PATHOGENESIS OF ACUTE VESICULAR STOMATITIS INFECTION IN EXPERIMENTALLY INFECTED CATTLE

by

JANILDO LUDOLF REIS JR

(Under the Direction of Corrie C. Brown)

ABSTRACT

Vesicular stomatitis (VS) is a contagious and vector-borne disease of livestock caused by vesicular stomatitis virus (VSV). Vesicle formation in the oral mucosa, and in the skin of the coronary band (CB), teats, prepuce and vulva characterize the disease. Transmission occurs via direct animal-to-animal contact or via biting and non-biting insects. The pathogenesis is poorly understood. Viremia is rarely reported in livestock, viral replication kinetics in tissue is unknown, the role of insect bite in infection has not been established, and the detailed immune response to VSV acute infection has not been described. To address these questions steers were inoculated with VSV via scarification (SC) and black fly bite (FB) in the CB and in the skin of the neck. Animals were euthanized from 12 to 120 hours post infection (HPI). Multiple tissue samples and serum were collected for virus isolation and detection of numerous T helper 1 and 2 cytokines. Samples were also processed for immunohistochemistry to characterize the inflammatory

cell infiltration. This study describes that VSV replicates to high copy numbers in specific anatomic regions, such as the CB, but fails to replicate and to cause lesions in the skin of the neck. Viral replication in the CB peaks between 24 and 48 HPI, with detection of replicating virus restricted to 24 HPI in the lymph nodes. Lesions are more prominent and develop faster in FB-inoculated animals. There is up-regulation of MHC II in SC-inoculated animals, with lack of MHC II up-regulation in the FB-inoculated animals, which may explain why lesions are more severe with FB. Marked T helper 1 cytokines are present at 48 HPI in the CB of infected animals, which comes immediately after the peak of virus replication. Strong and acute antiviral cytokine response may prevent the virus from systemic spread (viremia). Viral antigen presentation to B cell through follicular dendritic cells occurs as early as 72 HPI. This finding correlates with the fact that neutralizing antibodies are produced early in VSV infection. The results of this study add new data to better understand VS pathogenesis and might be instrumental for disease control and prevention.

INDEX WORDS: Black flies; cattle; cytokines; immunohistochemistry; *in situ* hybridization; pathogenesis; vesicular stomatitis virus.

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by

JANILDO LUDOLF REIS JR

DVM, Universidade Estadual do Norte Fluminense, Brazil, 1999

MS, Universidade Federal de Minas Gerais, Brazil, 2001

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

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by

JANILDO LUDOLF REIS JR

Major Professor:	Corrie C. Brown
Committee:	Elizabeth W. Howerth
	Daniel G. Mead
	Elizabeth W. Uhl

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2010

DEDICATION

To my wife, Aline, for her sacrifices and fullest support during these last years. I also dedicate this dissertation to my parents, Cidlea and Janildo, for their endless encouragement.

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CHAPTER 1

INTRODUCTION

Vesicular stomatitis (VS), a disease caused by Vesicular Stomatitis viruses (VSV), is an important contagious and vector-borne disease described in a wide range of animals, including livestock, wild, and laboratory animals.[1] Rare cases have also been reported in humans in endemic areas or in laboratory accidents.[2] The disease occurs from South[3] and Central America,[4] where it is endemic, to the Western USA,[5] where sporadic outbreaks are reported at 3 to 5 year intervals. The VSV are single-stranded, negative sense RNA viruses in the Genus Vesiculovirus, Family Rhabdoviridae.[6] The two major VSV serotypes of importance in animal health are vesicular stomatitis New Jersey virus (VSNJV) and vesicular stomatitis Indiana virus (VSIV).[6] The former is more frequently reported in US and Central America, and the latter with its related serological groups Cocal[7] and Alagoas[8] are endemic in South America.

The disease is especially important in cattle, horses and pigs, causing vesicular lesion in the coronary bands of the feet, oral cavity, muzzle, lips, teats, prepuce and vulva.[1] The lesions are indistinguishable from those found in cattle and pigs infected with foot-and-mouth disease (FMD) virus, and so all cases need to be investigated thoroughly. The viruses can spread quickly and have an important economic impact due to the high morbidity rates resulting in quarantine, animal movement restrictions, and decreases in production of meat and milk.[9] The presence of VS in a region can interfere

with the international trade of animals and their products, such as meat, milk, semen, embryos and biological products.

The pathogenesis of VSV infection in livestock is not completely understood. To the best of the authors' knowledge, viremia in livestock has been described only once, in 1926 by Cotton,[10] with no other posterior studies describing viral systemic spread. Furthermore, the viral kinetics in the tissues of infected animals has not been reported, the role of insect bites in VSV infection is unknown, and the host immune response has not yet been characterized.

Therefore, the aims of this study were:

1 - To track temporal viral replication and distribution in tissues during early infection of cattle inoculated with VSNJV in the coronary bands (CB) via scarification (SC) and black fly bite (FB);

2 – To compare lesion development in cattle inoculated with VSNJV via scarification and black fly bite;

3 – To characterize the cellular inflammatory infiltration in VSNJV-inoculated coronary bands and draining lymph nodes from SC- and FB-inoculated animals

4 – To characterize and compare the cytokine response of SC- and FB-inoculated steers during early VSNJV infection.

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CHAPTER 2

LITERATURE REVIEW

TRANSMISSION AND PATHOGENESIS OF VESICULAR STOMATITIS VIRUSES.*

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*Janildo L. Reis Jr, Danny Mead, Luis L. Rodriguez and Corrie C. Brown. *Braz J Vet Pathol*, 2009, 2(1), 49 - 58

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ABSTRACT

Vesicular Stomatitis (VS) is caused by Vesicular stomatitis viruses (VSV), negative single stranded RNA arthropod-borne members of the Family Rhabdoviridae. The VSV virion is composed of the host derived plasma membrane, the envelope, and an internal ribonucleoprotein core. The envelope contains a transmembrane glycoprotein, the G protein, which mediates viral entry and exit from the cell. The ribonucleoprotein core contains the viral genome encased within the nucleocapsid protein, the N protein. The large protein (L), the nucleocapsid (N) and the phosphoprotein (P) have key roles in viral replication. The matrix protein (M protein), located between the envelope and the nucleocapsid core, participates in viral assembly, and particle budding. The VSV serotypes involved with disease in livestock are New Jersey and Indiana 1, 2 and 3. Serotypes New Jersey and Indiana 1 occur from USA through Central America to much of South America. Serotype Indiana 3 (or Alagoas) occurs in the North, Northeast and Central Brazil. The serotype Indiana 2 (or Cocal), occurs in Southern Brazil and in Argentina. Outbreaks of VS in Brazil in recent years have resulted in severe economic losses. The clinical disease in horses, cattle, and pigs is characterized by vesicles in tongue, planum nasale, planum rostrale, coronary bands (CB) of the feet, prepuce, and teats. Subclinical disease, with seroconversion and lack of vesicle formation is common under natural conditions and can be induced experimentally depending on the site of inoculation. VSV infection typically involves cytolytic infection of mammalian host cells at specific sites of inoculation. Transmission can occur via infected insect bite but

animal-to-animal contact could also be important in within-herd spread. There is some evidence to suggest that biting insects may play a role in the pathogenesis of VSV infection, although mechanisms of pathogenesis are not well understood. Viral spread seems to stop at the draining lymph nodes with no viremia. As a well-known *in vitro* producer of interferon, it is hypothesized that the host immune response to VSV infection may limit viral spread. A better understanding of pathogenic aspects could allow development of prevention and disease control strategies.

Key words: VSV, vesicular stomatitis, transmission, biting insects, pathogenesis.

INTRODUCTION

Vesicular stomatitis viruses (VSV), the causative agent of vesicular stomatitis (VS), cause clinical disease in multiple livestock species. The viruses can spread quickly and have an important economic impact due to the high morbidity rates resulting in quarantine, animal movement restrictions, and decreases in production of meat and milk. Another important aspect of the disease is the fact that VS in cows and swine is clinically indistinguishable from foot-and-mouth disease, and so all cases need to be investigated thoroughly. This is particularly important in South America where programs to eradicate foot-and-mouth disease have been implemented. The presence of VS in a region can interfere with the international trade of animals and their products, such as meat, milk, semen, embryos and biotechnological products. Vesicular stomatitis is restricted to the

Americas with specific serotypes usually occurring in defined regions. Vesicular stomatitis was previously considered a List A OIE disease, which carried with it mandatory international reporting requirements and severe trade restrictions, but now it has been reclassified as one of the more than 100 "listed diseases" according to the new OIE scheme. Nevertheless, countries continue to monitor the presence of VS, and its presence can result in severe economic damage due to quarantines or trade embargoes. The purpose of this paper is to provide a review of the literature on vesicular stomatitis focusing on the transmission and pathogenetic mechanisms.

ETIOLOGY

Vesicular stomatitis viruses are enveloped, nonsegmented, single negative-stranded RNA arthropod-borne viruses (arboviruses) in the Genus Vesiculovirus, Family Rhabdoviridae.[1] They have a large bullet-shaped (65-185nm) virion, and are the prototype virus for the family. The viruses have been extensively studied in vitro in numerous laboratories, largely as a tool to investigate the production of interferon. However, the natural cycles and pathogenesis of the disease caused by the various serotypes in livestock have not been as well characterized.[2] There are two major serotypes of VSV: New Jersey (VSNJV) and Indiana (VSIV). The serotype Indiana has been subdivided into three distinct serological groups. Indiana type 1 represents the classical Indiana strains.[3] Cocal virus (COCV) is the prototype virus of the Indiana 2 subtype and was originally isolated from mites collected from rice rats in Trinidad in

1961 and in northern Brazil in 1962.[4] Alagoas virus (VSAV) is the prototype virus of the Indiana 3 subtype and was first isolated from a mule in Alagoas, Brazil, in 1964.[5] There are other vesiculoviruses that infect humans and that will cause lesions when experimentally inoculated into domestic animals, but with no importance to date in natural outbreaks in livestock. These include Piry virus originally isolated from an opossum (*Didelphis albiventris*) in Brazil, Chandipura virus first isolated from a human in India, and Isfahan virus isolated from sandflies and humans in Iran.[6-8] These three viruses will not be discussed further in this review.

The virion structure of VSV (Fig. 2.1), like all rhabdoviruses, is composed of an external phospholipid bilayer membrane (envelope) derived from the host cell and an internal ribonucleoprotein core. The envelope contains the viral glycoprotein or G protein, an important transmembrane protein which forms an array of 400 trimeric spikes. G protein is synthesized by membrane-bound ribosomes, associates with the chaperones BiP and calnexin that assist in correct folding, and eventually undergoes glycosylation and acylation as it moves to the Golgi apparatus. Once correctly folded and glycosylated, G protein migrates to the cell membrane.[1,9] G protein mediates cell recognition and fusion.[10,11] It is also important in determining specificity in induction of neutralizing antibodies according to serotype[12] and virulence among serotypes,[11] where New Jersey is more virulent than Indiana, purportedly due to differences in pH dependent infectivity.[13] The nucleocapsid core is composed of the viral genome tightly encased within the nucleocapsid protein (N). N proteins are arranged as beads on a string along the viral RNA (Fig. 1), which form an RNase-resistant core environment. Because the VSV genome is negative stranded RNA, its replication relies on a viral RNA-dependent RNA

polymerase, composed of the large (L) and phosphoprotein (P) protein molecules. The N-protein RNA complex interacts with P-L complex during viral transcription and replication. P protein in combination with the L protein forms the viral transcriptase-replicase complex. When P protein undergoes polymerization, it forms trimers that are required for binding of L and N-RNA complex to form the active transcriptase. The matrix protein (M) is located between the internal surface of the envelope and the nucleocapsid core. It has numerous functions, such as condensation of the nucleocapsid during assembly and viral particle budding. The viral N, L, P and M proteins do not change their composition among particles, whereas the transmembrane G protein can vary greatly among and within serotypes.[1]

The replication cycle of VSV is typical for most of the negative-stranded RNA viruses[14] (Fig. 2.2). Following attachment, penetration, and uncoating the viral nucleocapsid is released within the cytoplasm. The viral genome, which is encased with the N protein, serves as a template for initial transcription by the virion RNA-dependent RNA polymerase, resulting in synthesis of leader RNA and all five viral mRNAs for the N, P, M, G, and L genes. Once viral proteins are synthesized from primary transcripts, viral genome is replicated with synthesis of full-length positive strand RNA (antigenome), which serves as a template for synthesis of negative-strand RNA (genome). Genome encapsidation occurs at the same time as its replication. The early events of the replication cycle (attachment, penetration, uncoating, and primary transcription) take place within the first few hours postinfection. The remaining steps of the replication cycle (genome replication, secondary transcription and assembly) take 12 to 18 hours.[14] It has been proposed by some investigators that VSV uses a phosphatidyl

serine as a cellular receptor.[15] However others suggest that VSV does not use phosphatidyl serine, because in most of the cells these molecules are found in inner portions of the plasma membrane and consequently are not available to act as a receptor for viral entry.[16] VSV attachment to host cells may use nonspecific electrostatic and hydrophobic interactions.[17] Lower pH also plays an important role in VSV attachment as well as viral membrane fusion.[14] Lower pH alters G protein conformation which is required for fusion. As for many other viruses, VSV penetration uses clathrin-dependent endocytosis into coated vesicles. The vesicle loses its clathrin coat and becomes an endosome, subsequently the pH drops below 6.5 and G protein mediates fusion of the endosome membrane with the viral envelope. This fusion leads to the release of the nucleocapsid into the cytoplasm where M protein dissociates from the nucleocapsid, which is necessary for viral RNA synthesis to occur.[14] The primary transcription mediated by the virion-associated RNA-dependent RNA polymerase occurs in the absence of protein synthesis. For transcription viral genome associated with N protein is used as a template and requires L and P proteins. When P is phosphorylated, it forms P protein trimers that bind to L protein resulting in polymerase activity.[1] Transcription begins at the 3' end of the genome, where a 47-nucleotide RNA called leader is first synthesized.[1]

HOST RANGE AND CLINICAL SIGNS

Vesicular stomatitis viruses have a wide host range in animals, causing natural vesicular disease in equidae (horse, mule and donkey), cattle and pigs. However, the host range is considerably wider and evidence of infection with the various serotypes has been detected in a number of species, including South American camelids and many wild rodents. Serological surveys have shown that the virus circulates in bats and tamarin monkeys in Bahia State, Brazil.[18] Experimental infection has been successful in domestic rodents, deer, raccoons, bobcats, and primates. Sheep and goats are more resistant and have rarely been affected.[2,7] Vesicular stomatitis is a zoonotic disease, and people have become ill through both natural infection[19] with reported cases of encephalitis[20] and via laboratory accidents.[19,21] In ruminants and pigs, the clinical signs of VS resemble those of foot-and-mouth disease (FMD), and are characterized by initial vesicular formations that progress to erosions and ulcerations on the tongue, palate, gum, lips, snout (swine), teats, prepuce, interdigital space and coronary band. Humans usually do not present with vesicle formations; mild influenza-like signs are most commonly reported.[22] Rodents develop systemic disease with viremia and central nervous system lesions with no vesicle formation on the skin.[23-25]

Because of the similarity to FMD in clinical and pathological presentation in cattle and pigs,[9] a thorough and precise investigation must be done in all suspected VS cases, in order to rule out FMD.[9] Furthermore, dual VSV and FMDV infections have been described from some herds of cattle and concomitant infection has been experimentally reproduced.[6] In pigs, VS also has to be distinguished from other vesicular

diseases, including swine vesicular disease.[6] The latter has never been described in the Americas, but recent outbreaks in Europe have raised worldwide concern as a potential emergent threat.

