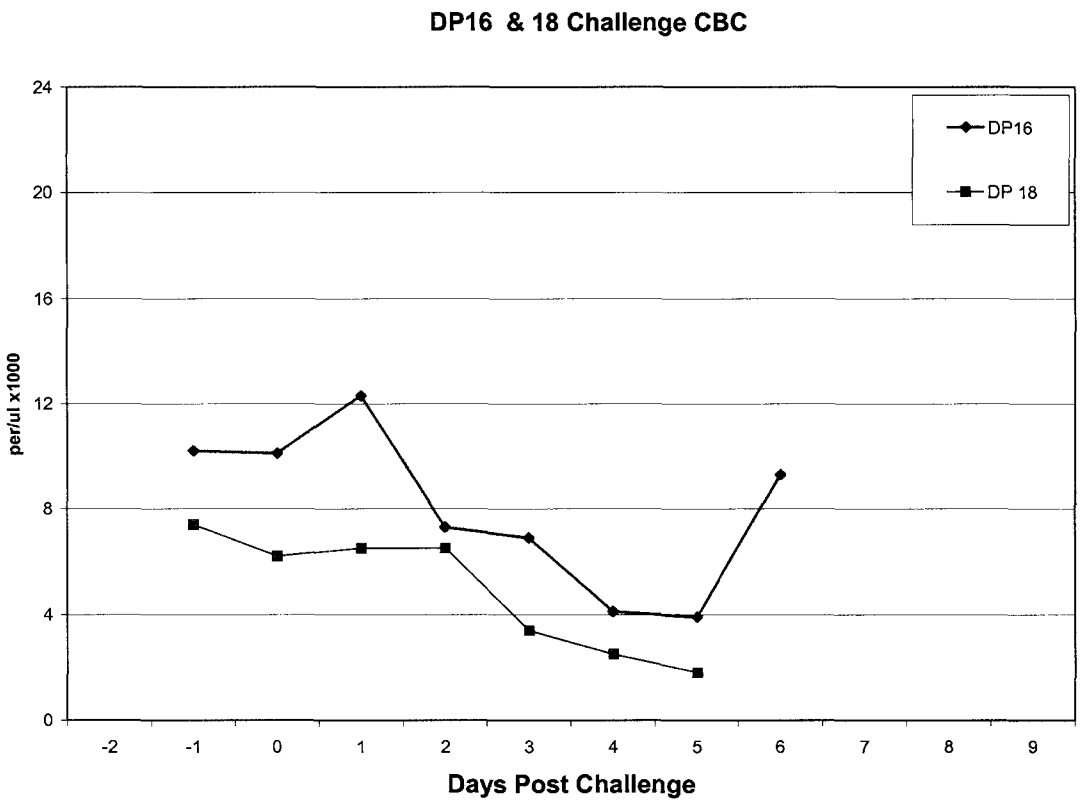

FIGURE 2.9. White blood cell counts of DP16 and 18, after inoculation with TrD VEEV.

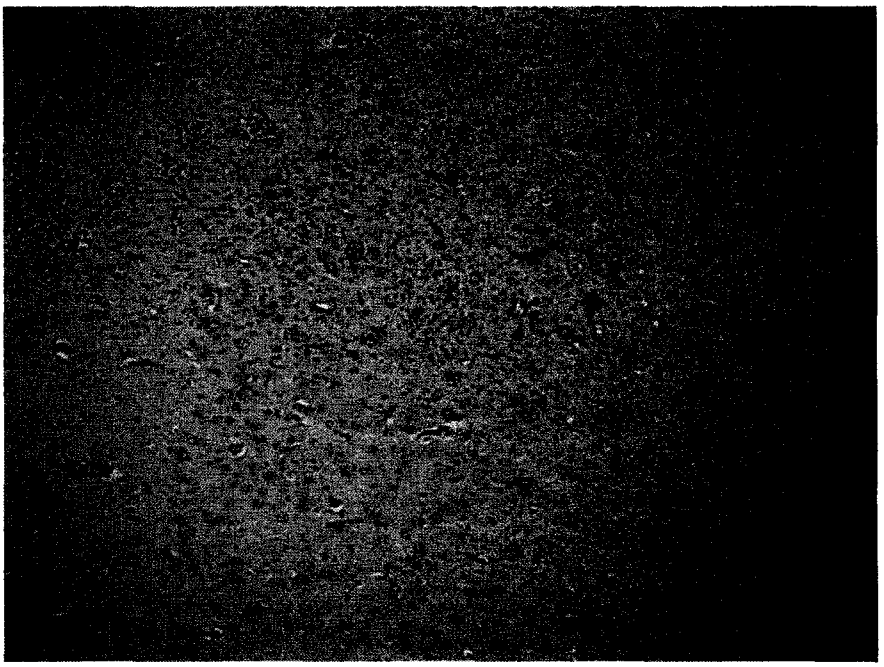

FIGURE 2.10. DP 16 Posterior Cerebrum, IHC Fast Red stain x200.

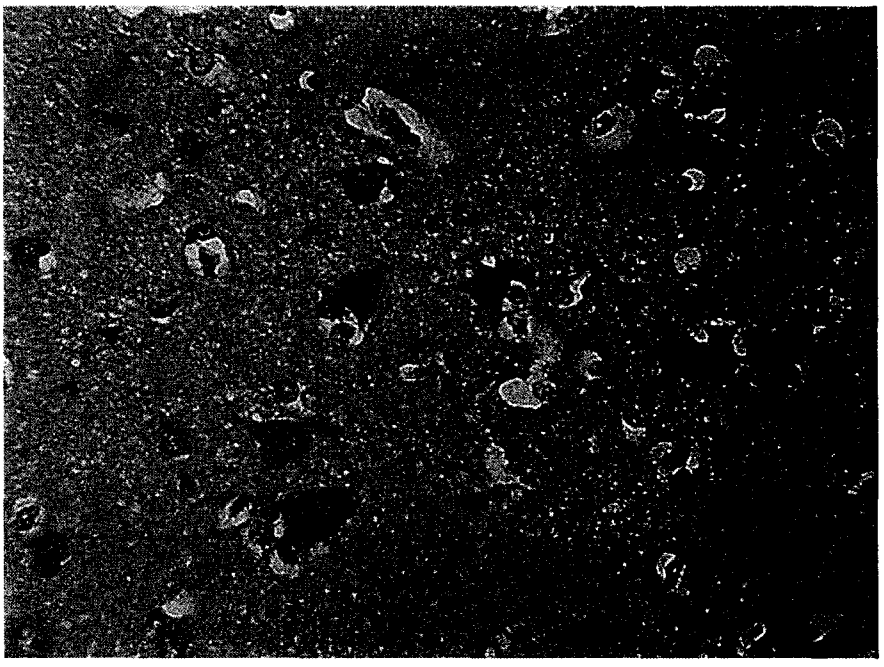

FIGURE 2.11. DP 18 anterior cerebrum, Fast Red IHC staining x400.

DISCUSSION

Bone Marrow appears to be an important tissue in the replication of TrD VEEV in horses. Twenty-four hours after challenge, the bone marrow was second in viral titer magnitude ($10^{2.5}$ PFU/g), surpassed only by the draining lymph nodes ($10^{6.1}$ PFU/g). By day two post infection, the highest virus titers were found in the bone marrow ($10^{8.0}$ & $10^{6.2}$ PFU/g) (Figure 2.12). On the fourth day after infection, the highest viral titers were, again, found in the bone marrow ($10^{6.2}$ PFU/g) in MT22, while in MT34 the highest titers were found in the olfactory bulb and tract ($10^{6.5}$ & $10^{6.8}$ PFU/g tissue, respectively) and second highest titers in the bone marrow ($10^{6.2}$ PFU/g). Even by day six post infection when horses exhibited neurological signs consistent with encephalitis, the bone marrow still had a high level of infectious virus ($10^{3.6}$ & $10^{3.4}$ PFU/g), equal to the titers in the central nervous system ($10^{1.7\text{-}4.4}$ PFU/g).

Comparisons of the peak bone marrow virus titers and the peak viremia titers suggest that the virus was replicating in the bone marrow. In the four horses necropsied at two and four days (MT21, MT33, MT22 and MT34) the peak bone marrow titers (10^{8}, $10^{6.2}$, $10^{8.6}$, and $10^{6.2}$ PFU/g, respectively) were all higher than the peak viremia titer reached at any time during infection. After initial seeding by viremia, the bone marrow may actually contribute to increased magnitude of the viremia titer, although the lymph nodes probably also contribute to the magnitude of viremia.

Results from previous research on IAB VEEV in horses either did not look for virus in bone marrow or found significantly lower titers of infectious virus. One study mentions that the bone marrow was almost depleted pathologically of hematopoietic

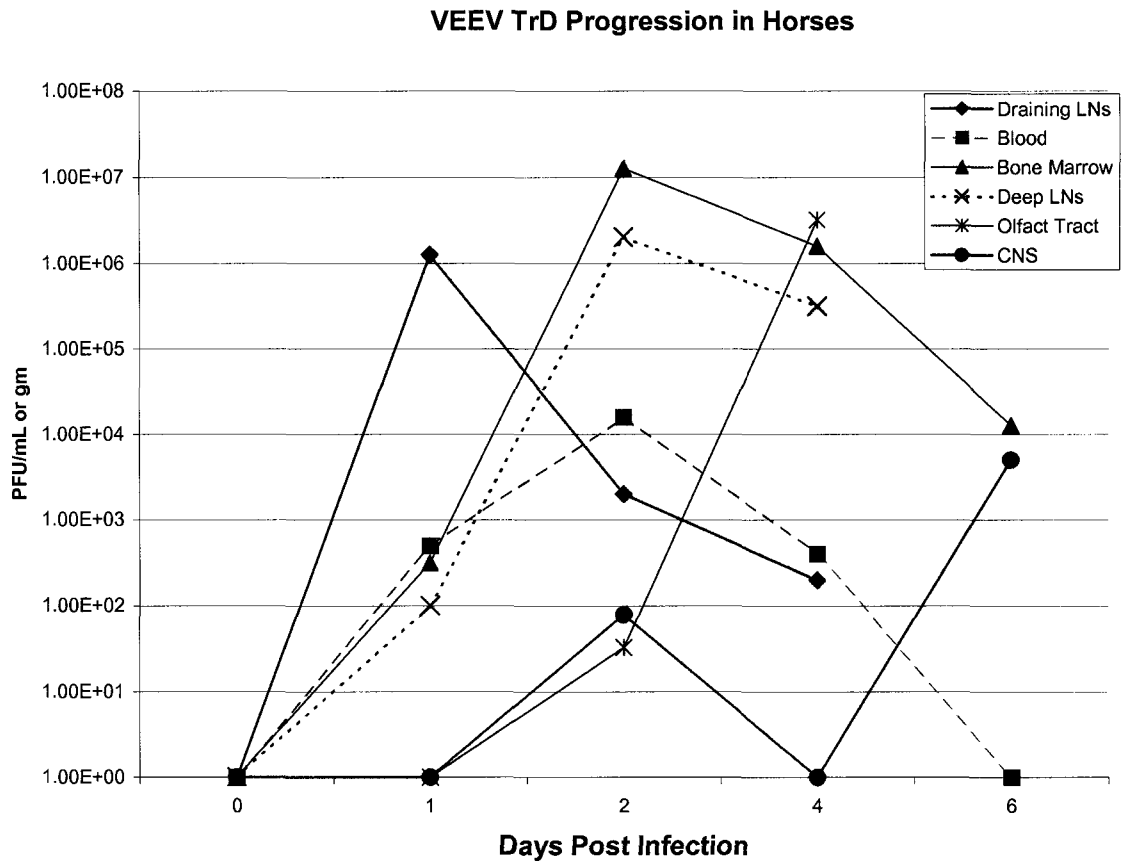

Figure 2.12. TrD VEE virus titrated in Vero cells from tissues (PFU/gram) or sera (PFU/mL). Each data point represents the mean of values from two horses, except data points on day 4 which represents a single horse.

