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DISSERTATION

SALIVARY POTENTIATION OF VESICULAR STOMATITIS NEW JERSEY VIRUS INFECTION

SUBMITTED BY

KIRSTEN HILL LIMESAND

DEPARTMENT OF MICROBIOLOGY

IN PARTIAL FULFILLMENT OFTHE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KIRSTEN H. LIMESAND ENTITLED "SALIVARY POTENTIATION OF VESICULAR STOMATITIS NEW JERSEY VIRUS INFECTION" BE ACCEPTED AS FULFILLING, IN PART, REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

COMMITTEE ON GRADUATE WORK:

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ABSTRACT OF DISSERTATION

SALIVARY POTENTIATION OF VESICULAR STOMATITIS NEW JERSEY VIRUS INFECTION

Saliva of arthropod vectors has been shown to modulate vertebrate host hemostatic and immunologic functions. Vesicular stomatitis New Jersey virus (VSNJ) infection in mice was potentiated when delivered by engorging *Aedes triseriatus* mosquitoes as compared to when delivered by virus injections. Ninety-four percent of the three-week-old mice bitten by infected mosquitoes developed antibody, whereas antibody was detected in only 13% of inoculated mice. Adult mice (73%) developed neutralizing antibody when fed upon by infected mosquitoes, but only 11% developed antibody when virus was injected.

Mosquito salivary gland homogenate (SGH) also enhanced VSNJ infection in mouse fibroblast cells, which express interferon. Cells treated with mosquito SGH for four hours demonstrated a significant enhancement in viral replication over time. Treatment with a mosquito vasodilator, sialokinin I, did not significantly affect viral replication over time. Treatment of infected mouse fibroblast cells with interferon α/β antibody altered the virus growth curve from that of untreated cells, but this effect was not significant. Total RNA from mouse fibroblast cells was analyzed by ribonuclease protection assay (RPA) for interferon (IFN)-$\alpha 2$; there was a significant difference in interferon production at six and eighteen hours post-infection between SGH treated and untreated cells.

Anatomic and molecular events in the virus introduction site were analyzed in mice exposed to mosquito feeding or needle inoculation. Examination of mosquito feeding sites revealed a diffuse cellular infiltrate of neutrophils, eosinophils, mast cells, lymphocytes, and macrophages. In contrast, in injection sites, the cellular infiltrate was only comprised of neutrophils. RNA extracted from these sites was also examined for the presence of viral replication by RT/PCR and IFN-α2 production by RPA. Viral replication was not detected in the mosquito feeding or injection sites. IFN-α2 was induced by infected mosquito feeding, uninfected mosquito feeding, virus injection and tissue culture medium injection; however, no significant differences were detected. After mouse footpad inoculation or mosquito bloodfeeding, total RNA from draining lymph nodes was examined for viral transcripts and production of IL-4 and IFN-γ cytokines. Popliteal lymph nodes of mice exposed to VSNJ infected mosquitoes and inguinal lymph nodes of mice injected with VSNJ were positive for viral transcripts 96 hours post-infection. IL-4 was produced in the popliteal lymph nodes in mice exposed to mosquito feeding, but not in mice injected with virus. IFN-γ was not detected in any of the lymph nodes examined.

The development of a Th2 response after mosquito feeding may have an important impact on the salivary potentiation of viral infection. Understanding the arthropod-vertebrate interface may lead to a greater perception of the role of arthropods in VSNJ epizootics and the development of more efficacious vaccines.

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Dedication

This work is dedicated to my late grandmother Violet Soderholm Hill for her everlasting devotion. While teaching me the domestic skills of cooking and sewing, she also imparted her sense of fairness and kindness to all. Her impeccable character demonstrated the kind of person we all should strive to mimic. She had a lasting impression on my life and I have truly missed her.

Table of Contents

List of Tables

List of Figures

Chapter One

Literature Review

Introduction

Vesicular stomatitis (VS) is a disease that primarily affects horses and cattle (Hanson, 1952). This disease is very important in the Southwestern United States due the significant economic impact it causes to producers and indirectly to the surrounding communities. Periodic epizootics of VS have affected the southwestern states of Texas, New Mexico, Arizona, and Colorado approximately every ten years (Letchworth *et al.*, 1999). Animal agriculture, which can be devastated by infectious diseases, is an important industry in these states. For example, during an epizootic in 1995, millions of dollars were lost in this region due to declines in trade, cancellations of rodeos and county fair events, and loss of tourism due to the cancellation of the US Olympic Equestrian Trials in Denver, Colorado (Tabachnick, 2000; Carter, 1995).

The etiologic agent in many of these VS outbreaks was VS New Jersey (VSNJ) virus. This virus has been isolated from open vesicles on cattle and horses, from a variety of bloodfeeding arthropods, and from fomites such as milking machines (Corn *et al.*,

1990; Comer *et al.* 1990; Webb and Holbrook 1989; Sudia *et al.* 1967; Strozzi and Ramos-Soco 1953). The natural transmission cycle of VSNJ is currently unknown. Mechanical transmission by arthropods has been proposed as the main mode of epizootic transmission (Francy *et al.* 1988). However, it is difficult to envision how this mode of transmission could account for long-term maintenance and transmission. Biological transmission by arthropods has also been proposed as a main transmission route due to the concentration of clinical cases during the summer months and the absence of cases after the first freeze (Webb and Holbrook 1989). However, clinical cases do not demonstrate a detectable viremia, which would be required for infection of a bloodfeeding arthropod. Nonetheless, most investigators feel that arthropods do play a major role in VS epizootics in the American West (Tabachnick, 2000; Letchworth, *et al.* 1999).

During the process of bloodfeeding, the vector salivates into the wound site. Salivary proteins affect the vertebrate host's haemostasis and immune responses. Consequently, transmission of vector-borne pathogens is more complex than injection of the pathogen alone. Typically, laboratory inoculations of arboviruses have involved injecting high concentrations of virus with a syringe, which certainly does not mimic the natural cycle of transmission. Indeed, there are now many examples of salivary potentiation of the infectivity of vector-borne pathogens (Ribeiro, 1989; Ribeiro, 1987). Thus, understanding the roles of arthropod saliva in bloodfeeding and especially in pathogen transmission is critical to the study of arthropod-borne diseases.

Arbovirus Transmission Cycles

In classic biological transmission cycles of vector-borne pathogens, the bloodfeeding arthropod acquires the pathogen from the blood of the vertebrate host. The pathogen then replicates or develops in the arthropod host during the extrinsic incubation period. After the pathogen reaches the salivary glands of the arthropod, transmission to a susceptible vertebrate host ensues with the next bloodfeeding (Woodring *et al.* 1996). The pathogen then replicates or develops in the vertebrate host during the intrinsic incubation period. If the vertebrate host develops a sufficient titer of the pathogen in the bloodstream, other bloodfeeding arthropods can acquire the pathogen, the cycle is repeated, and the pathogen is amplified and maintained. In mechanical transmission, the proboscis or other mouthparts of the arthropod becomes contaminated with the pathogen, and transmission occurs when this arthropod probes or feeds upon a new vertebrate host (Woodring *et al.* 1996). This type of transmission occurs very quickly due to the absence of pathogen replication or development in the invertebrate host. Finally, horizontal transmission can also occur when an infected arthropod transmits the pathogen directly to another arthropod during co-feeding on a vertebrate host (Jones *et al.* 1987; Mead *et al.* 2000).

Molecular Biology of Vesicular Stomatitis Viruses

Vesicular stomatitis (VS) viruses are classified in the family *Rhabdoviridae* and the genus *Vesiculovirus* (Wagner and Rose 1996). More than 70 rhabdoviruses of vertebrates and well over 100 rhabdoviruses of plants have been identified (Jackson *et al.* 1987). Virions are bullet shaped and approximately 180 nm in length and 75 nm in

width. The genome is single stranded, negative sense RNA (11,161 nucleotides) that is protected by nucleoproteins. The genomic structure of the virus consists of a nonsegmented RNA that encodes five genes with intergenic spaces between each gene. Transcription begins at the 3' end of the genome with a 48- nucleotide leader RNA, yielding N, P, M, G and L mRNAs. A gradient of mRNA abundance for each gene is produced by a termination of transcription at the gene junctions, followed by 70-80% polymerase reinitiation at the next gene (Iverson and Rose 1981).

The coding assignments for each gene have been described. The nucleocapsid protein, encoded by the N gene, tightly encases the RNA genome. The viral transcriptase is encoded within the L and P genes and is included in the ribonucleoprotein (RNP) core. The matrix protein, encoded by the M gene, serves as the link between the RNP core and the glycoprotein spikes. Finally, the glycoprotein, encoded by the G gene, serves as the epitope for receptor binding and immune response targeting. VS viruses have a very broad host range in part because phosphotidylserine is utilized as the viral receptor for entry into cells (Wagner and Rose 1996).

History of Outbreaks of Vesicular Stomatitis Viruses

Early outbreaks of "sore tongue" in 1801, 1802, and 1817 helped to define the clinical symptoms of VS that are used today (Letchworth *et al.* 1999). One of the earliest epidemics of VS was recorded in 1862 in army horses during the Civil War (Nichol, 1986). Cases of VS in Europe were reported during World War I, which were attributed to importation of the viruses from the United States (Hanson, 1952). The next major

epizootic in the US occurred in 1916; the disease spread rapidly from Colorado to the East Coast. Subsequently, epizootics have occurred on a 10 to 15 year basis.

One of the first well-documented epizootics of VSNJ virus was in 1949. Clinical cases were detected in three large livestock areas in late spring (May). The epidemic exploded in August and September and finally died out in October (Letchworth *et al.* 1999). In 1982, a major epizootic of VSNJ occurred in Colorado and other Western States (Letchworth *et al.* 1999; Francy *et al.* 1988; Webb *et al.* 1987). VS emerged again in 1995 in the southwestern states (Bridges *et al.* 1997). The first cases were detected in horses in New Mexico in May. By October, the virus had spread to Arizona, Texas, Utah, Wyoming, and Colorado. Quarantines were imposed on the movement of animals within a 10-mile radius of premises experiencing infection. Thirty-nine states and multiple foreign governments imposed restrictions on the importation of animals from Colorado (Bridges *et al.* 1997). This same epizootic also caused the US Olympic Equestrian Trials in Denver to be canceled (Carter, 1995). In 1997, the first clinical case of VS Indiana (VSI) in thirty years was reported in New Mexico and subsequent cases were reported in Utah, Arizona, and Colorado (McCluskey *et al.* 1999). VSI is endemic in Mexico and areas of Central and South America, but it is unknown if this virus was introduced into the United States by importing animal products or if the virus is present in an unknown reservoir host. VSNJ virus was also reported in the summer of 1997, infecting predominantly horses (88% of cases investigated) in New Mexico, Arizona, Texas, Utah, Wyoming, and Colorado (McCluskey *et al.* 1999). In 1998, 130 positive premises were reported in New Mexico, Arizona, Colorado, and Texas for VSNJ or VSI

viruses (Vesicular Stomatitis Weekly Updates 1999). Ninety-four percent of these sites reported a single animal affected (Hurd *et al.* 1999).

Epidemiology of Vesicular Stomatitis Viruses

VSNJ is endemic in Central America and Mexico and random epizootics have swept through the western U.S. approximately every ten years. Two different epidemiological patterns of VSNJ are present in the United States (Webb and Holbrook 1989; Webb *et al.* 1987).

The first and most well known epidemiological pattern is that of sporadic seasonal emergence of the virus. Molecular epidemiological evidence suggests that the virus emerges in Mexico and rapidly spreads to cause explosive epizootics in cattle, horses, and swine throughout the Southwestern States (Nichol, 1987). Epizootics move along riverine environments into Colorado. Virus activity ceases after killing frosts (Webb and Holbrook 1989). In some instances, the epizootics spread to involve the Eastern United States. Genomic analyses indicate that the VSNJ virus sequence is diverse within a small endemic region in Central America (Nichol, 1988). Sequence analysis of the phosphoprotein (P) from various VS isolates, across diverse geographical locations, indicates a correlation with a south to north trend from Panama to the United States. Unexpectedly, there is no correlation with time of virus isolation. VSNJ virus is the first example of a punctuated equilibrium evolutionary pattern (Nichol *et al.* 1993). Genetic stasis within a geographical setting may be influenced by replication constraints within vertebrate and invertebrate hosts (Nichol, 1987).

There may be an enzootic cycle of VS in the United States. Epidemic investigations in Colorado revealed anti-VSNJ antibodies in elk from Rocky Mountain National Park (Webb and Holbrook 1989) and in rodents from premises with VS cases (Webb *et al.* 1987). The latter may be significant because sand flies inhabit rodent burrows and could thus serve as vectors in an enzootic cycle. A well-characterized model of an endemic cycle of VSNJ is present on Ossabaw Island, off the coast of Georgia. Isolation of VSNJ from pools of *Lutzomyia shannoni* correlates with the period of seroconversion in sentinel swine (Corn *et al.* 1990). However, molecular epidemiological evidence did not support the involvement of this virus source in the Southwestern U.S. epizootics (Nichol, 1987). In addition, the virus can be transovarially transmitted by *Lutzomyia shannoni*, which may indicate an additional maintenance mechanism (Comer *et al.* 1992). VSI is also efficiently transovarially transmitted by South and Central American *Lutzomyia* species (Tesh *et al.* 1972). Thus infected vectors could maintain virus from season to season.

In Central America, a principal risk factor for VS in cattle is the presence of sand flies on the premises (Alsuhaibani, 1990). Recently, a report examined management risk factors for VS in the Southwestern United States (Hurd *et al.* 1999). Factors such as livestock movement (including the introduction of new animals), interactions with wildlife, proximity to other livestock and rodent populations had no effect on disease risk. However, reported increases in biting insect populations and proximity to running water (less than 0.25 miles) did yield significant increases in disease risk (Hurd *et al.* 1999). Although the role of arthropods in the epidemic cycle in the Southwestern US is still being debated, there is little doubt that vector transmission may play a significant role in epidemic transmission cycles.

Transmission Cycle of Vesicular Stomatitis Viruses

The transmission cycle of these viruses is currently unclear. Seasonal epizootics and isolation of virus from sand flies, black flies, and mosquitoes have indicated arthropod transmission (Francy *et al.* 1988; Liu and Zee 1976). More cases of VS occur on sites near woodland pastures, rivers, and lakes in comparison to open plains. During epizootics, virus is clearly transmitted directly and indirectly via fomites to susceptible animals (Webb and Holbrook 1989). Transmission between animals occurs by virus-infected aerosols and secretions (Knight and Messer 1983). Molecular epidemiologic techniques have revealed that VS can also be transported long distances via animal movement (Nichol, 1986). In addition, VS has been isolated from a large number and variety of arthropods during epizootic investigations (Tesh *et al.* 1972; Francy *et al.* 1988; Walton *et al.* 1987; Suarez, 1967; Sudia *et al.* 1967). In Colorado, isolations have been made from a variety of culicoid species, simulids, mosquitoes, face flies, and other flies. Whether these are mechanical or biological vectors remains to be determined. Titers of VSNJ virus in vesicles and lesions can be significant; thus arthropods may become contaminated with the virus and mechanically transmit it (Francy *et al.* 1988). However, many arthropod species are permissive for the replication of VS viruses and could serve as biological vectors during epizootics (Webb and Holbrook 1989; Walton *et al.* 1987). Under laboratory conditions, black flies (*Simulium vittatum*), sand flies (*Lutzomyia shannoni*), and biting midges (*Culicoides variipennis sonorensis*) have been demonstrated as competent vectors of VSNJ virus (Weaver *et al.* 1992; Mead *et al.* 1997; Tabachnick 2000). In explosive epidemics, both mechanical and biological transmissions probably occur.

The role of arthropods in VS epidemics in the US remains controversial. The difficulty of demonstrating viremia in vertebrate hosts indicates a lack of vector transmission. A large number of vertebrate species have been inoculated experimentally with VS, but viremia and lesions are extremely difficult to demonstrate (Webb and Holbrook 1989; Gibbs, 1984). Proponents of vector transmission stress the association of VS epizootics with riverine environments, wet pastures and vegetation, and the effect of frosts on disease transmission (Webb *et al.* 1987).

Demonstration of "non-viremic" transmission of VSNJ between black flies suggests an alternate mode of horizontal transmission of VS viruses (Mead *et al.* 2000). In this study, infected black flies (*Simulium vittatum*) co-fed with uninfected black flies on *Peromycus maniculatus* mice (age six weeks or six months). In the first bloodfeeding trial on six-week old mice, seventeen percent (3/18) of the uninfected black flies that engorged with infected flies became infected. The second bloodfeeding trial used six-month old mice and sequential feeding over three days. On day one, fourteen percent (2/14) of the uninfected black flies became infected. On day 2 and 3, twenty-six percent (4/15) and twenty-five percent (3/12) of the uninfected black flies became infected, respectively. Thus, detectable viremia in naturally infected cattle and horses may not be necessary for horizontal transmission of VSNJ virus.

Clinical Symptoms of Vesicular Stomatitis

Clinical signs of VS are indistinguishable from Foot and Mouth Disease (FMD). The potential for spread and its impact on agriculture has resulted in VS being categorized as a List A disease by the Office of International Epizootics (Vesicular

Stomatitis Weekly Updates 1999). Because VS viruses are designated as a List A disease, infections must be reported to state and federal officials. Restrictions on trade and movement of livestock from VS infected areas and the loss of milk production are the cause of major economic losses. Characteristics of this disease include: vesicle formation and erosions of oral mucosa, tongue, teats, and coronary bands of the hoof (Knight and Messer 1983). Other signs associated with vesicular stomatitis include fever, anorexia, weight loss, and lameness. Fifty percent of clinical cases demonstrated lesions in the interdigital spaces and 2-10% of dairy cows present with lesions in the udder region (Hanson, 1952). Virus titers present in the fluid of vesicular lesions range from 10^{4-6} plaque-forming units (Comer *et al.* 1990). In range animals, the disease is self-limiting and recovery is not complicated by sequela, which makes direct losses associated difficult to document. VS outbreaks in dairies can result in decreased lactation and secondary mastitis. Direct costs to dairy farmers in the 1982 epizootic were estimated to be between $97 and $253 per clinical case (Goodger *et al.* 1985). Adult cattle seem to be more susceptible to clinical disease than young calves, which frequently only have a subclinical response (Goodger *et al.* 1985; Hanson, 1952). Morbidity rates can range from 1-100%, but mortality rates are very low (Vanleeuwen *et al.* 1995; Thurmond *et al.* 1987). Virus attack rates have been reported as high as 96% in dairy farms, where contact transmission is more frequent (Webb and Holbrook 1989).

Various animals present at the sites of VS outbreaks exhibit antibody to the virus, but primarily cattle and horses display clinical signs. In earlier times, differential diagnosis of VS infections was obtained by inoculation of horses, because they are not

susceptible to infection with FMD virus but are to infection with VS viruses (Letchworth *et al.* 1999).

Experimentally, animals can be re-infected within 30-60 days after recovery, which indicates virus specific immunity is of short duration (Hanson, 1952). Antibody in animals infected in the field disappears within six months. Neutralizing antibodies protect against re-infection, but antibody titers can fluctuate up to 1000 fold within a given month (Letchworth *et al.* 1999; Thurmond *et al.* 1987). Neutralizing antibodies alone do not protect cattle in endemic areas; animals have presented with neutralizing antibody titers before the onset of clinical symptoms (Letchworth *et al.* 1999). This suggests that vaccines should be targeted to mucosal surfaces (IgA) instead of systemically (IgG).

Currently there is no broadly used vaccine against VS. During epizootics, the United States Department of Agriculture (USDA) has provided killed virus vaccine, but the distribution was limited to states with confirmed cases. Only one study has examined the efficacy of this vaccine; two intramuscular injections yielded low titers of antibodies against the viral N protein (Webb and Holbrook 1989). The addition of IFN-γ to purified viral glycoprotein resulted in a five-fold increase in neutralizing titer after cattle had been boosted. Animals vaccinated with high doses of IFN-γ and purified glycoprotein, then challenged intraligually with 10^2 PFU of VSNJ virus, did not develop lesions (Yilma *et al.* 1989; Anderson *et al.* 1988). New technologies are currently being investigated in the development of an efficacious vaccine.

In vivo Pathology of Vesicular Stomatitis in Cattle and Horses

Histopathological lesions associated with VS infection involve the epithelial cells of the oral mucosa and skin that are lysed by viral infection followed by severe interstitial edema (Letchworth *et al.* 1999). Vesicles emerged above the basal layer of tissue and infiltration of neutrophils and monocytes promoted necrotic tissue clearance. These vesicles burst after 48 hours due to mechanical forces of the mouth. During this period, animals demonstrated a transient fever. The epithelial layer sloughs and virus shed from these lesions may titer as high as 10^9 PFU/ml (Letchworth *et al.* 1999). Oral lesions heal in 2-3 weeks, whereas udder and foot lesions may take longer. Scarification may occur due to coarse roughage and hard feed pellets. Natural infection of pregnant mares revealed that the virus does not cross the placenta or cause abortions (Letchworth *et al.* 1999).

In vitro Cytopathology of Vesicular Stomatitis viruses in Mammalian Cells

In vertebrate cell culture (baby hamster kidney and Vero), cytopathic effect is rapid and very destructive. Specific inhibition of cellular gene expression leads to destruction of the cellular cytoskeleton and apoptosis (Letchworth *et al.* 1999). Much of the cytopathic effect of VS virus infection has been attributed to the M protein. The M protein targets and inhibits transport of proteins into the nucleus and transport of cellular mRNA out of the nucleus. Viral mRNA becomes the predominant message and is exclusively translated by the cellular ribosomes. The role of M protein in cellular pathology is to cause cell rounding and detachment by depolymerizing actin. This occurs

within the first hour of infection. Soon after, tubulin and vitmentin fibers are targeted (within 3-4 hours post-infection), which causes a breakdown in actin stress fibers, microtubules and intermediate filaments. The breakdowns of the cellular skeleton and cell death are apparently critical for release of the virus into the environment (Letchworth *et al.* 1999).

Mouse Model for Vesicular Stomatitis Viruses

VSNJ virus is not a natural pathogen in mice and does not induce clinical lesions similar to those caused in horses and cattle. Pathogenesis of VS in the mouse model is age dependent (Skinner, 1957b; Hanson, 1952). Young mice, less than three weeks of age, develop encephalitis and die when infected with virus by any route (Skinner, 1957a; Sabin and Olitsky 1937). Adult mice develop encephalitis only when injected by intranasal or intracranial routes (Hanson, 1952). Peripheral injection of virus (10^7 minimal cerebral lethal dose) into adult mice does not result in virus replication (Sabin and Olitsky 1937) and presumably results in immediate clearance by innate immune responses (Gobet *et al.* 1988). A heat labile factor in normal mouse serum (presumed to be complement) is capable of inactivating low doses of VS viruses (Gobet *et al.* 1988).