The incubation period for VSV is variable but usually vesicles are visible within 24-72h after virus inoculation.[26-28] Coinciding with the period of vesicle formation, infected animals are febrile and anorexic. Recovery typically occurs within two to three weeks of vesicle formation, but there can be severe secondary infections that can result in laminitis in horses, and severe mastitis and teat scarring in dairy cattle.[6,7]

EPIDEMIOLOGY

Infection rates are variable among outbreaks; morbidity can be as high as 96%. In cattle and horses mortality is negligible.[29] However, as previously mentioned, in horses laminitis can develop as a result of VSV infection and this may lead to euthanasia.

Vesicular stomatitis is only present in the American continent[2,6] (Fig. 2.3). The New Jersey serotype accounts for 80% of the outbreaks in the U.S., and Indiana 1 for the remainder.[30] In the U.S., the southwest and southeast are the areas where VS has been reported most frequently, with fewer reports in other regions in this country.[31] The New Jersey serotype is also the most important serotype in Central America, from southern Mexico to Panama[22] and in the northwestern part of South America, such as Bolivia, Colombia, Ecuador, Peru and Venezuela. In 2007, the disease was reported in 497 herds

from these countries. Colombia reported 391 outbreaks where 364 were due to New Jersey and 27 due to Indiana 1 serotype.[32]

In Brazil and Argentina, clinical disease associated with New Jersey and Indiana 1 serotypes has not been reported. Outbreaks of VS in these countries are caused by viruses serologically classified as Indiana 2 and 3 serotypes.[33] VSV-IN 3 occurs in regions of Brazil at 1-2 year intervals and VSV-IN 2 outbreaks occur sporadically in southern Brazil and Argentina.[33,34] A serological survey from 112 equidae from the Brazilian States of Pernambuco, Bahia, Goais and Rio Grande do Sul revealed 2.6% reactivity for the New Jersey serotype.[18] Therefore, even though no clinical disease associated with this serotype has been reported in Brazil, it is presumed that either VSNJV or a closely related and possibly undescribed vesiculovirus is circulating in those States. Vesicular stomatitis virus Indiana 2 has been isolated from outbreaks in Sao Paulo State, in 1966 in Rancheria and in 1979 in Ribeirao Preto. Other large outbreaks due to the Indiana 2 serotype were reported in 1998 in Santa Catarina and Parana States, in southern Brazil (http://www.panaftosa.org.br). The Indiana 3 serotype outbreaks are more frequently reported in northeast Brazil[35] and for a long time it was believed that this serotype was restricted in geographic distribution to this particular region. A virus related to Indiana 3 was isolated from naturally infected phebotomine sand flies in Colombia in 1986[36] with serological evidence of viral circulation among livestock, but no clinical disease caused by Indiana 3 has been reported outside Brazil.

Outbreaks of VS occurred in Europe during the First World War and in South Africa from 1884 to 1943 from horses exported from the US.[9] Vesicular stomatitis no longer occurs in these areas.

TRANSMISSION AND PATHOGENESIS

Knowledge concerning the natural cycle and pathogenesis of VSV remains incomplete. Vesicular stomatitis virus transmission can occur in a number of ways and these are depicted in Fig. 2.4. Under natural conditions, insect vector transmission and direct contact have been described. Experimentally, various routes have been tried with success, including intranasal, intradermal, intravenous,[26] scarification of the skin[28] or oral mucosa[27], animal-to-animal contact[27] and biological and mechanical insect vector transmission.[37] However, studies with VSNJV have demonstrated that the clinical outcome of these routes of inoculation varies considerably. Livestock can be experimentally infected with subsequent clinical disease (vesicle formation) when injection is intradermal at the coronary bands, planum nasale or oral cavity.[26,28,38,39] Intranasal instillation, intravenous or intradermal injection at the ear[26], scarification of the flank skin [28] or insect bite at the flank skin[40] results in subclinical (lack of vesicle formation) infection, with serocorversion. Viral shedding from tonsils is known to occur in pigs. [26,27] Also, virus inoculation via insect bite at sites where lesions are not observed in cattle result in subclinical infection.[41]

In general lesion development is restricted to the site of inoculation. However, in some studies, pigs inoculated at the snout via intradermal injection developed vesicles at the coronary bands as well.[27,38] The authors of this study suggested a short-term viremia was present, even though it was not detected in their experiment or in any other in domestic animals to date. Because vesicles promptly developed at the site of the inoculation (snout) in those experiments, the authors alternatively suggest that virus was

mechanically introduced by the snout vesicle making contact with an abraded epidermal site (coronary band) or by shedding though saliva from tonsil infection.

In animal-to-animal contact studies,[26,27] naïve pigs became infected when housed with pigs inoculated with VSNJV intradermally on the snout. Contact pigs shed virus as early as 1 day after contact with inoculated animals. Contact animals developed lesions on coronary bands or snout. In these same experiments, another group of animals was inoculated on the coronary bands with less severe vesicle formation than observed in the group inoculated at the snout. Naïve animals housed with this group (inoculated on the coronary band) failed to develop clinical disease or seroconvert.[26] These studies demonstrate that the development of vesicular lesions is important for animal-to-animal contact transmission.[26] They also demonstrated the importance of the site of the inoculation regarding the severity of lesion development and the fact that in order to have efficient animal-to-animal contact transmission prominent lesions (vesicles) are necessary.

Subclinical disease is frequently reported under both natural and experimental conditions. Epidemiological data reveals that up to 90% of animals within endemic herds can be seropositive for VSV with only 10% of animals presenting the typical clinical signs (vesicles) [41,42]. One possible explanation is that insect site predilections for feeding are at the flank, ear, and periocular areas, where viral inoculation does not result in vesicle formation.[37] The role of subclinical disease in transmission via insect or via animal-to-animal contact is not completely known. Some investigators suggest that it could serve as source of transmission and spread of the virus in natural conditions.[40] However, it has been shown in experimental cases that it is necessary to have marked

clinical disease, with evident vesicle formation, to successfully transmit the virus via animal-to-animal contact[26,27] and also animal-to-insect.[37]

As with other arbovirus transmission cycles, insects are part of the natural life cycle of VSV. In 1969, Tesh and colleagues detected VSV Indiana 1 serotype in naturally infected sand flies (Diptera: *Psychodidae*).[22] Numerous other reports of VSV infecting hematophagous insects such as black flies (Diptera: Simuliidea),[43-45] mosquitoes[46] (Diptera: culicidae), and *Culicoides* (Diptera: Ceratopogonidae)[47] have since been published. Experimentally, only sand flies have been shown to be capable of transovarial transmission[22,44]. Studies have demonstrated that black flies are competent VSNJV vectors with transmission resulting in the development of clinical disease in mice[44] and pigs.[45] In one study, cows developed specific neutralizing antibodies following the bite of VSNJV-infected midges (*Culicoides sonorensis*) feeding on flank skin.[40] But in this experiment, although there was seroconversion, lesions were not observed and virus was never isolated from collected samples. In 2000, Mead and colleagues demonstrated horizontal transmission of VSNJV in black flies.[48] This report shows that uninfected flies became infected when co-feeding with infected flies on the same non-viremic host. This finding provides a potential mechanism for maintenance of the virus in nature since viremia has not been reported in naturally or experimentally infected livestock.

A few studies have been done regarding pathogenesis of VSV in laboratory animals and some investigations have been done in livestock. With the limited information available, the mechanisms of the disease appear to be markedly different between rodents and livestock. As already mentioned, viremia has not been documented

in naturally or experimentally infected livestock, and vesicular lesions tend to develop at the inoculation site on specific cutaneous or mucocutaneous regions.[26-28,39] In contrast, viremia with encephalitis and meningitis are expected findings in rodents.[23,24] Cornish et al (2001) infected young and adult *Peromyscus maniculatus* (deer mice), which is a natural host, via two routes - intranasal and intradermal - at the base of the tail.[24] In this experiment, all ages of mice inoculated intranasally developed encephalitis and meningitis. However, in the group inoculated intradermally, young animals were the only ones to develop lesions in the central nervous system. No cutaneous lesions (vesicles) were observed in any of the two experimental groups.

There are no studies focusing on the early pathological and host response events of VSV infections in livestock. In experiments done with swine and horses, animals were followed past the point of seroconversion or after viral shedding had ceased. At this time point, vesicular lesions were in healing stages and so no information was gained about the microscopic events occurring during the vesicular formation stage.[26,27] Howerth et al (2006) inoculated the VSNJV and Indiana 1 serotypes independently in two groups of horses on the tongue, chin and lip, and euthanized the animals at postinoculation days 12 to 15.[39] There was partial to full reepithelization at the primary lesion (original site of inoculation) and superficial ulceration at secondary oral lesions (commonly observed when inoculation site is the oral cavity). Histologically, there was perivascular infiltration of plasma cells and lymphocytes with lesser numbers of neutrophils. Immunophenotyping revealed a mixture of BLA36 (which recognizes B lymphocytes), CD3 (which recognizes T lymphocytes) and MAC387 (which recognizes histiocytes)

positive cells. Vesicular stomatitis virus antigens were not detected at the same sites, probably because samples were collected at a late stage of the disease.

The ability of VSV to induce interferon (IFN) production has raised the attention of several investigators, [49-53] who use VSV as a tool in various laboratory techniques for assessing interferon production. Isolates of VSV vary widely within and among serotypes in their ability to induce type I IFN production in chicken embryo cells.[49] In general VSNJV serotypes tend to induce higher levels of IFN when compared with Indiana isolates.[49] However, to date there are no studies assessing IFN or other cytokine production in livestock following any of the described inoculation methods, either fly bite or scarification. A question that can be raised is whether the infected host has a different cytokine response when inoculated via fly bite as compared with scarification. A recent review of arboviral diseases describes how mosquito saliva enhances transmission and development of disease for numerous arboviral agents. [54] Specifically, mosquito salivary gland extract or mosquito bites enhance transmission and infection of different arboviruses in different systems[55-57] including VSV *in vitro*[58] and *in vivo* (mice).[59] Intradermal *Aedes aegypti* salivary gland extract and Sindbis virus co-inoculation in mice downregulated IFN-gamma and IFN-beta at 24 and 72 hours postinoculation.[60] This same experiment demonstrated upregulation of IL-4 and IL-10. The ability of mosquito saliva to downregulate the expression of T_H1 associated antiviral cytokines and to upregulate T_H2 associated cytokines in many arboviral diseases suggests that it would be worthwhile to determine if biting insects may be exerting similar effects in VSV-infected livestock. The generation of baseline data of host response regarding cytokine expression

in cows inoculated via scarification and via fly bite could lead to a better understanding of the pathogenesis of VS in livestock.

Preliminary results from a recent comparative study suggest that black flies have a facilitating component for viral replication.[61] In this experiment two Holstein steers were inoculated at the coronary bands with VSNJV, one was inoculated via scarification and the other via infected black fly bite. Euthanasia and tissue collection were performed at 24 hours post-inoculation with coronary bands and local draining lymph nodes processed by *in situ* hybridization using an anti-sense riboprobe for VSV. Intense cytoplasmic staining of keratinocytes from the stratum spinosum as well as in scattered inflammatory cells in the superficial dermis was observed in the coronary bands from both animals. However, the draining lymph nodes of the steer inoculated with black flies revealed markedly higher number of positive cells when compared with the scarified animal, which presented scant positively staining cells. Positive *in situ* hybridization cells were mainly distributed within capsular and cortical sinuses in both animals. This study has shown for the first time *in situ* viral transcription and/or replication in livestock tissues at the inoculation site and most importantly at distant organs, draining lymph nodes (popliteal). This may be an indication that black flies have a component that enhances viral replication.

CONCLUSION

Although much is known about the VSV in cell culture, and in laboratory animals, and its abilities to produce IFN *in vitro*, the disease in livestock is much less well understood. Further exploration of transmission and pathogenesis in the natural host could improve our abilities to control this economically important disease of livestock in this hemisphere.

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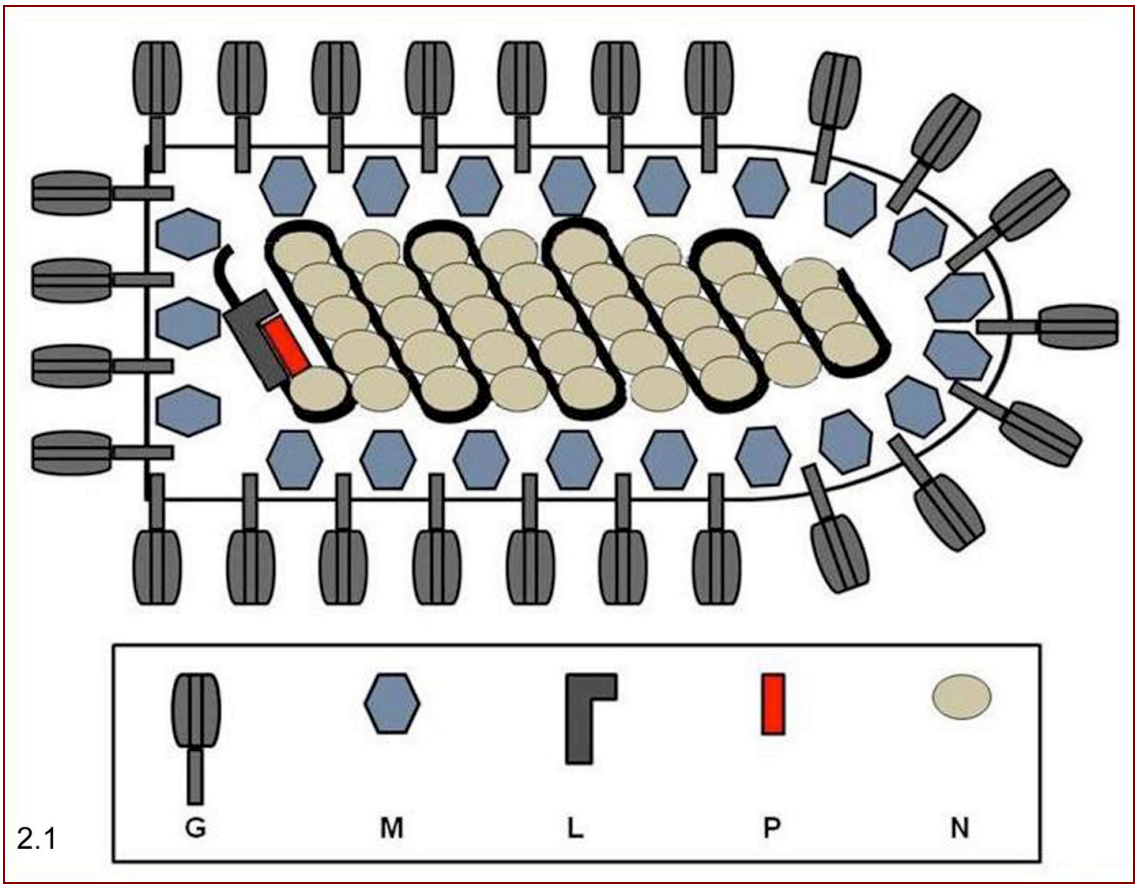

Fig. 2.1: Adapted schematic structure of VSV.[1] Note nucleocapsid core containing the single-stranded RNA, the N protein and the complex L and P protein. The envelope contains the transmembrane the G protein and the M protein in its inner surface.