cells (Kissling et al, 1956) but, bone marrow is not in the list of tissues assays for virus. In another study, bone marrow was not collected for virus titration on the two horses challenged with GJ9-1BJ, a IAB VEEV subtype (Henderson et al, 1971).

One reason that other studies found little or no virus in the bone marrow may have been due to clearance of the virus before necropsy. In mice, virus is cleared from the tissues, except CNS, one to two days after clearance of viremia (Charles et al, 2001; Jackson et al, 1991). This same scenario is seen in mice infected with mouse-pox virus (Fenner, 1948). In horses challenged with IAB VEEV, viremia is cleared by around day five pi (Walton et al, 1973; Kissling et al, 1956). One study noted an inverse relationship in the time period between clearance of viremia and necropsy, and the ability to isolate virus from tissues (Kissling et al, 1956). Another study reported isolating IAB VEEV only from the brain and kidney in horses, which were necropsied one to four days after the disappearance of viremia (Walton et al, 1972). Viral titers (10^7 LD_{50}) in the bone marrow of mice sacrificed at 48 hours post challenge (Tasker et al, 1962) were comparable to this study in horses. For this thesis, horses were necropsied during the viremic phase of infection. This should have allowed tissue samples to be obtained prior to virus clearance.

Four days after inoculation with TrD VEEV, the olfactory bulbs and olfactory tracts contained more virus and viral antigen than the cerebrum and cerebellum. At two days post infection, the two cerebrum samples and olfactory bulb sample virus titers were nearly the same ($10^{1.6}$, $10^{2.1}$ and $10^{1.8}$ PFU/g, respectively). These early viral titers in the CNS may have been the result of mechanical seeding of virus from the blood. At four days post infection no virus was found in the cerebrum of

MT34, while $10^{4.6}$ PFU/g were found in the cerebrum of MT22. MT22 was the horse whose viremia increased the entire four days of infection, in contrast to all other infected horses, and peaked at $10^{6.9}$ PFU/mL, the highest viremic titer noted in all horses. Additional evidence that suggests the olfactory tract contained virus at 4 days post challenge while the anterior and posterior cerebrum contained no detectable virus was the indirect IFA on MT22. The IFA of MT22's olfactory tract showed several aggregates of virus infected cells per slide, while all six assayed slides from the cerebrum were negative. This evidence suggests that the olfactory bulb and olfactory tract contained more virus than the cerebrum at four days after inoculation in these two horses. This may indicate that either the virus replicates to a higher magnitude in the olfactory bulb and tract than in the cerebrum, or it may mean that these two tissues are infected before the cerebrum. Infection of the olfactory system before the cerebrum suggests that infection of the CNS is through the olfactory nerve. One possible reason for infection of the olfactory tract prior to the cerebrum is that the infection initiates in the olfactory bulb and moves in a retrograde manner through the olfactory tract to the CNS along nervous tissue. This route of entry of VEEV into the CNS has been suggested in mice after subcutaneous inoculations (Charles et al, 1995), and after intranasal inoculation for mouse hepatitis virus (Barnett and Perlman, 1993), pseudorabies (Babic et al, 1994) and rabies viruses (Johnson, 1965). Previous studies in horses would not have detected this evidence because serial early pathogenic studies have not been completed in horses. This study provides evidence that the route of CNS infection may be through the olfactory nerve, but this evidence is based on tissues from only three horses so further study is warranted.

Viral titers in the dental pulp and trigeminal nerve suggest viral replication in these tissues. Viral titers in the dental pulp and trigeminal nerve, which innervates the dental structures, were equal to the viremic levels at two days post inoculation (Figure 2.13). By six days post inoculation, viremia was not detectable while the dental pulp still contained $10^{3.2}$ PFU/gram. This level of virus titers in the dental pulp suggests viral replication in the tissue rather than mechanical seeding. Seeding of the dental pulp with retrograde movement up the trigeminal nerve has been suggested in mice (Charles et al, 1995).

Data suggest viral replication occurred initially in the draining lymph nodes and deep lymph nodes. By 24 hours post challenge, the draining lymph nodes contained viral titers of $10^{6.1}$ PFU/gram, which were the highest viral titers of any tissue at this time. By two days post challenge, virus was detectable in the deep and distant lymph nodes. At four days post challenge, only the bone marrow ($10^{7.1}$ PFU/gram) had higher viral titer than the deep lymph nodes ($10^{3.0\text{-}6.0}$ PFU/gram). The high viral titers at 24 hours post infection in the draining lymph nodes support the model of VEEV replication in the local lymph node with spread to other lymph nodes (Gleiser et al, 1961, Grieder et al, 1995). VEEV may spread to other lymph nodes through either the lymphatic system or the blood (Charles et al, 2001).

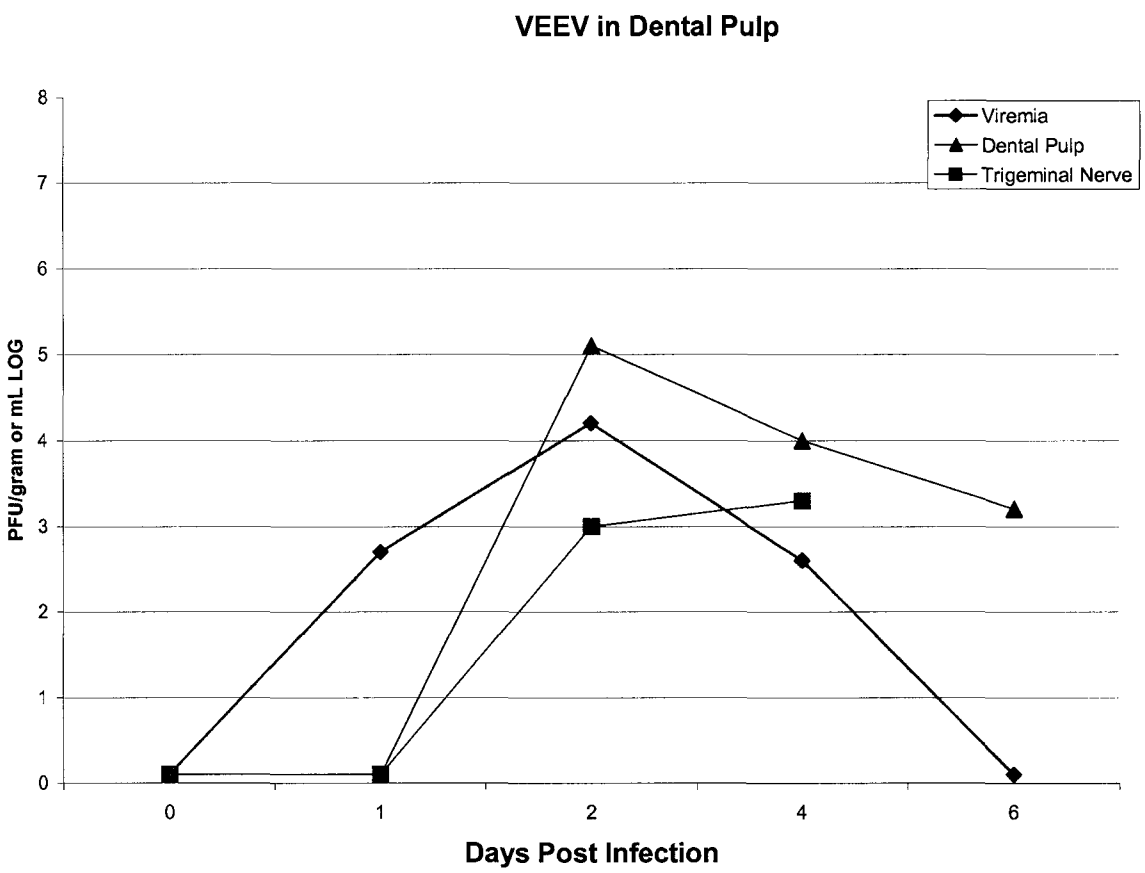

Figure 2.13. VEEV titers in the blood, dental pulp and trigeminal nerves of horses inoculated with TrD. Data are from two horses sacrificed at each time point.

SUMMARY

Evidence from this study suggests that the early pathogenesis of VEEV in horses is similar to that seen in mice. Draining lymph nodes are the first major tissues infected, followed by infection of other lymph nodes and viremia. A novel finding from this study was the high viral titers in the bone marrow of horses. This suggests the bone marrow may be one of the more important tissues in viral replication. Additional evidence from this thesis indicates viral titers are detected earlier and to higher levels in the olfactory tract than in the cerebrum, which suggests that the olfactory nerve serves as an infection route for virus into the CNS.