Innate Immune Responses Against Vesicular Stomatitis Virus Infection

Early non-specific immune responses control the replication and spread of many viruses. Among these early immune responses is the induction of complement, production of cytokines such as interferon (IFN) and tumor necrosis factor alpha (TNF-α), and activation of natural killer (NK) cells. Activation of these nonspecific responses is crucial

for downstream events that lead to acquired immunity. In particular, induction of certain cytokines by innate immune responses can control the endogenous T cell responses to the infecting agent. Promotion of Th1 or Th2 pathways by innate immunity can have a significant effect on the pathogen (Biron, 1998).

VS viruses are very sensitive to IFN α/β mediated anti-viral effects. During virus replication, a double stranded RNA intermediate is produced that stimulates the production of IFN-α/β (Burke, 1981). Virus replication is not required for VS to induce IFN; specifically only 10% of the VS genome must be transcribed in order to induce IFN (Marcus and Sekellick 1987; Marcus and Sekellick 1980). Induced IFN-α/β is secreted from infected cells. It signals itself (autocrine) and the surrounding cells (paracrine) to produce anti-viral defense genes. This signaling cascade induces the production of numerous proteins such as Mx, 2,5'A oligoadenylate synthetase, and 2',5'A dependent RNase (Bovolenta *et al.* 1995). Each of these proteins can target different stages of virus infection. For example, MxA inhibits the accumulation of viral transcripts either by directly binding to the viral polymerase complex or indirectly by reducing the mRNA stability (Staeheli and Pavlovic 1991).

Treatment of cells with interferon can yield different responses. One study demonstrated a decrease in transport of the viral (VSI) glycoprotein from the Golgi complex to the plasma membrane (Singh *et al.* 1988). Another study revealed that the major site of interferon action was at the level of primary viral transcription (Belkowski and Sen 1987). These examples demonstrate the broad activities activated by interferon.

Blocking IFN α/β by antibodies or in knockout mice decreases the survival of mice infected with VS viruses (Steinhoff *et al.* 1995). Interestingly, IFN α/β receptor knockout

mice, primed with UV inactivated virus or recombinant vaccinia viruses expressing the VS glycoprotein, are protected from intravenous challenge. Adoptive transfer of virus specific antibody also protects mice from challenge; however, transfer of specific memory B and T-cells does not produce neutralizing antibodies quickly enough to confer protection. This indicates that early immune responses are critical for the control of VS virus infection, and once the virus is allowed to replicate, the infected mice eventually die (Steinhoff *et al.* 1995).

Natural killer (NK) cells are also an important aspect of innate immunity, because of their cytolytic activity against cells infected with virus. NK cells can recognize and kill VS infected murine cells (Letchworth *et al.* 1999). Activation of NK cells is primarily by IFN-α/β and can be detected one day after VS infection (Stitz *et al.* 1985). Activated NK cells release IFN-γ that is essential for T-cells and acquired immunity. NK cells and IFN-α/β are believed to be responsible for termination of many acute viral infections (Playfair, 1995).

Another important aspect of innate immunity is the production of TNF-α. Production of this cytokine results in the induction of ICAM-1 expression on keratinocyes (Kondo and Sauder 1997). ICAM-1 in return induces the production of chemokines that function in the recruitment of neutrophils, eosinophils, macrophages, and lymphocytes to the site. TNF-α is a key mediator of inflammation and the mammalian host's response to injury or invasion by pathogens (Kondo and Sauder 1997). TNF inhibits the replication of some viruses *in vitro*, but there is no evidence of this protective effect *in vivo* (Rubin, 1992). Provocatively, an anti-TNF factor has been described in mosquito saliva *in vitro*,

and the potential role of anti-TNF on transmission efficiency needs be assessed *in vivo* (Bissonnette *et al.* 1993).

Recently the role of nitric oxide (NO) in the anti-viral response has been reviewed (Reiss and Komatsu 1998). NO is a neurotransmitter that functions in the central nervous system and is synthesized by a group of enzymes called NO synthetases. NO synthetase is induced in astrocytes and microglia cells after injury. NO production also increases the levels of TNF–α and interleukin-1β. IL-1β has been shown to enhance the ability of IL-12 to induce IFN-γ production by NK cells (Ahmed and Biron 1999). Increased expression of NO is correlated with decreased viral titer (Bi *et al.* 1995). Treatment of a neuroblastoma cell line with NO for 30 minutes or six hours before VS virus infection results in a 100-fold reduction of viral titer (Bi and Reiss 1995).

Acquired Immunity Against Vesicular Stomatitis Virus Infection

Since neutralizing antibody against VS viruses is an important indicator of protection from challenge in mice, induction of humoral immunity is critical. VS viruses induce an early dose dependent T cell-independent B cell response in mice; antibody can be detected as early as four days after infection (Freer *et al.* 1994; Charan *et al.* 1986). Activation of B cells is dependent on the structure of the viral glycoprotein. The densely packed paracristalline structure on the viral envelope induces a B cell response without the help of T cells; however, random distribution of the viral glycoprotein as seen on the surface of infected cells requires T cell help to induce a B cell response (Bachmann *et al.* 1995). Mice infected with live VS produce a primary neutralizing antibody response more efficiently (with doses $>10^2$ PFU) than those injected with UV-inactivated VS

(>10^6 PFU) or purified VS glycoprotein G (equivalent to 10^7 PFU) (Gobet *et al.* 1988). Very low doses of VS (< 10^2 PFU) fail to prime mice, presumably due to the inactivation abilities of the alternative complement cascade (Gobet *et al.* 1988). Mice incapable of generating a B cell response develop a paralytic infection after footpad injection (Gobet *et al.* 1988). These studies demonstrate the importance of neutralizing antibody in controlling VS infections in the mouse model. Neutralizing antibodies are specific for the viral serotype. Non-neutralizing antibodies; however, recognize both VSNJ and VSI viruses (Lefrancois, 1984).

While neutralizing antibodies are specific for viral serotype, cytotoxic lymphocytes (CTL) recognize and kill cells infected with VSNJ or VSI viruses (Rosenthal *et al.* 1983). This cross-reactivity is due to the similarities in the nucleocapsid protein between serotypes. CTLs are predominantly reactive against the nucleocapsid protein verses the glycoprotein. CTL responses peak approximately six to seven days after infection (Zinkernagel *et al.* 1978) and last for four months (Bachmann *et al.* 1994b). Intravenous inoculations of vaccina virus expressing the viral nucleocapsid protein into mice induced CTL responses 30 times greater than intraperitoneal and subcutaneous injections (Bachmann *et al.* 1994b).

Role of Saliva in Bloodfeeding

Acquisition of a bloodmeal by arthropods is a complex and dynamic process. The arthropod vector uses its saliva to locate and acquire a bloodmeal. Blood ingested by female mosquitoes is metabolized and serves as an important energy source and substrate for oviposition proteins. Certain challenges are presented to bloodfeeding arthropods such

as location of vascular tissue, prevention of haemostasis, and modulating vertebrate immune system components (James, 1994). The proteins secreted in vector saliva primarily function in addressing these challenges. Vector-borne pathogens exploit these phenomena to more efficiently infect vectors. Salivary potentiation and salivary activated transmission (SAT) are two terms that have been used to describe the ability of vector saliva to influence transmission of pathogens.

Many of the molecules in arthropod saliva that function during bloodfeeding have been described (Champagne, 1994; James, 1994; Ribeiro, 1989; Ribeiro, 1987a). These proteins are produced only in adult female mosquitoes and are stored in the ascinar space of the cell until a suitable host is located. Bloodfeeding components must combat the vertebrate responses of vasoconstriction, platelet aggregation and coagulation in order to ensure engorgement.

During bloodfeeding, the host experiences cell injury at the bite site. This injury leads to the activation of platelets, cells involved in the innate immune response (e.g. NK cells), and blood vessel contraction. The vector counteracts these activities by releasing pharmacological molecules in the saliva. These molecules, which are produced in the distal lateral lobes of the female mosquito salivary gland, include: apyrase, vasodilators, anti-coagulants, immune modulators, and a number of other proteins (James, 1994).

The role of apyrase in bloodfeeding has been extensively described (James, 1994). ADP is released as a result of cell injury at the site and activates platelet aggregation. Activated platelets and damaged cells release ATP, which enhances the local inflammation and induces polymorphonulear cells to degranulate (Ribeiro, 1987b). Apyrase inhibits this response by breaking down ATP and ADP to AMP and inorganic

phosphate. Apyrase also assists the vector in locating blood thereby shortening the time the vector spends probing. Interestingly, mosquitoes infected with *Plasmodium* spp. have a five-fold reduction in the level of apyrase in the salivary glands with no reduction in the total volume of saliva secreted. This causes mosquitoes to increase probing times, and the number of interrupted feedings increases (Champagne, 1994; Rossignol *et al.* 1984). Finally, apyrase is found in all arthropods that acquire a bloodmeal, emphasizing its evolutionary importance.

The bloodfeeding vector must also combat the haemostasis and immune capabilities of the host. The coagulation cascade is a complex sequence of events that has many steps that arthropod saliva could target. Vasodilators counteract vessel contraction and may play a role in immune modulation. Vasodilators differ from one arthropod to another, and thus there is a long list of proteins that exhibit similar functions. Sand flies produce maxadilan, mosquitoes produce sialokinin, ticks produce prostaglandin E_2 (PGE_2) (Champagne, 1994), and the vasodilators from *Culicoides* and *Simulium* are in the early stages of description (Perez de Leon *et al.* 1997; Cupp and Cupp 1997; Cross *et al.* 1993). Sialokinin from *Aedes* mosquitoes was the first detection of a tachykinin in insects. Characteristics include a combination of functions similar to substance P and neurokinin. Both substance P and neurokinin are members of the tachykinin family of proteins and function in the enhancement of primary antibody responses. *Anopheles* mosquitoes rely on the destruction of endogenous vasoconstrictors (e.g. serotonin and noradrenaline) by a salivary heme protein (Champagne, 1994).

Dipteran Models of Salivary Potentiation

One of the best-studied examples of salivary potentiation is the interaction between *Leishmania* parasites and its vector sand flies. Pioneering studies by Titus and Ribeiro (1988) revealed that infection of vertebrate hosts was enormously enhanced by inclusion of sand fly saliva with purified *Leishmania* parasites. In the presence of sand fly salivary glands, only a few parasites were necessary to establish infection and induced lesions in the footpads of mice. In the absence of saliva, >1,000 purified parasites were required to establish an active lesion in the vertebrate host, and even then the amount of parasites was negligible. Sand fly saliva has been shown to contain a protein called *Leishmania* enhancing factor (LEF). This factor was demonstrated to enhance experimental *Leishmania* infection when the parasite was injected with sand fly salivary gland lysate. Increased parasite survival and development of more severe disease resulted from this enhancing effect. This effect was not seen when the parasites were injected alone. Further studies have determined LEF to be the same protein as erythema inducing factor (EIF), which has been demonstrated to be maxadilan, (Titus and Ribeiro 1990).

Enhancement of infection by components of arthropod saliva is now recognized in a number of other models. White tailed deer and chipmunks, bitten by *Aedes triseriatus* mosquitoes infected with LaCrosse (LAC) virus, had viremias of longer duration and greater amplitude than those infected by viral injections alone (Osorio *et al.* 1996). In addition, adult mice fed upon by LAC infected mosquitoes developed encephalitis and die, while those injected with tissue culture propagated virus survived (Higgs unpublished data). Similar experiments with Cache Valley virus (CVV), mice, and *Aedes*

triseriatus mosquitoes revealed that injections of CVV into sites where uninfected *Aedes triseriatus* had fed resulted in enhanced viremia and induction of virus specific antibody (Edwards *et al.* 1998). This enhancing effect could be demonstrated up to four hours after uninfected mosquito feeding. CVV is a natural pathogen of sheep, so the effect of salivary potentiation was tested in groups of pregnant sheep (N=4). Three different groups were studied: 1) Injection of CVV, 2) Injection of CVV plus mosquito saliva, and 3) Injection of CVV plus mosquito saliva followed by daily administration of saliva. Abortion (50% of ewes) was only detected in the cohort of sheep that were infected with virus in saliva and received additional saliva post infection. This was the first demonstration of CVV induced abortion in domestic animals following a normal route of infection; this suggests that continuous arthropod feeding on range animals could dramatically affect the pathogenesis of arboviruses. In addition, viremia titers were consistently higher in sheep infected with infected salivary glands (Edwards unpublished data).

Non-dipteran Models of Salivary Potentiation

Another example of salivary potentiation, non-viremic transmission, has been described with Thogoto virus (*Orthmyxoviridae*) and ticks (Jones *et al.* 1987). In the case of non-viremic transmission, an infected tick feeding in the same proximity as an uninfected tick can transmit the virus without the induction of a detectable viremia in the vertebrate host. Non-viremic transmission of Thogoto virus can only be replicated with metastriate ixodid ticks and not by other haematophagous arthropods (e.g. mosquitoes) (Jones *et al.* 1992a; Jones *et al.* 1992b). Salivary activated transmission in ticks has also been shown for louping ill virus (*Flaviviridae*) and tick-borne encephalitis viruses

(*Flaviviridae*) (Labuda *et al.* 1993). As noted previously, non-viremic transmission of VSNJ virus has been recently demonstrated with black flies (Mead *et al.* 2000). This was the first report of this type of transmission by a dipteran species.

Immunomodulation by Salivary Proteins

Dipteran Models

Maxadilan, from sand fly saliva, is one of the most potent vasodilators known and has a secondary function in modulation of the immune response. Maxadilan has a number of immunomodulating activities, including: preventing macrophages from becoming activated by IFN-γ, downregulating macrophage ability to present peptides, inhibiting NO and H_2O_2 production, and preventing secretion of a number of cytokines including TNF-α (Soares *et al.* 1998; Donnelly *et al.* 1998; Hall and Titus 1995; Theodos and Titus 1993). This immunomodulation by sand fly saliva has been shown to be effective for four days (Theodos and Titus 1993). Ironically, sand fly salivary gland lysate contains substances that are chemotactic to murine monocytes (Anjili *et al.* 1995). Macrophages are the target cells for *Leishmania* parasite replication and development. If vector saliva inhibits the killing ability of macrophages attracted to the bite site, the *Leishmania* parasite can exploit this effect resulting in enhancement of infection (Titus and Ribeiro 1990; Ribeiro, 1987). Sand fly saliva also induces the production of Th2 cytokines, which aid in the exacerbation of *Leishmania* infection (Nabors *et al.* 1995). The knowledge from these experiments has led to the development of new animal model systems for *Leishmania* spp. that do not readily infect mice (Lima and Titus 1996).

The mosquito vasodilator, sialokinin, can also perturb the Th1/Th2 responses. Injecting purified sialokinin I peptide into mice results in a significant increase in Th2 cytokines (IL-4 and IL-10) and a decrease in Th1 cytokines (IFN-γ and IL-2) (Zeidner *et al.* 1999). While modulation of Th1/Th2 responses exacerbates parasitic infections, the effect on viral infections is currently unknown.

Simulium spp. salivary proteins also affect immune cell responses. Salivary gland extract activities include: interfering with antigen presentation, suppressing T cell proliferation, and enhancing humoral antibody titer to a foreign antigen (Cross *et al.* 1993). These activities may influence horizontal transmission of VSNJ to co-feeding black flies (Mead *et al.* 2000).

Non-dipteran Models

Ticks are characterized as extended bloodfeeders, because they feed for days to weeks, in contrast to the dipterans, which feed quickly. During this extended feeding, factors in tick saliva immunosuppress the host in the feeding site, and the transmitted pathogens are not efficiently cleared by the host (vertebrate) immune response. Ticks have evolved especially diverse and complex systems to counter innate and acquired host defenses. Tick salivary gland homogenate contains abundant anti-protease activity, which prevents activation of the alternative pathway of complement (Wikel *et al.* 1994). Other salivary proteins include a kinase, which inhibits bradykinin and the host pain response, and a prostacyclin, which inhibits the degranulation of host leukocytes (Wikel *et al.* 1994). The tick vasodilator prostaglandin E_2 (PGE_2) has been the subject of considerable research. PGE_2 inhibits cytokine production from Th1 cells and also

inhibits the activation of macrophages, neutrophils, platelets, and mast cells (Champagne, 1994). Tick salivary gland extract (SGE) treatment of spleenocyte cultures results in a 94% reduction of interferon induction by polyinosinic-polycytidylic acid (Poly I:C), and salivary activated transmission (SAT) of Thogoto virus in one third of A2G (Mx1+) mice (Dessens and Nuttall 1998; Kopecky and Kuthejlova 1998). Treatment of L929 cells with tick SGE results in a decrease in interferon action and ~100 fold increase in VSI titer at 16 and 23 hours post-infection (Hajnicka *et al.* 1998; Hajnicka *et al.* 2000). Other innate immune responses affected by tick SGE include: suppressing cytotoxic activities of NK cells, decreasing TNF-α, and inhibiting production of NO by macrophages (Kopecky and Kuthejlova 1998). Providing TNF-α to *Borrelia* susceptible mice, and then exposing the mice to tick feeding results in a 95% protection rate (Zeidner *et al.* 1996). TNF-α is also important in anti-viral defenses and activation of polymorphonuclear lymphocytes (Wikel *et al.* 1994). Modulation of innate immunity may provide a favorable environment for virus transmission (Hajnicka *et al.* 1998).

Acquired immune responses are also affected by tick saliva. Suppression of macrophage cytokines occurs within 48-144 hours after feeding. These cytokines include IL-2, IL-1, and IFN-γ (Wikel *et al.* 1994). Providing IL-2 or IFN-γ to mice susceptible to *Borrelia* infection and then exposing them to tick feeding results in 55-70% protection rate (Zeidner *et al.* 1996). IL-2 is important in autocrine stimulation of T lymphocyte growth. IFN-γ is important for anti-viral functions by induction of MHC class I and II expression (Wikel *et al.* 1994). IL-1 is important in neutrophil chemotaxis and stimulation of IL-2 receptor expression (Wikel *et al.* 1994). Reduction of macrophage

functions also contributed to a loss in the ability to generate primary antibody responses (Wikel *et al.* 1994).

Systemic Responses to Arthropod Bloodfeeding

It has been hypothesized that the immune system of vertebrates that are frequently bitten by arthropods may be less capable of combating infectious diseases (Titus, 1998). Studies at the Arthropod-borne Animal Diseases Research Laboratory (ABADRL-Laramie, WY) examined the immune response of livestock naturally exposed in the field to continuous arthropod bloodfeeding in nature. Livestock were exposed to as many as 100,000 mosquitoes, black flies, and a variety of other arthropods per steer during a 3-4 week period. These animals exhibited significant differences in the immune response at the local site of feeding (inhibition of T and B cells and nitric oxide production) as well as systemic effects (slower IgM and IgG responses, monocytopenia, etc) (Tabachnick, 2000). Similarly, injection of sand fly salivary gland lysate plus antigen resulted in a systemic immunosuppression of antibody response against the antigen for one week in young adult mice (Titus, 1998). While further studies are needed to examine the role of arthropod saliva in systemic immunomodulation, these studies reveal the importance of a competent immune system in combating common pathogens.

In vivo Response to Bloodfeeding

Histological Response

The consequences of bloodfeeding to the host are complex and may impact the nonspecific defenses and the immune system of the host. Neutrophils are immediately

recruited to the site of injury to clear debris. Langerhan cells migrate from the skin to present antigen to lymphocytes in the draining lymph nodes (Randolf and Nuttall 1994).

Histological characterization of *Leishmania* lesions enhanced by sand fly saliva confirmed the increased lesion size and parasite load from earlier studies. At 21 days post-infection, saliva-free lesions progressed to small, organized granulomas of epitheloid macrophages with few parasites and fewer eosinophils and neutrophils. In contrast, addition of saliva caused extensive, poorly organized accumulations of parasitized epitheloid macrophages with persistent neutrophils and eosinophils. The inflammatory response early in infection was quantitatively and qualitatively similar in both groups. Cellular infiltrate into lesions consisted of: activated histiocytes, disseminated population of eosinophils, and scattered neutrophils and lymphocytes. This marked difference in lesions demonstrated the exacerbation of a controlled infection to a chronic, uncontrolled infection (Donnelly *et al.* 1998).

The histology of tick feeding sites has also been examined to characterize the host response. Cellular infiltrate after tick feeding and migration of these cells from the feeding site could be beneficial to virus dissemination. Detection of TBE viral antigen in Langerhan cells, migratory monocyte/macrophages, and neutrophils from skin explants exposed to tick feeding indicates a potential vehicle for viral dissemination (Labuda *et al.* 1996). These cells are also involved in the host response to tick infestation. Activation of complement components C3 and C5 leads to the induction of mast cell degranulation and edema (Ribeiro, 1987b). Degranulated mast cells and basophils are suggestive of immediate hypersensitivity reactions with eosinophils in a regulatory role (Wikel, 1982). While unnatural hosts developed effective anti-tick immunity, natural tick-host

associations are characterized by the absence or partial expression of tick resistance despite a significant reaction at the attachment site. This effect was attributed to the ability of tick saliva to inhibit various neutrophil activities including phagocytosis (Ribeiro *et al.* 1990).

Host Immune Response to Vector Salivary Proteins

Defining the host response to arthropod salivary proteins could be important in understanding how to prevent the modulation of the immune response. Only a few studies have examined the immune response against mosquito feeding. The first report of protective immunization with mosquito antigens was in the development of a malaria vaccine. Seventy percent of mice immunized with normal salivary gland material alone were protect from *Plasmodium* challenge (Alger *et al.* 1972).

Initial exposure of humans to *Aedes aegypti* feeding resulted in no immediate cutaneous response other than a tiny red spot at the bite site. After 20-24 hours a marked delayed response occurred and characteristic wheal and flare reaction (Mellanby, 1946). Repeated exposure to biting insects results in hypersensitivity to their antigens. This response is described in two phases. The immediate hypersensitivity phase is characterized by an erythematous, edematous reaction with the infiltration of polymorphonuclear cells. The delayed response is characterized as lymphocytic and an eosinophilic infiltrate follows one day later (Edman and Spielman 1988). When hypersensitive guinea pigs are exposed to *Aedes aegypti* feeding, an infiltration of predominantly neutrophils (66-99%) with eosinophils as the remaining cell type follows (French, 1972).

Sera collected from children who resided in Finland, Iceland, Kenya, or Mexico were analyzed for antibodies against salivary gland proteins. There was a high occurrence of IgE and IgG4 antibodies to mosquito saliva in the children from Finland, Kenya, and Mexico, but children from Iceland did not show either antibody subtype. Initial exposure to mosquito bites did not result in a response, but after subsequent bites there was an immediate reaction. The IgE anti-saliva antibodies were involved in cutaneous whealing, edema, and induced the release of histamine and other mediators from mast cells (Reunala *et al.* 1994).