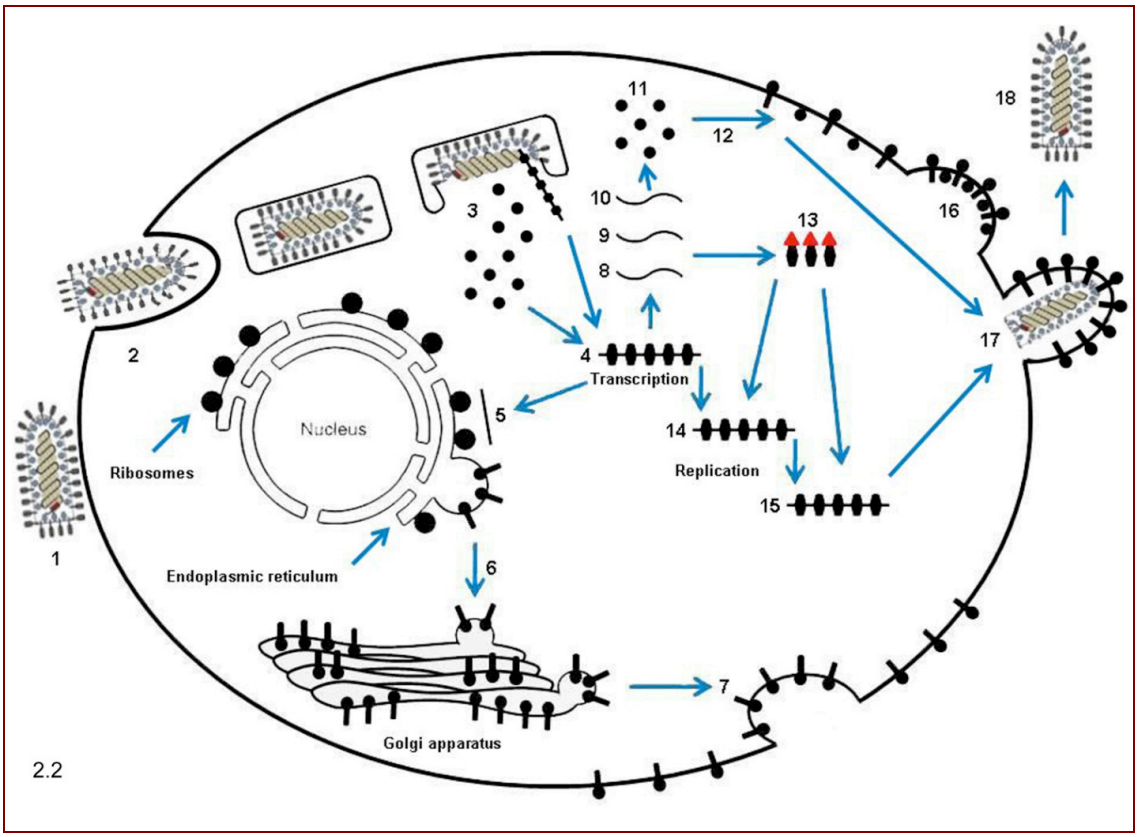

Fig. 2.2: Adapted diagram of the life cycle of VSV.[1] 1 – Binding to cell surface; 2 – Endocytosis; 3 – Fusion and uncoating; 4 – Transcription from (-) genomic RNA; 5 – G mRNA; 6 - Synthesis and glycosylation of G protein; 7 – Delivery of G protein to plasma membrane; 8 – N mRNA; 9 – P mRNA; 10 - M mRNA; 11 – M protein; 12 – M protein migration to the inner plasmatic membrane; 13 – N:P complex; 14 – (+) Replicate intermediate; 15 – (-) Progeny genome; 16 – Formation of bud site; 17 – Budding, and 18 – Progeny virion.

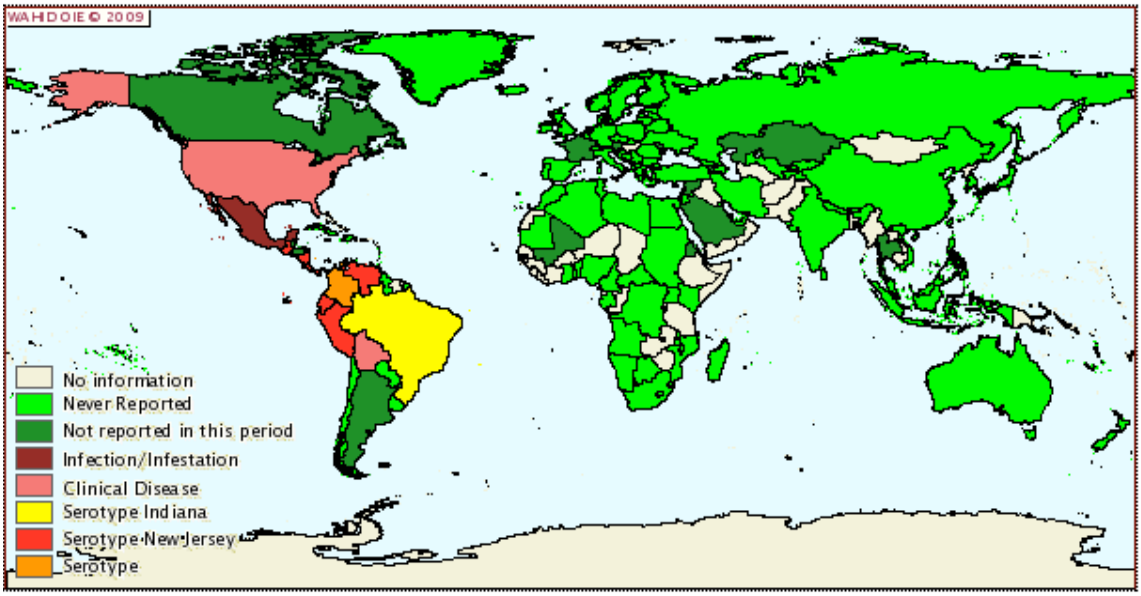

Fig. 2.3: Geographical distribution of vesicular stomatitis virus according to the World Organization for Animal Health (OIE) for the period of January to June of 2006.[62]

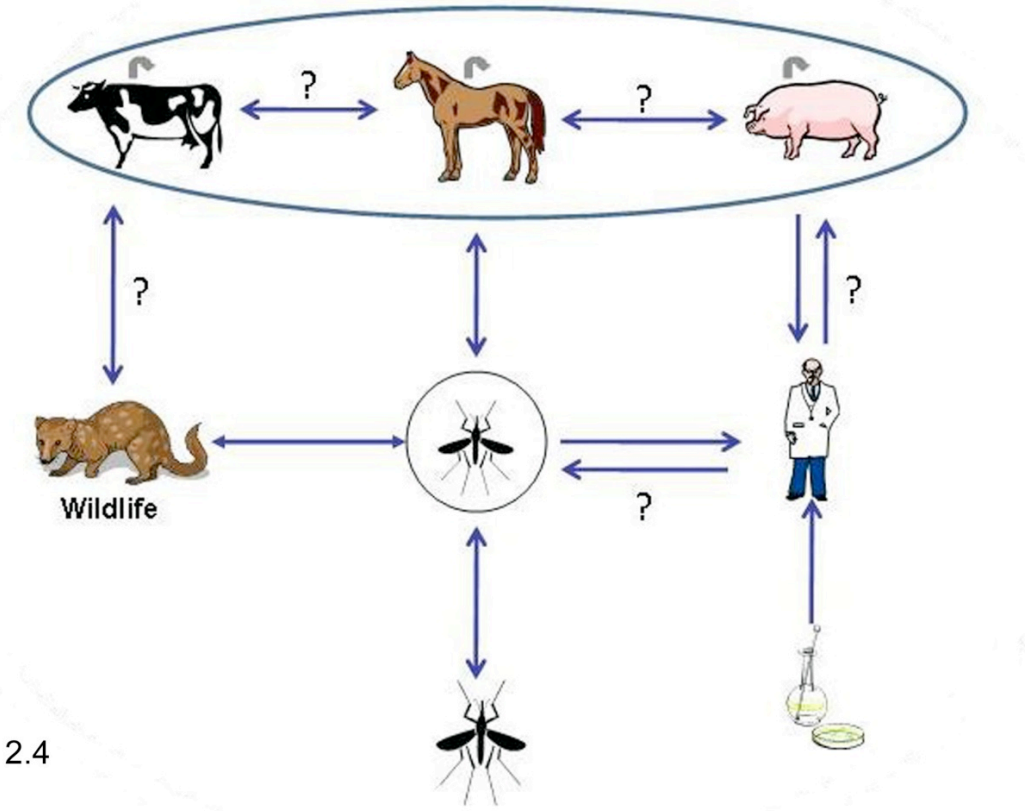

Fig. 2.4: Proposed natural cycle of vesicular stomatitis viruses based on current scientific information. Arrows indicate direction of virus transmission. Question marks indicate transmission routes presumed to occur but not documented in current literature.

CHAPTER 3

LESION DEVELOPMENT AND REPLICATION KINETICS DURING EARLY INFECTION IN CATTLE INOCULATED WITH VESICULAR STOMATITIS NEW JERSEY VIRUS VIA SCARIFICATION AND BLACK FLY (SIMULIUM VITTATUM) BITE.

J.L. Reis Jr., L.L. Rodriguez, D.G. Mead, G. Smoliga, and C.C. Brown. *Vet. Path*, published online first (ahead of print) in September 21, 2010.

ABSTRACT

Vesicular stomatitis viruses are the causative agents of vesicular stomatitis, an economically important contagious disease of livestock that occurs in North, Central and South America. Little is known regarding the early stages of infection in natural hosts. Twelve adult Holstein steers were inoculated with *Vesicular stomatitis New Jersey virus* (VSNJV) on the coronary bands (CB) of the feet via scarification (SC) or by VSNJV-infected black fly *(Simulium vittatum)* bite (FB). Three additional animals were inoculated on the neck skin using FB. Clinical disease and lesion development were assessed daily and animals were euthanized from 12 hours postinfection (HPI) through 120 HPI. The animals inoculated in the neck failed to develop any clinical signs or gross lesions and VSNJV was detected neither by *in situ* hybridization (ISH) nor by immunohistochemistry (IHC). Lesions on the CB were more severe in the animals infected by FB than by SC. In both groups, peak VSNJV replication occurred between 24 and 48 HPI in keratinocytes of the CB, as evidenced by ISH and IHC. There was evidence of viral replication limited to the first 24 HPI in the local draining lymph nodes, as seen through ISH. Successful infection via FB required logarithmically less virus than with the SC technique, suggesting that components in black fly saliva may facilitate VSNJV transmission and infection in cattle. The lack of lesion development in the neck with the same method of inoculation used in the CB suggests that specific characteristics of the CB epithelium may facilitate VSNJV infection.

Key words: Vesicular stomatitis virus, livestock, pathogenesis, early infection, black fly.

INTRODUCTION

Vesicular stomatitis New Jersey virus (VSNJV) is an arthropod-borne virus (arbovirus) in the family *Rhabdoviridae*, genus *Vesiculovirus*. Geographic distribution of VSNJV is restricted to the Americas, occurring from South America to the western United States[1] and is one of the causative agents of vesicular stomatitis (VS), a contagious and vectorborne disease of livestock. Although VSNJV infects a wide range of species including wildlife,[2] laboratory and domestic animals,[1] the natural disease is only reported in cattle, pigs, and horses. The disease is clinically indistinguishable from other viral vesicular diseases, especially foot-and-mouth disease when cattle and pigs are affected.

Vesicular stomatitis is characterized by fever from 48 to 72 hours post-inoculation (HPI) with noticeable vesicle formation between 48 and 72 HPI. Vesicles can occur in the oral cavity (dorsal tongue epithelium, hard palate and gums), muzzle, snout, lip, coronary bands (CB) of the feet, teats and prepuce.[1] Vesicles can rupture with consequent viral shedding and potential animal-to-animal contact transmission.[3] Lesions tend to heal around the second week after vesicle formation. Transmission also occurs by actively infected biting insects, such as black flies (*Simulium* spp.),[4] sand flies (*Lutzomyia* spp.)[5] and biting midges (*Culicoides* spp.).[6] VSNJV has been isolated from non-biting insects during disease outbreaks[7] and it is believed they might play a role in mechanical transmission of the virus during epizootics.

The detailed pathogenesis of VSNJV infection is unclear. Clinical disease can be consistently reproduced experimentally by application of at least 10^7 $TCID_{50}$/animal to

scarified regions where lesions naturally occur.[8] When VSNJV is inoculated in haired skin of the neck,[8] flank,[8] or ear,[9] lesions do not occur but animals may seroconvert and/or shed virus through the tonsils (subclinical infection). Even though no lesions develop in those haired skin sites, it has been demonstrated that there can be limited viral replication.[10] Also, noninfected black flies physically separated from VSNJV-infected black flies became infected while co-feeding at these sites on a nonviremic host.[11] However, none of the previous studies used ISH or IHC to detect virus in those areas.

Compared to inoculation via scarification (SC), feeding of VSNJV-infected black flies at sites where vesicle formation normally occurs will reproduce the disease,[4] although the amount of virus contained within the feeding flies is far less than what is required to reproduce disease via SC.[8] Previous studies investigating the pathogenesis of VSNJV in experimentally infected livestock have focused on the clinical course and late stages of the disease[12, 13] following infection via SC or intradermal injection. However, the early events of experimental or natural VSNJV infection in livestock, including virus replication kinetics and distribution in tissues have not been documented.

The present study was designed to sequentially assess and describe lesion development, virus distribution and replication kinetics in cattle tissues during the early phases of VSNJV infection. Additional objectives were to compare insect bite transmission (FB) to direct inoculation (SC), and to assess the presence of virus inoculated in susceptible versus non-susceptible regions of skin via FB.

MATERIALS AND METHODS

All experimental infections and all methods utilizing non-fixed samples were conducted at the USDA-ARS Plum Island Animal Disease Center under biosafely level (BSL)-3Ag conditions. Tissues fixed in formalin were transferred to the Department of Pathology at the University of Georgia, for histological processing, immunohistochemistry and *in situ* hybridization studies.

The virus utilized was a VSNJV field strain (95COB) isolated from a cow during an outbreak in Colorado, USA, in 1995. This virus was responsible for a large VS outbreak in 1995 and has been used previously to successfully reproduce clinical disease in cattle.[8]

Fifteen 150-250 kg Holstein steers were used in this experiment. Scarification inoculation was done on seven animals, six scarified with virus and one with cell culture media only. Eight animals were inoculated by FB – seven with infected flies and one with noninfected flies. Three steers had flies feed on the neck skin whereas for the other five FB animals, inoculation was on the CB. All animals had hair from the area of inoculation removed with clippers followed by shaving 24 hours prior to the inoculation time. They were sedated with an intramuscular injection of 0.3 mg/kg of xylazine prior to the inoculation, and sedation was reversed after inoculation by intravenous injection of 2 mg/kg of tolazoline.

Inoculation by scarification

A plastic bifurcated needle (Duotip Tests, Lincoln Diagnostics, Decatur IL) served as the inoculating tool and was used as previously described.[8] The line of scarification was approximately 8 cm long and located on the lateral aspect of the limbs, on the haired skin of the CB immediately proximal to the thick non-haired skin (Fig. 3.1). The inoculum contained 10^7 $TCID_{50}$ of VSVNJ in 50 µl of cell culture media with 1% antibiotic/antimycotic as previously described;[8] virus-free medium was used as the negative control. The scarification site was created with approximately 25 skin pricks, then the inoculum or virus-free medium was applied with a pipette. The procedure was repeated, with 25 additional skin pricks and application of additional medium. In experimental animals, the viral inoculum was applied to the right front and rear CB, while the left front and rear CB were scarified and treated with virus-free medium. In the mock-inoculated animal, the right front and rear CB were scarified and treated with virus-free medium, while the left front and rear CB were left intact. The mock- inoculated animal was euthanized at 24 HPI and the virus-infected animals were euthanized at 12, 24, 48, 72, 96 and 120 HPI.