CHAPTER III

EARLY INTERFERON RESPONSE IN HORSES CHALLENGED WITH VENEZUELAN EQUINE ENCEPHALITIS VIRUS

INTRODUCTION

Alpha and beta interferons (IFN –α/β) have been shown to be an important factor in the early innate immune response to virus challenge. Mice without a functional IFN response succumb to avirulent virus challenge (Grieder & Vogel, 1999; Hwang et al, 1995; Muller et al, 1994), yet IFN alone is not sufficient in repelling severe or fatal challenge with many viruses. Mice treated with IFN, IFN inducers, or PEGylated IFN have improved survival times, but still succumb to viral challenge (Lukaszewski & Brooks, 2000). In humans infected with Ebola virus, a profound IFN response has been equated with a poor outcome (Villinger et al, 1999). It is theorized that for resistance to many viral infections to be successful, a combination of early

innate immunity and adaptive immunity are required. The innate immunity limits the spread of virus until the acquired adaptive immunity can eliminate the viral infection. This study attempts to examine potential differences in IFN response in horses to avirulent and virulent VEEV challenge, and the IFN response to a live-attenuated VEEV vaccination.

The speed and sensitivity of interferon assays can be varied by changing the parameters of cell concentration, virus concentration, length of incubation, and time of challenge (Rubinstein et al, 1981). Test results can be obtained in as little as 16 hours, if the right parameters are used, but a rapid test decreases sensitivity. For this research, assay sensitivity was deemed more important than speed, so established cell monolayers were used, with an overnight incubation of interferon samples and a low MOI of challenge virus.

SPECIFIC AIM: Characterize the early serum IFN response in horses to epizootic and enzootic VEEV challenge and to inoculation with a live-attenuated VEEV vaccine.

HYPOTHESIS: The interferon response in horses is influenced by a complex set of mechanisms, but primarily by the amount of viremia present, thus the more abundant IFN response will be observed in subjects that have the higher and longer viremic titers typical of epizootic virus challenge. Enzootic and vaccine exposures will result in a low, but detectable IFN response.

MATERIALS AND METHODS

Animals

Preparation of all horses in this portion of research was accomplished as described in chapter two of this thesis. A brief description is repeated here for convenience of the reader. Adult horses were tested negative for antibodies to WEE, EEE and VEE viruses. Temperature recorders were implanted in the left flank of each horse 10-14 days prior to challenge, and horses entered into the BSL-3 facility. All animal use was approved by the CSU Animal Use Committee and the Code of Federal Regulations #9 of the US Department of Agriculture was followed.

Viruses

Four different VEE virus strains were used in this portion of research:

- Trinidad donkey strain (TrD), an equine-virulent IAB subtype virus.
- 64A99, a IE subtype virus that appears to be avirulent in horses; it was originally isolated in mice from a pool of mosquitoes collected in Mexico in 1964 (Scherer et al, 1971; Dietz et al, 1978).
- 93-42124 (Mex32), a IE subtype virus originally obtained in the Chiapas state of Mexico during the 1993 IE epizootic, from CNS tissue of a horse that died (Oberste et al, 1998).
- V3526, a live, attenuated vaccine strain of virus (Davis et al, 1995).

Challenge and Collection of Specimens

The first group of eleven horses was challenged with the IAB TrD strain (five horses), or the IE 64AA99 IE strain (three horses), or the IE Mex32 strain (three horses) of VEE virus. The horses were allowed to progress at least two weeks post challenge or until clinical signs of encephalitic disease were evident, before they were humanely euthanized. Challenge was by subcutaneous injection of either 10^4, $10^{4.3}$, or $10^{5.4}$ plaque forming units (PFU) of TrD, 64A99 or Mex32, respectively. Serum, blood for complete blood counts (CBC), and temperature measurements were collected the day before and the day of challenge. After challenge, sera were collected twice daily for ten days. For serum, the blood was allowed to clot for four to eight hours, centrifuged, separated, and stored at -80°C until IFN assays could be performed.

A second group of 12 horses was vaccinated against VEE virus, with one of two dilutions of the attenuated VEEV strain V3526 (Table 3.1). Baseline sera, CBCs, and temperatures were taken before vaccination, the same as the first group of horses. Twenty-eight days after the vaccination, the horses were challenged with either the TrD or 64A99 strain of VEE virus. Sera and rectal temperatures were taken twice daily for 10 days after vaccination, and also for 10 days after challenge. CBCs were taken once a day for 10 days after vaccination and 10 days after challenge.

IFN assays

Interferon assays were completed in duplicate or triplicate using a modified technique based on previously described interferon assays (Rubinstein et al, 1981; Familletti et al, 1981). In this modified technique, MDBK (Madin-Darby bovine

Table 3.1

List of Horses Challenged with VEEV

ID Number	Sex	Age Years	Vaccine log 10 PFU	Challenge Virus	Challenge log 10 PFU	Euthanize days pi
DP 16	F	3	—	IAB (TrD)	4	5.5
DP 17	CM	3	—	IAB (TrD)	4	6
DP 18	CM	11	—	IAB (TrD)	4	5.5
DP 37	F	4	—	IAB (TrD)	4	8
DP 38	F	8	—	IAB (TrD)	4	6
DP 29	F	6	—	IE (64A99)	4.3	18
DP 30	CM	14	—	IE (64A99)	4.3	17
DP 31	F	3	—	IE (64A99)	4.3	17
DP 6	F	8	5	IAB (TrD)	4	15
DP 7	CM	3	5	IAB (TrD)	4	14
DP 8	CM	5	5	IAB (TrD)	4	19
DP 9	F	10	5	IAB (TrD)	4	19
DP 10	CM	6	3	IAB TrD	4	19
DP 11	F	4	3	IAB (TrD)	4	16
DP 12	CM	7	5	IAB (TrD)	4	20
DP 13	F	3	3	IAB (TrD)	4	20
Dp 14	F	8	3	IAB (TrD)	4	16
DP 15	F	2	3	IAB (TrD)	4	20
DP 22	F	3	5	IE (64A99)	4.3	14
DP 27	F	3	3	IE (64A99)	4.3	18
SW 8	F	2	—	IE (Mex32)	5.4	7
SW 46	CM	1	—	IE (Mex32)	5.4	10
SW 47	CM	1	—	IE (Mex32)	5.4	7
MT 31	F	1.5	—	IAB (TrD)	3.3	1
MT 32	M	1.5	—	IAB (TrD)	3.3	1
MT 21	CM	1	—	IAB (TrD)	3.3	2
MT 33	M	2	—	IAB (TrD)	3.3	2
MT 22	F	1	—	IAB (TrD)	3.3	4
MT 34	M	2	—	IAB (TrD)	3.3	4

M = Male F = Female CM = Castrated Male

Table 3.1. List of horses challenged with VEEV. Vaccine dose (V3526) and challenge dose of VEEV strain is given in log 10 PFU.

kidney) cells (epithelial origin) (Madin & Darby, 1958) were grown to confluency, exposed to samples purported to contain IFN, then challenged with vesicular stomatitis virus (VSV). The MDBK cells were used between the third and eighth passages.

To make the IFN assay plates, MDBK cells were trypsinized and diluted in growth medium (DMEM supplemented with 7.5% FBS, penicillin, streptomycin, and amphotericin B) to a concentration of approximately 50-55 x 10^3 cells per 100µl. Each 96-well plate was seeded with 100 µL of cell suspension per well and cells allowed to grow to confluency. Growth medium was replaced prior to the addition of serum samples.

Twenty-five international units/100 µL of human interferon A served as a positive control in each assay. Sera were heat-inactivated at 56°C for 30 minutes. The samples and positive control, in triplicate, were diluted two-fold from 1:2 to 1:128 in the 96-well plates containing cell monolayers. After overnight incubation, these were challenged with 1600 PFU VSV (Indiana strain). Each 96-well plate also contained five wells without IFN and seven wells without virus, as additional controls.

After addition of VSV, the plates were incubated for 44-56 hours. The plates were fixed and stained when cells in all control wells were determined to have approximately 95% or greater cytopathic effects. To fix and stain, the plates were immersed in 70% methanol with 0.5% (w/v) crystal violet for 10-15 minutes, washed under running water, and allowed to air dry.

The interferon titer was assessed as the reciprocal of the dilution that represented protection of greater than 50% of the cell monolayers. Initially, plate assessment was verified by microscopic examination of wells, to ensure they corresponded with visual

assessments. After three sets of IFN assays, the microscopic assessments were discontinued as they were consistent with the gross visual assessments.

RESULTS

Reproducibility of the IFN assays varied by up to four-fold for the same sample. Others have encountered similar problems in serum IFN assays (Marquardt et al, 1992). Indirect measures of IFN induction have been used for VEEV in mice (Schoneboom, et al, 2000) and EHV-1 in horses (Bridges & Edington, 1986) to alleviate this problem. Indirect measures include assay of IFN mRNA expression by RT-PCR (Schoneboom, et al, 2000), and assay of IFN produced in peripheral blood mononuclear cells obtained from challenged subjects (Marquardt et al, 1992).

On trial assays, it was determined that assay results were affected by the passage history of the MDBK cells and the confluency of the cells before addition of sample. To get consistent results, cells were used between the fourth and eighth passages, the cells were passed early in confluency, and same final concentration of cells was added to each well. Additionally, cell monolayers containing VSV and no protective IFN were included in each plate to verify consistent cell cytopathic effects.

Five unvaccinated horses (DP16, DP17, DP18, DP37, and DP38) were challenged subcutaneously with 10^4 PFU of Trinidad Donkey VEEV (IAB) and allowed to progress until clinical signs of encephalitis were evident. All five developed a detectable peripheral blood IFN response, with peak titers between 320 IU/mL (DP18, DP37, and DP38) to 1280 IU/mL (DP16) of serum (Table 3.2). Serum IFN was first

Table 3.2

IFN Titers in Horses Challenged with TrD VEEV

	Hours Post Challenge												
Horse #	0	12	24	36	48	60	72	84	96	108	120	132	144
DP 16	<	<	30	320	480	640	640	1280	1280	960	320	40	<
DP 17	<	<	10	60	160	160	480	480	320	96	30	<	<
DP 18	<	<	40	40	80	160	320	240	80	20	<	<	<
DP 37	<	<	<	320	240	240	160	80	50	20	<	<	<
DP 38	<	<	<	80	240	320	320	320	80	30	<	<	<
Average	<	<	16	164	240	304	384	480	362	225	70	8	<

< not detected

TABLE 3.2. Serum IFN levels in five horses challenged with IAB (TrD) VEE virus. Numbers indicate the reciprocal of the IFN titer that protected 50% of MDBK cells.

detectable at 24 hours post challenge in 3 of 5 (60%) animals. IFN became detectable with the remaining two animal's sera at the next sample (36 hours). Serum IFN levels remained detectable through 4.5 days post challenge for three horses (DP18, DP37, DP38), through 5 days for one horse (DP17), and through 5.5 days for one horse (DP16). The length, in days, of detectable IFN ranged from 3.5 to 5.0 days, with an average length of 4.1 days.