The effects of anti-mosquito antibody ingestion by the mosquito have been examined. Different strains of mice were immunized with mosquito whole body extract and each strain produced different levels of antibody. Mosquitoes feeding on mice with high levels of antibody demonstrated significant levels of mortality by day two post-feeding. Mosquitoes that fed on mice with intermediate levels of antibody did not show high levels of mortality until day five post-feeding. Finally, mosquitoes that fed on mice with low levels of antibody did not show any change in mortality in comparison to controls. There were no significant differences detected in feeding success between mosquitoes feeding on immunized or control mice (Hatfield, 1988).

Such studies suggest that the host response to mosquito feeding could be incorporated into vaccine strategies against arthropod-borne pathogens. This could represent a novel approach to the control of vector-borne diseases (Titus and Ribeiro 1990). Clearly, arthropod transmission is more complex than "flying syringes" delivering pathogens, and more knowledge on the vector-vertebrate interface is needed to address the increasing problem of vector-borne diseases.

Summary and Hypotheses

Vector saliva clearly potentiates transmission of *Leishmania* and tick-borne pathogens, but little work has examined diptera-borne pathogens. The transmission cycle of VSNJ in the Southwestern United States remains unknown, but epidemiological information suggests that VSNJ is an arbovirus. Contradicting this fact is that inoculation of virus into animals, especially adults, does not result in infection and viremia, a prerequisite for an arbovirus cycle. However, none of these infections was attempted using vector transmission. The vector/vertebrate interface was examined in these studies using VSNJ and the mouse model. In the second chapter, the hypothesis that mosquito saliva can potentiate VSNJ infection in mice is examined. In the third chapter, the hypothesis that mosquito saliva modulates the virus infection by targeting interferon α/β is examined *in vitro.* Finally in the fourth chapter, anatomic and molecular events in infection site are compared and contrasted. Interferon production in the feeding and injection sites of mosquito fed and needle inoculated mice is also examined. These studies hopefully will provide insight into the complex transmission cycle of VSNJ virus.

Chapter II

Mosquito Saliva Potentiates Vesicular Stomatitis New Jersey Virus Infection in Mice

Introduction:

Understanding the roles of arthropod saliva in bloodfeeding and especially in parasite transmission is critical to the study of arthropod-borne diseases. Transmission of these agents is more complex than injecting the pathogen alone. Studies by Titus and Riberio (1988) demonstrated the immune-modulating effect of arthropod saliva during pathogen transmission. Injection of *Leishmania* promastigotes plus ½ of a sand fly salivary gland into mice resulted in increased lesion size and parasite load when compared to injection of parasites alone. Many of the molecular and biochemical components of arthropod saliva that function during bloodfeeding have been described (Champagne, 1994; James, 1994; Ribeiro, 1987a; Ribeiro, 1989; Titus and Ribeiro, 1990). These components also play an enhancing role in pathogenesis of arthropod–borne diseases (Wikel, 1982). For example, the protein maxadilan from sand fly saliva has been demonstrated to be a potent vasodilator as well as an inhibitor of macrophage functions (Qireshi *et al.* 1996; Theodos and Titus, 1993). Similar immune modulators

have been described in mosquito and tick salivary gland extracts (Bissonnette *et al.* 1993; Jones *et al.* 1992; Wikel *et al.* 1994; Zeidner *et al.* 1999).

Salivary potentiation by mosquitoes has also been described. Animals exposed to LaCrosse virus (LAC) infected *Aedes triseriatus* mosquitoes have viremias of longer duration and greater amplitude than those infected by viral injections alone (Osorio *et al.* 1996). When fed upon by infected mosquitoes many adult mice develop encephalitis and die, while those injected with tissue culture propagated virus survive (Higgs unpublished data). Similar experiments with Cache Valley virus (CVV), mice, and *Aedes triseriatus* mosquitoes revealed that injections of CVV into sites where *Aedes triseriatus* had fed (up to four hours previously) resulted in enhanced viremia and virus specific antibody (Edwards *et al.* 1998).

Vesicular stomatitis New Jersey (VSNJ) virus is in the genus *Vesiculovirus,* family *Rhabdoviridae*, and order *Mononegavirales* (Nichol, 1986; Wagner and Rose, 1996). Clinically VS is indistinguishable from Foot and Mouth Disease, and epizootics of VSNJ affect the Southwestern United States approximately every ten years. While VS does not cause high mortality rates, the economic losses to producers can be devastating (Thurmond *et al.* 1987; Vanleeuwen *et al.* 1995). The transmission cycle of this virus is currently unclear. Seasonal epizootics and isolation of virus from sand flies, black flies, and mosquitoes have indicated arthropod transmission (Liu and Zee, 1976; Francy *et al.* 1988).

Pathogenesis of VS in mice is age dependent (Hanson, 1952; Skinner, 1957a). Young mice, under three weeks of age, develop encephalitis and die when infected with virus by any route (Sabin and Olitsky, 1937; Skinner, 1957b). In contrast, adult mice

develop encephalitis only when injected by intranasal or intracranial routes (Hanson, 1952). Peripheral injection of virus (10^7 minimal cerebral lethal dose) into adult mice results in no virus replication (Sabin and Olitsky, 1937). Presumably, the innate immune response immediately clears the injected viral particles, and the memory immune response is not induced (Gobet *et al.* 1988).

Studies were conducted to test the hypothesis that VSNJ infection could be potentiated by arthropod saliva. Mice in three different age groups (three days, three weeks, or >1 eight months) were exposed to VSNJ infected mosquitoes or were injected with an equivalent dose of VSNJ (titer 1.5-3 logs). Mice were monitored for the presence of clinical symptoms leading to death, viremia at two days PI, and the presence of specific anti-VSNJ neutralizing antibody at seven and fourteen days PI.

Materials and Methods:

Virus and Cells

A VSNJ field isolate obtained from USDA-ABADRL laboratory in Laramie, Wyoming was used in all experiments. This virus was isolated from *Culicoides variipennis* during the 1985 Colorado epizootic. Propagation and serum neutralization tests were performed using Vero (green monkey kidney) cells originally obtained from ATCC. Cells were maintained in Leibovitz-15 medium supplemented with 5% fetal bovine serum and 100U penicillin and100µg streptomycin per ml.

Mosquitoes

Aedes triseriatus mosquitoes were maintained as described by Higgs and Beaty (1996). To ensure that 100% of mosquitoes were infected, each one was intrathoracically

inoculated with 1-2 $\log_{10}TCID_{50}$ tissue culture propagated virus as described by Cupp et al (1992). Mosquitoes were maintained for five days post-inoculation before transmission attempts. Mosquitoes (N=5) were aspirated into separate cartons and deprived of sugar 24 hours before bloodfeeding. After allowing a group of mosquitoes to feed, the number of engorged mosquitoes was recorded. To confirm that the mosquitoes were infected, impression smears of mosquito heads were fixed in acetone and assayed by indirect immunofluorescence with a primary polyclonal antibody against VSNJ (Ab #VS2129) provided by the Centers for Disease Control and Prevention, Division of Vector-borne Infectious Diseases, Ft. Collins, CO. In certain groups of mice, uninfected mosquitoes were allowed to feed on exposed mice 1-3 days post-infection to examine the effect of continuous mosquito feeding on antibody development as well as for detection of viremia in mice.

Saliva collection

Mosquito saliva was collected as previously described (Higgs *et al.* 1998) with slight modifications. Briefly, at five days post-inoculation the mosquito legs and wings were removed. The mosquito proboscis was placed into a 10µl capillary tube (Fisher Scientific) filled with type B immersion oil (R. P. Cargille Laboratories, Inc. Cedar Grove, NJ), and mosquitoes were allowed to salivate for 2-4 hours. Saliva was expelled into 30µl L-15, vortexed, and centrifuged for 2-3 minutes at 10,000-x g to transfer the saliva from the oil into the L-15. Samples were titrated by two fold dilutions in triplicate on Vero cells and tissue culture infectious dose endpoints ($TCID_{50}$) were determined (Karber, 1931). Heads of individual mosquitoes were assayed by IFA to confirm infection.

Mice

Mice (ICR strain from Harlan-Sprague) in three different age groups were used: three-day-old suckling, three week, and adult (older than eight months). Pregnant females were ordered, and three days after birth the suckling mice were infected by mosquito feeding or by injection of virus (dose=3 $\log_{10}TCID_{50}$). Three-week old and retired breeders were used for the other two age groups. Mice were restrained according to Edwards *et al* (1998). Briefly, mice were placed onto a wooden block and covered with a wire mesh screen. A small feeding area along the back was exposed to mosquitoes. Injections were delivered into a similar area on the mouse. Mice were bled at day 2, 7 and 14 post infection from the retroorbital sinus. All experiments were conducted within a BSL-3 facility approved by the USDA and were approved by the Colorado State University Animal Care and Use Committee (# 96-143A-02).

Viral assays:

Virus Isolation:

Day 2 post-infection sera were assayed for the presence of viremia by limiting dilution on Vero cells. Sera were diluted 10 fold in 96 well plates and 100µl of cell concentration ($4x10^5$ cells/ml) was added to each well. Cells were maintained for seven days and examined for CPE daily. $TCID_{50}$ endpoint titers were calculated by the Karber method (Karber, 1931).

Serum Neutralization:

All sera were tested for neutralizing antibody by limiting dilution, starting with 1:10 dilution of sera and titrating 2 fold across 96 well plates. VSNJ virus (200 $TCID_{50}$) was added to each well, followed by back titrations of the virus dilution to confirm titer.

Diluted serum and virus were incubated at 37°C for one hour. One hundred microliters of Vero cells (concentration = $4x10^5$ cells/ml) was added to each well. Plates were examined at days two and three to determine neutralizing endpoints of each serum sample. Negative controls consisted of sera from mice injected with tissue culture medium and mice fed upon by uninfected mosquitoes. The positive control consisted of Ab #VS2129, which consistently had an endpoint titer >160.

Indirect Immunoflourescent Antibody Staining:

IFA analysis was performed on mosquito head impression smears as previously described (Higgs *et al.* 1998). Briefly, slides with impression smears of mosquito heads were fixed in acetone for ten minutes at –20°C. Primary antibody consisted of polyclonal mouse ascitic fluid (Ab #VS2129) used at a 1:5000 dilution. Slides were incubated for 45 minutes in a humid chamber at 37°C after each step. Biotinylated sheep anti-mouse antibody (Amersham) was used as secondary antibody with Evan's blue counterstain (both at 1:200 dilution). Fluoroscein-streptavidin (Amersham) was used for detection (1:200 dilution). Slides were mounted with coverslips (Sigma) and DABCO (Lernen Laboratories) and examined using an Olympus BH-2 Fluorescent Scope.

Organ titrations:

Suckling mice showing severe clinical symptoms were euthanized and selected organs were homogenized with a pestle in 400 µl cell culture medium. Homogenate (100 µl) was titrated by ten fold dilutions in triplicate on Vero cells. $TCID_{50}$ endpoint titers were determined seven days later and calculated by the Karber method (1931).

Statistical analysis:

Neutralization titers were transformed to the log scale and multiple comparisons (F-protected LSD test) were generated using the SAS program (SAS Institute, 1993). Significance was determined with $p \leq 0.05$.

Results:

VSNJ content in saliva:

Mosquito saliva (N=12) was titrated to estimate the amount of virus delivered by arthropod bite. Five days post-inoculation, mosquito saliva was collected as described in materials and methods. Titers ranged from 0.0-4.5 $\log_{10}TCID_{50}$ with a mean titer of 2.0 $\log_{10}TCID_{50}$ per saliva sample. Mosquito infection was confirmed by IFA analysis of head tissues.

Suckling mice:

Suckling mice at three days of age were either fed upon by infected mosquitoes or injected (dose=3 $\log_{10}TCID_{50}$) with VSNJ. All of the mice (9/9 mosquito fed and 10/10 injected mice) developed encephalitis between two and three days post exposure. Selected organs from animals within both the mosquito fed and injected groups were titrated for the presence of virus. Mean titers for liver, spleen, kidney and lung ranged between 3.0-5.3 $\log_{10}TCID_{50}$ with no significant differences between groups. The highest titers of virus were found in the brain and blood (mean titers of 8.3 $\log_{10}TCID_{50}$ and 6.5 $\log_{10}TCID_{50}$ respectively). Interestingly, 2/2 mice died that were only probed by infected mosquitoes (no mosquitoes in the respective groups imbibed blood).

Three-week-old mice:

Mouse groups that were fed upon by infected mosquitoes had seroconversion rates that ranged from 85.7-100% (31/33) at day fourteen (Table 1). Seroconversion was defined as $\geq$ four-fold increase in antibody titer between acute and convalescent sera. However, in mice injected with the high dose (3 $\log_{10}TCID_{50}$) the seroconversion rate ranged from 13-25% (9/40) and the low dose (1.5 $\log_{10}TCID_{50}$) mouse group did not have any seroconversions (0/30). The remaining mice exposed to infected mosquitoes (2/33) had low neutralization titers (titer = 20). Comparison of day 7 antibody titers (Table 1) revealed a significant difference between the mice fed upon by mosquitoes only one day (66.7%) and mice that were fed upon by mosquitoes for consecutive days (30% for both groups). One mouse from the mosquito fed group exhibited encephalitic symptoms on day 8 and was subsequently euthanized. Virus was isolated from the brain sample of this mouse. Virus isolation attempts from day two post-infection sera were not successful.

Adult mice:

Results for the adult cohort of mice were similar to those with the three- week old cohort (Table 2). Mice that were fed upon by infected mosquitoes had high seroconversion rates ranging from 50-100% (16/22) on day fourteen. In comparison, seroconversion rates of mice injected with either the high (22%; 4/18) or the low (0%; 0/19) dose of virus were lower. In the remaining mice exposed to infected mosquitoes (6/22), three out of six had low neutralization titers (titer = 20) and two out of six were

only probed. One mouse exposed on consecutive days of infected mosquito probing seroconverted. None of the adult mice developed clinical symptoms. Virus isolation tests of day two post-infection sera were negative. Additional delivery of saliva by infected or uninfected mosquitoes did not influence serum neutralization titers or recovery of virus in day 2 sera.

Time to engorgement comparison between infected mosquitoes and uninfected mosquitoes:

An interesting observation was noted concerning engorgement rates of infected and uninfected mosquitoes. Virus infected mosquitoes exhibited 19.0-44.6% daily engorgement rates (mean rate =30.5%; 84/275) over an extended period of time (1-1.3 hours). In contrast, 84.7-95% of uninfected mosquitoes (mean rate =90%; 122/135) engorged during a much shorter time (15-20 minutes). Mosquitoes intrathoracically inoculated with cell culture medium were also examined to determine if this manipulation caused an adverse effect on bloodfeeding. Five days post inoculation, 90% of these mosquitoes fed in 15 minutes.

Neutralizing Antibody Titers- Three week old

	<10-20	40-160	>320	% Sero-conversion
Day 7				
Fed upon by infected mosquitoes (day 1)	5	9	1	66.7
Fed upon by infected mosquitoes (day 1-4)	7	3	0	30.0
Fed upon by infected mosquitoes (day 1) and uninfected mosquitoes (day2-4)	7	3	0	30.0
Injection dose 3.0 logs	23	9	0	28.1
Injection dose 3.0 logs followed by uninfected mosquito feed (days 2-4)	8	0	0	0
Injection dose 1.5 logs	20	0	0	0
Injection dose 1.5 logs followed by uninfected mosquito feed (day 2-4)	10	0	0	0
Injected with L15 medium	10	0	0	0
Fed upon by uninfected mosquitoes	10	0	0	0
Day 14				
Fed upon by infected mosquitoes (day 1)	2	7	5	85.7
Fed upon by infected mosquitoes (day 1-4)	0	3	7	100
Fed upon by infected mosquitoes (day 1) and uninfected mosquitoes (day2-4)	0	3	6	100
Injection dose 3.0 logs	24	0	8	25
Injection dose 3.0 logs followed by uninfected mosquito feed (days 2-4)	7	1	0	13
Injection dose 1.5 logs	20	0	0	0
Injection dose 1.5 logs followed by uninfected mosquito feed (days 2-4)	10	0	0	0
Injected with L15 medium	10	0	0	0
Fed upon by uninfected mosquitoes	10	0	0	0

Table 1: Neutralization titers for three-week age group
Sera from day seven and fourteen post-exposure were assayed for VSNJ neutralizing antibody as described in the materials and methods section. Number of mice per antibody titer range is listed for each group.

Neutralizing Antibody Titers- Adult

	<10-20	40-160	>320	% Sero-conversion
Day 7				
Fed upon by infected mosquitoes (day 1)	5	2	0	28.6
Fed upon by infected mosquitoes (day 1-4)	6	3	0	33.3
Fed upon by infected mosquitoes (day 1) and uninfected mosquitoes (day2-4)	4	4	0	50
Injection dose 3.0 logs	8	1	0	11.1
Injection dose 3.0 logs followed by uninfected mosquito feed (days 2-4)	9	0	0	0
Injection dose 1.5 logs	9	0	0	0
Injection dose 1.5 logs followed by uninfected mosquito feed (days 2-4)	10	0	0	0
Injected with L15 medium	5	0	0	0
Fed upon by uninfected mosquitoes	3	0	0	0
Day 14				
Fed upon by infected mosquitoes (day 1)	2	2	2	67
Fed upon by infected mosquitoes (day 1-4)	0	4	4	100
Fed upon by infected mosquitoes (day 1) and uninfected mosquitoes (day2-4)	4	0	4	50
Injection dose 3.0 logs	7	2	0	22
Injection dose 3.0 logs followed by uninfected mosquito feed (days 2-4)	7	2	0	22
Injection dose 1.5 logs	9	0	0	0
Injection dose 1.5 logs followed by uninfected mosquito feed (days 2-4)	10	0	0	0
Injected with L15 medium	5	0	0	0
Fed upon by uninfected mosquitoes	3	0	0	0

Table 2: Neutralization titers for adult age group
Sera from day seven and fourteen post-exposure were assayed for VSNJ neutralizing antibody as described in the materials and methods section. Number of mice per antibody titer range is listed for each group.

Summary of Day 14 Seroconversion Rates

	%Seroconversion
Three-week old	
Fed upon by infected mosquitoes	94*
Injection dose 3.0-1.5 logs	13
Injected with L15 medium	0
Fed upon by uninfected mosquitoes	0
Adult mice	
Fed upon by infected mosquitoes	73*
Injection dose 3.0-1.5 logs	11
Injected with L15 medium	0
Fed upon by uninfected mosquitoes	0

* indicates statistical significance within respective age group

Table 3: Summary of Day 14 Seroconversion Rates
Analysis of mice infected by mosquito bite revealed no significant differences in seroconversion rates by F-protected LSD test; so data from these three groups of mice were combined for the final analysis. Additionally, mice that were injected with high or low doses of virus did not differ significantly in their seroconversion rates; and data from these four groups of mice were combined. Data were analyzed by F-protected LSD tests to compare seroconversion rates in mosquito fed mice, injected mice, and negative control mice.

Discussion:

Mosquito delivery of VSNJ potentiates virus infection in mice older than three weeks. The seroconversion rates in mice exposed to VSNJ infected mosquitoes and in the injected mice at day fourteen differ statistically for the three-week and adult age groups (Table 3). Previous studies have reported that VS does not replicate to a detectable level in adult mice (Sabin and Olitsky, 1937), and the older two mouse cohorts injected in this study confirm that result. However, mosquito transmission of VSNJ potentiates infection as seen by the higher seroconversion rates. The mechanisms by which vector saliva conditions the permissiveness of hosts to viral infection are not yet understood. Perhaps while feeding, the mosquito saliva induces a local immunosuppression, and the virus is not cleared by innate immune functions (Cross *et al.*, 1994; Schmidt *et al.*, 1995). Survival and possibly replication of VSNJ would lead to T-cell priming and activation of memory immune responses as reflected by development of serum neutralizing antibodies.

Additional mosquito feeding experiments on days 2-4 were designed to analyze the impact of supplemental saliva on the virus infection or on the mouse immune response. Examination of day seven seroconversion rates revealed a significant difference within the three-week age group ($p<0.05$). Mice only fed upon by infected mosquitoes on day one had a seroconversion rate of 62.5%. In comparison, mice that were initially infected by mosquito feeding and exposed to additional mosquito feeds (infected or uninfected) on days 2-4 had a seroconversion rate of 30% for both groups. By day fourteen, these mice seroconverted. However, three-week old mice injected with high or low doses of virus did not demonstrate this delay in neutralizing antibody. Further

analysis revealed no significant differences in day seven neutralizing antibody titers in the adult age group. This delay in neutralizing antibody detection for one age group and not the other is somewhat perplexing. It is possible that some sort of systemic immune modulation occurs when animals are continuously bombarded by arthropod feeding as hypothesized by Tabachnick (2000). Studies at the Arthropod-borne Animal Diseases Research Laboratory (ABADRL-Laramie-WY) have examined exposure of livestock to arthropod bloodfeeding in nature. Livestock were exposed to as many as 100,000 mosquitoes, black flies, and a variety of other arthropods per steer during a 3-4 week period. These animals exhibited significant differences in the immune response at the local site of feeding (inhibition of T and B cells and nitric oxide production) as well as systemic effects (slower IgM and IgG responses, monocytopenia, etc).

It is noteworthy that mosquito probing alone lead to the death of 2/2 three-day-old mice. This demonstrates that virus can be transmitted while the mosquitoes are searching for blood without the need for engorgement. Gubler and Rosen (1976) established that probing mosquitoes transmit as efficiently as mosquitoes feeding to repletion. In contrast, in the older two mice groups, only one mouse seroconverted out of three mice that were probed. The adult mouse that seroconverted was exposed to consecutive (days 1-4) probing by infected mosquitoes. This may indicate a threshold level of saliva needed to potentiate VSNJ infection.

The absence of detectable viremia in three-week and adult cohorts of mice is not surprising. Serum viremia in clinically ill animals has been difficult to detect in both field and laboratory studies (Webb and Holbrook, 1989). However, high virus titers are present in open vesicles on epithelial surfaces that are easy targets for arthropod feeding.

These observations may lend credence to the possibility of non-viremic transmission of VSNJ during epizootics in the Southwestern U.S (Mead *et al.* 2000).

Infected mosquitoes took significantly longer to engorge than uninfected mosquitoes. Viral replication within the salivary glands may add an additional burden on the invertebrate host and subsequently cause extended probing/feeding. Grimstad et al (1980) examined this phenomenon in LaCrosse infected *Aedes triseriatus* mosquitoes. Mosquitoes were monitored for the number of probes and the amount of blood imbibed, if any. Infected mosquitoes tended to probe more times than uninfected mosquitoes and exhibited a reduced frequency of total engorgement. This phenomenon may be caused by virus particles obstructing the salivary ducts (Grimstad *et al.* 1980). Pathological effects of salivary gland infection by viruses might also influence gland physiology and integrity. For example, electron microscopic analysis of Semliki Forest virus infected *Aedes aegypti* salivary glands revealed significant pathology (Lam and Marshall, 1968). Feeding to engorgement prolongs host contacts and increases pathogen distribution in the host population. These studies, along with this study, suggest that arbovirus infection of vector salivary glands may influence vector-borne disease epidemiology.