Inoculation by fly bite

Inoculation by fly bite was done as in previous studies[10]. Briefly, black flies were intrathoracically injected, as previously described,[14] with approximately 1 µl containing

10^7 $TCID_{50}$ of VSNJV. Infected black flies were allowed to feed on the designated CB of four animals and on sites on the necks of three animals. For the CB animals, 30 caged VSNJV-infected black flies were allowed to feed for approximately 20 min at the lateral aspect of the right front and rear CB (Fig 3.2). Cages were placed in a position to allow flies to access either haired or glabrous CB skin. In general flies probed or bit in the haired skin, approximately 1 cm proximal to the thick glabrous CB skin. The same number of uninfected black flies was allowed to feed for approximately 20 min at the lateral aspect of the left front and rear CB. These four FB CB experimental animals were euthanized at 24, 48 and 72 and 96 HPI. One mock-inoculated animal was treated with uninfected black flies on the right CB, with no treatment at the left CB, and euthanized at 24 HPI. The same methodology was used in the group of the 3 neck-inoculated animals. Infected black flies were allowed to feed on the right side of the neck and uninfected black flies were allowed to feed on the left side of the neck. Of these three steers inoculated in the neck, one animal was euthanized at 24 HPI and two animals at 72 HPI.

Analysis of black flies

As a quality control for the fly bite inoculation, all flies were tested for the presence or absence of mammalian blood by SYBR-Green real-time PCR (RT-PCR) for mammalian DNA (Power SYBR Green PCR Master Mix ABI #4367659) after feeding on cattle. Briefly, nucleic acid was extracted from each individual fly as described below for tissues. RT-PCR was conducted using primers designed specifically to target the 16s

mammalian rDNA gene. Forward primer sequence: 5' CCTGTTTACCAAAAACATCAC 3', reverse primer sequence: 5' AYTGTCGATAKGRACTCTWRARTAG 3' (Invitrogen, Carlsbad CA, USA). Primers were used at a final concentration of 300 nM per 25 µl reaction. The reaction was performed using 8 µl (per 25 µl reaction) of Power SYBR Green PCR Master Mix (Applied Biosystems #4367659). The amount of nucleic acid template was 2.5 µl per reaction, which was performed on an ABI 7000 sequence detection system using the following cycling conditions: 94°C for 2.5 min, and 40 cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 60 sec, and a final 72°C hold for 5 min. After the RT-PCR, a melt curve was performed to detect the Tm of SYBR Green-bound DNA products. Products with a Tm of approximately 80°C were considered positive for mammalian DNA.

The blood meal analysis was done in all flies used in this study and determined that approximately 20% of the flies (which corresponds to about 6 flies) fed during the period of mock and viral inoculation (data not shown).

Samples

Samples collected immediately after euthanasia included lateral aspect of all four CB and potential lesions extending from the original site of the inoculation, right and left neck skin, and local draining lymph nodes (LN) including prescapular, axillary, popliteal, and prefemoral. In addition, spleen, tongue and tonsils were also collected. Approximately 30 mg of collected tissues were quick-frozen in liquid nitrogen for later

transfer to -70^{0}C for virus isolation (VI) and reverse transcriptase RT-PCR (rRT-PCR) for detection of live virus and viral nucleic acid respectively. Additionally, serum was collected just prior to euthanasia for VI and rRT-PCR. Sections <1 cm thick of the remaining tissues were fixed in a 10% buffered formalin solution, for histopathology, immunohistochemistry and *in situ* hybridization. The whole neck and CB skin at and around fly cage location and scarified areas were collected and trimmed for histopathologic evaluation. For each of these sites, there were four tissue cassettes collected, each with three samples of epidermis. All four HE-stained slides from these blocks (equaling at least twelve sections from each site) were examined and a minimum of two slides (six sections) were selected and corresponding sections analyzed by ISH and IHC. A semi-quantitative score was attributed to the ISH and IHC slides: negative (N) was assigned for no signal; + for minimal to mild or faint signal; ++ for a moderately discrete but localized signal, and +++ for a prominent and diffuse signal.

Real time reverse transcriptase PCR (rRT-PCR)

Tissues were thawed and immediately macerated by adding two 5 mm stainless steel beads (Qiagen, Catalog No. 69989) and 0.9 ml of MEM-25mM HEPES, and shaken in a TissueLyser bead beater (Qiagen) for 2 min at a frequency of 22/s. After maceration, 50 µl of sample were transferred to 96-well plates (King Fisher No. 97002540) containing 150 µl lysis/binding solution. RNA was then extracted using Ambion's MagMax-96 Viral RNA Isolation Kit (Ambion, Catalog No. 1836) on a King Fisher-96

Magnetic Particle Processor (Thermo Electron Corp.). Briefly, after the initial 5 min lysis/binding step, the RNA sample underwent a series of four washing steps, a drying step, and a final elution. RNA was eluted in a final volume of 25 µl. At each of the above steps, RNA was magnetically bound to the beads contained in the lysis/binding solution and was transferred to the different extraction solutions. The extracted RNA was analyzed by rRT-PCR using 2.5 µl of RNA on the ABI 7000 as described below. The remaining macerated tissue was clarified (1000 g for 2 min at 4°C) and the supernatant was cleared of possible bacterial contamination using centrifuge tube filters (Spin-X, Costar). Samples were then stored at -70°C for VI. The rRT-PCR was performed using primers and probe designed specifically for the nucleocapsid region of isolate used for the inoculations (95COB). Forward primer sequence was 5' GCACTTCCTGATGGGAAATCA 3', reverse primer sequence was 5' GGGAAGCCATTTATCATCCTCA 3' (Invitrogen, Carlsbad CA, USA), and a 6-Carboxyfluorescein (6-FAM)-labeled probe 5' ACCCTGACCGTTCTG 3' (Applied Biosystems, Foster City, CA, USA) was used. Primers were used at a final concentration of 300 nM, and probe used at 100 nM per 25 µl reaction. The reaction was performed using the Taqman EZ RT-PCR Core Reagents (Applied Biosystems #N808-0236) at the following volumes/concentrations per 25 µL reaction: 5 µl of 5x buffer, 3 µl of 25 mM Mn $(OAc)_2$, 3 µl dNTPs (combined 1:1:1:1 by volume), 1 µl rTth, 0.25 µl Amperase, and 9.7 µl H_20. The amount of RNA template was 2.5 µl per reaction, which was performed on an ABI 7000 sequence detection system using the following cycling conditions: 60°C for 25 min, 95°C for 2 min, and 40 cycles of 95°C for 10 sec, and 60°C for 1 min. Results were expressed as cycle threshold (CT) values. A CT value <40 was considered

positive for the presence of virus. Negative control consisted of all rRT-PCR reagents and water in place of template.

Virus Isolation (VI)

A 250 µl aliquot of the supernatant described above was placed in individual T-25 tissue-culture flasks containing a monolayer of VERO cells. The virus was allowed to adsorb for 1 h at 37°C on a rocker plate, followed by addition of 5 ml of maintenance media with 3 subsequent days of incubation at the same temperature. Flasks were examined under an inverted microscope to observe cytopathic effect (CPE) at 24, 48 and 72 HPI. After 72 HPI, CPE-positive samples were stored at -70°C for rRT-PCR and 250 µl supernatant from each CPE-negative sample was transferred to a new culture flask (second passage). After 72 HPI all flasks (CPE positive or negative) from the second passage and all CPE-positive materials from the first passage were processed for rRT-PCR.

Histopathology and immunohistochemistry

All formalin fixed samples were routinely embedded in paraffin, cut at 3-4 µm thickness, stained with HE for histological examination to assess inflammatory reaction, extent of microvesicle formation, and any other pathologic changes.

For immunohistochemistry (IHC), deparaffinized sections were subjected to antigen retrieval with 80 μg/ml of proteinase K for 15 min at 37°C, followed by blocking of non-specific epitopes. Then samples were incubated overnight at 4°C with primary antibody, anti-VSNJV polyclonal, a mouse hyperimmune ascitic fluid (kindly provided by Dr. Robert Tesh, Department of Pathology, Medical Branch, University of Texas, Galveston), at a dilution of 1:1800. Primary antibody was followed by incubation with an alkaline phosphatase-linked polymer system (LabVision). The reaction was revealed using Vector Red chromogen. The slides were lightly counterstained with Mayer's hematoxylin then cover-slipped using Permount for permanent record. For each IHC protocol anti-VSV antibody was applied to a left (mock-inoculated side) CB or neck skin and popliteal or prefemoral lymph nodes for negative control. Additionally each IHC protocol had a slide containing a section of CB skin with a recognizable histological vesicular lesion or a lymph node draining a CB with lesion. For these sections normal mouse serum was used instead of the anti-VSV antibody. Selected lymph node sections were additionally subjected to an IHC protocol to highlight dendritic cells, using the same procedure outlined above, except that the primary antibody was anti-CD1b(monoclonal ascites fluid antibody - VMRD catalog number TH97A) at a dilution of 1:100.

***In situ* hybridization (ISH)**

Negative sense riboprobes were used in order to detect positive-stranded (replication) viral RNA. The riboprobes consisted of 410 bases corresponding to the

nucleoprotein (N) gene using methods previously described.[15] The following primers were used to produce the N gene construct, 5'-GAA GAT GGT CTT GAC TTC TTT G-3' (forward) and 5'-CGA GTT GAT CTT AGC AAG AGT G-3' (reverse). The N gene segment was cloned and amplified in a pGEM-4Z vector (Promega, Madson, WI). The vector was digested with BglII restriction enzyme to create a template for the negative sense probe, and digoxigenin-labeled nucleotides were added during transcription of the probe. Incorporation of the digoxigenin was verified by dot blot. Sections from tissues that were positive either on VI or on rRT-PCR were processed for ISH as previously described[16]. Tissue sections were treated with 100 μg/ml of proteinase K solution then incubated with the negative sense VSNJV riboprobe (corresponding to the N gene) at 42°C for 12 h, followed by incubation with anti-digoxigenin conjugated to alkaline phosphatase (1:300). Negative controls included noninfected tissues. The reaction was visualized using tetrazolium-based (NBT-BCIP) chromogen. The slides were lightly counterstained with Mayer's hematoxylin then cover-slipped with Permount for permanent record.

RESULTS

Animals inoculated by FB on the neck

None of the animals inoculated in the neck skin developed fever or any other clinical sign of disease. They had only multiple small (approximately 1 mm in diameter)

focal areas of hyperemia at the site of FB in both right and left (infected and uninfected FB) neck skin.

Histologically, the superficial dermis had minimal to mild foci of inflammatory infiltrates in the right and left neck skin of all three steers. Dermal infiltrates were slightly more severe in the animal euthanized at 24 HPI, and were composed of small numbers of macrophages with fewer neutrophils. There were no lesions in the epidermis, except for the animal euthanized at 24 HPI that had scant small (approximately 0.5 mm in diameter) focal areas of epidermal necrosis within the FB region in both right and left sides.

There were very limited positive results by any of the techniques used to detect virus in these tissues. Inoculation site skin in the 24 HPI animal was positive by both VI and rRT-PCR on the right (infected) side. At 72 HPI, one animal out of the two euthanized animals at this time had inoculated neck skin positive on rRT-PCR only, with no viral detection with VI, ISH or IHC. At no time were the tissues of any of these three animals positive by IHC or ISH, nor any evidence of epidermal disruption seen on HE. All other tissues as well as serum collected from these animals were consistently negative for the presence of virus by all techniques (data not shown).

Clinical and gross findings in animals inoculated in CB

All experimentally infected animals in this group developed fever (over 39.2°C) and mild depression between 24 and 48 HPI. No other clinical abnormalities were noted.

In the case of the SC-inoculated animals, at 12 HPI, the only lesion was a mild hyperemia along the line of inoculation. Subtle gross lesions, including mild edema and hyperemia, were present at the site of inoculation at 24 HPI in both SC- and FB-inoculated animals. At 48 HPI there was moderate edema and hyperemia restricted to the site of SC. However, the FB-inoculated steer euthanized at the same time had much more prominent hyperemia and swelling of the right (infected) rear CB, with a few 1 to 2 cm diameter, dark and circumscribed areas of ulcerations that coincided with the sites where the flies were feeding. In addition, in a region beyond where the cage was positioned, in the right rear interdigital space, this animal had marked vesicular formation, characterized by a focally extensive white and elevated area that exuded serous fluid when cut. No gross lesions were present at 48 HPI in the right (infected) front FB-inoculated CB, and analysis of fly blood meals revealed that no flies fed on this foot. At 72 and 96 HPI, the right CB infected by SC had moderate hyperemia and edema, multifocal areas of hemorrhage and multiple 2 to 3 mm ulcerations corresponding to zones of SC. A serous fluid frequently oozed from the small ulcers. However, at the same time points (72 and 96 HPI) the lesions in the FB-inoculated animals were more severe, with diffuse edema, hyperemia and a few dark 2 to 3 cm diameter ulcerated areas corresponding to where fly cages were positioned at the time of inoculation. In these FB-inoculated animals, lesions extended far beyond the site of FB with vesicles reaching the interdigital space. At 120 HPI, the SC lesions were similar to or slightly more severe than those seen in the 96 HPI SC steer. In the mock-inoculated animals CBs developed a mild hyperemia at the site of SC or FB at 24 HPI and occasionally at 48 HPI. The 96 HPI FB and the 120 HPI SC had moderately to markedly enlarged popliteal and prescapular LNs.

Histological findings in animals inoculated in CB

There was only one SC-inoculated steer that was euthanized at 12 HPI. In this animal in both right and left (virus- and mock-inoculated respectively) CB, small areas of serocellular crusting with underlying epidermal disruption developed, characterized by poorly defined focal eosinophilic areas with loss of definition of cellular borders in the upper strata spinosum and granulosum. There was focal loss of the epidermis at the areas of scarification (Fig. 3.3a). There was mild to moderate focal neutrophilic exocytosis in the epidermis. The superficial dermis had focal perivascular infiltration of numerous neutrophils and fewer macrophages.

At 24 HPI, both the SC- and FB-inoculated animals presented large coalescing areas of mild to moderate intercellular edema (spongiosis) with stretching of intercellular bridges and shrunken keratinocytes in the deep stratum spinosum (Fig. 3.4a). Cells from the strata basale and granulosum were consistently spared at this time. There was mild neutrophilic exocytosis throughout the affected epidermis, with moderate numbers of neutrophils and macrophages around perivascular areas of the superficial dermis.

At 48 HPI in the SC-inoculated animal, the narrow zone of inoculated haired CB skin had necrosis of epithelium of the epidermis and hair follicles with accompanying neutrophilic infiltration, the thicker nonhaired skin closer to the hoof had severe intercellular edema and hypereosinophilic, pyknotic and shrunken keratinocytes limited to the stratum spinosum (Fig. 3.5a). Multifocal areas of this thick glabrous skin formed cavitations partially filled with numerous neutrophils, fewer macrophages, cell debris and acantholytic cells. Similar to what was observed at 24 HPI, the strata basale and

granulosum were consistently spared. The superficial dermis had moderate perivascular to interstitial infiltration of numerous macrophages and neutrophils, with fewer lymphocytes.

The FB-inoculated steer at 48 HPI had minimal lesions in the right front CB whereas the right rear CB had severe epidermal disruption affecting a much larger zone of haired skin. These lesions were more severe than those observed in the SC-inoculated animal at this time. Hair follicles were frequently effaced by marked epidermal necrosis and accumulation of degenerated neutrophils. In the nonhaired thick skin (located immediately distal the haired skin but not in contact with the flies), there were extensive areas of full-thickness necrosis from the stratum basale to the stratum granulosum, with much greater damage than what was seen in the SC-inoculated animal at this time point. Within the stratum spinosum were multiple large coalescing cavitations filled with numerous neutrophils, few macrophages, cell debris and occasional acantholytic cells. There was cleft formation at the stratum basale, with separation of this layer from the stratum spinosum. The dermis had moderate to severe perivascular to interstitial infiltration of numerous macrophages and neutrophils, with fewer lymphocytes and infrequent mast cells.