Serum viremia was detectable in all five animals, with a peak range from $10^{3.7}$ PFU/mL (DP38) to $10^{4.7}$ PFU/mL (DP18, DP37) (Table 3.3). Viremia was first detectable at 24 hours for 3 horses (60%), the same three horses (DP16, DP17, DP18) in which IFN was first detectable at 24 hours. The other two horses (DP37, DP38) had detectable viremia at 36 hours post challenge, the same time that IFN was first detectable in these horses. Viremia remained detectable through 4.5 days for two horses (DP16, DP18), through the fourth day post challenge for two horses (DP17, DP37) and through 3.5 days post challenge for one horse (DP38). Time span for detectable viremia ranged from 2.5 days (DP38), to four days (DP16, DP18). Viremia in the other two horses was detectable for 3.0 and 3.5 days (DP17, DP37). Average time span for detectable viremia titers in the five horses was 3.4 days.

In all horses except one (DP18), detectable IFN lasted longer than detectable viremia levels. In the one horse (DP18), both viremia and IFN were detectable the same length of time (4.0 days). In three of the horses, the last detectable IFN sample was taken 24 hours after the last detectable viremia sample.

Serum samples were obtained from twelve adult horses that had been vaccinated with an attenuated VEEV vaccine (V3526). Six of twelve horses (50%) had low levels

Table 3.3

Viremic Titers in Horses Challenged with TrD VEEV

	Hours Post Challenge										
Horse	**0**	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**	**108**	**120**
DP16	<	<	2.0	3.0	3.6	3.9	4.0	4.2	4.1	2.7	<
DP17	<	<	1.2	2.6	3.6	4.4	4.1	4.0	0.6	<	<
DP18	<	<	1.7	3.6	3.7	4.4	4.4	4.7	3.5	2.5	<
DP37	<	<	<	2.9	4.0	4.5	4.7	4.1	2.3	<	<
DP38	<	<	<	3.5	3.6	3.4	1.3	3.7	<	<	<
Average	<	<	1.6	3.3	3.7	4.3	4.3	4.3	3.5	2.2	<

log 10 / mL < = not detectable

TABLE 3.3. Serum viremia levels in five horses challenged with IAB (TrD) VEE virus.

of serum IFN between one and four days post vaccination (Table 3.4). Peak IFN titers ranged from 20 IU/mL to 80 IU/mL serum in the six horses. None of the six horses had detectable IFN titers for longer than 48 hours. Four horses had detectable IFN titers for 24 hours, one for 36 hours and one for 48 hours. Average length of detectable IFN for the six positive horses was 30 hours. Viremia was not detected in any of the horses from day zero to day ten post challenge.

Serum samples were also obtained from seven horses previously vaccinated with V3526 virus and challenged four weeks later with TrD VEEV, four horses challenged with $10^{4.0}$ PFU of TrD, and three horses with $10^{4.3}$ PFU of strain 64A99. None of the seven vaccinated horses had detectable IFN titers between zero and seven days post challenge with virulent VEEV.

Three unvaccinated horses (WEE, EEE and VEE seronegative) were challenged with the 64A99 strain of VEEV. None of the three horses exhibited clinical signs of infection. Virus was not detected by Vero cell inoculation, RT-PCR, or quantitative RT-PCR (See chapter 4) on blood samples taken twice daily through ten days. All three horses responded with detectable levels of serum IFN. Peak levels of IFN ranged from 210-320 IU/mL (Table 3.5). Initial detection of IFN from these three horses was at 24 hours for one horse, and 36 hours for the other two horses. Duration of detectable IFN was three days (72 hours) for two horses, and 2.5 days for one horse (60 hours) for an average of 2.8 days.

Three additional unvaccinated horses were challenged with $10^{5.4}$ PFU of the Mex32 strain of IE VEEV. All three horses challenged with the Mex32 virus became ill, with clinical signs consistent with encephalitis (Bowen, unpublished data). IFN was not detected in blood samples from any of these horses through day ten post infection.

Tables 3.4 & 3.5

IFN Titers in Horses Challenged with VEEV vaccine strain V3526

	Hours Post Vaccination									
	0	12	24	36	48	60	72	84	96	108
10	<	<	<	20	20	20	<	<	<	<
11	<	<	<	<	20	30	<	<	<	<
12	<	<	<	10	20	<	<	<	<	<
15	<	<	<	<	20	20	<	<	<	<
22	<	<	<	80	10	<	<	<	<	<
27	<	<	<	20	40	60	20	<	<	<

< not detected

TABLE 3.4. IFN titers in six horses that had detectable titers after VEEV (V3526) vaccination. Six additional horses had no detectable titers and are not shown. Number represents the reciprocal titer that provides 50% protection against VSV challenge.

IFN Titers in Horses Challenged with IE 64A99

	Hours Post Infection									
Horse	0	12	24	36	48	60	72	84	96	108
DP29	<	<	80	240	80	20	20	13	<	<
DP30	<	<	<	20	210	130	66	20	<	<
DP31	<	<		266	320	53	66	20	10	<

< not detected

TABLE 3.5. IFN Titers after avirulent IE VEEV challenge. Number represents the reciprocal titer that provides 50% protection against VSV challenge.

Viremia levels were low and transient with all titers below 100 PFU/mL and lasting only for 12-36 hours (data not shown).

DISCUSSION

Viremia

In this study, viremia titers for five TrD VEEV infected horses peaked between day 2.5 and 3.5 with maximum titers of $10^{3.4}$-$10^{4.7}$ PFU/mL, which is consistent with previous studies. These previous studies used a variety of challenge doses with peak viremic titers ranging from 0 to $10^{8.2}$ PFU/mL, the majority in the 10^{4}-10^{7} PFU/mL range. For example, one study challenged four horses with between $10^{4.9\text{-}5.6}$ $SMICLD_{50}$ (suckling mouse intracranial lethal dose) of IAB VEEV and obtained peak titers on day 2 or 3 of $10^{4.2\text{-}6.2}$ $SMICLD_{50}$/mL (Henderson et al, 1971). In another study, 16 horses were challenged with between $10^{3.8\text{-}5.5}$ $SMICLD_{50}$ of IAB or IC VEEV and peak titers of $10^{0\text{-}8.2}$ $SMICLD_{50}$/mL noted around day 2.5 post infection. Peak titers were not reported individually, but apparently most were in the $10^{4.5\text{-}5.5}$ $SMICLD_{50}$ /mL range (Walton et al, 1973). In a third study, five horses were challenged with between $10^{1.5\text{-}4.6}$ $SMICLD_{50}$ of virulent (strain unknown) VEEV, and peak viremic titers of $10^{2.8\text{-}7.5}$ $SMICLD_{50}$/mL noted between days 2 and 5 (Kissling et al, 1956). Differences in peak viremic titers may be due to different VEEV strains, passage history, challenge dose, or the immune response of the animal. In the present study, horses were challenged with $10^{4.0}$ PFU of TrD VEEV, a dose similar to what three to ten VEEV infected mosquitoes would potentially inject (Turell & Spielman, 1992).

The initiation, peak titers and length of serum IFN production in horses challenged with TrD VEEV correlated with the initiation, peak titers and length of viremia. In all five horses challenged with TrD VEEV, in the sample in which IFN was first detected, virus was also first detected. Both IFN and virus were initially detected at 24 hours post challenge in three horses, and at 36 hours post challenge in the remaining two horses. In four of five horses, both IFN and viremia titers peaked within 12 hours of each other. In the fifth animal (DP37), the peak IFN titer was the first detectable IFN titer, and the peak viremia titer was 36 hours later. When the IFN and viremia titers were averaged for all five horses, both titers peaked at the same time point (Figure 3.1). The length of detectable serum IFN ranged from 3.5 to 5 days. The length in time of detectable viremia ranged from 2.5 to 4 days, or an average of 24 hours less than detectable IFN. Thus, the initiation, peak titers, and length of serum IFN production and viremia can be correlated to each other in horses challenged with TrD VEEV. Similar results are seen in other studies with other viruses. Interferon titers were examined in adult horses challenged with a DNA virus, equine herpes virus subtype 1 (EHV-1). IFN titers were first detectable at 24 hours post infection, peaked at about 100 IU/mL at day four post infection, and slowly decreased through day 14 post infection (Bridges & Edington, 1986). Individual horse results were not given, only an average of the six horses. In mice challenged with virulent IAB and attenuated VEEV (V3043), IFN expression and viremia peaked around 18-24 hours pi (Charles et al, 2001; White et al, 2001). In these studies, both serum IFN and viremia levels peaked 2-4 days after infection, and then both declined, the same as noted in this research. The data from these studies suggests that serum IFN and viremia influence each other.