Salivary potentiation of VS infection in adult mice by mosquito saliva may provide insight into the complex natural transmission cycle of VS. Future studies are aimed at defining anatomic and immunologic determinants of this phenomenon. Understanding the role of salivary proteins in arbovirus transmission will provide insight into the pathogenesis of all vector-borne pathogens and perhaps for developing more efficacious vaccines.

Chapter III

The Effect of Mosquito Salivary Gland Treatment on Vesicular Stomatitis New Jersey virus Replication and Interferon α/β Expression *in vitro*

Introduction:

Vesicular Stomatitis (VS) is a disease that primarily affects cattle and horses. Animals stricken with this disease present clinical symptoms that include vesicles on the oral mucosa and udder region, inappetence, and sometimes lameness (Knight and Messer 1983). Periodic epizootics of VS New Jersey (VSNJ) virus spread through the southwestern United States approximately every ten years. Recently these epizootics have been more frequent for unknown reasons. Epizootics of VS cause severe economic losses to producers through the loss of milk production and restrictions on transport of animals. While the natural transmission cycle is currently unclear, arthropod transmission has been implicated due to the strong association of exposure to arthropods and clinical cases (Francy *et al.* 1988; Liu and Zee 1976). During bloodfeeding, arthropods transmit pathogens in the saliva into the feeding site. This saliva has been

demonstrated to prevent haemostasis in the vertebrate host as well as to potentiate pathogen infection (James, 1994).

Various models have described the broadly defined phenomenon of salivary potentiation. Studies by Titus and Ribeiro (1988) first demonstrated the ability of saliva to alter pathogen infection in the vertebrate host. White tailed deer and chipmunks, exposed to LaCrosse infected *Aedes triseriatus* mosquitoes, had higher and longer viremias than infected by intramuscular injection (Osorio *et al.* 1996). Tick saliva can potentiate pathogen transmission because of its ability to modulate innate immune responses. In particular, tick salivary gland extracts (SGE) have been shown to inhibit complement and interferon (Kopecky and Kuthejlova 1998; Wikel *et al.* 1994). Modulation of innate immune responses would have a significant impact on transmission of viruses.

VSNJ virus infection in mice is age dependent (Hanson, 1952). In mice less than three weeks of age encephalitis develops after injection with virus by any route. Older mice develop encephalitis only when injected intracranially or intranasally. Peripheral injection of virus into adult mice does not result in detectable replication and apparently results in clearance of virus by innate immune responses (Sabin and Olitsky 1937). Interferon α/β is induced after 10% of the VS genome is transcribed (Marcus and Sekellick 1987; Marcus and Sekellick 1980). Secretion of interferon by infected cells causes the surrounding cells to establish an anti-viral state. Interferon production also leads to the activation of downstream immune responses and plays an important role in viral clearance.

Viruses have evolved numerous strategies to evade innate immunity. For example, interferon is strongly induced by double stranded RNA and induces surrounding cells to transcribe anti-viral genes. Some viruses have evolved strategies to block the anti-viral response in surrounding cells. Hepatitis C virus, for example, encodes a nonstructural and a structural protein that selectively interact with interferon induced proteins (Taylor *et al.* 1999). Arthropod saliva could also inhibit innate immune responses. Bloodfeeding ticks salivate into the feeding site and proteins in saliva inhibited complement and interferon action. The block in interferon activity by tick saliva is hypothesized to result from perturbation of the interferon receptor, thus preventing the inductions of anti-viral responses in surrounding cells (Hajnicka *et al.* 2000). This inhibition would create a local immunosuppression at the feeding site and a possible advantage to a transmitted virus. Tick salivary gland extracts enhanced VS-Indiana virus production in mouse fibroblast cells (Hajnicka *et al.* 1998). Suppression of interferon action may be responsible for this increase in virus production.

Studies were conducted to test the hypothesis that mosquito salivary gland homogenate (SGH) would enhance VSNJ infection in mouse fibroblast cells, which express interferon. This enhancement, theoretically, would not be seen in cells that have a deletion in the interferon gene (Vero). Both cell lines were treated with mosquito SGH four hours before infecting with a low dose of VSNJ virus. Culture medium was assayed by end point virus titration at predetermined times after infection and virus titers were correlated with interferon induction as measured by ribonuclease protection assay.

Materials and Methods:

Virus and Cells:

A VSNJ virus field isolate obtained from USDA-ABADRL laboratory in Laramie, Wyoming was used in all experiments. This virus was isolated from *Culicoides variipennis* during the 1985 Colorado epizootic. Virus was propagated in Vero (green monkey kidney) cells originally obtained from ATCC. Vero cells were maintained in Leibovitz-15 medium supplemented with 5% fetal bovine serum and 100U penicillin and 100μg streptomycin per ml. Mouse fibroblast cells (L929) were purchased from ATCC. Cells were maintained in L15 medium supplemented with 10% fetal bovine serum and 100U penicillin and 100μg streptomycin. Cells were grown to confluence in 24 well plates for each experiment and six wells were in each treatment group.

Mosquitoes:

Aedes triseriatus mosquitoes were maintained in previously described conditions (Higgs and Beaty, 1996). Five days after eclosion, salivary glands were dissected and approximately 100 glands were placed in 50 μl sterile PBS supplemented with 100U penicillin and 100μg streptomycin. Glands were sonicated in an ice water bath (Aquasonic Model 75D, VWR Scientific) for five minutes, and stored at –80°C. Total protein was calculated by Bradford assay. Glands were diluted to a final protein concentration of 1mg/ml and termed salivary gland homogenate (SGH).

Treatment of cells:

Salivary gland homogenate (25μl) was added to each well four hours before infection. Recombinant sialokinin was synthesized at Colorado State University based on published sequences (Zeidner *et al.* 1999) . Cells were treated with 100ng or 1000ng sialokinin four hours before virus infection. Anti-interferon α/β antibody was purchased from Accurate Biochemical (Westbury, NY). One hundred units of antibody was added to each well of the culture at six hours post-infection and an additional ten units was added at each time point. One neutralization unit is the amount of antiserum required to neutralize one unit of mouse interferon α/β to a 50% endpoint. Six wells of a 24 well plate were treated for each treatment group.

Virus Infection:

Cells were infected at multiplicity of infection (MOI) of 0.44 after treatment. One hundred microliters of supernatant was taken at 4hr, 6hr, 8hr, 12hr, 18hr, 24hr, 36hr and 48hr post-infection. One hundred microliters of fresh medium was replaced at each time point.

RNA isolation and Ribonuclease Protection assay:

L929 cells, from six wells within a treatment group and within the respective time point, were collected together into 1 ml RNAWiz (Ambion, Inc; Austin, TX). RNA was isolated according to reagent protocol. Samples were treated with ten units of DNase enzyme and incubated at 37°C for 30 minutes. The ribonuclease protection assay (RPA) III kit (Ambion) was used to quantify RNA transcripts. Total RNA transcripts were probed using the interferon-α2 and β-actin genes. Total RNA and RNA probes were hybridized in solution at 42°C overnight. Remaining single stranded products were

degraded with 1:100 dilution of RNase A and T1 provided in the RPA kit. Bright Star biotinylated RNA Century markers were used to confirm sizes of transcripts (Ambion). Double stranded RNA products were electrophoresed (125V) using a six-percent polyacrylamide/8M urea gel (1mm thick) for 1½ hour. Samples were electro-blotted onto positively charged membrane (Ambion) at 50V for two hours at 4°C. Membranes were cross-linked (UV Stratalinker 1800, Stratagene), and the non-isotopic Bright Star Biodetect kit (Ambion) was used to detect biotinylated products. Two-minute exposures the day after detection were used in all concentration analysis. Autoradiographs were analyzed using the Gel Expert software (Nucleotech Corp., San Mateo, CA). Background correction was set as the area around each individual interferon band.

Probes:

Mouse β-actin control DNA was provided in the Maxi-script kit (Ambion) and was *in vitro* transcribed to produce the RNA probe used in the analysis. Interferon-α2 message was reverse transcribed with an oligo dT primer and amplified by PCR using forward primer 5'GCTGCTTGGAATGCAACCCTCCTAGAC3' and reverse primer 5'GACCACCTCCCAGGCACAGGGGC3' (Stratagene). RNA template was derived from a two- week old mouse skin twenty-four hours after injection of 2.5 logs of VSNJ virus. The resulting fragment was subcloned into the pCR2.1 cloning vector (Invitrogen). The purified plasmid was commercially sequenced to verify the insert was the interferon α2 gene product (Genbank accession number NM 010503) (Davis Sequencing, Davis, CA). Template preparation involved overnight linearization of the plasmid with BamHI restriction enzyme and subsequent treatment with proteinase K (final concentration 100μg/ml) for one hour at 37°C. DNA template was purified by phenol/chloroform

extraction and precipitated with ethanol (Sambrook *et al.* 1989). RNA probe was synthesized from the T7 promoter by run-off *in vitro* transcription (Maxi-script, Ambion), which incorporated biotin-16-uridine-5'-triphosphate (Boehringer Mannheim). Both probes were gel purified by polyacrylamide gel electrophoresis and eluted overnight (25°C) in the probe elution buffer provided in the RPA kit (Ambion). Probes were then aliquoted and stored at –80°C. Six hundred picograms of each probe were used in the RNA hybridization. Non-isotopic detection and exposure to x-ray film were performed as described above.

Statistical Analyses:

Virus growth curves were analyzed using the mixed procedure and least squared means programs in the SAS software (SAS Institute, 1993). Comparisons of relative mRNA levels were analyzed using the Student's t-test function in the Microsoft Excel program. Significance was set at $p<0.05$.

Results:

Specificity analysis of RPA probes

Biotinylated RNA probes were tested against a variety of control RNA samples (Figure 3.1). Lane one consisted of RNA from L929 cells stimulated with polyinosinic-polycytidylic acid (Poly I:C, Sigma) and probed for interferon-α2. Protected RNA fragments were visualized at ~300 and ~400 bases. Lane two consisted of an *in vitro* transcribed complement to the interferon-α2 probe. The resulting protected RNA fragment corresponded to the correct size (~400 bases; arrow A). The smaller band (~300 bases; arrow B) may be the result of premature termination products in the *in vitro*

transcription reaction or a cross reaction with other interferon-α mRNA species. Lane three consisted of mouse liver RNA provided in the Maxi-script kit and probed for β-actin. The resulting protected fragment corresponded to the proper size (~250 bases; arrow C) and a slight amount of undigested probe (~300 bases). The interferon band density (400 base product), divided by the β-actin band density (250 base product), was used to calculate relative interferon mRNA levels. Lane four consisted of unstimulated L929 cells probed for interferon-α2 and β-actin.

Analysis of Salivary Gland Homogenate Treatment of L929 and Vero Cells

Virus growth curves in SGH treated and untreated L929 cells are depicted in Figure 3.2A. In cells treated with SGH, infectious virus could be detected as early as 12 hours post-infection (pi). In contrast, infectious virus was detected at 18 hours pi in untreated cells. This "shift" in the virus propagation cycle by treatment with mosquito salivary glands progressed throughout the time course. At 48 hours pi there was a 10-fold difference in titer between SGH treated and untreated cells. The overall treatment effect between SGH treated and untreated cells differed significantly ($p=0.0039$). In comparing treatment groups at individual time points, SGH treatment of L929 cells at 18, 24, 36, and 48 hours differed significantly from untreated controls ($p<0.05$).

Virus growth curves in Vero cells were very different from those in L929 cells (Figure 3.2B). No significant differences in virus growth were detected between SGH treated and untreated cells ($p= 0.4781$). Infectious virus was detectable at 12 hours pi in both treatment groups, and titered 6.52 $\log_{10}TCID_{50}/100$ µl and 6.68 $\log_{10}TCID_{50}/100$ µl at 48 hr p.i. for the treated cells and untreated cells, respectively.

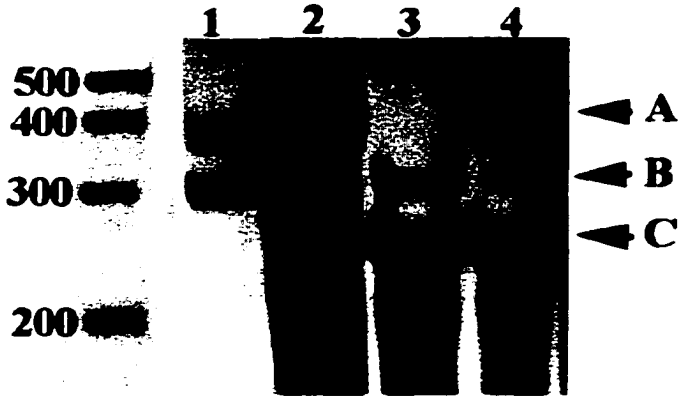
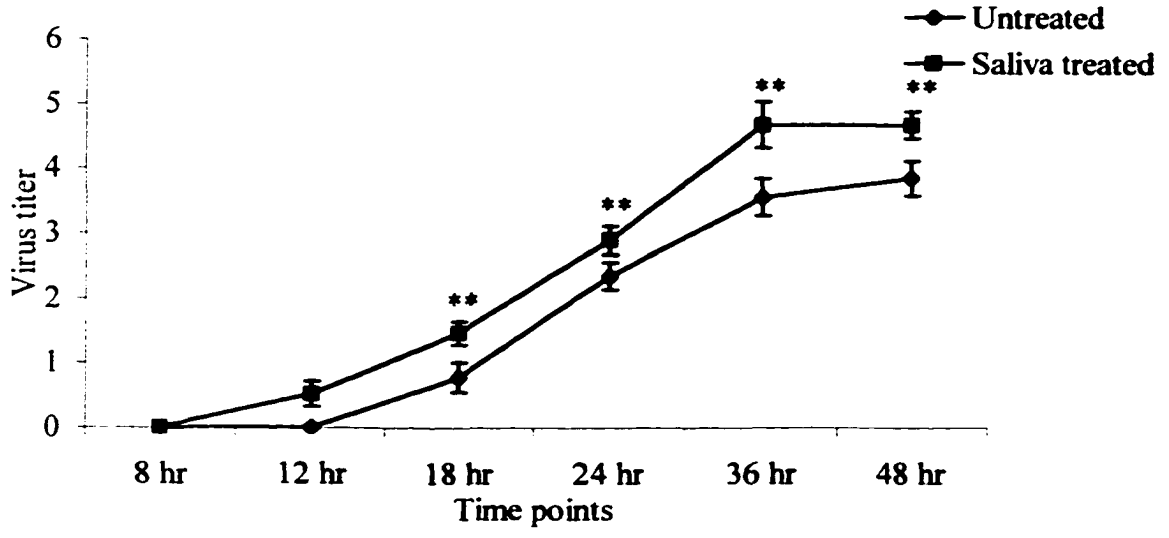


Figure 3.1: Specificity analysis of RPA probes.
Total RNA, from stimulated and unstimultaed L929 cells, was probed with the interferon-α2 and β-actin probes to test the specificity of each RPA probe. Arrow A designates the interferon-α2 transcript at the predicted size, arrow B designates an intermediate band corresponding to full length β-actin probe or cross-reactivity with another interferon-α transcript, and arrow C designates the β-actin protected transcript at the predicted size. Lane one represents RNA extracted from L929 cells stimulated with Poly I:C for twenty-four hours and probed for interferon-α2 message. Lane two represents an *in vitro* transcribed complement to interferon-α2 probed for interferon. Lane three represents liver RNA probed for β-actin. Lane four represents RNA extracted from uninduced L929 cells probed for interferon-α2 and β-actin messages.

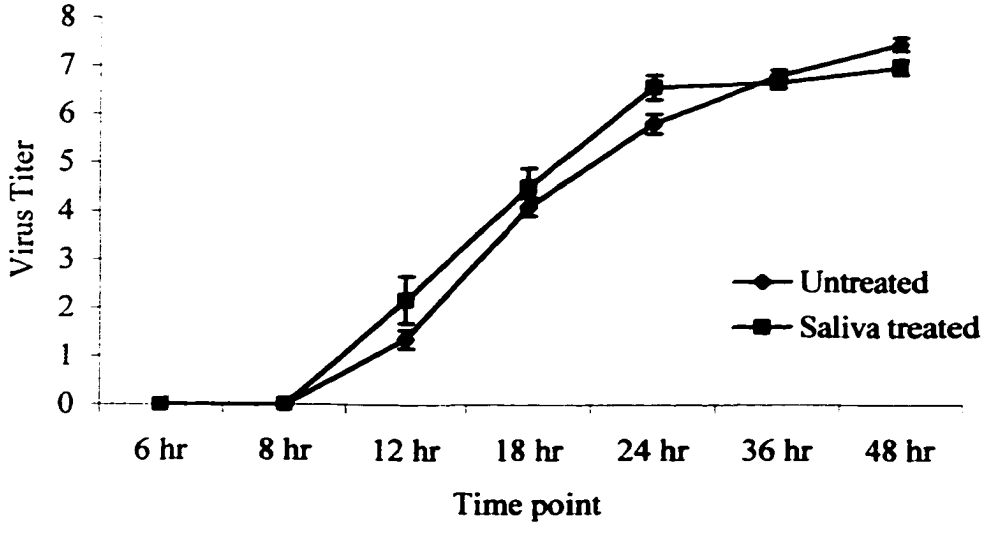
Figure 3.2: VSNJ virus replication in SGH treated or untreated L929 or Vero cells.
(A) L929 cells were treated with mosquito SGH for four hours before VSNJ virus infection. Cell supernatant at various times post-infection was assayed by endpoint virus titration. Infections were repeated at least three times and all data points are represented in graph. Virus titer is expressed in $\log_{10}TCID_{50}/100$ µl sample. Error bars represent the standard error of the mean for each time point. Asterisks designate time points that differ significantly.

(B) VSNJ virus replication in saliva treated or untreated Vero cells. Vero cells were treated with mosquito SGH for four hours before VSNJ virus infection. Cell supernatant at various times post-infection was also assayed by endpoint virus titration. Infections were repeated at least three times and all data points are represented in graph. Virus titer is expressed in $\log_{10}TCID_{50}/100$ µl sample.

A. L929 Time Course



B. Vero Time Course



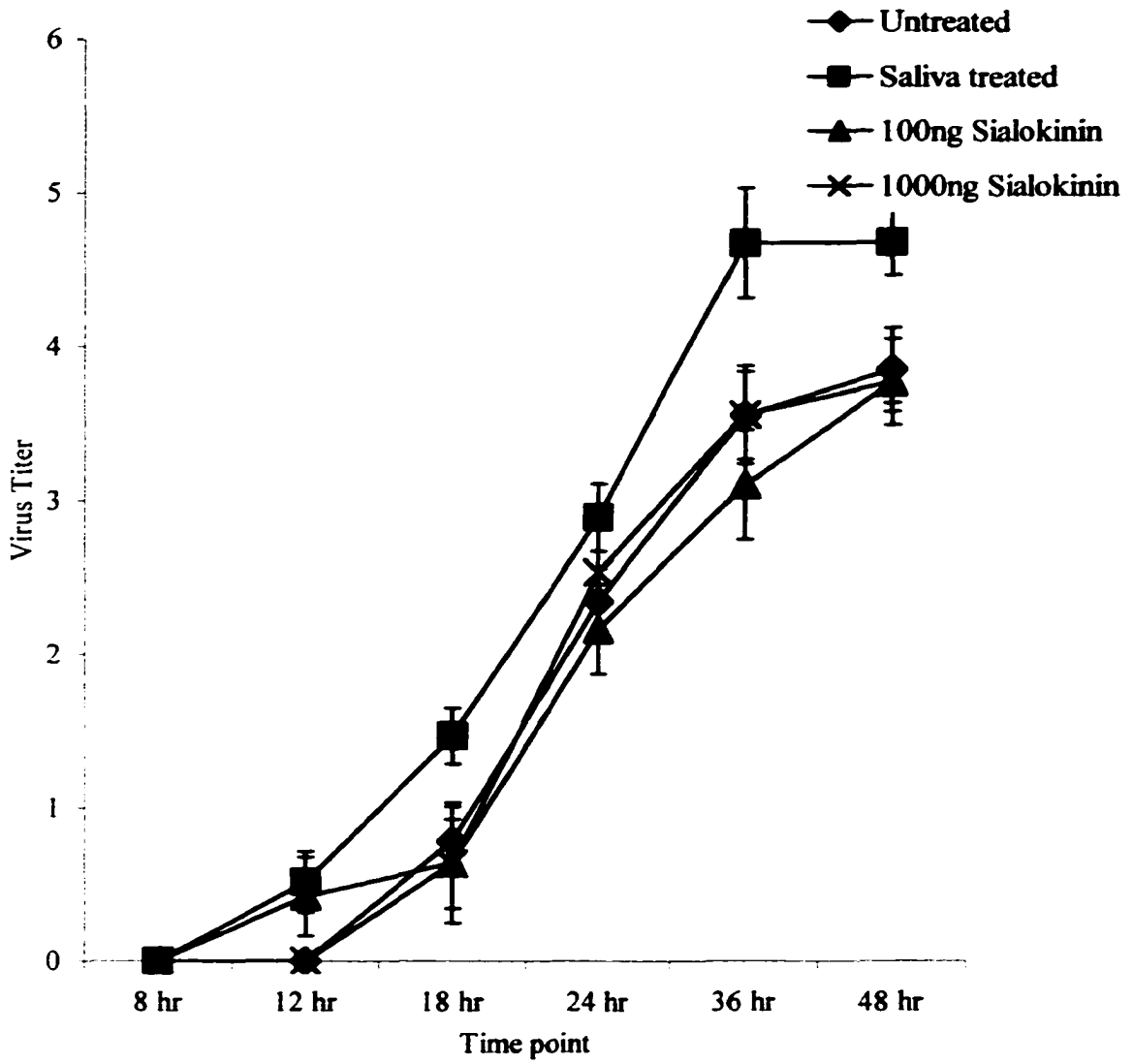


Figure 3.3: VSNJ virus replication in sialokinin treated L929 cells. L929 cells were treated with sialokinin for four hours before VSNJ virus infection. Cell supernatant at various times post-infection was assayed by endpoint virus titration. Infections were repeated at least three times and all data points are represented in graph. Virus growth curves for SGH treated and untreated L929 cells are identical to Figure 3.2A. Virus titer is expressed in $\log_{10}TCID_{50}/100$ µl sample. Error bars represent the standard error of the mean for each time point. There was no significant difference in virus growth kinetics between sialokinin treated and untreated L929 cells.

Analysis of Sialokinin Treatment of L929 Cells

L929 cells were treated for four hours with 100 ng or 1000 ng recombinant sialokinin. Virus growth curves in sialokinin treated cells (Figure 3.3) and untreated cells did not differ significantly. Infectious virus was detected at 12 hours pi in the cells treated with 100ng sialokinin, similar to that in SGH treated cells. However, over time this treatment group exhibited virus titers similar to the untreated group. The viral growth curve for the cells treated with 1000ng sialokinin mirrored the untreated cells at all time points examined.