By 72 HPI, there was extensive necrosis of the thick nonhaired skin at the CB of the SC-inoculated animal, characterized by large pale eosinophilic areas, with loss of cellular detail (Fig. 3.6a). Areas of necrosis were centered in the stratum spinosum, spared the stratum basale, and extended to but did not efface the stratum granulosum. There were numerous neutrophils infiltrating through the intercellular spaces around necrotic keratinocytes and filling areas where there was loss of keratinocytes. The dermis

had moderate to severe perivascular to interstitial infiltration of numerous macrophages and neutrophils, with fewer lymphocytes. The animal inoculated by FB had similar lesions to those observed in the FB-inoculated steer at 48 HPI, with more extensive areas of necrosis and loss of epidermis.

At 96 HPI, epidermal lesions in the animals inoculated by either method were similar. There was full thickness necrosis, cleft formations at the level under the stratum basale, with frequent loss of the entire epidermis of the CB (ulceration).

At 120 HPI, lesions were similar to those observed at 96 HPI, but with evidence of initial epidermal regeneration, in which the basal layer contained numerous and prominent digitiform projections composed of hyperplastic epithelial cells extending into the dermis.

The lymph nodes draining the right (inoculated) CBs presented with nonspecific reactive changes. No difference was observed between SC- and FB-inoculated animals. Mild paracortical hyperplasia started at 24 HPI, with gradual development of more paracortical and also medullary cord hyperplasia from 72 to 120 HPI.

Detection of virus in animals inoculated in the coronary bands

All results from animals inoculated in the CBs are shown in Table 1. Detection of VSNJV by VI and rRT-PCR was restricted to the site of inoculation and draining LNs. All samples from mock-inoculated animals, and left mock-inoculated CB (on the left side), tongue, tonsil, spleen and serum from experimental animals were consistently

negative by these techniques in all animals using either method of inoculation. For the right CBs (inoculated side), positive VI and low rRT-PCR cycle threshold (CT) values were positive at all times post-inoculation, from 12 to 120 HPI, in both FB- and SC-inoculated groups of animals. However, the right front CB from a steer sampled at 48 HPI, where inoculation with infected black flies was attempted, was negative on VI, and only weakly positive (high CT value) on the rRT-PCR. Flies from this site were negative for mammalian DNA, indicating that flies fed very poorly, if at all. For the draining lymph nodes of inoculated animals, live virus was isolated as early as 12 HPI in a scarified animal and no later than 72 HPI in both animal groups. The rRT-PCR results from the draining lymph nodes tended to have much higher CT values (lesser amount of virus) than the inoculated CBs.

In situ hybridization for animals inoculated in CB

Using the negative sense probe which is complementary to the viral mRNA and antigenome strand (which are both sense/positive strands) for the N gene resulted in segmentally diffuse staining of the keratinocyte cytoplasm in the inoculated CBs. The SC-inoculated steer at 12 HPI, which had ISH signal from the stratum basale extending up into the mid stratum spinosum (Fig. 3.3b). Signal distribution and intensity varied from darkly distinct small foci at 12 HPI to extensive coalescing large areas at 24 HPI (Fig. 3.4b) for both methods of inoculation. The signal had the same intensity at 48 HPI, but the positive keratinocytes were scattered within (Fig. 3.5b) or located around the

periphery of necrotic areas (vesicle) for both SC and FB animals. At 72 HPI, both SC- and FB-inoculated animals had positive keratinocytes with fainter but diffuse signal throughout the cytoplasm (Fig. 3.6b). When comparing the two methods of inoculation at 48 HPI, the location of ISH labeling was distinct between SC- (Fig. 3.7) and FB-inoculated (Fig. 3.8) animals. For both, the ISH signal was scattered within the stratum spinosum and restricted to the CB epithelium. However, in the FB-inoculated steer, ISH signal was deeper, occurring in the stratum basale of the CB and expanding beyond the fly feeding area into the interdigital region, with cleft formation under the level of the stratum basale of both CB and interdigital skin (Fig. 3.8). ISH-positive keratinocytes were present throughout the necrotic epidermis. At 96 HPI, SC- and FB-inoculated animals had faint diffuse signal in the cytoplasm of keratinocytes, as did the SC-inoculated animal at 120 HPI. Positivity was multifocal and located more frequently at the periphery of necrotic areas.

Use of a negative sense probe to detect replicating virus in the draining LNs highlighted scattered large cells with abundant cytoplasm, morphology suggestive of dendritic cells or macrophages, in subcapsular areas of cortex and paracortex regions at 24 HPI only in both SC- and FB-inoculated animals (Fig. 3.9).

Immunohistochemistry in animals inoculated in the coronary bands

There was signal representing viral protein in the cytoplasm and in the plasma membrane of keratinocytes in the inoculated CB. Signal was consistently restricted to the

epidermis. Epidermal location of the signal followed in a similar fashion with the development of the histological lesion described above, except for the 12 HPI scarified steer, which had IHC signal from the stratum basale up through the mid stratum spinosum (similar to the ISH staining). The IHC signal was present at later time points than ISH signal but was otherwise similar in the epidermis, but had many similarities to that seen with negative sense riboprobe ISH. That is, staining intensity varied from focal and intense, being restricted to small foci at 12 HPI (Fig. 3.3c) through to more extensive, affecting large coalescing areas from 24 until 72 HPI (Figs. 3.4c, 3.5c, and 3.6c), after which it became fainter and more localized at 96 and 120 HPI. However, the patterns of IHC and ISH diverged in the lymph nodes. In contrast to what was observed with ISH, there was IHC positivity until 120 HPI in the draining lymph nodes, with positive cells in the cortical subcapsular regions at 24 HPI, and in perifollicular and follicular regions at 48, 72 (Fig. 3.10), 96 and 120 HPI. On sections of 24 HPI lymph nodes, IHC specific for dendritic cells (anti-CD1b antibody) highlighted the same types of cells in the same areas as were positive by ISH at 24 HPI (data not shown).

DISCUSSION

In this study, acute clinical disease and typical lesion development was successfully reproduced in cattle experimentally infected with VSNJV by application of virus to a scarified region on the CB, and by feeding of VSNJV-infected black flies at the CB. The kinetics of viral replication and dissemination that occur during acute stages of

VSNJV infection were analyzed using IHC for detection of viral protein, VI, rRT-PCR, and ISH for detection of viral replication (negative sense riboprobe ISH). Intense ISH signal was observed in CB keratinocytes from 24 to 48 HPI using the negative-sense riboprobe, which recognizes mRNA and replicative intermediate RNA, indicating that viral replication is taking place in these cells. In the same tissues, IHC labeling of viral proteins was observed beginning at 12 HPI and increasing through 72 HPI, with the signal becoming more localized and faint from 96 to 120 HPI. Therefore, these results demonstrate for the first time that the *in vivo* peak of viral replication occurs between 24 and 48 HPI and demonstrate clearly that the keratinocytes from the CB are permissive cells for early viral replication. The fact that the intensity of IHC signal persists until 72 HPI, with faint ISH labeling at that time is likely associated with the natural cycle of viral replication, with virus replicating early, and viral protein remaining in the cells longer. The presence of virus and therefore potential viral shedding from CBs persisted to the end of the experiment, 120HPI, as shown by VI and rRT-PCR.

In the local draining lymph nodes of animals inoculated in the CBs, whether cattle were infected by SC or FB, replicating VSNJV was detected for the first time via ISH (negative sense riboprobe), and was restricted to the 24 HPI time point. ISH signal was scattered and distributed predominantly in the subcapsular areas within individual cells with morphology suggestive of macrophages or dendritic cells. On adjacent sections anti-CD1b antibody highlighted cells with similar morphology and distribution of those containing replicating virus at 24 HPI. Morphologic features and anti-CD1b labeling in subcapsular sinuses strongly suggests that these cells are dentritic cells. With the negative sense riboprobe, any positive signal represents the presence of positive sense

RNA, which would be either a replicative intermediate of the virus or viral mRNA. In either case positive ISH signal is an indication of active viral replication in the lymph node. In the LNs, live virus or its nucleic acid was detectable by VI and/or rRT-PCR for up to 72 or 120 HPI respectively. However, neither of these parameters provides definitive evidence that the virus is replicating there, as virus could be passively carried to the lymph node. So this study is the first to show VSV replication beyond the site of the vesicle in cattle.

Both SC and FB inoculation methods resulted in disease when introduced at the CB, and the character and time course of infection had some definite similarities. However, there were also notable differences, with more severe and extensive gross and histological lesions in the FB-inoculated animals.

Feeding of infected black flies at the CBs could reproduce the disease, although the amount of virus contained within the total FB inoculum is far less than with SC. Previous pilot studies demonstrated that inoculation of virus via SC with smaller amounts than the 10^7 $TCID_{50}$/animal, as used in this study, did not consistently produce clinical disease in cattle (L. Rodriguez, personal communication) or pigs.[17] The amount of VSNJV shed in saliva when a black fly is probing and blood feeding is not known; however, in previous studies that used an in vitro method to collect fly saliva,[12] VSNJV titers between $10^{1.5}$ and $10^{2.34}$ plaque forming units (pfu)/ml were found in the saliva of black flies infected in the same manner as in our experiment. Flies probably cannot inoculate much more than 10-30 µl of saliva each. In this experiment blood meal analysis revealed that only 6 flies out of 30 per cage participated in feeding. Consequently the total amount of inoculated fly saliva is very low. Although it is

technically not feasible to quantitatively compare pfu with $TCID_{50}$ it would seem that a total dose of less than 200 pfu is considerably less than 10^7 $TCID_{50}$. Even with this lower dose, the lesion severity increased earlier and more rapidly in the FB compared to the SC animals. Therefore, it appears that infection by FB can be successful with markedly decreased amounts of virus, compared to SC, which suggests that there may be a facilitating factor supplied by the insect inoculation.

In this study, we also inoculated virus into neck skin using FB and examined the tissues using VI, HE, IHC, and ISH. Previous VSNJV studies in cattle[8] with SC of the CBs and flank skin could produce lesions only in the inoculated CBs, with no lesion development in the flank or in any other tissue.[8] However, it has been shown that noninfected black flies physically separated from VSNJV-infected black flies became infected while co-feeding at these non-vesicular sites on the same nonviremic host.[11] Our findings confirmed the lack of gross lesion development and demonstrated no histological viral-associated lesions. In addition, none of the multiple examined sections were positive either by IHC or ISH, although there was limited positivity using VI and rRT-PCR. Therefore, presence of the virus in this type of tissue is probably short and transient, although enough to be transmitted to other flies, with no resultant vesicle development or detectable virus by ISH or IHC.

Lesions developed only when the inoculation was in the CB and were restricted to the site of inoculation (CB), with viral replication, and dissemination to the draining lymph node. These data indicate that the characteristics of the epithelium may well play a pivotal role in susceptibility to the virus infection.

The epidermis of the CBs as well as the epidermis of other areas where vesicular lesions develop, such as oral cavity (tongue, hard palate and gums), muzzle, teats and prepuce share similar histological and physiological features. These areas are unhaired, have thicker epidermis with a prominent stratum spinosum and are more subjected to mechanical stress. These areas require higher amounts and more specialized intercellular adhesion molecules for proper tissue cohesiveness. In contrast, the neck and the flank skin are haired and have a markedly thinner epidermis compared to those areas where VSNJV replicates and forms vesicles, with less mechanical stress and therefore decreased concentrations of intercellular bridges. Comparing the morphophysiological features of the anatomical locations where VSNJV lesions develop to those sites where vesicles do not form, it seems plausible that the virus uses either cell membrane receptors (perhaps intercellular junction molecules) for viral entry and/or uses part of specialized cell pathways, for viral replication.

In summary, our study demonstrates that VSNJV replicates successfully and extensively in the keratinocytes from specific anatomical locations with thick unhaired skin. Vesicular lesions were limited to the site of inoculation with peak of viral replication between 24 and 48 HPI in the CB. Replicating virus was detected in draining lymph nodes but only at 24 HPI. Lesion development was markedly more severe and extensive when the virus was transmitted via fly bite which suggests that components in the saliva facilitate VSNJV infection.

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Table 3.1: Detection of VSNJV by virus isolation (VI), reverse transcriptase real time PCR (rRT-PCR), *in situ* hybridization (ISH) and immunohistochemistry (IHC) in steers inoculated by scarification or fly bite in the coronary bands (CB) and euthanized at different times post-inoculation (HPI).

	Time	12 HPI				24 HPI				48 HPI				72 HPI				96 HPI				120 HPI			
	Tissue	VI	rRT-PCR	ISH	IHC	VI	rRT-PCR	ISH	IHC	VI	rRT-PCR	ISH	IHC	VI	rRT-PCR	ISH	IHC	VI	rRT-PCR	ISH	IHC	VI	rRT-PCR	ISH	IHC
SCARIFICATION	Right front CB	P	29	+++	+++	P	21	+++	+++	P	17	++	++	P	23	+	++	P	16	N	++	P	18	N	+
	Right axillary	N	0	N	N	N	33	+	+	P	33	N	+	N	0	N	N	N	0	N	N	N	36	N	N
	Right prescapular	P	37	N	N	P	39	N	++	P	28	N	+	N	0	N	N	N	34	N	N	N	33	N	+++
	Right rear CB	P	23	+++	+++	P	25	+++	+++	P	17	++	++	P	17	+	++	P	16	+	N	P	18	+	+
	Right popliteal	P	28	N	N	P	30	++	++	N	0	N	+	P	22	N	++	N	31	N	++	N	34	N	+++
	Right prefemoral	N	0	N	N	N	0	N	N	N	0	N	N	N	0	N	N	N	0	N	+	N	0	N	N
	Right prefemoral	N	0	N	N	N	0	N	N	N	0	N	N	N	0	N	N	N	0	N	+	N	0	N	N
FLY BITE	Right front CB	NA	NA	NA	NA	P	29	+++	+++	N	33	N	+++	P	36	N	N	P	19	N	+	NA	NA	NA	NA
	Right axillary	NA	NA	NA	NA	N	0	N	N	N	0	N	N	N	39	N	N	N	37	N	N	NA	NA	NA	NA
	Right prescapular	NA	NA	NA	NA	P	33	+	++	N	0	N	N	N	0	N	N	N	28	N	+++	NA	NA	NA	NA
	Right rear CB	NA	NA	NA	NA	P	29	+++	+++	P	16	+++	+++	P	18	+	+	P	19	+	+	NA	NA	NA	NA
	Right popliteal	NA	NA	NA	NA	P	34	+++	+++	P	32	N	+++	P	39	N	N	N	33	N	+++	NA	NA	NA	NA
	Right prefemoral	NA	NA	NA	NA	P	33	+	N	N	0	N	N	N	0	N	N	N	0	N	N	NA	NA	NA	NA
	Right prefemoral	NA	NA	NA	NA	P	33	+	N	N	0	N	N	N	0	N	N	N	0	N	N	NA	NA	NA	NA

P = positive; N = negative; NA = Not applicable (there were no animals in those time points); NP = not processed. rRT-PCR results expressed as cycle threshold value (CT value < 40 was considered positive for the presence of virus.