Figure 3.1

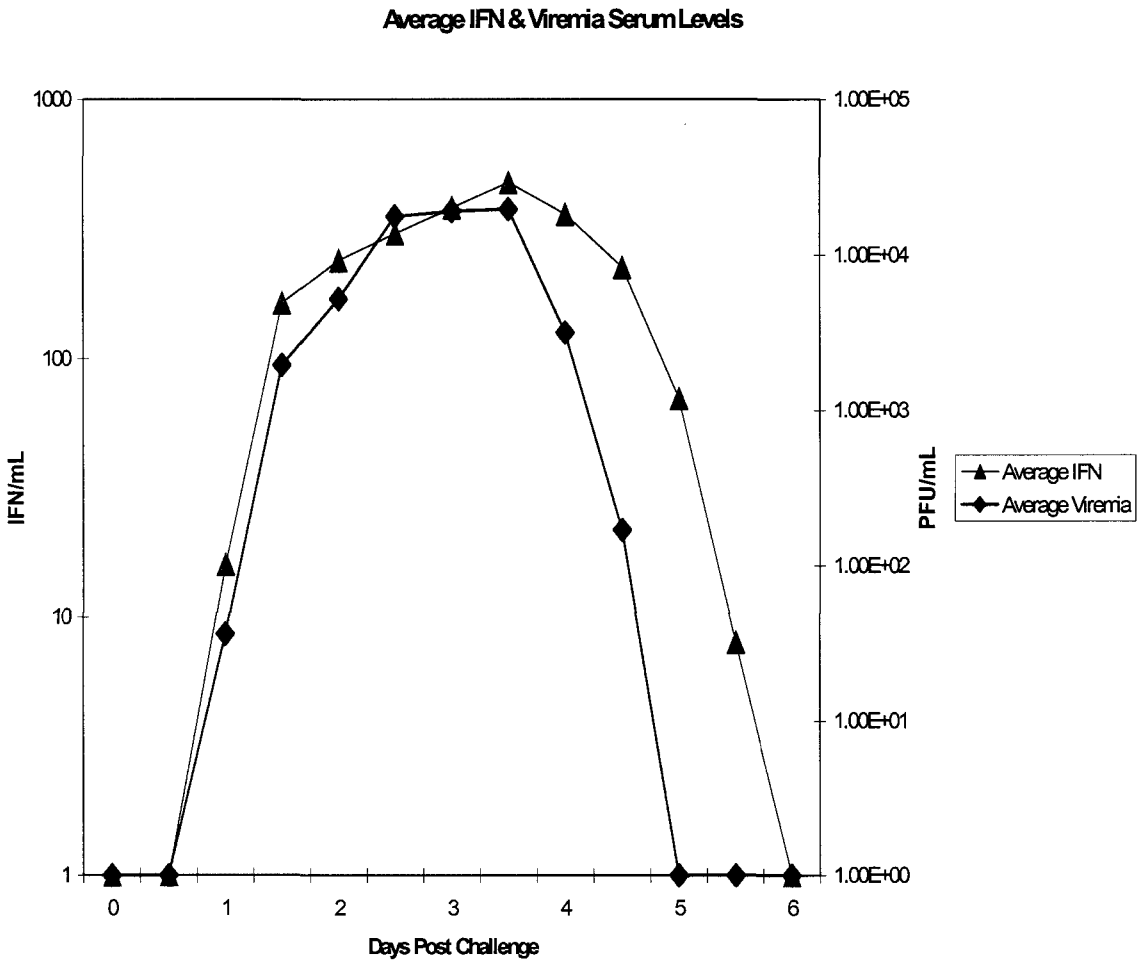

FIGURE 3.1 Average serum levels of viremia and IFN in five horses challenge with the TrD strain VEE virus.

Detectable viremia in horses challenged with TrD VEEV disappeared before loss of detectable IFN. In three of five animals challenged with TrD VEEV, the disappearance of detectable viremia preceded the loss of detectable serum IFN by 24 hours, while in one horse, the difference was 12 hours (Table 3.2, Figure 3.1). In the sixth horse, the disappearance of both detectable IFN and viremia were at the same time point. In this study, detectable IFN levels remained until viremia was eliminated. Double-stranded RNA should remain in the infected cells until the cessation of viral replication. While IFN is important in the overall defense against viral infection, IFN acts only indirectly in clearance by preventing the infection of additional cells, while IgM and phagocytic cells are directly involved in clearing VEEV (Charles et al, 2001). The evidence from this research indicates that IFN production continues until viremia is eliminated.

Vaccination with attenuated VEEV resulted in a low but detectable IFN response in 50% of horses. IFN titers were detectable in six of twelve horses vaccinated with 10^3 or 10^5 PFU of attenuated V3526 VEEV. V3526 has been shown to replicate in mosquitoes (Turell et al, 1999), mammalian cells (Davis et al, 1995) and in mice (Ludwig et al, 2001). Mice, injected subcutaneously with the attenuated V3526 VEEV, developed viremia that peaked at about 200 PFU/mL (Ludwig et al, 2001). No viremia was detected in horses after inoculation with the attenuated V3526 strain (Bowen, unpublished data). This does not preclude the possibility that virus replication occurred at low levels locally in the lymph nodes. In mice challenged with a different attenuated VEEV strain (V3032), the virus replicated normally in the draining lymph node and apparently spread like virulent VEEV to other lymphoid organs, with little or no viremia detected (Grieder et al, 1995). Additionally, detectable viremia is not a

prerequisite for induction of IFN during virus infection, since IFN is produced almost immediately at the site of infection (Baron, 1963; Baron et al, 1992).

The magnitude of IFN response did not correlate with the magnitude of viremia when comparing virulent IE and avirulent IE VEEV challenge in horses. IFN response was evident in all three horses challenged with the avirulent IE 64A99 VEEV strain. Viremia was not detectable in any of these horses. In contrast, IFN was not detected in three horses challenged with the virulent IE Mex32 strain of VEEV, while all three had detectable low viremic titers. There are several possibilities to explain this observation. The level of IFN present may be below the detection limits of the IFN assay. The low number of test subjects may not truly represent the response of the population to virulent IE VEEV. The virulent IE strain may have mechanisms to inhibit IFN production or signaling.

Viruses have several strategies to counteract or take advantage of chemokines such as IFN. These include producing homologs of IFN receptors that competitively bind IFN, inhibition of IFN synthesis, and inhibition of gene induction by IFN. Vesicular stomatitis (Ferran & Lucas-Lenard, 1997) and hepatitis B (Weaver et al, 1998) inhibit the synthesis of IFN. Myxoma viruses encode a homolog of the IFN receptor which effectively binds to IFN molecules, preventing the activation of cells by IFN (Alcami & Smith, 1995; McFadden et al, 1995). Murine polymoviruses and vesicular stomatitis inhibit the Jak-STAT pathway (Weihua et al, 1998). The Epstein-Barr virus EBNA1 oncogene blocks gene induction in cells (Kanda et al, 1992). The virulent IE VEEV strain may have a mechanism to inhibit the production of IFN, since IFN was not detectable, although viremia was present. Other viruses, such as TrD VEEV, may incite a high IFN response and still produce severe or fatal disease. In

humans infected with Ebola virus, elevated IFN levels are an indicator of a poor outcome (Villinger, et al, 1999).

SUMMARY

Evidence from this research suggests that the length and magnitude of IFN responses can be correlated with length and magnitude of viremia in horses challenged with the Trinidad Donkey (TrD) isolate of VEEV, a IAB subtype. It may be that when detectable IFN titers are produced in response to virus infection, IFN levels remain elevated until detectable virus is eliminated. While the TrD strain of VEEV induced an IFN response in horses, the virulent IE subtype did not consistently stimulate a response detectable in the serum. The virulent Mex93 strain of IE VEEV produced low levels of viremia, yet did not induce a detectable IFN response, although the outcomes of these infections were severe. In contrast, the avirulent IE 64A99 strain induced an IFN response in horses although viremia was not detectable.

CHAPTER IV

DETECTION OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS IN SERUM

INTRODUCTION

Diagnosis of VEEV infection in horses and humans typically relies on clinical signs, development of antibodies in paired sera, or if the patient dies, virus isolation from CNS tissues (Merck, 1998; Office International des Epizooties, 1996). In horses, virus isolation from serum samples is possible only if samples are obtained early during the infection (Casals et al, 1943: Weaver et al, 1996), but early non-specific clinical signs of increased body temperature along with depression or anorexia are not pathognomonic (specific) for viral infection. By the time viral infection is suspected, the viremia may have been cleared. Additionally, successful virus isolation depends on development of cytopathic effects in cell cultures or clinical illness in intracerebrally inoculated mice (Office International des Epizooties, 1996) both very time-consuming.

For these reasons, VEEV infections in horses are often diagnosed after recovery of the patient, or death of the patient. Although no specific treatment presently exists for alphavirus infections, several treatments are under investigation (Coppenhaver et al, 1995; Lukaszewski & Brooks, 2000). Successful treatments in horses and humans will rely on rapid diagnosis and early intervention. Additionally, rapid diagnosis is needed to prevent the further spread of VEEV epizootics through early vaccination, quarantine, restricted horse movement, and mosquito control. Thus, a technique for rapid diagnosis of VEEV infection is needed. Reverse-transcriptase PCR (RT-PCR) techniques have been developed for the rapid detection of alphaviruses, including the medically important VEE viruses IAB, IC, ID and IE (Pfeffer et al, 1997; Brightwell et al, 1998; Linssen et al, 2000). Quantitative (real-time) RT-PCR techniques would reduce the diagnostic time even further. A Taqman real-time RT-PCR assay has recently been developed for the detection of EEE and WEE viruses (Lambert et al, 2003). The aim of this study was to develop a quantitative (real-time)RT-PCR (qRT-PCR) assay for the rapid diagnosis of VEEV in horse sera.

HYPOTHESIS: VEE virus RNA in horse sera can be detected and quantified with a quantitative real-time PCR (qRT-PCR) assay. The resulting qRT-PCR detection should be more sensitive than virus isolation in cell culture, due to the presence of non-infective virus particles which are detectable by qRT-PCR.

AIM: Develop a rapid, sensitive and accurate qRT-PCR to detect the E2 gene of VEE IAB virus in horse sera.

MATERIALS AND METHODS

RNA Isolation

A two tube, real-time qRT-PCR was used to amplify viral RNA isolated from horse sera, whole blood, tissues and Vero cell culture medium. Vero cell culture monolayers were inoculated with MOI ~ 0.01 of Trinidad Donkey (TrD) IAB VEE virus and incubated until approximately 75% of the cells showed CPE. The medium, along with viral isolation positive tissue and blood samples, was used as a positive control. Whole blood and serum were collected at various time points from infected animals. Whole blood was divided into equal parts, one-half diluted 1:2 in RNAlater (Ambion, Austin, TX) and the other half undiluted, then both stored at -80°C. Sera were stored undiluted at -80°C. Tissues collected sterilely at necropsy were placed in 0.5 mL of RNAlater and immediately put on dry ice, until transfer into -80°C freezer.

For RNA isolation from tissues, the tissues were cut and between 0.15-0.20 grams ground in a sterile Ten Broeck glass grinder (with 2.0 mL of BA-1 medium (MEM supplemented with 1% bovine serum albumin, 350 μL sodium bicarbonate in 0.05 M Tris, pH 7.6). The weight of each tissue was recorded, and dilution factor determined. The tissue homogenates were centrifuged for five minutes and the resulting supernatants collected for RNA isolation. A 250 μL sample of each serum, blood or tissue homogenate in BA-1 was added to 0.75 mL Tri-Reagent (Tri-Reagent BD for whole blood and serum) to which 2.5 μL of PolyAcryl carrier (Molecular Research Center, Cincinnati, OH) was added. Tissue RNA was isolated in three aliquots (750 μL

total), serum RNA in two aliquots (500 μL total) and combined in a later step. Cell culture medium RNA, isolated from 100 μL aliquots, was added to 0.75 mL Tri-Reagent with 2.5 μL of Poly Acryl carrier. The RNA-TriReagent-PolyAcryl mixtures were vortexed and 0.1 mL of 1-bromo-3-chloropropane (BCP) added. Tubes were centrifuged for 10 minutes at 12,000 g in a cold rotor, to separate the mixture into RNA, DNA and protein fractions. The RNA fractions were pipetted into a clean, RNAse free microcentrifuge tubes with 0.5 mL of isopropanol. The outside of the tubes were rinsed in 10% bleach solution and taken out of BSL-3. The tubes were then centrifuged at 12,000 x g to precipitate the RNA. RNA pellets from separate aliquots were combined and washed with 1 mL of ethanol. The isolated RNA was stored at –20°C until the reverse transcriptase (RT) procedure could be performed.