Analysis of IFN-α/β Neutralization Treatment in L929 Cells

Six hours post-infection, L929 cells were treated with 100 units of interferon α/β antibody per well. Due to the reduction of total antibody at each time point, ten units of antibody were added back into the culture at each time point. Infectious virus was detected at 18 hours post-infection, similar to the untreated cells. The viral growth curve in L929 cells treated with IFN antibody (Figure 3.4), ranged between the treated and untreated cells. The overall treatment effect of interferon α/β antibody did not differ significantly from SGH treated cells ($p=0.2170$) or untreated cells ($p=0.1517$).

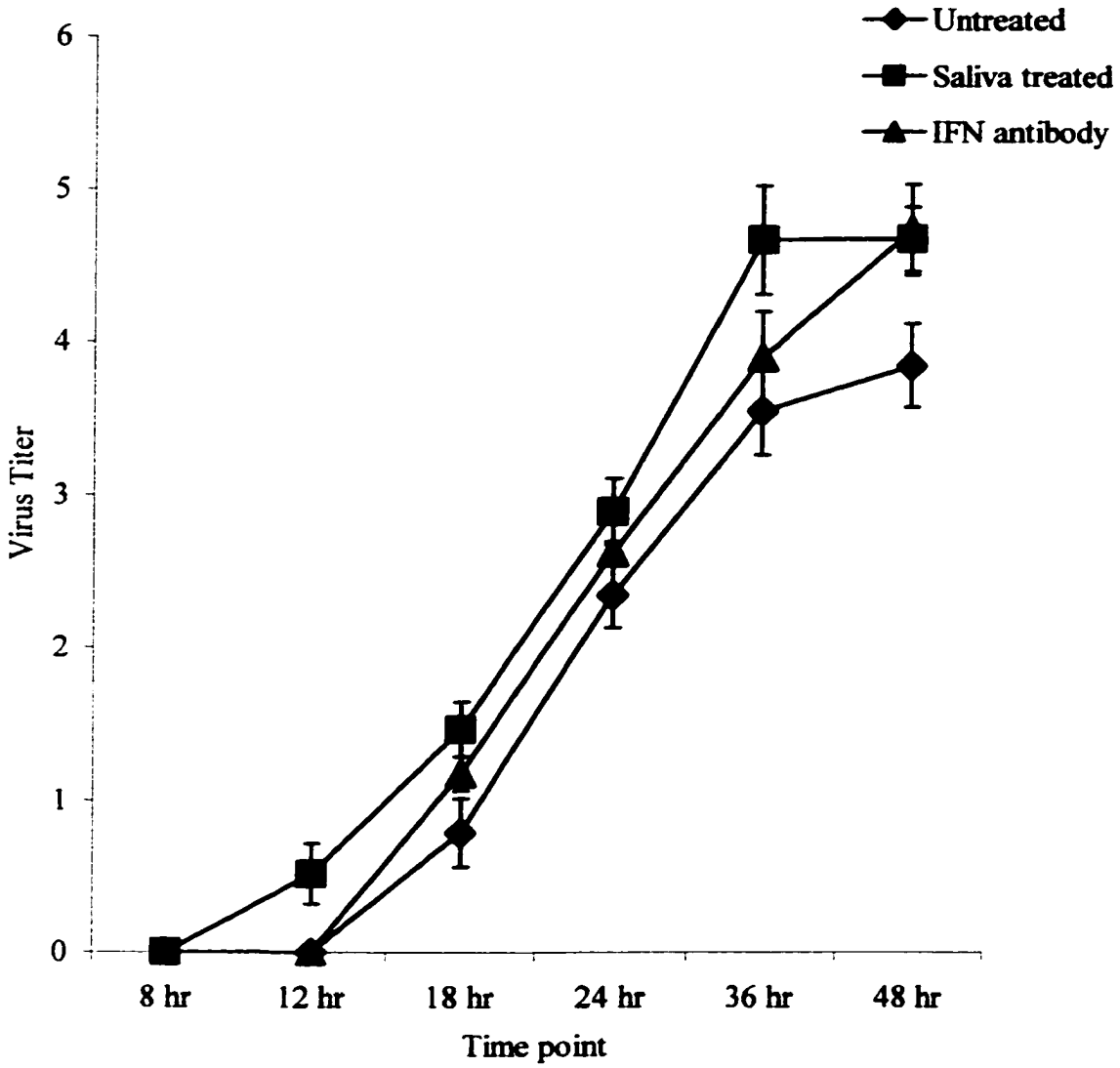
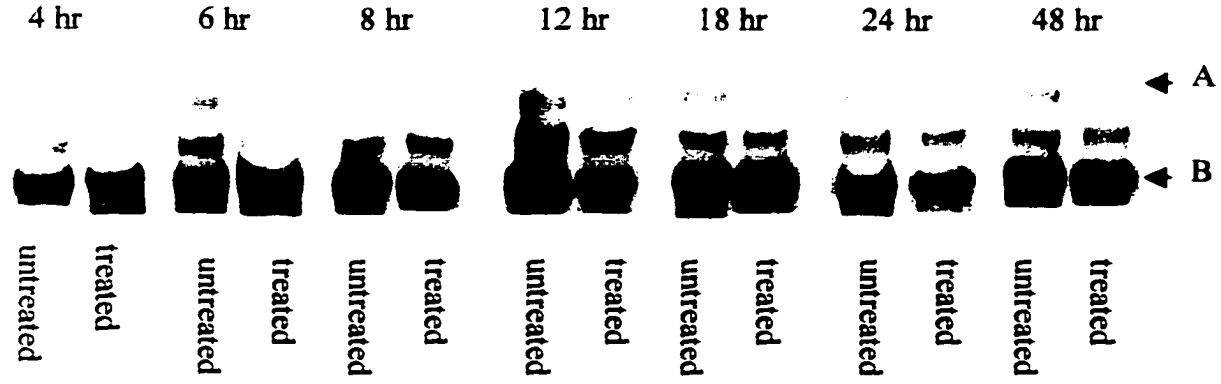


Figure 3.4:VSNJ replication in L929 cells treated with interferon α/β antibody.

L929 cells infected with VSNJ were treated with interferon α/β antibody six hours post-infection. Cell supernatant at various times post-infection was assayed by endpoint virus titration. Infections were repeated at least three times and all data points are represented in graph. Virus growth curves for SGH treated and untreated cells are identical to Figure 3.2A. Virus titer is expressed in $\log_{10}TCID_{50}/100$ µl sample. Error bars represent the standard error of the mean for each time point. The overall treatment effect of interferon α/β antibody did not differ significantly from SGH treated cells ($p=0.2170$) or untreated cells ($p=0.1517$).

A.



B.

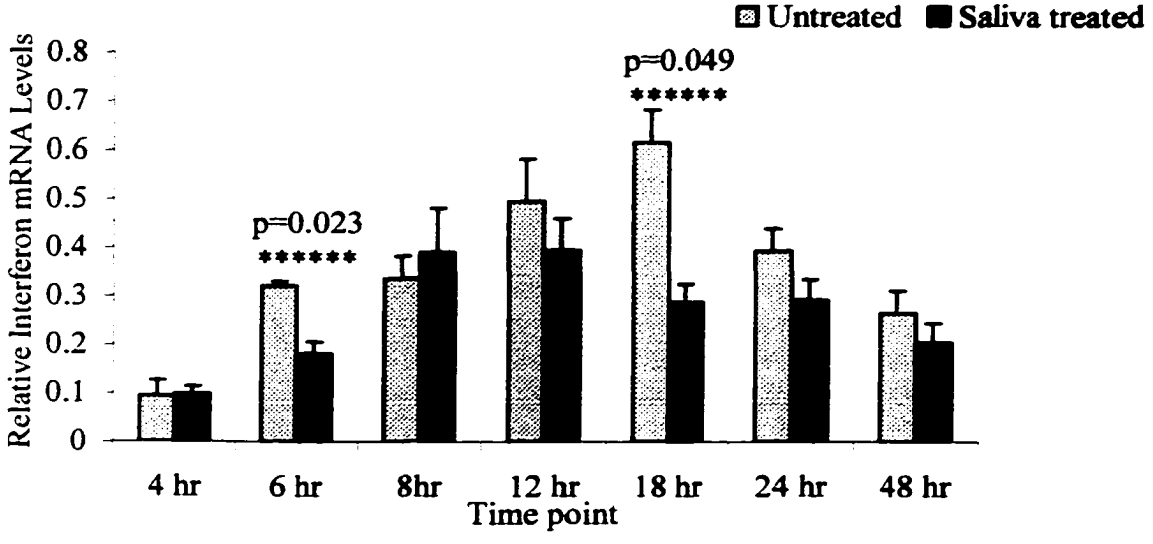


Figure 3.5: RPA analysis of interferon-α2 mRNA levels.
(A.) Two-minute exposures of RPA autoradiographs the day after detection were used in all concentration analysis in the Gel Expert program. Background correction was set as the area around each individual interferon band. Arrowhead A designates the interferon-α2 band and arrowhead B designated the β-actin band used in the densitometric analysis.

(B.) The interferon band density score was divided by the β-actin band density score to calculate relative mRNA levels. Error bars represent the standard error of the mean from three separate experiments. Evaluation by student's t-test revealed a significant difference (designated by asterisks) at 6 hours pi (p= 0.023) and 18 hours post-infection (p=0.049) between SGH treated and untreated L929 cells.

RPA Analysis of Relative IFN-α2 mRNA Levels in L929 Cells

Ribonuclease protection assays (RPA) were utilized to determine the relative levels of interferon mRNA in L929 cells at various time points after infection. Total RNA was probed for IFN-α2 and β-actin messages. Autoradiographs were analyzed using Gel Expert software to compare densities of both bands. The interferon band density score was divided by the β-actin band density score for each sample and graphed as relative mRNA level (Figure 3.5). IFN induction was suppressed in the SGH treated L929 cells early after infection (four and six hours) as compared to untreated controls. Also, IFN induction in SGH treated L929 cells did not reach comparable levels as untreated controls. Evaluation by student's t-test revealed a significant difference at 6 hours pi ($p= 0.023$) and 18 hours post-infection ($p=0.049$). The remaining time points did not differ significantly from each other.

Discussion:

Mosquito transmission of VSNJ virus potentiates infection in mice older than three weeks (Chapter II). The mechanism of this enhancement could result from direct effects on the virus or indirect effects resulting from modulation of the vertebrate immune response. Due to the lack of detectable replication of VS viruses in older mice, an *in vitro* approach was used to assess the effect of mosquito SGH on mouse fibroblast cells. Treatment of mouse fibroblast cells with mosquito SGH resulted in a significant difference in the viral growth curve as compared to untreated controls (Figure 3.2A). This effect did not occur in cells lacking interferon (Vero). This indicates that the

enhancing effect of mosquito saliva may not be a direct effect on the virus, but rather a modulation of the host interferon response.

The mosquito SGH treatments differed slightly from the previous report on tick SGE enhancement of VS-Indiana (Hajnicka *et al.* 1998). Treatment of mouse fibroblast cells with mosquito SGH was reduced to four hours instead of the 24-hour treatment with tick SGE. This was done because the enhancing effects of mosquito feeding on a vertebrate host lasts about four hours (Edwards *et al.* 1998). Also, the bloodfeeding characteristics between mosquitoes and hard ticks are very different, which prompted the change in treatment. A low multiplicity of infection was used in these experiments to mimic the low dose of virus naturally transmitted by mosquitoes (Chapter II). Cells infected with lower multiplicities of infection (0.01-1.0 PFU/cell) produce higher levels of interferon than cells infected at higher multiplicities (more than 10 PFU/cell) (Nishiyama *et al.* 1978).

Sialokinin I is a mosquito vasodilator responsible for preventing blood vessel constriction during bloodfeeding. Injection of this protein has also been demonstrated to shift a mouse immune response from a Th1 profile to a Th2 profile (Zeidner *et al.* 1999). Treatment of mouse fibroblast cells with 1000 ng or 100 ng doses of sialokinin did not result in a significant difference in virus growth from the untreated control. This may indicate that sialokinin I is not solely responsible for the modulation demonstrated by the complete salivary gland homogenate or it may act in synergy with another protein present in mosquito saliva. It is also possible sialokinin targets immune cells and this effect would be lost in an *in vitro* system comprised only of mouse fibroblast cells.

L929 cells were treated with antibodies to interferon α/β to determine if mosquito saliva modulated interferon induction, thereby enhancing virus infection. Concentrations of antibody used were similar to those used by Nishiyama *et al.* (1978). Units of interferon in the cell supernatant were determined at predetermined times after VSNJ infection. Actinomycin D treatment of cells was used to reveal the rate at which interferon was transcribed. Transcription of interferon began at six hours post-infection, and 10U of protein was detected in the supernatant at eight hours post-infection (Nishiyama *et al.* 1978). Overall, IFN α/β antibody treatment of L929 cells did not result in statistically significant different titers of virus. Antibody treatment altered the growth curve kinetically higher from that in untreated cells, but not to the level in cells treated with complete SGH (Figure3.4). This may indicate only a partial neutralization of IFN α/β, or SGH may have a more potent affect on IFN induction in L929 cells.

The effect of mosquito SGH on interferon induction was analyzed using ribonuclease protection assay. Total RNA, from SGH treated and untreated cells, was analyzed at various times after virus infection. For untreated controls, maximum interferon production was at 18 hours post-infection. Interestingly, Newcastle disease virus induced maximum production of interferon-α2 mRNA in L929 cells fifteen hours after infection (Hoss-Homfeld *et al.* 1989). In addition, the rapid onset and rapid decline of interferon production were similar to previous results. In contrast, treatment with mosquito SGH inhibited the production of interferon over time (Figure 3.5). This reduction was significant (Student's t-test; $p<0.05$) at six and eighteen hours post-infection. Inhibition of interferon induction by SGH early in infection seems to correlate with the increase in viral titer. Tick SGE modulated interferon action in a biological

assay (Hajnicka *et al.* 1998); however, the mechanism of inhibition is currently unclear. It has been hypothesized that arthropod saliva may act at the level of receptor interaction, thus preventing induction of the anti-viral response in surrounding cells. Alternatively, the modulation in interferon mRNA production may be a result of lower levels induced in the infected cell.

The effect of arthropod saliva on innate immune functions may be critical in the transmission of many pathogens. Innate immunity is the first line of defense against invading pathogens in a vertebrate host. Modulation of this defense would provide a selective environment for the introduced pathogen, especially viruses. While the natural transmission cycle of VS may be unclear, modulation of IFN α/β by mosquito saliva may facilitate both contact and vector-borne transmission.

Chapter IV

Comparison of Mosquito Bloodfeeding sites to Virus Injection sites: Analysis of Virus Replication, Interferon alpha Induction and Histopathology

Introduction:

Studies of viral pathogenesis *in vivo* have largely followed large dose injections of virus by needle and syringe. However, this route of administration does not depict the natural transmission of most viruses, especially arthropod-borne viruses. Arthropod saliva contains many pharmacological factors that prevent haemostasis and that modulate the vertebrate immune response (James, 1994).

Titus and Riberio (1988) first demonstrated the ability of arthropod saliva to alter pathogen infection in the vertebrate host. These studies revealed sand fly salivary proteins could suppress macrophage functions such as presentation of antigen (Hall and Titus 1995; Donnelly *et al.*, 1998; Soares *et al.* 1998). Delivery of sand fly saliva also shifted the cytokine production from characteristic Th1 cytokines (IL-2 and IFN-γ) to

characteristic Th2 cytokines (IL-4 and IL-5). The Th1 or Th2 cytokine profile differentiates the mouse response to infection. Characteristically Th1 responses are generated after a viral infection, and Th2 responses are generated during allergic reactions and after parasitic infections (Seder and Mosmann 1998). Interestingly, some Th2 cytokines negatively regulate Th1 responses, which may exacerbate pathogen infections. For example, generation of a Th2 response can exacerbate *Leishmania* infection in mice (Nabors *et al.* 1996).

The role of arthropods in the transmission cycle of vesicular stomatitis New Jersey (VSNJ) virus remains unclear. This virus has been isolated from a number of arthropod species, and it is believed that these same arthropods have a significant role in epizootics (Tesh *et al.* 1992; Francy *et al.* 1988; Walton *et al.* 1987; Suarez, 1967; Sudia *et al.* 1967). Defining the pathogenesis of this virus has been difficult, due to the absence of a small animal model. Although VSNJ infects young mice, a peripheral injection of VSNJ virus into adult mice does not result in detectable virus replication (Sabin and Olitsky 1937). Low doses of virus may be neutralized by complement immediately after injection into adult mice (Gobet *et al.* 1988). However, transmission of VSNJ by mosquitoes results in a potentiation of virus infection in the mouse model (Chapter II). The determinants of *in vivo* enhancement of infection remain to be elucidated. Mosquito feeding could enhance VS infection by inducing anatomic changes at the feeding site, modulating murine innate immune responses, or directing the murine acquired immune responses. Induction of Th2 responses by mosquito saliva may account for the potentiation of VSNJ infection.

Materials and Methods:

Virus:

A VSNJ field isolate obtained from USDA-ABADRL laboratory in Laramie, Wyoming was used in all experiments. This virus was isolated from *Culicoides variipennis* during the 1985 Colorado epizootic. Virus seed stocks were propagated in Vero (green monkey kidney) cells originally obtained from ATCC. Cells were maintained in Leibovitz-15 medium supplemented with 5% fetal bovine serum and 100U penicillin and100μg streptomycin per ml.

Mice:

ICR mice (age 6-8 weeks) were purchased from Harlan-Sprague. Small areas along the backs of mice were clipped at the site of virus injection or mosquito feeding to allow for easier tissue collection. Mice were divided into five cohorts and treated as follows: 1. Fed upon by VSNJ infected mosquitoes, 2. Fed upon by uninfected mosquitoes, 3. Injected with a viral dose of three logs $TCID_{50}$ (volume=50μl) of VSNJ, 4. Injected with tissue culture medium (volume=50μl) and 5. Untreated mice. The number of mice per time point for each analysis is presented in Table 4.1. Virus dilutions were back titrated to confirm doses. After infection with VSNJ virus, mice were maintained under BSL-3 conditions. Before euthanization, mice were bled from the retroorbital sinus to assay for viremia. Six to eight week old mice were also used in the lymph node evaluations (Table 4.2). Mice were infected by allowing mosquitoes (N=10) to feed on one footpad or by injection of 2 logs into the footpad (volume =10μl).

Mouse Group	6 hr	12 hr	18 hr	24 hr	48 hr	72 hr
RNA Isolation						
Fed upon by VSNJ infected mosquitoes	5	5	5	5	5	5
Fed upon by uninfected mosquitoes	3	3	3	3	3	3
Injected with VSNJ (dose=3 logs)	5	5	5	5	5	5
Injected with medium	3	3	3	3	3	3
Naïve	1	1	1	1	1	1
Paraffin Embedded						
Fed upon by VSNJ infected mosquitoes	5	5	5	5	5	5
Fed upon by uninfected mosquitoes	2	2	2	2	2	2
Injected with VSNJ (dose=3 logs)	5	5	5	5	5	5
Injected with medium	2	2	2	2	2	2
Naïve	4	4	4	4	4	4

Table 4.1: Number of mice and time points sampled for analysis of skin tissue.

Mouse Group	48 hr	72 hr	96 hr
Fed upon by infected mosquitoes	3	3	3
Fed upon by uninfected mosquitoes	3	3	3
Injected with VSNJ (dose=2 logs)	3	3	3
Naïve	3	3	3

Table 4.2: Number of mice and time points sampled for RT/PCR analysis of lymph nodes

Mosquitoes:

Aedes triseriatus mosquitoes were maintained under previously described conditions (Higgs and Beaty, 1996). Adults were intrathoracically inoculated with 1-2 $\log_{10}TCID_{50}$ tissue culture propagated virus followed by an intrinsic incubation period of five days (Cupp *et al.* 1992). Mosquitoes (N=5) were allowed to feed on a small area on the back of experimental mice.

Virus titration:

Serum samples collected at each time point were assayed for the presence of viremia by limiting dilution on Vero cells. Sera were diluted 10-fold in 96 well plates, and 100µl of cell concentration ($4x10^5$ cells/ml) was added to each well. Cells were maintained for seven days and examined for cytopathic effect daily.

Tissue collection:

Skin:

Skin tissue samples were taken at 6, 12, 18, 24, 48, and 72 hours post-infection (pi). Hemorrhaging sites (up to 24 hours) from mosquito bloodfeeding aided in tissue collection (Figure 4.1). For H&E staining, skin tissue samples were placed in 10% formaldehyde/PBS for 24 hours and then transferred to 70% EtOH. Tissues were stored in 70% EtOH until embedded into paraffin by the Pathology Diagnostic Laboratory at Colorado State University. Skin tissue samples for RNA isolation were immediately placed into tubes of liquid nitrogen, which were maintained on dry ice. Tissues were triturated with a cold mortar and pestle and placed into 1ml of RNAWiz (Ambion). The samples were then homogenized in a sonicating water bath for five minutes at 50°C. Samples were stored at –80°C until RNA isolation.

Figure 4.1: Gross examination of mouse skin at mosquito feeding induced hemorrhage sites.
Murine skin tissue twelve hours post mosquito feeding. Arrows designate hemorrhage sites resulting from mosquito feeding.

Lymph Node:

Popliteal and inguinal lymph nodes were isolated separately from each mouse at respective time points and placed into 0.5 ml RNAWiz (Ambion). Tissues were triturated with a small pestle and homogenized in sonicating water bath for five minutes at 50°C. All three lymph nodes (popliteal or inguinal) at each time point were pooled for RT/PCR analysis.

Tissue Sectioning and Hematoxylin and Eosin (H&E) staining:

Skin tissue was serially sectioned (10 micron) through feeding and injection sites, and sections were mounted onto positively charged microscope slides (Superfrost Plus, Fisher). Slides were dried overnight at 37°C. Tissue sections were rehydrated through an ethanol series (100%, 95%, 70%, and 50%) and stained with hematoxylin for 20-30 seconds. After washing in water, sections were stained in eosin for 20-30 seconds. Slides were dipped in 95% and 100% EtOH eight times and mounted with Permount.