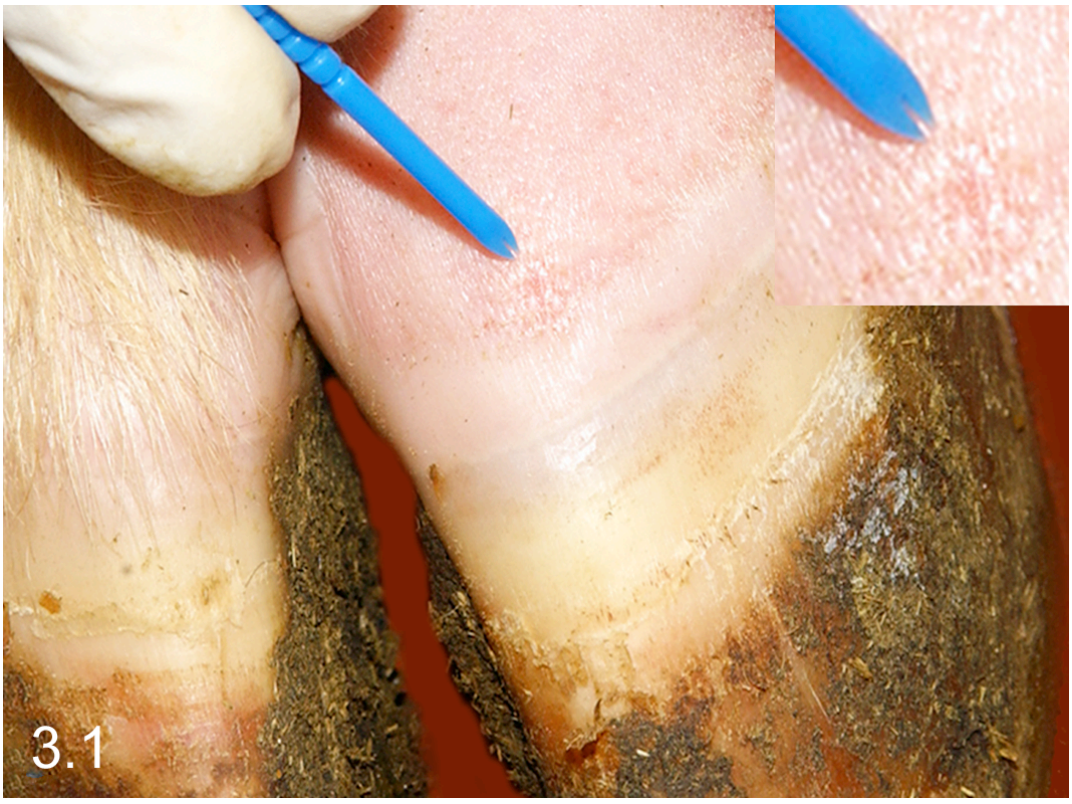


Fig. 3.1: Coronary band. Inoculation via scarification, using bifurcated needle. Inset: close up view of the bifurcated edge of the needle.

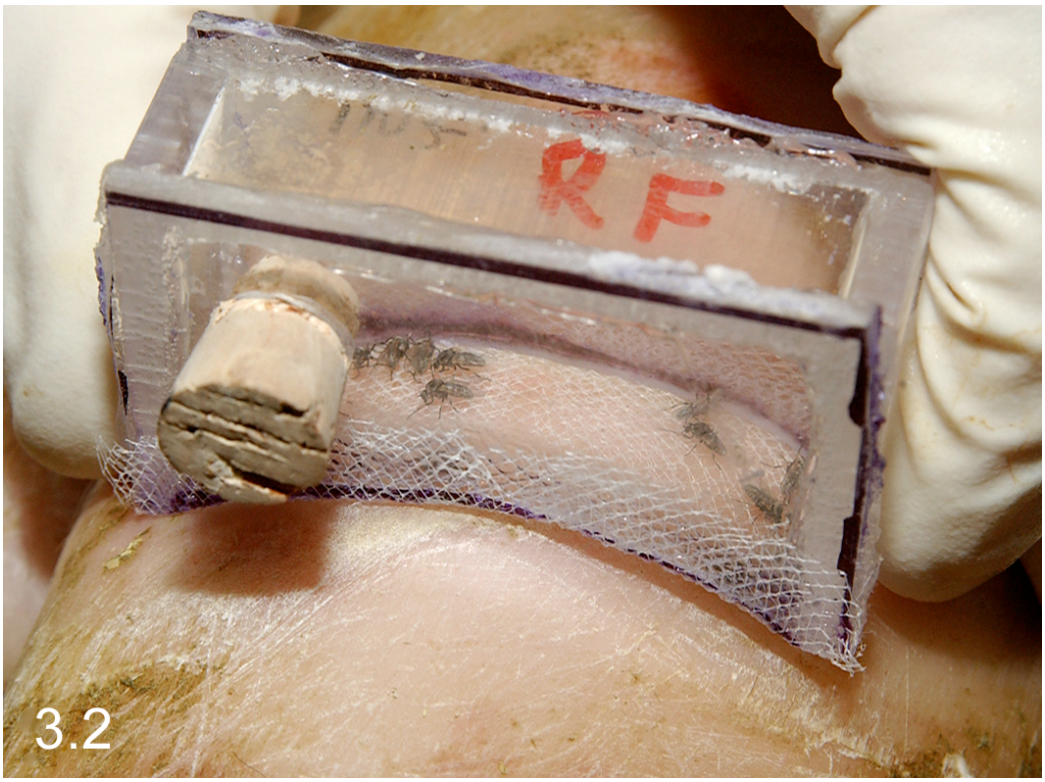


Fig. 3.2: Coronary band. Inoculation via fly bite, with VSNJV-inoculated black flies in cage.

Figs. 3.3 to 3.6: Coronary band. Histologic sections of the coronary bands from cattle experimentally infected with VSNJV, 12 to 72 hours postinfection (HPI), (a) with HE

stain, (b) *in situ* hybridization for presence of viral replication (negative sense digoxigenin-labeled riboprobe, anti-digoxigenin-antibody, alkaline phosphatase, NBT/BCIP) and (c) immunohistochemistry for viral protein (anti-VSNJV polyclonal antibody, alkaline phosphatase-linked polymer system, Vector Red substrate, hematoxylin counterstain).

Fig. 3.3 12 HPI, SC. a: Focal area of epidermal disruption due to scarification. B, c: Discrete and intense focus of ISH (b) and IHC (c) signal.

Fig. 3.4. 24 HPI, SC. a: Focally extensive spongiosis of the stratum spinosum with scattered individual loss of keratinocytes with initial vesicle formation. **b, c:** Intense and diffuse ISH (b) and IHC (c) signal in the cells of stratum spinosum.

Fig. 3.5. 48 HPI, SC. a: Marked epidermal disruption, with necrosis, large cavitations and loss of the stratum spinosum. Note that the stratum basale is spared. **b:** Localized ISH signal within necrotic stratum spinosum. **c:** Intense and diffuse IHC signal of the stratum spinosum.

Fig. 3.6. 72 HPI, SC. a: Marked epidermal disruption, with diffuse coagulative necrosis of the stratum spinosum. **b:** Faint and diffuse ISH signal in necrotic stratum spinosum. **c:** Intense and diffuse IHC signal of the stratum spinosum.

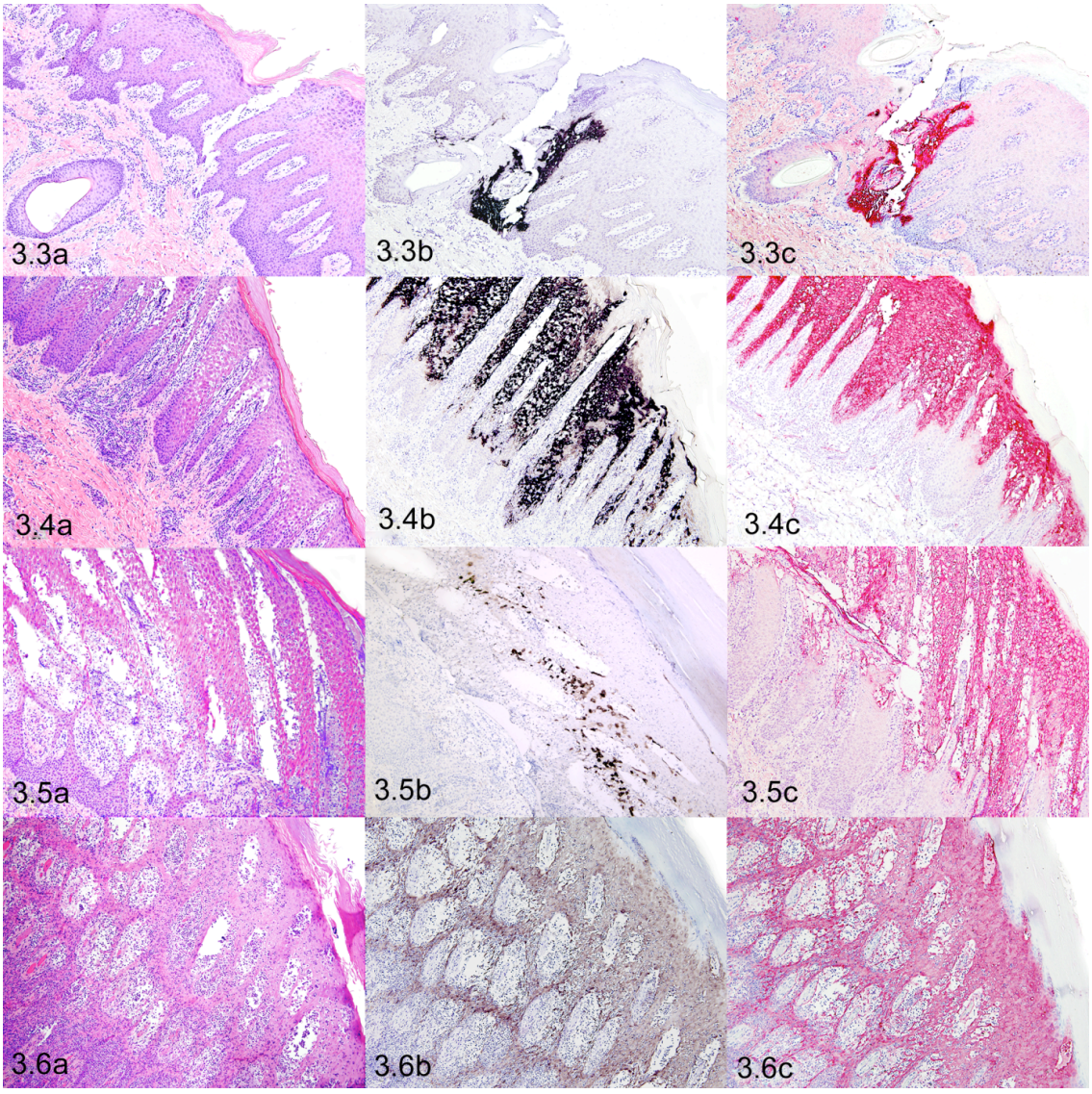
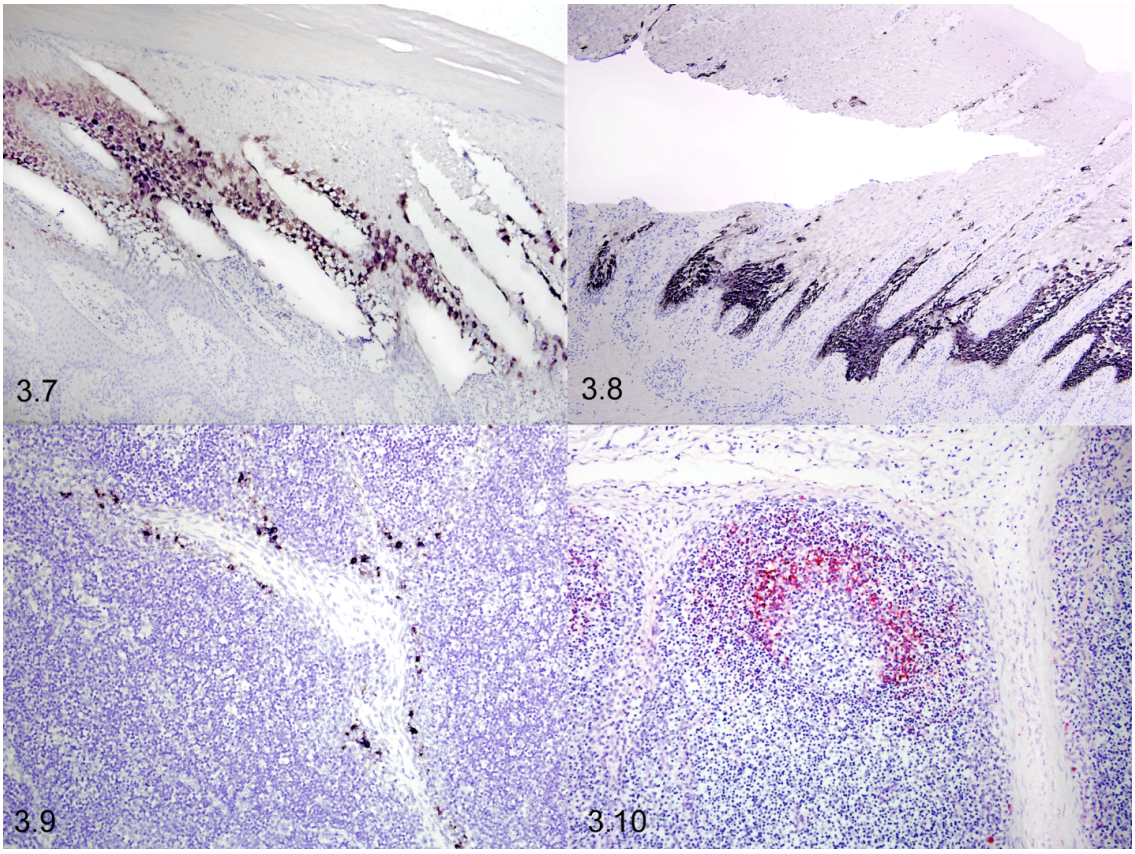

3.3a
3.3b
3.3c
3.4a
3.4b
3.4c
3.5a
3.5b
3.5c
3.6a
3.6b
3.6c

Fig. 3.7. 48 HPI, SC: Coronary band. *In situ* hybridization localizes replicating virus to the upper stratum spinosum, with sparing of the stratum basale (negative sense digoxigenin-labeled riboprobe, anti-digoxigenin-alkaline phosphatase, NBT/BCIP).

Fig. 3.8. 48 HPI, FB: Interdigital skin. *In situ* hybridization localizes replicating virus predominantly in the deeper stratum spinosum and stratum basale, (negative sense digoxigenin-labeled riboprobe, anti-digoxigenin-alkaline phosphatase, NBT/BCIP).

Fig. 3.9. 24 HPI, FB: Popliteal lymph node. *In situ* hybridization localizes replicating virus within inflammatory cells in subcapular areas, (negative sense digoxigenin-labeled riboprobe, anti-digoxigenin-alkaline phosphatase, NBT/BCIP).

Fig. 3.10. 72 HPI, SC: Popliteal lymph node. Immunohistochemistry localizes viral protein to the marginal zone of the lymphoid follicles, (alkaline phosphatase-linked polymer system, Vector Red substrate, hematoxylin counterstain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CHAPTER 4

$WC1^+$ γδ T LYMPHOCYTES AND CLASS II MHC IN VESICULAR STOMATITIS NEW JERSEY VIRUS EARLY INFECTION IN CATTLE INOCULATED VIA SCARIFICATION AND BLACK FLY (*SIMULIUM VITTATUM*) BITE.*

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*J.L. Reis Jr., A.M. Rodriguez, L.L. Rodriguez, D.G. Mead, and C.C. Brown. *To be submitted to vet pathol.*

ABSTRACT

Vesicular stomatitis New Jersey virus (VSNJV) is the causative agent of vesicular stomatitis (VS), a contagious and vector-borne disease of livestock. The pathogenesis of VSNJV is not completely understood and the cellular immune response in tissue of infected animals has not been characterized. In the current report, we performed an immunohistochemical study using different antibodies to target inflammatory cells in coronary band (CB) and draining lymph nodes of steers inoculated with VSNJV via scarification (SC) and black fly bite (FB). Animals were euthanized at 24, 48 and 96 HPI. Throughout the experiment the majority of inflammatory cells within areas of vesicle formation in the CB were $MAC387^+$, with higher numbers of neutrophils at 24 HPI and an increase of macrophages towards to 96 HPI. The second most common population of cells were $CD3^+$ and it appears that most were $WC1^+$, indicating that $WC1^+$ γδ T cells are the main T lymphocytes to infiltrate the VSNJV lesion. Up-regulation of MHC II was present in the SC-inoculated animals. However, no MHC II up-regulation was observed in the FB-inoculated animals. This finding needs to be further investigated since this might explain why VSNJV lesions are more severe with FB. More prominent detection of follicular dendritic cells (FDC) was present at the end of the experiment. The location of these cells coincides with the areas of VSNJV antigen detection at 72 HPI in the lymph nodes, which suggests that viral antigen is being presented to B cells at that time.