Synthesis of cDNA

For the RT procedure, the stored RNA pellet was centrifuged at 7,500 g for five minutes, the supernatant carefully removed, and the tubes allowed to air-dry. RNA pellets were then dissolved in 20 μL of a 1:5 mixture of RNAsecure™ (Ambion, Austin, TX) and DEPC water. Two microliters of positive control and 10 μL of each unknown sample RNA solution were used to obtain cDNA. Initially, six RT reactions, three with random hexamers and three with oligo (dT) primers were completed and the products run on 1% agarose gel electrophoresis with 10 μg of 200 base pair ladder, to determine the best primer. All reactions followed the suggested RT procedure, for reactions containing less than 5 μg of RNA and using the oligo (dT) primer, from the SuperScript™ First-Strand Synthesis System (Invitrogen Carlsbad, CA), in a 24 μL

reaction. RT reaction conditions: 42°C for 50 minutes with a 15 minute, 70°C incubation to inactivate RT. RNase H (Invitrogen, Carlsbad, CA) was added after final extension, and incubated according to manufacturer's instructions, to eliminate the RNA strand from RNA/DNA hybrids. The cDNA was stored at -20°C until further use.

Constructing Primers and Probe

Preparing primers and a probe for a Taqman qRT-PCR involved several steps. First, the RNA sequence for the Trinidad Donkey strain of VEE virus was obtained from the NCBI web site (www.ncbi.nlm.nih.gov/blast). Then several forward primers were manually designed, in the E2 region of VEEV TrD RNA in the general area of 8300-8500nt. These primers were constructed based on the following criteria (Dieffenbach et al, 1995; Baumforth et al, 1999; Bustin, 2000): 16-22 nt in length; 40-60% GC content; desired melting temperature of 58-60°C; if possible, no more than two GC pairs were to be in the last five nucleotides on the 3' end of the primers; a C or G on both ends was desirable; no runs of >4 of identical nucleotides. Melting temperature and GC content were obtained from the on-line Qiagen Oligo Toolkit (http://oligos.qiagen.com/oligos /toolkit). After designing several primers, they were checked for self-complementary binding on the Qiagen Oligo Toolkit web site. Two forward primers that best met the desired criteria were selected. Each of these two was verified, on the NCBI blast search, to match the E2 region of the IAB VEE virus and were checked to see that no mammalian genes were matched. Four potential probes were manually designed as close as possible to the 3' end of the two primers, according to the following criteria (Baumforth et al, 1999; Dieffenbach et al, 1995; Bustin, 2000):

18-26 nt in length, 50-60% GC content, no G nucleotide on the 5' end, melting temperature 8-10 °C above the primers, no runs of >4 identical nucleotides, and more Cs than Gs. The four potential probes were checked for self-complementary binding and dimer formation with the primers on the Qiagen Oligo Toolkit web site. The two best probes were then checked on NCBI Blast site for binding to the VEE TrD strain virus E2 region. The two forward primers and two probes were entered as individual pairs into the on-line Primer3 program (www.genome.wi.mit.edu/cgibin/primer/ primer3) to obtain suggested reverse primers. Several reverse primers were suggested, and these were manually screened for meeting the above criteria for primers while keeping the amplicon size between 75-150 base pairs. They were also screened on the Qiagen Oligo Toolkit web site for self-complementary binding and dimer formation. One primer pair, that produced a 115 base pair amplicon, appeared to best meet all criteria. The selected forward primer was '5-GAACTGCGAGCAATG GTC-3' at position 8371, and reverse primer '5-TCTCTGCTGGTTTTCTGTCG-3' at position 8486. The primers were constructed at Invitrogen Life Technologies (Rockville, MD), tested in a PCR reaction and verified with gel electrophoresis to amplify a 115 bp product. The probe '5-TCGCCAATGTGACGTTCCCA-3' starting at position 8412, was then ordered with a FAM-6 fluorescent reporter dye (FAM-6) on the 3' end and the quencher 6-carboxy-tetramethyl-rhodamine (TAMRA) on the 5' end. Another PCR test was conducted with probe included to ensure the probe did not interfere with amplification of cDNA.

In retrospect a more efficient method to select primers and a probe for a Taqman system, may have been to select a working probe first, then design primers around the

probe. The method used here of selecting the forward primer, a probe, and then the reverse primer was used to obtain a large selection of amplicon sizes to choose from, and thus keep the amplicon size near 100 base pairs.

Real-time Taqman PCR

To select the most efficient reagent concentrations and annealing temperature for the selected primers, numerous trial PCR experiments were conducted at various temperature gradients on a Robocycler (Stratagene, La Jolla, CA). Literature reviews suggested annealing temperatures of approximately 5-7 degrees Celsius below calculated primer melting temperatures (Baumforth et al 1999; Bustin, 2001). Tested annealing temperatures ranged from 44-54°C in two degree steps.

A plasmid with a VEEV E2 cDNA insert was obtained from Dr. Ann Powers at CDC to serve as a known standard for the Taqman assay. The plasmid was constructed by cloning the E2 structural gene of TrD VEEV into a pBluescript II SK(+) plasmid (Powers et al, 2000). Concentrations of circular and linearized plasmids were determined photometrically to be 0.2 μg/μl and verified by gel electrophoresis with known amounts of a 1 Kb ladder after single digestion with restriction enzymes Sst1, Xho1 and EcoR1. The number of molecules was then calculated based on a plasmid length of 6800 bp and an average weight/nucleotide of 336 g/mol (Linssen et al, 2000). This yielded a molecular weight of 7.6×10^{-9} ng per molecule and 2.6×10^{10} molecules per μL. The plasmids were serially diluted from 4.0×10^{8} to 4.0×10^{0}, and stored at -20C°.

Amplification trials with various concentrations of $MgCl_2$ and probes at various temperature settings were run on the iCycler thermocycler (Bio-Rad Hercules, CA) to determine the most efficient concentrations and temperatures. All trial assays and subsequent unknown samples were run on the iCycler in 96 well microtiter plates. Tested $MgCl_2$ concentrations ranged from 3 mM to 6 mM in 1 mM steps, while tested primer concentrations ranged from 0.4 μM to 1.2 μM in 0.2 μM steps. Optimal concentrations were 4 and 5 mM $MgCl_2$ and 0.8 μM primer concentration. These optimal concentrations were then used in assays with serial 10-fold dilutions of control cDNA and linearized plasmids (10^6 to 10^{-2} copies per well) to determine the reproducibility and sensitivity of real-time PCR detection of VEE virus E2 cDNA. Final triplicate assays each contained a volume of 25 μL with 4.5 mM $MgCl_2$, 0.8 μM forward and reverse primers (8371 and 8486), and 1.25 μM fluorogenic probe. Each also contained either 1 μL of plasmid or cDNA or 4 μL of RNA from test serum samples. Final reaction conditions were: one minute at 95°C, 45 cycles of 94°C for 30 s, 48°C for 20 s and 70°C for 45 s.

RESULTS

Four restriction enzymes, EcoR1, Sal1, Not1 and BamH1, each cut at a single site in the VEEV pBluescript II SK (+) plasmid (Figure 4.1). The Xho1 enzyme cut the plasmid twice. PCR and gel electrophoresis of the EcoR1 and Not1 products indicated PCR amplification, verifying the E2 gene was still intact in the linearized plasmid in the

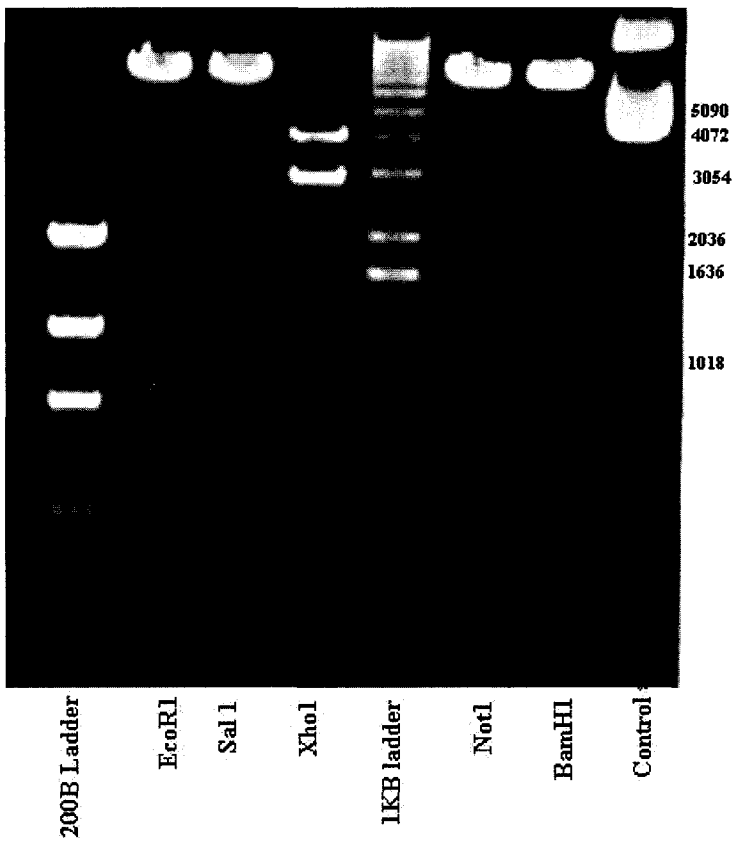

Figure 4.1. Linearization of pBluescript II SK(+) plasmid with VEEV E2 insert. Two ladders are included with the products of five restriction enzymes and VEEV plasmid. All restriction enzymes produced one cut in plasmid except Xho1 which produced two cuts.

region enclosed by the primers (not shown). EcoR1 RE was utilized in the remaining assays.

Efficiency of the real-time PCR reaction was explored with different concentrations of $MgCl_2$ and primers. Efficiencies were between 90-110% with 0.8 μM of primers and 5 μM $MgCl_2$ (Table 4.1). A PCR efficiency of 107% and slope of -3.2 were obtained with 3-5 μM $MgCl_2$ when combined with 0.8 μM primer.