RNA isolation and RT/PCR:

RNA was isolated from the skin and lymph node tissues using the RNAWiz reagent according to the manufacturer's suggestions (Ambion). One microgram of total RNA was assayed by reverse transcriptase/polymerase chain amplification of the viral nucleocapsid message. The forward primer was 5'TACAGTTAAGAGAATCATT3', and the reverse primer was 5'TTGTCAATTTCTTGGCCTGG3'. The PCR program consisted of denaturation at 95°C for five minutes followed by 25 cycles of amplification; 95°C for one minute, 52°C for one minute, 72°C for two minutes, and a final extension at 72°C for seven minutes. Mouse lymph node RNA, at each time point, was analyzed for IL-4 and IFN-γ production by RT/PCR by Dr. Nord Zeidner (Division of Vector-borne

Infectious Diseases, Centers for Disease Control, Ft. Collins, CO). The sequence of the primers utilized in the PCR reactions were designed according to previously published results by Mbow et al. (1998). The PCR program consisted of denaturation at 95°C for five minutes followed by 40 cycles of amplification; 95°C for forty-five seconds, 56°C for one minute, 72°C for two minutes, and a final extension at 72°C for seven minutes.

Ribonuclease Protection assay:

All RNA samples were treated with ten units of RNase-free DNase enzyme (Ambion) and incubated at 37°C for 30 minutes. The ribonuclease protection assay (RPA) III kit (Ambion) was used to quantitate RNA transcripts. Total RNA transcripts were probed using the interferon-α2 and β-actin genes. After overnight RNA hybridation at 42°C, RNA was degraded with RNase A and T1 (1:100 dilution) provided in the RPA kit. Bright Star biotinylated RNA Century markers were used to confirm sizes of transcripts (Ambion). Six-percent polyacrylamide/8 M urea gel (1mm thick) was used to separate double stranded RNA products. Samples were electro-blotted (Bio-Rad) to positively charged membranes (Ambion) at 50V for two hours at 4°C. Membranes were cross-linked, and hybrids were detected non-isotopically using the Bright Star kit (Ambion). The day after detection, concentrations were determined using two-minute exposures. Autoradiographs were then analyzed using the Gel Expert software (Nucleotech Corp., San Mateo, CA). Background correction was set as the area around individual interferon bands. Specificities of RNA probes for interferon-α2 and β-actin are described in Chapter III (Figure 3.1). Interferon-α2 band density was divided by the β-actin band density to calculate relative mRNA levels.

Probes:

Mouse β-actin control DNA was provided in the Maxi-script kit (Ambion) and was *in vitro* transcribed to produce the RNA probe used in the analysis. RNA template for interferon was derived from mouse (age = two weeks) skin twenty-four hours after injection of virus (dose = 2.5 logs). Interferon-α2 message was reverse transcribed with an oligo dT primer and amplified by PCR using the program described on page 71 (forward primer 5'GCTGCTTGGAATGCAACCCTCCTAGAC3' and reverse primer 5'GACCACCTCCCAGGCACAGGGGC3'). The resulting fragment was subcloned into the pCR2.1 cloning vector (Invitrogen). The purified plasmid was sequenced (Davis Sequencing) to verify the insert was the interferon α2 gene product (Genbank accession number NM 010503). Template preparation of the plasmid involved linearization with BamHI restriction enzyme and treatment with Proteinase K (final concentration 100μg/ml) for one hour at 37°C. DNA template was extracted using phenol/chloroform and precipitated with ethanol (Sambrook *et al.* 1989). The RNA probe was synthesized from the T7 promoter by run-off *in vitro* transcription (Maxi-script, Ambion). Both probes were DNase treated, gel purified by polyacrylamide gel electrophoresis, and eluted overnight (25°C) in the probe elution buffer provided in the RPA kit (Ambion). Probes were then aliquoted and stored at –80°C. Concentrations of probes for RNA hybridization were determined by serial ten-fold dilutions dotted onto positively charged membrane with a known standard (Ambion). After non-isotopic detection, six hundred picograms of each probe was used in the RNA hybridization. Non-isotopic detection and exposure to x-ray film were performed as described above.

Statistical Analysis:

Comparisons in relative mRNA levels were analyzed using the Student's t-test function in the Microsoft Excel program. Significance was set at $p \leq 0.05$.

Results:

Histological Analysis of Mosquito Feeding Sites:

At predetermined times after mosquito feeding (Table 1), mosquito induced lesions in mouse skin tissue were analyzed by examining histologically H&E stained serial tissue sections and results were compared to those obtained with naïve mouse tissue sections (Figure 4.2). At six hours post-infection (pi), a small, multi-focal infiltrate of cells was present in the epidermal and subcutaneous layers (Figure 4.3A and C). These cells primarily consisted of neutrophils, but eosinophils and mast cells were also present. At greater magnification, numerous cells were seen in the blood vessels and diapedising into the tissue (Figure 4.7A). Degranulation of mast cells was also detected (Figure 4.7A). At twelve hours pi, a moderate lesion was present in the dermal and subcutaneous regions. A diffuse infiltrate about twice the size of the six-hour lesion consisted of neutrophils, eosinophils and mast cells (Figure 4.4 A and C). Vessel hemorrhaging and acute inflammation around the blood vessels was also detected. At eighteen hours pi, a locally extensive lesion was present (Figure 4.5 A and C). In the dermal and subcutaneous tissue layers, considerable cellular infiltrate and inflammation was detected. The cellular infiltrate included: neutrophils, eosinophils, mast cells, lymphocytes, and macrophages (Figure 4.7 B-E). At twenty-four hours pi a moderate focal lesion was present (Figure 4.6 A and C). Small areas of vessel hemorrhaging were

still present (arrowhead). Remaining cells were primarily neutrophils, but the number of mast cells remained high. By forty-eight and seventy-two hours, no disruption in the tissue was detected. No discernable differences were detected between uninfected and infected mosquito bloodfeeding lesions (Figure 4.8 A and C).

Histological Analysis of Virus Injection Sites:

At predetermined times after injection (Table 1), histological analysis of lesions in mouse skin tissue were analyzed by examining H&E stained serial tissue sections and compared to untreated tissue sections (Figure 4.2). At six hours pi, a small lesion of neutrophils was present around the subcutaneous region, with some cellular infiltrate in the musculature (Figure 4.3 B and D). At twelve hours pi, the lesion had a heavier cellular infiltrate and a compact, organized structure (Figure 4.4 B and D). Cellular infiltrate consisted of neutrophils without detection of any other cell types. Again at eighteen hours pi, the lesions looked similar to the twelve-hour lesions: small, compact, and neutrophilic (Figure 4.5 B and D). By twenty-four hours pi, the lesion was reduced in size and the cells appeared to be migrating back into the blood vessel (Figure 4.6 B and D). Again at forty-eight and seventy-two hours pi, no tissue disruptions were detected. Interestingly, very few, if any, mast cells were present in these lesions at all time points examined. No discernable differences were detected between virus injected and L15 injected sites (Figure 4.8 B and D).

Analysis of virus replication:

Skin sites of infected mosquito feeding or virus injection were analyzed for virus replication by RT/PCR. The nucleocapsid gene was chosen because it is the first gene transcribed after virus infection. Viral messenger RNA was chosen as the analyte in

order to differentiate virus replicative forms from genomic RNA in virions. RNA samples from 6, 12, 18 and 24 hr time points were analyzed from all groups of mice. No viral message was detected from any of the skin samples. However, viral transcript was detected ninety-six hours pi in the popliteal lymph node of mice exposed to VSNJ infected mosquitoes. Virus was also detected ninety-six hours pi in the inguinal lymph node of mice injected with VSNJ, but not in the popliteal lymph node (Figure 4.9B). All other lymph node RNA samples were negative for virus replication (96 hour samples shown in Figure 4.9A).

Mice were also bled at each time point before being sacrificed. Serum samples were assayed for viremia by endpoint virus titration. No viremia was detected at any of the time points examined.

Analysis of IL-4 and IFN-γ mRNA within draining popliteal and inguinal lymph nodes:

Total RNA from pooled lymph nodes was analyzed by RT/PCR for IL-4 and IFN-γ production at predetermined times (Table 4.2). Popliteal lymph nodes from mice exposed to infected or uninfected mosquito feeding produced low levels of IL-4 at 48 hours p.i. and increasing levels were detected at 72 and 96 hours p.i. (Figure 4.10; Lanes 3, 4, 9, 8, 11, and 12). In contrast, no IL-4 production could be detected in the popliteal lymph nodes of untreated control mice and in mice injected with virus at all time points examined (Figure 4.10; Lanes 2, 5, 6, 9, 10, and 13). IL-4 cytokine expression was not detected in the inguinal lymph nodes from any of the mouse groups analyzed. Interferon-γ production was also absent in the popliteal and inguinal lymph nodes from all mouse groups at all time points examined. All lymph nodes were tested for β-actin production by RT/PCR to ensure RNA degradation had not occurred.

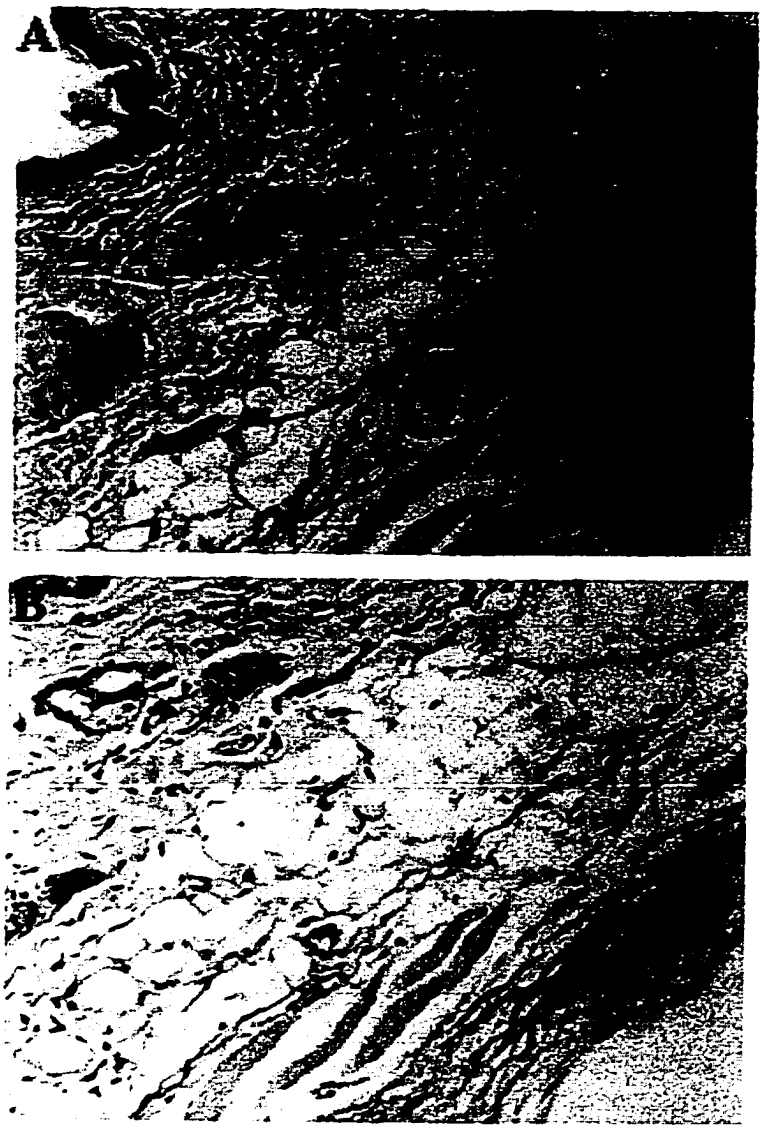


Figure 4.2: Normal mouse skin stained with hematoxylin and eosin (H&E). (A). Normal mouse skin tissue (100X). (B). Normal mouse skin tissue (200X).

Figure 4.3: Histological analysis of mouse skin by H&E staining six hours after mosquito feeding or injection.
Serial sections of mouse skin six hours after mosquito feeding were stained with H&E.
(A). Section of mouse skin fed upon by VSNJ infected mosquitoes. Top arrow designates epidermal lesion from mosquito probing and bottom arrow designates subcutaneous lesion after mosquito feeding.
(B). Section of mouse skin injected with VSNJ virus. Arrow designates subcutaneous neutrophillic infiltrate after injection.
(C). Section of mouse skin fed upon by VSNJ infected mosquitoes. Arrow designates infiltration of neutrophils and eosinophils into the lesion.
(D). Section of mouse skin injected with VSNJ virus. Arrow designates neutrophil infiltration around the muscle layer.
Panels A and B are 100X magnification. Panels C and D are 200X magnification.

Figure 4.4: Histological analysis of mouse skin by H&E staining twelve hours after mosquito feeding or injection.
Serial sections of mouse skin twelve hours after mosquito feeding were stained with H&E.
(A). Section of mouse skin fed upon by VSNJ infected mosquitoes. Arrow designates subcutaneous infiltration after mosquito feeding.
(B). Section of mouse skin injected with VSNJ virus. Arrow designates subcutaneous neutrophillic infiltrate after injection.
(C). Section of mouse skin fed upon by VSNJ infected mosquitoes. Arrow designates infiltration of neutrophils and eosinophils into the lesion.
(D). Section of mouse skin injected with VSNJ virus. Arrow designates the dense neutrophil infiltration around the injection site.
Panels A and B are 100X magnification. Panels C and D are 200X magnification.

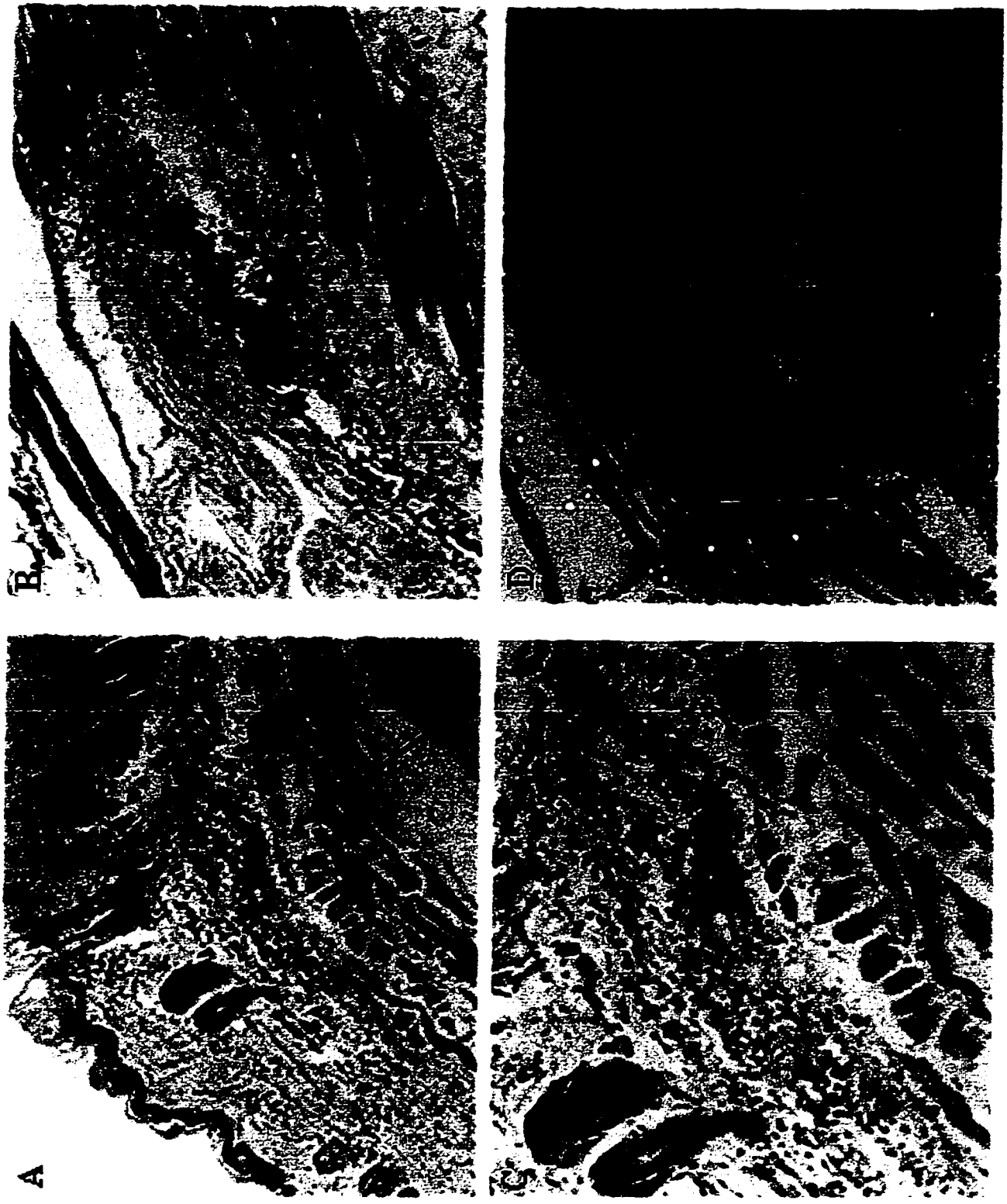


Figure 4.5: Histological analysis of mouse skin by H&E staining eighteen hours after mosquito feeding or injection.
Serial sections of mouse skin eighteen hours after mosquito feeding were stained with H&E.
(A). Section of mouse skin fed upon by VSNJ infected mosquitoes. Arrow designates extensive infiltration into the mosquito feeding site.
(B). Section of mouse skin injected with VSNJ virus. Arrow designates a small neutrophillic infiltrate after injection.
(C). Section of mouse skin fed upon by VSNJ infected mosquitoes. Arrow designates infiltration of a diverse number of cells into the lesion as well as severe inflammation and edema.
(D). Section of mouse skin injected with VSNJ virus. Arrow designates neutrophil infiltration around the subcutaneous layer. Arrowhead designates migration of the neutrophils to blood vessels.
Panels A and B are 100X magnification. Panels C and D are 200X magnification.

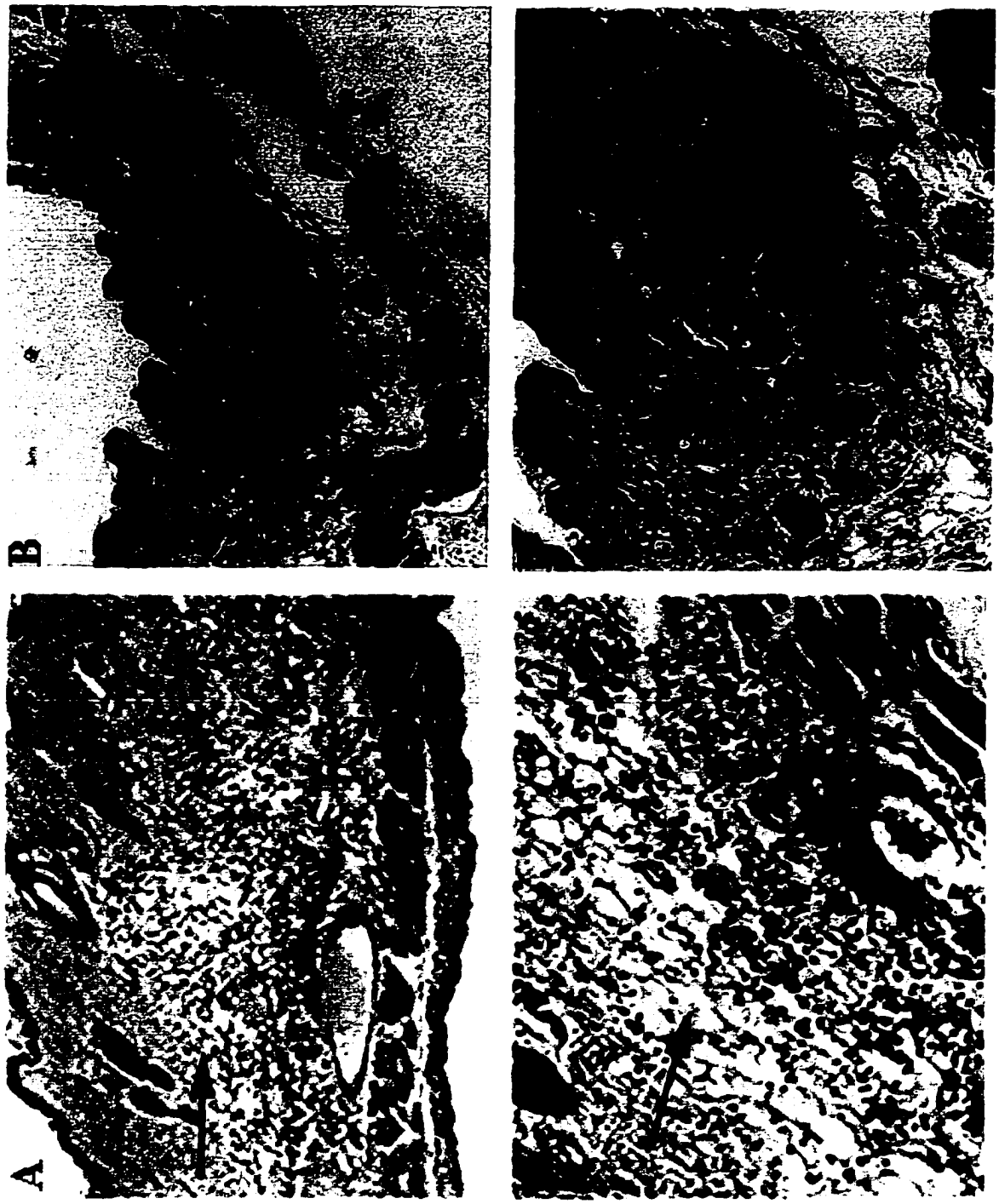


Figure 4.6: Histological analysis of mouse skin by H&E staining twenty-four hours after mosquito feeding or injection.
Serial sections of mouse skin twenty-four hours after mosquito feeding were stained with H&E.
(A). Section of mouse skin fed upon by VSNJ infected mosquitoes. Arrow designates extensive infiltration into the mosquito feeding site.
(B). Section of mouse skin injected with VSNJ virus. Arrow designates a small neutrophillic infiltrate after injection.
(C). Section of mouse skin fed upon by VSNJ infected mosquitoes. Arrow designates infiltration of a diverse number of cells into the lesion as well as severe inflammation and edema.
(D). Section of mouse skin injected with VSNJ virus. Arrow designates migration of the neutrophils to blood vessels.
Panels A and B are 100X magnification. Panels C and D are 200X magnification.

Figure 4.7: Histological analysis of cellular infiltrate into mouse skin by H&E staining six and eighteen hours after mosquito feeding.
(A). Section of mouse skin six hours post mosquito feeding (1000X). Left arrow designates numerous cells in blood vessel infiltrating into lesion. Center arrow designates a degranulating mast cell. Right arrow designates an eosinophil in the dermal tissue.
(B). Left arrow designates an eosinophil in the subcutaneous lesion. Right arrow designates a macrophage in the same lesion.
(C). Left arrow designates a neutrophil in the subcutaneous lesion. Right arrow designates a mast cell in the same lesion.
(D). Arrow designates an eosinophil in the dermal region of the lesion.
(E). Arrow designates the acute inflammation present throughout the lesion.
Panels B through E are sections of mouse skin eighteen hours after infected mosquito feeding (1000X).
(F). Untreated mouse skin tissue at 1000X magnification.