Key words: Vesicular stomatitis virus, pathogenesis, immunohistochemistry, γδ T lymphocytes, MHC II, catlle, black fly.

INTRODUCTION

Vesicular stomatitis New Jersey virus (VSNJV) is an arthropod-borne virus (arbovirus) in the family *Rhabdoviridae*, genus *Vesiculovirus* and is the causative agent of vesicular stomatitis (VS), a contagious and vector-borne disease of livestock. Although VSNJV infects a wide range of species including wildlife,[1] laboratory and domestic animals,[2] the natural disease is only reported in cattle, pigs, and horses. The disease is clinically indistinguishable from other viral vesicular diseases, especially foot-and-mouth disease when cattle and pigs are affected.

The pathogenesis of VSNJV, as well as other *vesicular stomatitis virus* (VSV) infections, is not completely understood. In a previous study,[3] we reported that VSNJV replicates extensively in the keratinocytes of the coronary bands (CB), with lesions limited to the site of inoculation and peak of viral replication between 24 and 48 HPI in the CB. Replicating virus was detected in draining lymph nodes but only at 24 HPI. In that same study, inflammatory cell infiltration peaked between 48 and 72 HPI, with cellular accumulation in epidermal areas within vesicle formation or in the superficial dermis underneath the vesicle in VSNJV-inoculated CB via scarification (SC) or black fly bite (FB).

Few reports have characterized cellular response in livestock infected with VSV. Howerth at al[4] reported infiltration of $CD3^+$ T lymphocytes and $MAC387^+$ cells (macrophages and neutrophils) in oral lesions at healing stage in horses inoculated with VSNJV and VSIV (vesicular stomatitis Indiana virus). Scherer et al[5] described, in a confocal microscopy study from acute infection in SC-inoculated cattle, co-localization

of VSNJV and MHC II antigens in the CB, but failed to co-localize VSNJV and MAC 387, suggesting that dendritic cells may play a role in VSNJV infection. Even though it is well known that γδ T cells are the major circulating lymphocyte population in ruminants and pigs,[6,7] the presence of these cells in VSV infection has not been determined. It is believed that γδ T cells play a large role in the innate immunity in these species,[8] but the exact mechanisms by which this happens are not totally understood.

Class II major histocompatibility complex (MHC II) is an integral transmembrane molecule required for the presentation of antigens to $CD4^+$ helper T cells[9] and γδ T cells.[10] MHC II is up-regulated during inflammatory processes and is constitutively expressed in professional antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages, and B lymphocytes,[11] and it is well known that it plays a key role in regulating host immune response. Dendritic cells are strategically present in lymphoid tissues, interstitium of most parenchymal organs and epithelia of skin, respiratory tract and intestine. They are divided into different subsets: the Langerhans cells of the epidermis (epidermal); dermal (interstitial) DC; interdigitating DC (thymus and in the paracortical areas of the lymph nodes), and; follicular dendritic cells ([FDC] fixed residents in the follicular centers of the lymph nodes).[12] Once an antigen is taken up, Langerhans cells lose their intercellular adhesion molecules and migrate to draining lymph nodes to activate naïve T helper lymphocytes in paracortical areas. DCs are the most effective APCs, due to their location and ability to initiate a T cell response. Activation of T helper cells results in subsequent clonal expansion and IFN-γ production, which in turn stimulates expression of MHC II by the APCs. Therefore, MHC II

expression in antigen presenting cells helps to link the innate and adaptive immune response and its up-regulation favors response against microbial infection.

Follicular dendritic cells are a particular subset of dendritic cells. They are fixed residents in lymphoid follicles, are not bone marrow-derived, and do not process antigen through class II MHC.[12] They capture antigen and display it to B cells in the form of immune complexes composed of either antigen and antibody, antigen and complement or all three molecule types.[13] Therefore, antigen display on FDC is instrumental in activation of B lymphocytes maturation in a T cell-independent antibody production fashion. [14]

Numerous reports[15,16] have described that insect saliva or insect feeding potentiates infection with various arboviruses. Likewise, our previous study[3] demonstrated that lesion development was markedly more severe and extensive between 48 and 96 HPI when VSNJV was transmitted via FB as compared to SC suggesting mechanisms or components of the insect bite inoculation that facilitate VSNJV infection.

The present study was designed to characterize, using immunohistochemistry in paraffin embedded sections, the inflammatory cellular response in the CB and lymph nodes of VSNJV-inoculated steers. The second objective of this study was to compare the cellular immune response in VSNJV-inoculated tissues via SC and FB.

MATERIALS AND METHODS

All experimental infections and all methods using non-fixed samples were conducted at the USDA-ARS Plum Island Animal Disease Center under biosafety level

(BSL)-3Ag conditions. Tissues fixed in formalin were transferred to the Department of Pathology at the University of Georgia, for subsequent immunohistochemistry studies.

Samples and animals

Samples used in the present study were collected from an experiment[3] that aimed to track viral *in situ* replication and distribution, and lesion development. Briefly, the virus utilized was a VSNJV field strain (95COB) isolated from a cow during an outbreak in Colorado, USA, in 1995, and successfully used in a previous study.[5] Seven Holstein steers from that experiment were used in this current study. As previously described,[3] the animals were inoculated on the right CB via SC and FB. Selected samples for processing in the current study were right front and rear CB, and right pre-scapular and popliteal lymph nodes from all inoculated animals. VSNJV-inoculated animals were euthanized at 24, 48 and 96 hours post-inoculation (HPI). A mock-inoculated animal was scarified on the front and rear right CB, followed by application of cell culture media free of virus. The aforementioned samples from all animals were collected immediately after euthanasia. Tissue sample sections no thicker than 1cm were fixed in a 10% buffered formalin solution and embedded in paraffin.

Immunohistochemistry (IHC)

Briefly, after deparaffinization, sections were subjected to antigen retrieval followed by blocking of non-specific epitopes with ultra V block (LabVision

Corporation). Then samples were incubated overnight at 4^0C with a primary antibody specific for an immune cell marker (CD79 for B cells; CD3 for T cells; WC1 for γδ T cells; MAC387 for macrophages and neutrophils; FDC for follicular dendritic cells; and CD1b for dendritic cells). The exact antibody used, its source, and the working dilutions are listed in Table 4.1. The following day, after washing, sections were incubated with an alkaline phosphatase-linked polymer system (LabVision Corporation). The reaction was revealed using Vector Red chromogen (Vector Laboratories). The slides were lightly counterstained with Mayer's hematoxylin then cover-slipped using Permount for permanent record.

For each IHC protocol primary antibodies were applied to CB or lymph node section from the mock-inoculated animal for noninfected negative tissue control. Additionally normal mouse serum was used as a primary antibody negative control.

A semi-quantitative score for the degree of antibody labeling was used: 0 = Negative (lack of any positive signal); + = Minimal, for only a few cells positive in the section; ++ = Mild, for a few high power fields HPFs with 1-10 positive cells; +++ = moderate, for many HPFs with more than 10 positive cells, and; ++++ = marked, for extensive positive signal, most HPFs have more than 10 positive cells.

RESULTS

Histopathology of lesions from VSNJV-infected cattle has already been described.[3] Briefly, inflammatory infiltrates are first evident at 24 HPI and increase to

dense accumulations at 48 and 72 HPI. They are most evident in the dermis but as vesicles develop, inflammatory cells also figure prominently in the epidermis. In our previous study, although grossly there were more severe areas of epidermal disruption in the FB-inoculated animals, histologically the degree and the type of inflammatory cells infiltration (mononuclear vs. neutrophils) were similar in both SC- and FB-inoculated animals.

The results of antibody labeling are depicted in table 4.2 and each is described briefly below.

Anti-CD79 antibody

Coronary bands of all animals, both inoculated and negative control, consistently lack CD79 signal. Positive control tissue, a lymph node from the noninfected animal, had numerous cells with cytoplasmic signal within the germinal center of lymphoid follicles.

Anti-CD3antibody

Cells positive for anti-CD3 antibody were present in moderate (+++) to marked (++++) numbers in all inoculated CB, except the right front CB of the FB-inoculated steer at 48 HPI (flies did not feed) where $CD3^+$ cells were only recorded as ++. For all

other animals, there did not seem to be a difference in the time postinfection, all were either +++ or ++++. The signal was present in the plasma membrane and cytoplasm of positive cells. The positive cells were more commonly infiltrating perivascular spaces and the interstitium of the superficial dermis underlying the vesicular lesions (Fig. 4.1), and less frequently were present in the epidermis within areas of vesicle formation. In comparison, the negative control animal, i.e., the one that was scarified with cell culture fluid, only, had rare (+) $CD3^+$ cells in the perivascular zones of the superficial dermis.

Anti-WC1 antibody

Cells positive for anti-WC-1 antibody were present in mild (++) to moderate numbers (+++) in all inoculated CB, except the right front CB of the FB-inoculated steer at 48 HPI (flies did not feed), which had relatively fewer $WC1^+$ cells. There did not seem to be a difference in the time postinfection, all were either ++ or +++, without any evidence of increase or decrease over time. The signal was present in the plasma membrane and cytoplasm. The number of positive cells was slightly lower, with almost the same distribution as that for $CD3^+$ cells – the $WC1^+$ cells were more commonly infiltrating perivascular spaces and the interstitium of the superficial dermis underlying the vesicular lesions (Fig. 4.2), and less frequently signal was present in the epidermis within areas of vesicle formation. In comparison, the negative control animal, i.e., the one that was scarified with cell culture fluid, only, had rare $WC1^+$ cells in the perivascular zones of the superficial dermis.

In the popliteal node from the mock-inoculated animal, there were moderate numbers of $WC1^+$ cells within the medullary sinuses. Compared to anti-WC1 antibody labeling from the lymph node of the mock-inoculated animal, lymph node from the VSNJV-inoculated animals had numerous $WC1^+$ cells within subcapsular sinuses (not present in the negative control) and higher number of positive cells in medullary sinuses.

Anti-MAC387 antibody

Cells positive for MAC387 were very numerous in most infected tissues examined. They were present only as minimal numbers of cells in the scarified negative control CB. In the virus-inoculated CB, they were abundant, especially at 24hpi. Signal was present in the cytoplasm. Cells were morphologically compatible with macrophages and neutrophils.

Positive cells could be seen infiltrating the epidermis in areas of vesicle formation and within the superficial dermis underlining those same areas, and multifocally in perivascular and periadnexal areas. Labeling had the same pattern but decreased from marked (++++) at 24 and 48 HPI to moderate (+++) at 96 HPI steer. Within the first 48 HPI, the majority of those positive cells were morphologically consistent with polymorphonuclear cells (neutrophils), with higher numbers of positive mononuclear cells (macrophages) at 96 HPI. Comparing lymph nodes draining VSNJV-inoculated CB to those draining mock-inoculated CB, there were greater numbers of $MAC387^+$ cells within subcapsular and medullary sinuses, with relatively fewer positive cells in

paracortical areas. The lymph nodes draining VSNJV-inoculated CB had moderate to marked numbers of MAC387+ cells at 24 HPI, with decreased numbers of positive cells at 48 and 96 HPI.

Anti-MHC II antibody

In the negative control animal, rare MHC II positive cells were present in and around hair follicles and sebaceous glands of the CB and rare to moderate numbers of positive cells in the lymph node were found throughout the paracortex and less frequently within germinal centers of the lymphoid follicles. MHC II positivity was in the cytoplasm and plasma membrane of mononuclear cells that had large vesicular nuclei and abundant cytoplasm, i.e., morphologically consistent with macrophages or dendritic cells. In the inoculated animals, in general, MHC II positivity was more prominent in the animals infected by SC as compared to the animals infected by FB. In the SC animals, MHC II positive cells increased from 24 through 96 HPI, and were present in the epidermis within areas of vesicle formation (Fig. 4.3), as well as in the superficial dermis underlying the vesicles and cells within and around hair follicles. In these animals, MHC II positive cells were also present in the lymph nodes – in paracortex, mantle and medullary sinuses. In comparison, CB from animals infected by FB had fewer MHC II positive cells, with none detectable at 24 HPI (Fig. 4.4), and in some CB at later time points, only mild to minimal MHC II positive cells. Similarly, lymph nodes from the FB-

infected animals had fewer MHC II cells although the distribution was similar to that described for the SC-inoculated steers.

Anti-follicular dendritic cells antibody (FDC)

The lymph node of the negative control animal had minimal anti-FDC labeling within mantle areas and in germinal centers of the lymphoid follicles. Anti-FDC antibody positivity was characterized as cytoplasmic and was also associated with plasma membrane. Positive cells generally had large amounts of cytoplasm, and a large oval vesiculated nucleus. In the inoculated animals, signal increased from minimal numbers of positive cells at 24 HPI, minimal to mild numbers at 48 HPI, and mild to moderate numbers at 96 HPI (Fig. 4.5), with the same distribution as observed in the negative control.

Anti-CD1b antibody

When present, anti-CD1b antibody positivity was in the cytoplasm and plasma membrane of mononuclear cells with large amounts of cytoplasm, and large oval vesicular nucleus.

The lymph node of the negative control animal had minimal anti-CD1b labeling within paracortical areas. In the inoculated animals, signal increased from minimal

numbers of positive cells at 24 HPI, mild to moderate numbers at 48 HPI, and moderate to marked numbers at 96 HPI (Fig. 4.6), with the same distribution as observed in the negative control.

DISCUSSION

In CB of cattle inoculated with VSNJV either by SC or FB, there were abundant cells expressing CD3, WC1, and MAC387 at the site of vesicle formation. Cells positive for MAC387 were plentiful at all time points. It was difficult to differentiate between macrophages and neutrophils with the MAC387 stain. However, it seemed that neutrophils were a prominent cell type during early stages of infection, as would be expected. But in addition, many of the MAC387 cells were macrophages morphologically, especially at the later time points. In the lesions, lymphocytes were also abundant. Using markers for T cell (CD3) and B cells (CD79), none were positive for CD79 and all were CD3 positive, so of the T cell lineage. Additionally, determining which type of T cell, the majority were positive for $WC1^+$ (a γδ T cell marker). Therefore, γδ T cells, which are $WC1^+$, $CD3^+$, $CD4^-$, and frequently $CD8^-$, seem to be the major T lymphocyte population to infiltrate areas of lesion development in the CB of both SC- or FB-inoculated steers. The minority of lymphocytes within the areas of inflammation are presumably $CD3^+$ $WC1^-$ and express either $CD4^+$ (helper T cells) or $CD8^+$ (cytotoxic T cells), or NK cells.