The reproducibility of the real-time assays was determined, using known plasmid dilutions of 4×10^5 to 4×10^{-1} copies/well in triplicate or quadruplicate, from four different assays conducted on different days (Figure 4.2). The threshold limit was set in the linear portion of the amplification curve above the noise band for all experiments. Student t-tests on the cycle count results indicated a significant difference between each serial dilution between 4.0×10^5 to 4×10^1 copies ($p<0.003$). The difference between dilutions containing 4.0×10^1 and 4×10^0 copies was $p<0.05$. There was no significant difference between the 4.0×10^0 and 4×10^{-1} copy dilutions ($p<0.26$) or lower dilutions. The 10^0 dilution correlated to a C_T of 39 and four copies of linearized plasmids/well which was acknowledged as the lower limit of detection. C_T values of greater than 39 (less than 4 copies) were considered negative in the remaining assays.

Correlation efficiency and amplification curves were determined for the known plasmid standards between 4×10^5- 10^0 copies (Figure 4.3). Earlier assays were conducted on the 4×10^{-1} & 10^{-2} copy dilutions, but these results were not linear, which verified the results of reproducibility studies. Results of the standard curve graph for

TABLE 4.1

Effects of different primer and $MgCl_2$ concentrations on the efficiency and standard curve of Taqman real-time RT-PCR.

$MgCl_2$ μM	Primers μM			
	0.4	**0.8**	**1.2**	**1.6**
3	-5 54%	-3.2 107%	-3.8 84%	
4	-4.2 72%	-3.2 105%	-5.4 54%	-6.1 46%
5	-3.3 100%	-3.2 106%	-3.4 97%	
6		-3 114%		

Table 4.1. Standard curve and efficiency of real-time PCR using linearized plasmids, containing VEEV E2 inserts, with varying amounts of $MgCl_2$ and primers. First number in block is the standard curve and second number is efficiency.

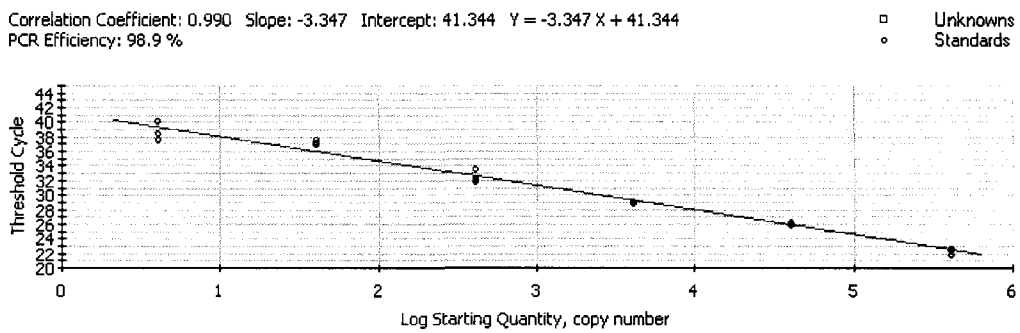

Figure 4.2. Standard curve obtained for 10-fold serial dilutions, (4×10^5, 10^4, 10^3, 10^2, 10^1, 10^0) per well, of linearized pBluescript II SK(+) plasmid containing VEEV E2 insert. C_T values were plotted against different plasmid dilutions assayed in triplicate. The 4×10^{-1} and 4×10^{-2} dilutions were also plotted, determined not to be linear and thus considered to be negative for all subsequent assays.

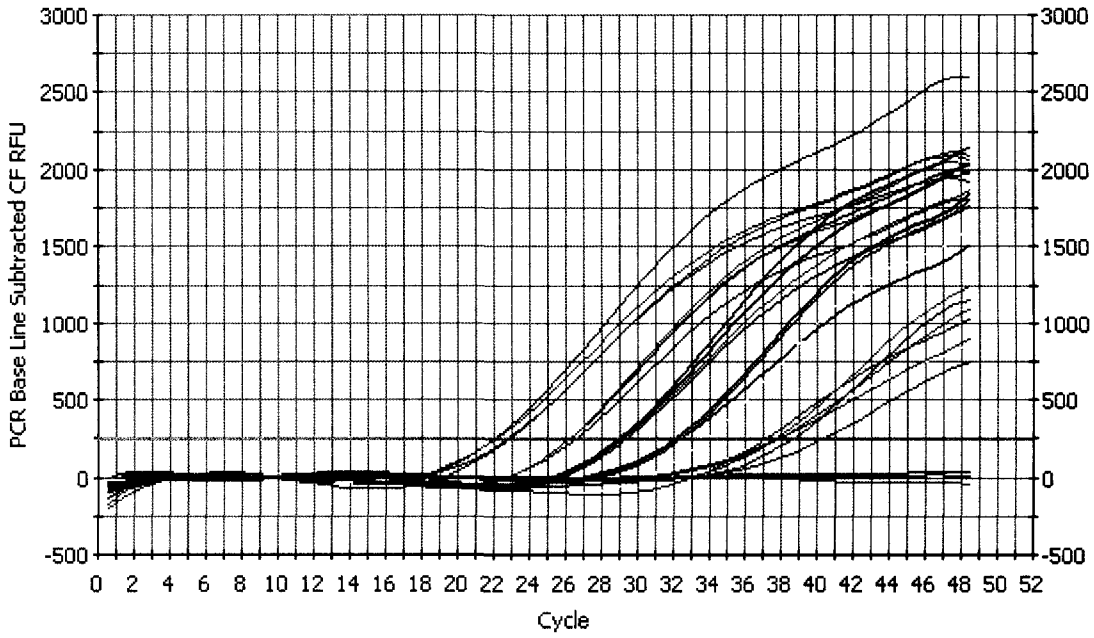

Figure 4.4. qRT-PCR graph showing amplification curves of known amounts (4 x10^5, 10^4, 10^3, 10^2, 10^1, 10^0 and negative controls) of linearized plasmid containing VEEV E2 insert. Baseline set at 235.

cDNA derived from culture medium of VEEV infected Vero cells resulted in slope of -3.014; correlation efficiency of 0.976 and PCR efficiency of 114% with a linear graph for dilutions of cDNA from 1 x 10^0 to 1 x 10^3 PFU-equivalent/well. The highest dilutions of cDNA were 1 x 10^3 PFU-equivalent/well. Dilutions at 1 x 10^{-1} copies/well and below were not detected consistently, the C_T values were over 40 and thus considered negative. This correlates to a lower limit of detection for qRT-PCR of one PFU-equivalent. Values lower than one could not be reproduced consistently using this procedure.

Serum samples from horses challenged with TrD VEEV were taken at 8 or 12 hour intervals. These samples were assayed by virus isolation on Vero cell culture and by qRT-PCR. Unknown sample C_T values were compared to the known control C_T values and the corresponding PFU-equivalence values were determined (Figures 4.4 and 4.5). For unknown samples, C_T values over 40 were considered negative, since known standard plasmid C_T values over 40 were not significantly different ($p>0.05$).

The ability of qRT-PCR to detect early viremia in horse sera was compared to the results from Vero cell VI assays. Serum samples from eleven horses were assayed by both procedures. In two horses (DP37 & MT34), viremia was detected by qRT-PCR assay prior to detection by cell culture VI assay (Table 4.2A/B). Detection by qRT-PCR was 12 hours (DP37) and 8 hours (MT34) earlier than by plaque assay. Viremia was not detected in fifteen vaccinated horses after challenge with TrD, by virus isolation nor qRT-PCR (Data not shown).

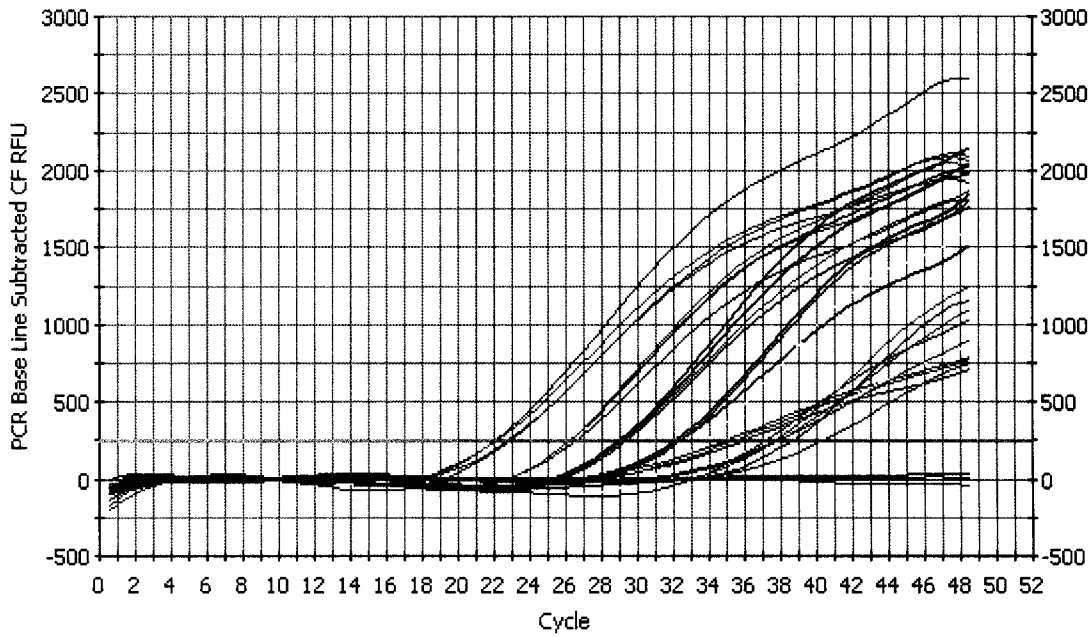

Figure 4.4. qRT-PCR graph of unknown cDNA titer from MT32 against the known plasmid controls. Threshold limit set at 235. Average known standard $4x10^2$ C_T value in graph was 32.1 with an unknown sample C_T value of 35.5 corresponding to 45 plasmids per well or 810 copies VEEV per mL sera.