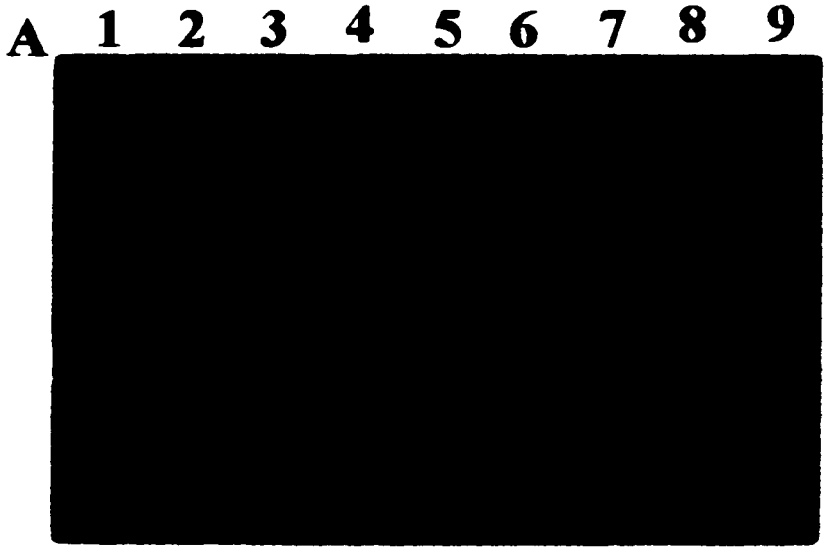
Figure 4.8: Histological analysis of mouse skin by H&E staining eighteen hours after mosquito feeding or injection.
(A). Section of mouse skin fed upon by VSNJ infected mosquitoes. Tissue pathology and cellular infiltration were similar in panel A and C.
(B). Section of mouse skin injected with virus. Tissue pathology and cellular infiltration were similar in panel B and D.
(C). Section of mouse skin fed upon by uninfected mosquitoes.
(D). Section of mouse skin injected with tissue culture medium.
All panels are 200X magnification.

Figure 4.9: Analysis of VSNJ replication at 96 hours pi in mouse lymph nodes by RT/PCR
Total RNA from lymph nodes was analyzed for viral replicative forms by RT/PCR.

A.) 1.5% Agarose gel of electrophoresed PCR products consisting of:
Lane 1, DNA Ladder;
Lane 2, untreated control popliteal lymph node;
Lane 3, untreated control inguinal lymph node;
Lane 4, popliteal lymph node from mice exposed to uninfected mosquito feeding;
Lane 5, inguinal lymph node from mice exposed to uninfected mosquito feeding;
Lane 6, inguinal lymph node from mice exposed to infected mosquito feeding;
Lane 7, popliteal lymph node from mice inoculated with virus;
Lane 8, RT negative control;
Lane 9, PCR negative control.

B.) 1.5% Agarose gel of electrophoresed PCR products consisting of:
Lane 1: DNA Ladder;
Lane 2: blank;
Lane 3: popliteal lymph node from mice exposed to infected mosquito without RT;
Lane 4: inguinal lymph node from mice inoculated with virus without RT;
Lane 5: blank;
Lane 6: popliteal lymph node from mice exposed to infected mosquito feeding;
Lane 7: inguinal lymph node from mice inoculated with virus;
Lane 8: blank.

Viral replicative forms were detected in lane 6 and 7, while the remaining lanes were negative.



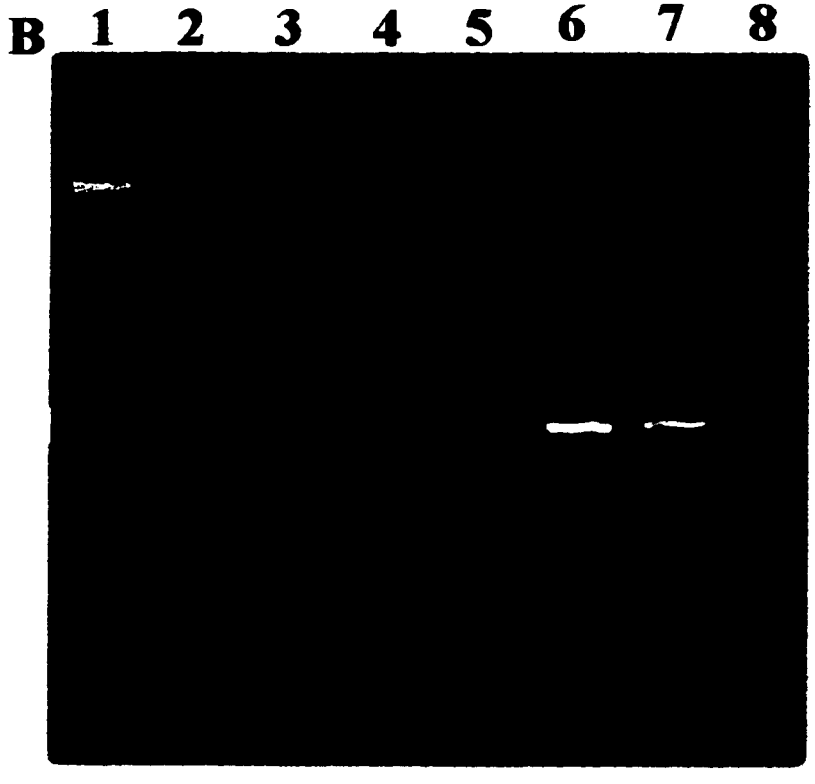


Figure 4.10: Analysis of IL-4 production by RT/PCR in popliteal lymph nodes at predetermined times after VSNJ infection.
Total RNA from mouse popliteal lymph nodes analyzed by RT/PCR for IL-4 production. 1.5% Agarose gel of electrophoresed PCR products consisting of:
Lane 1: Ladder
Lane 2: popliteal lymph nodes from untreated control mice, 48 hours p.i.
Lane 3: popliteal lymph nodes from mice exposed to uninfected mosquito feeding, 48 hours post-exposure
Lane 4: popliteal lymph nodes from mice exposed to infected mosquito feeding, 48 hours post-exposure
Lane 5: popliteal lymph nodes from mice injected with VSNJ, 48 hours p.i.
Lane 6: popliteal lymph nodes from untreated control mice, 72 hours p.i.
Lane 7: popliteal lymph nodes from mice exposed to uninfected mosquito feeding, 72 hours post-exposure
Lane 8 popliteal lymph nodes from mice exposed to infected mosquito feeding, 72 hours post-exposure
Lane 9: popliteal lymph nodes from mice injected with VSNJ, 72 hours p.i.
Lane 10: popliteal lymph nodes from untreated control mice, 96 hours p.i.
Lane 11: popliteal lymph nodes from mice exposed to uninfected mosquito feeding, 96 hours post-exposure
Lane 12 popliteal lymph nodes from mice exposed to infected mosquito feeding, 96 hours post-exposure
Lane 13: popliteal lymph nodes from mice injected with VSNJ, 96 hours p.i.
Lane 14: negative PCR control
Arrowhead designates predicted size of RT/PCR product.

Mice exposed to mosquito feeding (infected or uninfected) were positive for IL-4 induction.

Analysis of IFN-α transcripts:

Ribonuclease protection assays (RPA) were used to determine the relative levels of interferon mRNA in mouse skin tissue at various time points after infection. Ten micrograms of total RNA was probed for IFN-α2 and β-actin messages. Autoradiographs were analyzed using Gel Expert software to compare densities of both bands. Interferon band density score was divided by the β-actin band density score for each sample and graphed as relative mRNA level. Relative interferon levels of mice fed upon by VSNJ infected mosquitoes reached a peak at eighteen hours post-infection and the levels of interferon only dropped slightly during the time course (Figure 4.11). RPA was also used to determine relative interferon levels of mice injected with VSNJ, which reached a peak at twelve hours post-infection with a sharp decline in the remaining time points. Comparisons of relative interferon levels between mice fed upon by VSNJ infected mosquitoes and mice injected with virus at individual time points did not reveal any significant differences by Student's t-test. However, relative interferon levels in mice fed upon by VSNJ infected mosquitoes were significantly higher ($p<0.003$) than in mice that were only probed by these mosquitoes (Figure 4.12). Analysis of all treatment groups (Figure 4.13) did not reveal any significant differences in interferon induction between mice injected with VSNJ and mice injected with tissue culture medium. Similarly, there were no significant differences in interferon induction between mice fed upon by VSNJ infected mosquitoes and mice fed upon by uninfected mosquitoes.

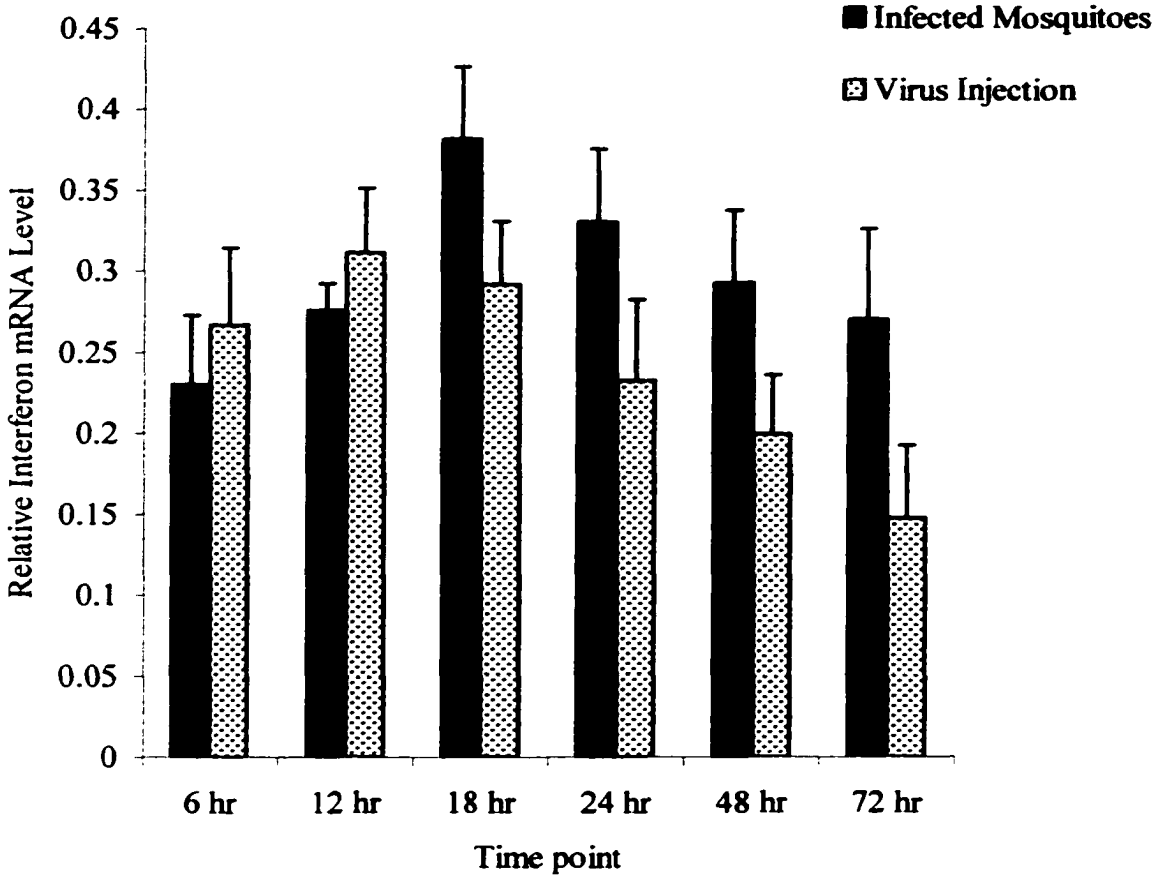


Figure 4.11: Analysis of interferon-α2 mRNA levels by RPA over time comparing mice fed upon by VSNJ infected mosquitoes and mice injected with VSNJ virus. Total RNA from mouse skin exposed to infected mosquito feeding or virus injection was analyzed for interferon induction. Two-minute exposures of autoradiographs the day after detection were used in all concentration analysis in the Gel Expert program. Background correction was set as the area around each individual interferon band. Interferon band density score was divided by the β-actin band density score to calculate relative mRNA levels. Error bars represent the standard error of the mean from mice analyzed in each time point. No significant differences by Student's t-test were detected between the two groups of mice.

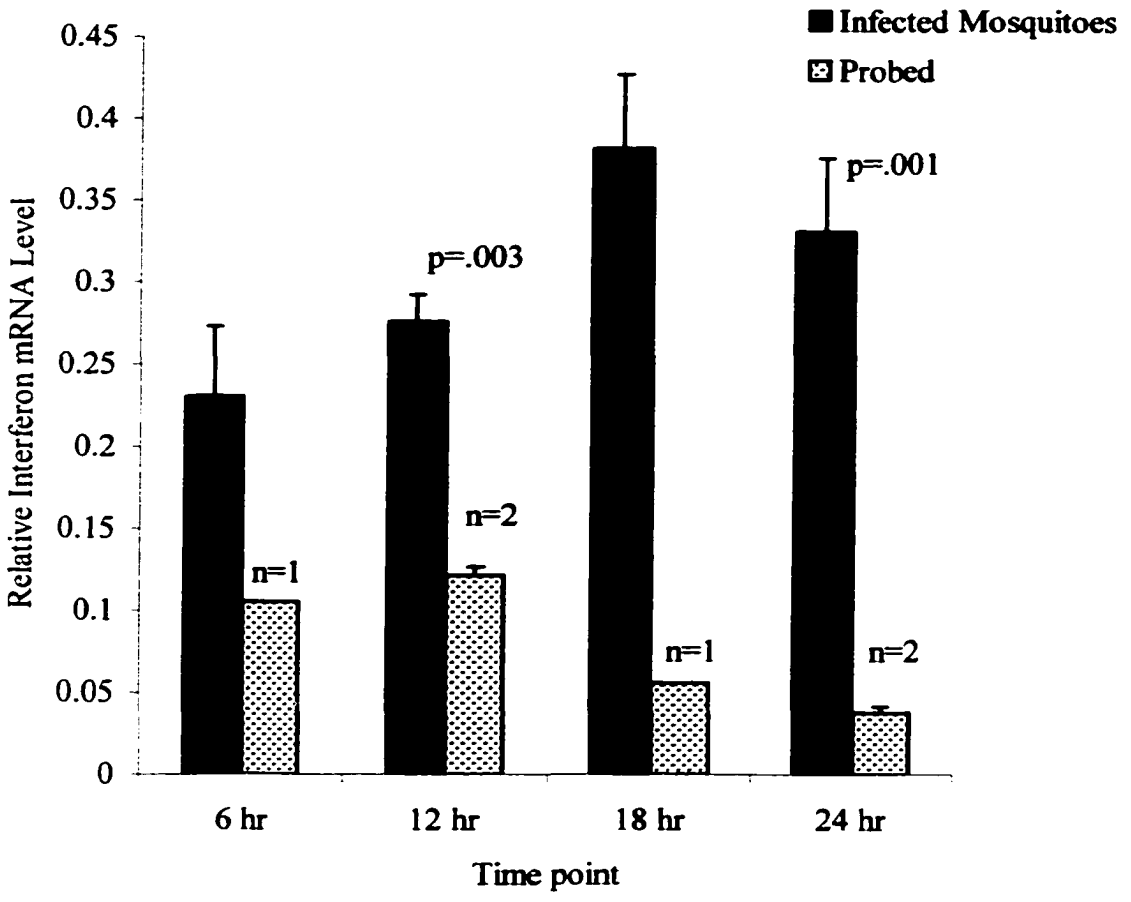


Figure 4.12: Analysis of interferon-α2 mRNA levels by RPA over time comparing mice fed upon by VSNJ infected mosquitoes and mice only probed by VSNJ infected mosquitoes.
Total RNA from mouse skin exposed to infected mosquito engorging or infected mosquito probing was analyzed for interferon induction. Two-minute exposures of autoradiographs the day after detection were used in all concentration analysis in the Gel Expert program. Background correction was set as the area around each individual interferon band. Interferon band density score was divided by the β-actin band density score to calculate relative mRNA levels. Error bars represent the standard error of the mean from mice analyzed in each time point. N values represent the number of mice probed within each time point. Statistical significance was determined using the Student's t-test function to compare 12 and 24 hour time points.

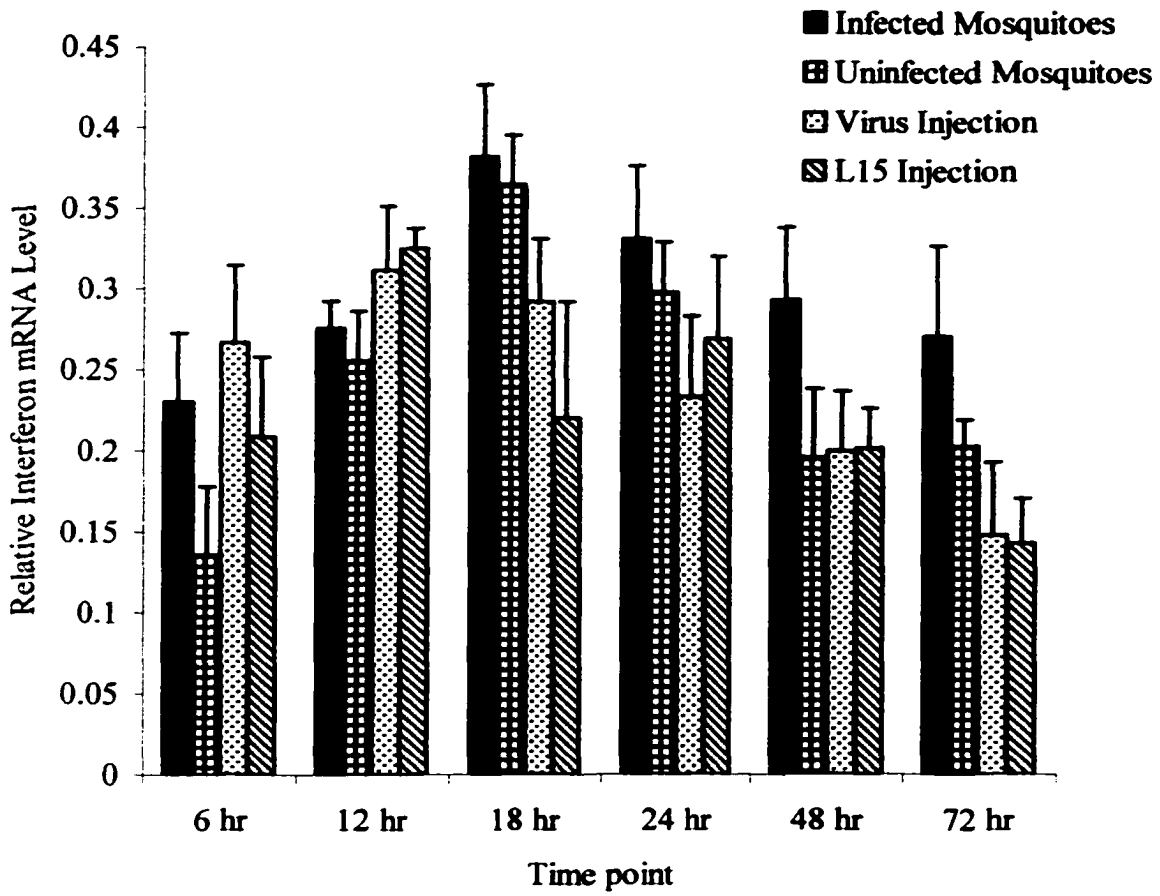


Figure 4.13: Analysis of interferon-α2 mRNA levels by RPA over time comparing mice from all treatment groups.
Total RNA from mouse skin exposed to infected mosquito feeding, uninfected mosquito feeding, cell culture medium injection or virus injection was analyzed for interferon induction. Two-minute exposures of autoradiographs the day after detection were used in all concentration analysis in the Gel Expert program. Background correction was set as the area around each individual interferon band. Interferon band density score was divided by the β-actin band density score to calculate relative mRNA levels. Error bars represent the standard error of the mean from mice analyzed in each time point. No significant differences by Student's t-test were detected between the groups of mice.

Discussion:

Mosquito delivery of VSNJ virus potentiates infection in mice older than three weeks. *In vivo* analyses were used to dissect the mechanism of this potentiation. Histological analysis of mosquito feeding sites within mouse skin revealed marked differences when compared to injected controls. Infiltration of a diverse number of cell types into mosquito bloodfeeding sites included neutrophils, mast cells, eosinophils, and macrophages.

Neutrophils initiate the progression of inflammation and were the predominant cell type in mosquito feeding and inoculation sites. The major functions of neutrophils in the inflammatory response are: participating in phagocytosis, releasing lytic granules, and producing chemotactic factors (Slauson and Cooper 1990a). It is hypothesized that the alternative complement pathway clears low doses of VSNJ injected into adult mice. Fragments from this pathway (C3bBb) preferentially attract neutrophils (Weller and Goetzl 1979). Neutrophils were also recruited into uninfected sites, which may be due to the phagocytosis function during the wound repair process.

At six hours post mosquito feeding, degranulation of mast cells could be seen in the dermal layers of the skin tissue. This degranulation can be caused by factors in mosquito saliva (Schleger *et al.* 1976), by complement activation products, by IgE antibody, or by certain cytokines (Metcalfe *et al.* 1992). Mast cells release histamine, which acts as a chemotactic agent for eosinophils *in vitro*. However, *in vivo* experiments did not reveal such chemotactic activity (Gleich *et al.* 1992). This may be significant because some species of mosquitoes contain an anti-histamine factor (Ribeiro, 1987b). Mast cells are intimately involved in acute inflammation, since the release of histamine

triggers smooth muscle contraction and edema formation (Metcalfe *et al.* 1992). Mast cells also release vasoactive amines (serotonin), which function in opening skin endothelial cell tight junctions and allowing the inflammatory cells to pass through the tissue (Mokhtarian and Griffin, 1984).

Eosinophils generally appear soon after the onset of mast cell mediated reactions and fragments from the classical complement pathway (C567) attract eosinophils to the site (Weller and Goetzl 1979). Interleukin 5 (IL-5) secretion by mast cells activates eosinophils, and these cells serve a regulatory role in immediate-type hypersensitivity reactions. This role includes inhibition of histamine release and phagocytosis of granules extruded by mast cells (Weller and Goetzl 1979). Infiltration of mast cells and eosinophils into mosquito feeding sites may indicate the generation of a Th2 cytokine response.