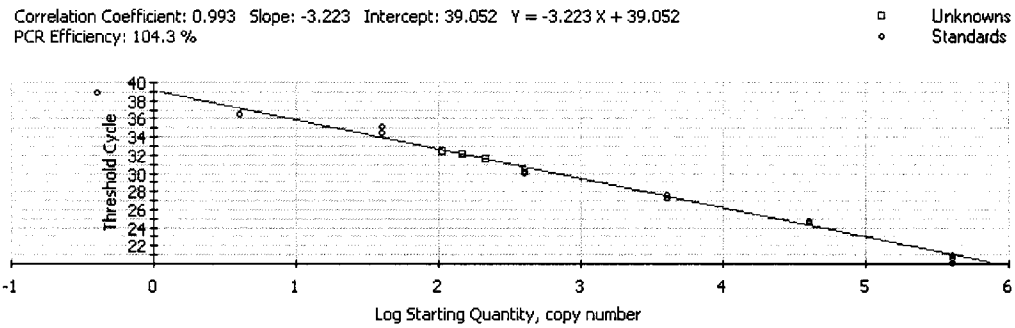

Figure 4.5. Example of unknown sera sample, #12 run in triplicate, plotted against known plasmid linear standard curve. Average C_T of 32.06 for the unknown sample equates to 150 VEEV copies per well.

TABLE 4.2 A & B

Detection of TrD VEEV in sera by virus isolation in cell culture and by Taqman qRT-PCR

Horse #	12 HRS VI	PI RT	24 HRS VI	PI RT	36 HRS VI	PI RT
DP16	-	-	+	+	+	+
DP17	-	-	+	+	+	+
DP18	-	-	+	+	+	+
DP37	-	-	-	+	+	+
DP38	-	-	-	-	+	+

Table 4.2A

Horse #	8 HRS VI	PI RT	16 HRS VI	PI RT	24 HRS VI	PI RT	32 HRS VI	PI RT
MT21	-	-	-	-	+	+	+	+
MT22	-	-	-	-	+	+	+	+
MT31	-	-	-	-	+	+	+	+
MT32	-	-	-	-	+	+	+	+
MT33	-	-	+	+	+	+	+	+
MT34	-	-	-	-	-	+	+	+

Table 4.2B

\- not detected
\+ detected

VI = virus isolation
RT = real-time PCR
PI = post infection

Table 4.2A/B. Detection of VEE virus in horse sera by plaque assay and viral RNA by qRT-PCR. Duplicate serum samples were assayed by each method at each time point and determined to be positive (+) or negative (–).

Results of qRT-PCR (copy number) and plaque assays (PFU) from identical serum samples were compared and the difference in $\log_{10}$ determined (Table 4.3). The two populations were significantly different ($p<.01$, student t-test). The average qRT-PCR titer for the population was 0.83 $\log_{10}$ higher than the virus isolation titer (95% confidence interval 1.18-0.48 $\log_{10}$). Clearly, the qRT-PCR titer reflects not only infectious virus but noninfectious virus, whereas the titer from plaque assay indicates only infectious virus.

DISCUSSION

Virus isolation by cell culture and intracerebral mouse inoculation are the current standard methods of virus isolation for detection of VEEV (Office International des Epizooties, 2000). Yet these methods require several days to obtain a diagnosis of VEEV infection. RT-PCR has been shown to be a reliable method of detecting RNA of VEEV grown in cell culture (Linssen, 2000), and can take less than one day. Detection of an RNA virus by qRT-PCR would decrease the detection time over RT-PCR by an hour or two. This study aimed to develop a rapid detection assay, qRT-PCR, for the RNA of Trinidad donkey (TrD) strain of VEEV in horse sera.

Preliminary research indicates Taqman qRT-PCR is sensitive, accurate and reproducible for the detection of RNA from the TrD strain of VEEV in horse sera. The qRT-PCR procedure was developed and tested for the ability to detect TrD E2 cDNA inserted into a Bluescript plasmid. Repeat assays of 1-5 $\log_{10}$ copies of

TABLE 4.3

Comparison of results of plaque assay and qRT-PCR serum titers

Sample #	VI Log 10	RT Log 10	Difference Log 10
1	3	4	1
2	3.9	4.5	0.6
3	4.3	4.8	0.5
4	2.6	3.4	0.8
5	4.4	5.1	0.7
6	3.6	4.7	1.1
7	4.4	5.4	1
8	4.7	5.6	0.9
9	1.8	2.6	0.8
10	3.1	4.3	1.2
11	2.2	3	0.8
12	3.7	3.2	-0.5
13	2.2	2.5	0.3
14	3.5	3.7	0.2
15	4.2	4.3	0.1
16	1.5	2.9	1.4
17	3.6	5.9	2.3
18	4.1	5.8	1.7
19	4.2	5	0.8
20	4.6	4.3	-0.3
21	5.4	6.6	1.2
22	5.9	7.5	1.6

Table 4.3. Comparison of results obtained by plaque and qRT-PCR assays on twenty-two known positive VEEV TrD sera. Each comparison is on an identical serum sample. Results and differences are given in $\log_{10}$ in third column. Average difference was 0.83.

linearized plasmids indicated good sensitivity and reproducibility down to a limit of 4 plasmids/well. This level of sensitivity and reproducibility were reached only after the use of RNAse H following the RT procedure, and linearization of plasmids with restriction enzymes. Both of these steps appear to have increased the efficiency of the PCR step and improved the overall sensitivity of the qRT-PCR.

On assays of less than 4 plasmids/well, reproducibility decreased below desired levels ($p > 0.05$). Random distribution may have adversely affected reproducibility at low titers. For example, random distribution of 120 copies of plasmids divided among three wells would give 40± copies of plasmid in each well while 12 copies of plasmids divided between three wells would result in 4± plasmid copies in the assayed wells. The percentage difference, between the possible 3 and 5 plasmid copies per well is much greater for the low titer levels and thus reproducibility is decreased.

After the qRT-PCR procedure was developed, isolated RNA from Vero cell cultures and horse sera was assayed for presence of TrD VEE virus RNA. Lower limits of detection for this qRT-PCR were 2.0 PFU-equivalent/mL, or about 0.7 PFU-equivalent/well, which is comparable to the findings with other viruses. The lower limit of detection for the cell culture assay was 5 PFU/mL. The published lower limits of detection using real-time PCRs include: respiratory syncytial virus, 0.06 $TCID_{50}$ (Dew-Maridor et al, 2004) and 0.34 $TCID_{50}$ (Van Elden et al, 2003); smallpox virus, 12 plasmid copies (Ibrahim et al, 2002); and 0.1 PFU West Nile virus (Lanciotti et al, 2000). Semi-nested PCRs have detected little as 1.2 PFU of TrD VEEV (Pfeffer et al, 1997).

Sensitivity and reproducibility of qRT-PCRs can be affected by several different factors such as source of cDNA, RNA extraction method, qRT-PCR technique, thermal cycling conditions, amplicon size and contamination. In this study, qRT-PCR assays of plasmid cDNA were more sensitive than assays from virus cDNA, as found in one other study (Ibrahim et al, 2003). In a comparison of RNA extraction methods, one study realized greater sensitivity using phenol chloroform extraction (Garcia et al, 2001). The present thesis explored different thermal cycling conditions and an amplicon size near 100 bp to improve PCR efficiency. Amplicon sizes substantially over 100 bases are not optimal and may decrease efficiency (Bustin, 2000). One-tube RT-PCR techniques decrease chances of contamination but adversely affect efficiency (Leutenegger et al, 1999). In summary, further research may improve the sensitivity and reproducibility of the qRT-PCR technique described here.

Titers of TrD VEEV in horse sera were significantly higher when assayed by qRT-PCR versus cell culture assays. A direct comparison of plaque assays and qRT-PCR on 22 positive serum samples indicated the qRT-PCR technique gave an average 0.83 log more copies than PFU in plaque assay. Other investigators have reported titers 1-3 logs higher are achieved by real-time PCR versus cell culture and mouse intracerebral inoculations with other viruses (Garcia et al, 2001; Houng et al, 2000; Garin et al, 2001). There are a couple possible reasons for the phenomena. One reason is virus titration by plaque assay detects only infectious virus particles, while real-time PCR can detect infectious and non-infectious virus RNA. Defective virus particles that are non-infectious have been described in a variety of virus types including DNA and RNA animal viruses (Huang et al, 1977; Von Magnus, 1954).

Of 78 serum samples taken in the early stages of viremia and assayed by both qRT-PCR and cell culture virus isolation, 76 samples showed concordance between both techniques for the presence or absence of virus. Two samples determined to be negative by cell culture were positive by qRT-PCR. The disparity on these two samples can be explained by the greater sensitivity of qRT-PCR assay. The lower limits of virus detection for plaque assay and qRT-PCR were 5 PFU/mL and 2 PFU/mL respectively and qRT-PCR assays on the two samples in question indicated 20 and 12 virus particles per mL. Overall, the qRT-PCR titers averaged 0.83 $\log_{10}$ copies of RNA higher than PFU detected by plaque assay. This agrees with other studies that obtained 10 to 1000-fold higher titers for qRT-PCR relative to virus isolation in cell culture (Garcia et al, 2001; Houng et al, 2000; Garin et al, 2001). Applying this increased titer to the two samples in question, the calculated PFUs would be 3 and 2 PFU/mL respectively for 20 and 12 copies of RNA detected by qRT-PCR. These titers, are both below the calculated lower limit of detection for virus isolation in cell culture.

Additional research is needed to determine the specificity of this qRT-PCR for virulent and non-virulent VEEV strains, other alphaviruses and other viruses. The ability of this assay to distinguish between VEEV, EEE, and WEE is important as a diagnostic tool. All three of these viruses occur in same geographical regions in both North and South America. The E2 portion of the TrD VEEV genome was amplified in an attempt to decrease cross-reactivity, since the E2 gene is one of the least conserved of the structural genes (Kinney et al, 1992; Wang et al, 1999). While use of the E2 gene may decrease cross-reactivity, it may increase the possibility of a mutated epizootic strain escaping detection by this assay. A set of antibodies against the E2 protein have

been able to distinguish between epizootic and enzootic VEE viruses (Roehrig and Bolin, 1997). Nonetheless, cross-reactivity for other viruses needs to be checked with an actual assay. NCBI search did not indicate the primers matched any other virus sequence, but matches still cannot be ruled out except with an actual qRT-PCR test. There may be matches to enzootic VEEV strains or other viruses that are not in the genebank. Future research may include a blind study using known positive samples of virulent and non-virulent VEEV along with other viruses, especially alphaviruses, to determine the specificity as well as sensitivity of this qRT-PCR.

SUMMARY

This study gives evidence that real-time PCR analysis can be used to rapidly detect the Trinidad donkey strain of VEE virus in horse sera. Results indicate that the sensitivity of the assay as described was 2 PFU/mL or 12 RNA genomic copies /mL. Future investigations are warranted to determine the specificity and potential cross-reactivity of this qRT-PCR for virulent and non-virulent VEE viruses and related viruses.

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