The infiltration of macrophages into the bite site by eighteen hours post mosquito feeding is very intriguing. Macrophages play a central role in regulating the immune response at an injury site and are a major source of IL-1 and TNF-α (Slauson and Cooper 1990b). TNF-α induces the migration of Langerhan' cells from the skin to the lymph node (Egan *et al.* 1996). TNF is a key mediator of inflammation and of the mammalian host's response to injury or invasion by pathogens (Kondo and Sauder 1997). Local concentrations of TNF-α bind to its receptor and induce the production of ICAM-1. ICAM-1 in turn induces chemokines, which attract neutrophils, eosinophils, macrophages, and lymphocytes to sites of injury (Krakauer *et al.* 1998). Macrophages are the prime target of immunomodulation by sand fly saliva (Hall and Titus 1995; Donnelly *et al.* 1998; Soares *et al.* 1998). Also, macrophages are the target cell for replication and

antibody-mediated enhancement of dengue viruses (Monath 1986). An important determinant of viral spread is the ability of the virus to replicate and/or survive in macrophages (Ahmed and Biron 1999).

At eighteen hours post mosquito feeding, all of these cells have been recruited into the feeding lesion. This response can still be detected at twenty-four hours post-feeding. In contrast, in the injected mice, lesions are small, condensed, and begin to dissipate by twenty-four hours post-injection. Mosquito bloodfeeding increases vascular permeability and damage, which is depicted in the inflammatory edema within the subcutaneous layer of the integument. The infiltration of these cells depicts the cellular response of the vertebrate host to mosquito salivary antigens because there are no qualitative differences between infected and uninfected mosquitoes. The importance of this infiltrate in salivary potentiation and transmission of arthropod-borne pathogens is unclear. It is possible that the innate immune response recognizes mosquito salivary proteins as a specific pathogen-associated molecular pattern and instructs the adaptive immune response to produce a Th2 cytokine profile (Medzhitov and Janeway, 1997). Recruitment of target cells, inhibition of antigen presentation, and modulation of Th1 cytokines by Th2 cytokines may provide a selective advantage for arthropod-borne pathogens.

The detection of viral transcripts in the popliteal lymph node, 96 hours after infected mosquito feeding, may be a direct result of the cellular infiltrate induced by mosquito feeding. No viral replication was detected at the mosquito feeding site so these cells may aid in sequestering and disseminating the virus to the draining lymph node. The presence of TBE viral antigen in Langerhan cells indicates a potential vehicle for

viral dissemination (Labuda *et al.* 1996). It is possible that the increase in seroconversion rates (Chapter II) correlates with an increase in virus dissemination and replication in the draining lymph node.

Changes induced in the skin during acute inflammation with respect to cellular and cytokine profiles are reflected in the draining lymph node (Egan *et al.* 1996). Production and secretion of IL-4 leads to a Th2 cytokine profile and increased antibody production (IgG1 and IgE). IL-4 was produced in the draining popliteal lymph nodes after mosquito (infected and uninfected) feeding at all time points examined (Figure 4.10). However, IL-4 was not produced in the inguinal lymph nodes in any of the mice examined. Th2 cytokine induction after arthropod feeding has also been demonstrated by Mbow et al. (1998) and Zeidner *et al.* (1999). Interferon-γ was not produced after mosquito feeding or viral injection in any of the lymph nodes examined. Similarly, IFN-γ was not produced in the draining lymph nodes of mice three days after tick feeding (Zeidner unpublished results). The absence of interferon-γ results in the production of IgG1 instead of IgG2a antibodies (Ahmed and Biron 1999).

The murine interferon response to uninfected mosquito feeding was similar to *in vitro* results (Chapter III). A characteristic bell shaped curve of induction followed by a rapid decline. However, the interferon response in mice exposed to infected mosquitoes was not suppressed early after infection not did it decline as rapidly as in the L929 cells (comparing Figure 3.5 to Figure 4.11). The presence of viral transcripts in the regional draining lymph node may continue to induce low levels of interferon. Interferon-α2 and α4 are predominately induced after VSNJ infection of L929 cells, but interferon-α1, α2, and α4 are induced in mouse macrophages (Hoss-Homeld *et al.*, 1989). It is also

possible that *in vivo* a different interferon-α gene is targeted by mosquito saliva instead of interferon-α2. The effect of probing mosquitoes on interferon induction was also examined in the earlier time points. Probing by infected mosquitoes did not induce interferon in mice nearly to the levels seen in mice that were fed upon by infected mosquitoes. While probing mosquitoes are as efficient in transmitting viral particles as fully engorged mosquitoes (Gubler and Rosen 1976), interferon induction may require a dosage of salivary proteins as well as transmitted virus. Induction of the innate immune response may require certain levels of antigen before activation and the cascade of downstream events.

The interferon response to injection differed slightly from the response after mosquito feeding. Levels of interferon in virus or tissue culture medium injected mice were induced at the first time point examined (six hours). After peaking at twelve hours pi, interferon levels dropped in the remaining time points examined. It is possible that interferon was immediately induced by the stress and trauma of injections, and thus a characteristic bell shaped curve was not observed. The rapid decline of interferon levels may be attributed to the lack of viral replication in the skin. However, there were no significant differences in interferon production between all four treatment groups within the time points examined (Figure 4.13). The presence of viral transcipts in the inguinal lymph node, 96 hours post inoculation, is somewhat perplexing (Figure 4.9). Drainage from the virus injection site would travel through the popliteal lymph node before reaching the inguinal lymph node. However, no viral transcripts could be detected in the popliteal lymph node after virus injection. It is possible that the popliteal lymph node only cleared a portion of the viral dose and the virus disseminated to the inguinal lymph

node or the popliteal lymph node was bypassed and the virus disseminated directly to the inguinal lymph node. It is possible virus dissemination and replication in a small number of animals is responsible for the low percentage of inoculated mice that seroconvert (Chapter II).

Salivary potentiation of VSNJ virus infection by arthropods may have a significant role in epizootics. The cellular infiltrate coupled with the induction of cytokines after mosquito feeding was qualitatively different from inoculations alone. Histological evaluation of mosquito feeding sites revealed an infiltrate of cells that function in the generation of a Th2 cytokine profile. Production of Th2 cytokines enhances antibody production (Seder and Mosmann 1998), which may explain the enhancement demonstrated in chapter two. Identification of virus replication in the lymph node draining infected mosquito feeding sites, along with a sustained level of interferon production at the feeding site, may indicate a productive infection. VSNJ virus dissemination to the lymph nodes needs to be analyzed in natural clinical cases.

Summary of Dissertation

Saliva of arthropod vectors can modulate vertebrate host immunologic functions in many ways. These studies were initiated to examine the hypothesis that mosquito saliva could enhance vesicular stomatitis New Jersey virus (VSNJ) infection. The role of arthropods in the epizootic cycle of VSNJ is currently unclear. The effect of vector mosquito feeding could be critical in the transmission and pathogenesis of VSNJ virus.

The ability of mosquitoes to potentiate VSNJ infection was investigated in a mouse model. Mice in three different age groups (three days, three weeks, or > eight months) were exposed to VSNJ-infected mosquitoes or were needle injected with an equivalent dose of VSNJ (titer 1.5-3 logs). Infection was monitored by examining serum for the presence of VSNJ at two days post-infection (PI) or for neutralizing antibody on days seven and fourteen PI. All three-day-old mice succumbed to viral infection by mosquito transmission or delivery by injection (Chapter II). Ninety-four percent of the three-week-old mice bitten by infected mosquitoes developed antibody, whereas antibody was detected in only 13% of inoculated mice (Table 2.1). Adult mice developed neutralizing antibody (73%) when fed upon by infected mosquitoes, but only 11% developed antibody when virus was injected (Table 2.2). All (2/2) suckling mice exposed to probing infected mosquitoes died; however, only 1/3 adult mouse seroconverted when exposed to probing infected mosquitoes. Day two serum samples

from three-week and adult age groups were negative by virus isolation. These data indicated that mosquito mediated delivery of VSNJ exacerbated virus infection in adult mice. Further studies were designed to examine this enhancement *in vitro* and *in vivo*.

The effects of mosquito saliva on VSNJ virus replication and interferon α/β expression *in vitro* were analyzed (Chapter III). Two cell lines were used in this study, mouse fibroblast cells (L929), which produce interferon, and Vero cells, which have a deletion in the interferon gene. Virus titers were determined by end point titration at predetermined times after virus infection in cells that were treated with mosquito salivary gland homogenate. These viral titers were compared to untreated controls. Salivary gland treatment of mouse fibroblast cells (L929) resulted in a significant difference in virus growth over time when compared to untreated controls (Figure 3.2). This enhancement in viral growth kinetics was demonstrated in the production of infectious virus at earlier time points and increasingly higher viral titers over time. In contrast, SGH treatment did not have a significant effect on Vero cells (interferon α/β deletion).

Various other treatments were analyzed to try to narrow the effects of SGH on L929 cells. Cells were treated with sialokinin I, a mosquito vasodilator, to determine if this salivary protein was responsible for the enhanced effect of virus replication. Virus growth curves using two different concentrations of sialokinin (100ng and 1000ng) treatment did not differ significantly from the untreated controls (Figure 3.3). Also, L929 cells were treated with interferon α/β antibody, challenged with VSNJ, and virus growth kinetics were determined and compared to those in SGH treated cells. Virus growth curves in interferon α/β antibody treated cells differed from those in SGH treated and untreated cells, but this change was not significant (Figure 3.4).

Ribonuclease protection assays (RPA) were used to analyze the relative interferon mRNA levels in SGH treated and untreated L929 cells. Induction of interferon-α2 in untreated cells followed a characteristic bell shaped curve with maximum production at eighteen hours after virus infection (Figure 3.5). In contrast, cells treated with SGH exhibited a modified bell shaped curve. The maximum interferon production in SGH treated cells was at eight and twelve hours after virus infection. There was a significant reduction in interferon-α2 at six and eighteen hours post-infection between the two treatment groups (Figure 3.5), which could account for the significant differences in virus growth kinetics (Figure 3.2).

Studies were conducted to investigate the anatomic and immune determinants of infection (Chapter IV). Mice were divided into five cohorts consisting of mice: fed upon by infected mosquitoes, fed upon by uninfected mosquitoes, injected with virus, injected with cell culture medium, and untreated (Table 4.1). Histologic examination, after mosquito feeding or injection of mice, revealed marked differences in cellular infiltrate and pathology. A diffuse cellular infiltrate into mosquito feeding lesions included: neutrophils, eosinophils, mast cells, lymphocytes and macrophages (Figures 4.3-4.6; panels A and C). In contrast, only a dense cellular infiltrate of neutrophils was detected in injection sites (Figures 4.3-4.6; panels B and D).

RNA extracted from these infection sites was examined for the presence of viral replication by RT/PCR and interferon-α2 production by RPA (Chapter IV). No virus replication could be detected in the feeding and injection sites at any of the time points examined. In addition, mouse serum samples were negative for viremia by endpoint titration at each time point. RPA analysis of mice exposed to mosquito feeding or

injection revealed a bell shaped curve of interferon induction. Mice exposed to mosquito (infected or uninfected) feeding exhibited maximum interferon production at eighteen hours post-feeding (Figure 4.13). Injected mice exhibited maximum interferon production at twelve hours post-injection (Figure 4.13). The level of interferon message in mice exposed to VSNJ infected mosquitoes did not appear to decline as rapidly as the other cohorts. However, the relative interferon mRNA levels between cohorts did not differ significantly at any of the time points examined. Interestingly, mice exposed to infected mosquito probing did not demonstrate an induction in interferon, which was significantly different from the mice exposed to infected mosquitoes feeding to repletion (Figure 4.12).

After mouse footpad inoculation or mosquito bloodfeeding, popliteal and inguinal draining lymph nodes were examined at predetermined times for viral transcripts and production of IL-4 and IFN-γ cytokine transcripts (Table 4.2). Mouse lymph nodes were pooled at each time point to acquire enough RNA for analysis. Pooled RNA from mice exposed to infected mosquito feeding was positive for viral transcripts in the popliteal lymph node 96 hours post-infection (Figure 4.9). The RNA pool from mice injected with VSNJ was positive for viral transcripts in the inguinal lymph node 96 hours post-infection. All other lymph nodes were negative for viral transcripts. Popliteal lymph nodes from mice exposed to mosquito feeding demonstrated an induction of IL-4, but not IFN-γ, over untreated controls (Figure 4.10). In contrast, mice injected with VSNJ (dose=2 logs$TCID_{50}$) did not show an induction of IL-4 or IFN-γ. Inguinal lymph nodes from all groups of mice did not produce either cytokine in all time points examined. The cellular infiltrate in the mosquito feeding site coupled with the induction of IL-4 in the

draining lymph nodes are highly suggestive of arthropod enhancement of Th2 cytokine responses. The effect of Th2 cytokines on viral infections is not as pronounced as the effect on parasitic infections; however, the rapid infiltration of a variety of cells may enhance viral dissemination.

In conclusion, arthropod saliva can potentiate the infection of various pathogens during transmission to the vertebrate host. The mechanisms of this effect are at the early stages of elucidation. The modulation of interferon-α by mosquito SGH in the *in vitro* experiments may indicate an important role in the transmission of many arthropod-borne pathogens (Chapter III). However, this effect on IFN could not be seen in the *in vivo* experiments (Chapter IV). The detection of replicative forms of VSNJ virus in the draining lymph nodes by RT/PCR may indicate a productive virus infection in mice that seroconvert. Also, the histological analysis differed significantly between mice exposed to mosquito feeding and injection. The cellular infiltrate in mosquito lesions was indicative of a Th2 response and may benefit viral pathogenesis by modulating Th1 responses. Production of IL-4, a characteristic Th2 cytokine, in the draining lymph node of mice exposed to mosquito feeding supports the histological results. Understanding the role of arthropod saliva in the pathogenesis of VSNJ could greatly impact prevention and control strategies.

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Appendix A

Rearing *Lutzomyia longipalpis*

Introduction:

Epizootics of vesicular stomatitis (VS) affect the southwestern United States approximately every ten years (Letchworth *et al.* 1999). It has been speculated that arthropods may have a significant role in epizootics (Tesh *et al.* 1992; Francy *et al.* 1988; Walton *et al.* 1987; Suarez, 1967; Sudia *et al.* 1967). A well-documented study on Ossabaw Island, off the coast of Georgia, has revealed an enzootic cycle of VS New Jersey (VSNJ) virus between *Lutzomyia shannoni* and an undefined vertebrate host. In these studies, pools of sand flies consisting of males or females were positive for VSNJ virus, and seroconversions of sentinel animals have occurred during the summer months (Comer *et al.* 1992; Corn *et al.* 1990). Also, this species of sand fly has demonstrated low levels (~1%) of transovarial transmission (TOT) of VSNJ virus to their offspring. Tesh (1972) reported a 34% TOT rate of VSI virus by other *Lutzomyia* spp. These examples of vertical transmission may provide an additional viral maintenance mechanism in nature (Tesh *et al.* 1972). The presence of VSNJ antibodies in sera collected from wildlife populations before and after an epizootic lent credence to a possible enzootic cycle in Colorado (Webb *et al.* 1987). Arthropod trapping studies near

Fort Collins, Colorado revealed the presence of *Lutzomyia apache, Lutzomyia oppidana,* and *Lutzomyia aquilonia* between May and September (Alsuhaibani, 1990).

Attempts to colonize these species were not successful, thus attempts were made to establish an already colonized strain of *Lutzomyia longipalpis* at AIDL. Colonization of sand flies from their natural habitat has been very difficult (Modi, 1997). The larval stages are terrestrial and difficult to find in nature. Studies on *Lutzomyia longipalpis* were conducted to establish protocols for rearing indigenous sand flies. The established colony of sand flies was to be used to study salivary potentiation and TOT of VSNJ. Protocols were developed including: preparing larval food, rearing larval stages, and infecting adults with VSNJ virus. Many observations were made during the rearing cycle. However, the attempt of long-term establishment was unsuccessful.

Materials and Methods:

Sand flies:

Lutzomyia longipalpis jacobena or *braziliensis* were obtained as eggs from the University of Texas Medical Branch, Galveston, TX. The sub-species title indicated the location of original collection.

Preparation of Larval Food:

Rabbit feces and rabbit chow was obtained from the Painter Center on Colorado State University main campus. Rabbit feces were cleared of all bedding debris. Chow and feces were mixed 1:1 and water was added until mixture was moist. Mixture was composted for three months. Composting consisted of the addition of water every day and mixing of the food. The mixture became quite moldy during this period. After

composting, the food was placed in a –80°C freezer for 24 hours to kill the bacteria and fungus. Next, a thin layer of food was spread into large pans and covered with aluminum foil. Holes were placed in the foil to allow for ventilation. Two to three times per week the food was kneaded. Gloved hands were lightly moistened with water to knead the food. It was important to keep the water to a minimum at this step because fungus would accumulate. After 2-3 weeks of kneading, the food was processed in a blender until it was the consistency of coffee. Blending was performed in a fume hood due to the dust created. Food was aliquoted into small Ziploc bags and stored at -20°C. Each bag was pre-tested in sand fly rearing containers to determine how quickly it would mold.

Preparation of rearing containers:

Containers were modeled according to the picture shown in Modi and Tesh (1983). Holes, approximately an inch apart, were drilled into round plastic containers. Containers were placed flat on counter and plaster of Paris was added. The layer of plaster, approximately an inch deep on the bottom of the container, was allowed to dry overnight. The centers of the lids were removed. White organdy was placed over the carton, secured with a rubber band, and the lids were screwed down. Containers were immersed in a pan of shallow water for about thirty minutes before use. Excess water was blotted from the inside of the container.

Cages and Bloodfeeding:

Eight-inch square cages were re-fitted with wire mesh with smaller mesh size and edges were covered with plastic to maintain humidity levels. After eclosion, adults were released from rearing containers into small cages. Adults were fed cotton balls soaked in 30% sucrose solution. Cotton balls were changed daily to prevent mold accumulation.

Five to seven days later, a restrained mouse was placed in the cage. Because sand flies are nocturnal feeders (Morrison *et al.* 1995), bloodfeeding was performed while the lights in the room were off or with a black plastic bag over the cage. One to two days after bloodfeeding, females were aspirated into the oviposition containers.

Oviposition containers:

Rearing containers were modified to oviposition containers by the addition of plaster coated microscope slides. Slides were placed vertically on a small rack and centered in the container. The slides provided crevice spaces that were ideal for egg laying.

Aspirating Adults:

Aspirators used for mosquitoes had too much suction and crushed sand flies. Pieces of cotton were placed into the pipette-modified aspirator to buffer the airflow. Aspirated sand flies were gently tapped out of the confined chamber.

Larval Rearing:

Sand fly eggs were white after oviposition, and the chorion melanized and hardened. Covered slides were removed from container and eggs were lightly brushed off using a paintbrush. The moisture level in container was maintained to ensure eggs would not dry out. The container was left at room temperature to minimize mold accumulation. Eggs generally hatched in seven days. It was very important to monitor the container at this time, because larval food could not be added until most of the eggs had hatched.

Once the L1 larvae emerged, a light dusting of dry food was added to the bottom of the container. L1 larvae were very small and characterized by two caudal hairs. This

stage was very sensitive to mold accumulation, so moisture content was be monitored frequently. Once larvae had ecdysed to the L2 stage, greater quantities of food were added (about half inch deep at this stage). This stage exhibited four caudal hairs (two long and two short) and became very active. Larvae were fed every other day, and the container was tapped, like a tambourine, to evenly distribute food and moisture levels. The container was monitored daily for larval activity. Careful monitoring of this stage was critical. A shortage of food would cause the larvae to eat each other; an excess of food would result in mold killing the larvae. Mites became a problem during this stage. Attempts to control mites included: autoclaving rearing containers between generations, killing the mites with a forceps, and transferring larvae to a fresh container. These were not successful in significantly reducing mite populations.

L3 larvae exhibited four caudal hairs (all similar length), and the activity level was very similar to the L2 stage. Feeding and monitoring was also very similar to the L2 larvae. Larvae in the final stage (L4) became inactive. These larvae rested on top of the food and were very sensitive to mold. This stage did not eat much food, so feeding was reduced to once a week. This stage has been shown to diapause, (Modi, 1997) so the larvae were monitored daily.

Pupal and Adult Care:

Pupal stages appeared as extended comas on the top of the food surface. Food was not added to the container at this time, not was it tapped, due to the fragility of pupae. This stage lasted for 5-10 days and was very sensitive to mold. If the mite population was great, the inactive pupae were fed upon.

Adults emerged from the pupae, and the male sand flies eclosed before the females. Cages were marked with the day of eclosion, and a new cage was started each day. Adults were maintained in the *Aedes triseriatus* room of the AIDL insectary (75°F; 75% humidity). After five to seven days, a mouse was restrained and provided as the bloodmeal source. Optimum feeding times were early morning or late evening, and the females were allowed to feed for about an hour. Adults were bloodfed two days in a row to ensure all females had fed. Care was taken to ensure that the mouse was well restrained, because a loose mouse would consume all the adults. The following day, all adults from a cage were aspirated into oviposition containers. Females were very fragile immediately after bloodfeeding and aspiration at this point occasionally resulted in death. Oviposition containers with covered slides provided crevice areas for adults, resulting in increased fecundity. After approximately seven days, all adults were removed and allowed to take a second bloodmeal. Containers with eggs were moved to a room with lower humidity (15-20%).

VSNJ Infection:

Attempts were made to infect *Lutzomyia longipalpis* with VSNJ virus. Numerous problems were encountered. Adults were very sensitive to cold and usually did not recover from anesthetization in the refrigerator. Needles used to intrathoracically inject virus were too large and resulted in death of the adults. Suckling mice 48 hours after VSNJ injection had viremias of 6.5 $\log_{10}TCID_{50}$ (chapter 2) and were used for bloodfeeding. Females used in this bloodfeed were in their second or third gonotrophic cycle and only 1/14 bloodfed. This engorged female was maintained for seven days and

a head impression smear was made. Head tissue was assayed by indirect immunofluorescence for VSNJ antigens.

Results:

Sand fly Fecundity:

After each gonotrophic cycle, eggs were brushed off the covered slides and counted. It has been reported that one female can lay up to 100 eggs, but under these conditions, the average was closer to 25-30 eggs per female in an oviposition container. Approximately 70% of the females died after the first oviposition, which was similar to previous reports (Chaniotis, 1986). Fecundity did not increase with concurrent gonotrophic cycles.

VSNJ Infection:

Suckling mice 48 hours post-infection were used as a viremic blood source for female sand flies. Only one female imbibed a bloodmeal and was maintained for seven days. Indirect immunofluorescence of head tissue from this female was negative for VSNJ antigens.

Discussion and Summary:

The importance of sand flies in enzootic cycles of VSNJ has been established; however, the role of sand flies in epizootic cycles of VSNJ remains unclear. In order to design experiments to examine the role of sand flies in salivary potentiation of VSNJ, the establishment of a colony of *Lutzomyia longipalpis* was attempted at AIDL. Despite numerous hours of labor and seven consecutive generations, the establishment of a long-

term colony was unsuccessful. In each generation, problems with mold, mites, and low fecundity seemed to expand until the eventual demise of the colony. Nevertheless, the protocols and procedures used to maintain the colonies are documented herein in order to facilitate future endeavors.

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