The role of γδ T cells in cell-mediated immunity has received increasing attention in recent years, but the exact mechanisms by which these cells operate in the ruminant immune response are not totally understood. It is well known that γδ T cells are the major circulating lymphocyte population in ruminants and pigs.[6,7] It is believed that they exert an important influence on innate immunity in these species. They can respond to stimulation via the T-cell receptor (TCR) and through CD3 stimulation.[17,18] Reports have demonstrated that γδ T cells are capable of all the responses shown by αβ T cells and that there is considerable overlap in function between the two populations.[19] Studies have shown that dendritic cells are more efficient than macrophages in stimulating γδ T cells[20] and it has been suggested that γδ T cells can be stimulated to produce IFN-γ via activation of the T-cell receptor (TCR).[17,18] *In vitro* studies[8,10] have demonstrated enhanced secretion of IFN-γ by bovine γδ T cells induced by coculture with *Mycobacterium bovis*-infected calf dendritic cells, through IL-12 and IL-18 produced by the dendritic cells. INF-γ is an important antiviral cytokine of the innate immune response and is known to mediate increased expression of MHC II on APCs and MHC I in a variety of cells inducing more efficient antigen processing and presentation to T cells.[12] Therefore we presume that γδ T cells are working in this same manner in VSNJV-infected cattle.

Marked upregulation of MHC II was observed in the SC-inoculated animals, in both CB as well as lymph nodes. Class II major histocompatibility complex is an integral transmembrane molecule required for the presentation of antigens to CD4 helper T cells[9] and γδ T cells.[10] This molecule is constitutively expressed in professional antigen presenting cells (APCs), dendritic cells and B lymphocytes,[11] and variably expressed in

macrophages.[12] Because the lesions in this study consistently had no B lymphocyte infiltration, it can be assumed that all of the MHC II positive cells are DCs and macrophages. It would be expected that in a viral infection, mononuclear cells would predominate, with macrophage infiltration and that these macrophages would have prominent expression of MHC II. This was all true for the animals infected via SC. A surprise finding was the relative lack of MHC II positive cells in the animals inoculated via FB. In fact, the FB-inoculated animals in many cases had decreased MHC II expression, even in comparison to the noninfected control animal. This association of MHC II down-regulation and FB helps explain why lesions are more severe in cattle inoculated via VSNJV-infected black fly bite than SC as previously reported.[3] Dendritic cells, thought to be the most efficient APC, present antigen bound to MHC II to naïve T helper lymphocytes leading to activation and clonal expansion of T helper lymphocytes with consequent production of IFN-γ and type 1 IFNs. Therefore, downregulation of MHC II may deregulate host T cell response favoring viral replication and consequently lesion development.

There was an increase in cells displaying FDC signal in the lymph nodes of infected animals, with increases correlating with time postinfection. This increase was equal comparing SC and FB. It is known that TNF-α stimulates the maturation of FDC and other DCs.[21] As expected in acute inflammatory processes, TNF-α is commonly produced in high amounts within the site of inflammation.[12] Therefore, we suggest that increase in detection towards the end of our experiment is likely associated with FDC maturation mediated by cytokines such as TNF-α. VSV is noted for its very acute nature and the production of antibodies very early in infection.[22] Follicular dendritic cells,

which are a particular subset of dendritic cells, do not express class II MHC[12] and they capture antigen to display to B cells.[13] In our previous study using anti-VSNJV antibody,[3] we demonstrated viral antigen localized in mantle areas of lymph nodes at late stages of the acute infection. Therefore, putting these findings together there is evidence that VSNJV antigen may be displayed on FDC and likely plays a role in B lymphocyte maturation for antibody production.

In a similar manner, there was increased expression of CD1b at later stages in VSNJV-infected steers. $CD1b^+$ cells are mainly present in paracortical areas and likely represent interdigitating dendritic cells or dermal dendritic cells from the CB that migrated to draining lymph nodes. In cattle CD1b molecules expression has been shown in paracortical dendritic cells in lymph nodes. [23] As previously reviewed[24] human dermal and interdigitating DCs, but not Langerhans cells, express CD1b, and its expression increases by external stimuli including microbial infections and various cytokines. CD1 is a family of nonpolymorphic major histocompatibility complex class I-like molecules well known for being capable of presenting mycobacterial lipid antigens to double-negative $CD4^- CD8^-$ as well as $CD8^+$ T cells.[25] However, it has also been shown that CD1 molecules can present other types of antigen[26] suggesting that CD1a, b, and c molecules may present antigens from a wider variety of microorganisms to T cells and this may be what happens in VSNJV infection in cattle. The upregulation of CD1b in the lymph nodes draining sites of VSNJV infection indicate that the dendritic cells are actively presenting antigen in the lymph node. This corresponds to our previous study, where we identified small amounts of replicating VSNJV in cells morphologically compatible with dendritic cells in the same nodes.

In summary, acute VSNJV infection in the site of vesicle formation in cattle inoculated via SC or FB is characterized by large numbers of neutrophils, macrophages and lymphocytes, with neutrophils predominating at the earlier time points and mononuclear cells predominating later. Lymphocytes were all of the T cell lineage, with most being γδ T lymphocytes. There is up-regulation of class II MHC in SC-inoculated animals. However, there was lack of MHC II up-regulation in the FB-inoculated steers. This latter finding needs to be further investigated as it seems to indicate that MHC II downregulation by the insect, and may explain the more extensive local lesions produced by FB inoculation.[3] Detection of follicular dendritic cells as well as CD1+ cells, likely expressed by dermal or interdigitating dendritic cells, increased in the lymph nodes through the study. Increased FDC signal might be associated with activated FDC presenting VSNJV antigen to B cells facilitating antibody production. Finally, higher detection of CD1b in both SC- and FB-inoculated animals may be related to maturation of dendritic cells in paracortical areas with antigen presentation to T cells.

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Table 4.1: List of antibodies with their respective antigen retrieval method and dilution used**.**

Antibody	Target	Type of antibody	Company/Origin	Catalog #	Antigen retrieval	Dilution
CD79αcy	B lymphocytes	Monoclonal mouse anti-human	Dako	M7051	Microwave EDTA	1:200
CD3	Pan T lymphocyte	Polyclonal rabbit anti-human	Dako	A0452	PK 60ug	1:300
WC1	γδ T lymphocytes	Monoclonal mouse anti-bovine	Serotec	MCA838S	DECOX, heat under pressure	1:100
MAC387	Neutrophils and macrophages	Monoclonal mouse anti-human	Dako	M0747	PK 60ug	1:800
MHC II	MCH II	Monoclonal mouse anti-bovine	VMRD	H42A	EDTA pressure	1:100
FDC	Follicular dendritic cells	Monoclonal mouse anti-human	Dako	M7157	Microwave EDTA	1:75
CD1b	Dendritic cells	Monoclonal mouse anti-bovine	Hope, JC Compton, UK	CC20	DECOX, heat under pressure	1:2

Table 4.2: Immunohistochemistry results according to different antibodies applied to coronary bands and lymph nodes of steers inoculated with VSVNJ via scarification or fly bite.

Steer		Tissue		CD79	CD3	WC1	MAC387	MHC II	FDC	CD1b
Mock	SC	CB	Rear	0	+	+	+	++		
		LN	POPL	++++	++++	++	+++	+++	+	+
24 HPI	SC	CB	Front	0	+++	+++	++++	+++		
			Rear	0	++++	+++	++++	+++		
		LN	PRES			++++	++++	+++	+	+
			POPL			++++	++++	+++	+	+
	FB	CB	Front	0	++++	+++	++++	0		
			Rear	0	+++	++	++++	0		
		LN	PRES			+++	++++	+	+	+
			POPL			++++	+++	+	+	+
48 HPI	SC	CB	Front	0	+++	++	++++	++++		
			Rear	0	++++	+++	+++	++++		
		LN	PRES			++++	++	++++	+	++
			POPL			+++	+++	+++	++	++
	FB	CB	Front*	0	++	++	++	++		
			Rear	0	++++	+++	++++	++		
		LN	PRES			++++	+++	+++	+	+++
			POPL			+++	+++	++	++	++
96 HPI	SC	CB	Front	0	+++	+++	+++	+++		
			Rear	0	+++	++	+++	+++		
		LN	PRES			+++	+++	+++	+++	++++
			POPL			+++	+++	++++	++	+++
	FB	CB	Front	0	+++	++	+++	++		
			Rear	0	++++	+++	++++	+		
		LN	PRES			++++	+++	+++	+++	++++
			POPL			+++	+++	++	+++	++

HPI = Hours post-inoculation; SC = Scarification; FB = Fly bite; CB = Coronary band; LN = Lymph node; PRES = Pre-scapular lymph node; POPL = Popliteal lymph node; 0 = Negative; + = Minimal (only a few cells positive in the section); ++ = Mild (a few high power fields [HPFs] have 1-10 positive cells; +++ = moderate (many HPFs have more than 10 positive cells), and; ++++ = marked (extensive positive signal, most HPFs have more than 10 positive cells).

Fig. 4.1. 24 HPI, SC. Coronary band. IHC localizes (in red) $CD3^+$ T lymphocytes in superficial dermis underlying vesicle formation (alkaline phosphatase-linked polymer system, Vector Red substrate, hematoxylin counterstain).

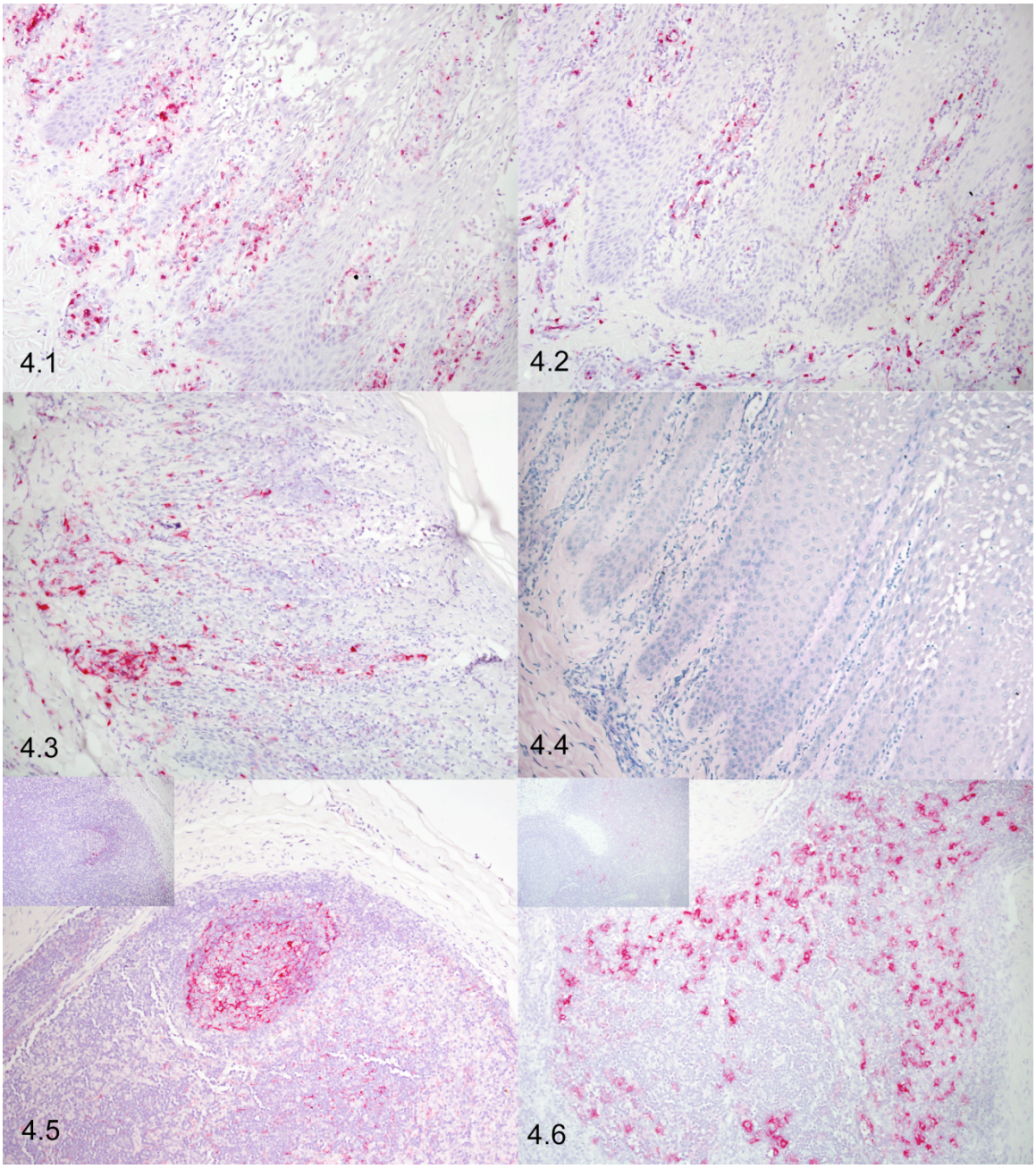
Fig. 4.2. 24 HPI, SC. Coronary band. IHC localizes (in red) $WC1^+$ T lymphocytes in superficial dermis underlying vesicle formation (alkaline phosphatase-linked polymer system, Vector Red substrate, hematoxylin counterstain).

Fig. 4.3. 24 HPI, SC. Coronary band. IHC localizes (in red) MHC II^+ cells in superficial dermis underlying vesicle formation (alkaline phosphatase-linked polymer system, Vector Red substrate, hematoxylin counterstain).

Fig. 4.4. 24 HPI, FB. Coronary band. IHC failed to localize MHC II^+ cells in superficial dermis underlying vesicle formation (alkaline phosphatase-linked polymer system, Vector Red substrate, hematoxylin counterstain).

Fig. 4.5. 72 HPI, SC. Lymph node. IHC localizes (in red) follicular dendritic cells in lymphoid follicles (alkaline phosphatase-linked polymer system, Vector Red substrate, hematoxylin counterstain).

Fig. 4.6. 72 HPI, SC. Lymph node. IHC localizes (in red) $CD1b^+$ cells in subcapsular and paracortical areas (alkaline phosphatase-linked polymer system, Vector Red substrate, hematoxylin counterstain).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CHAPTER 5

CYTOKINE RESPONSE AND APOPTOSIS IN VESICULAR STOMATITIS NEW JERSEY VIRUS EARLY INFECTION IN CATTLE INOCULATED VIA SCARIFICATION AND BLACK FLY *(SIMULIUM VITTATUM)* BITE.

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*J.L. Reis Jr., G. Smoliga, L.L. Rodriguez, D.G. Mead, and C.C. Brown. *To be submitted to Journal of Comparative Pathology*

ABSTRACT

Vesicular stomatitis New Jersey virus (VSNJV), the cause of vesicular stomatis, (VS) is an important contagious and vector-borne disease of livestock. The pathogenesis of VS is poorly understood. Although VSNJV is widely known for being a prominent inducer of type I interferons and a promoter of cell death through apoptosis, both *in vitro* and *in vivo* (mice), cytokine response and apoptosis have never been assessed in the natural livestock host. We measured expression of numerous T helper 1 (T_H1) and T helper 2 (T_H2) lymphocytes cytokines and active caspase 3 protein in the coronary bands (CB) and draining lymph nodes from steers inoculated VSNJV via scarification (SC) and black fly bite (FB). Animals were euthanized at 24, 48 and 96 hours post-infection (HPI). We report in this study marked T_H1 response at 48 HPI in both SC- and FB-inoculated animals. Detection of active caspase 3 was more prominent at later time points in the CB and lymph nodes of both SC and FB-inoculated animals. This strong and acute cytokine response, which coincides with peak of viral replication at 48 HPI in the CB, together with increase of caspase 3 indicates that VSNJV infection triggers an efficient host response that may be preventing the virus from systemic spread (viremia).

Key words: Black flies, cattle, cytokines, early infection, pathogenesis, vesicular stomatitis virus.

INTRODUCTION

Vesicular stomatitis virus (VSV) is an insect-transmitted and contagious virus that causes vesicular lesions in cattle, horses and swine.[1] Lesion development is limited to the site of inoculation/exposure and, to the best of the authors' knowledge, viremia in livestock has been described only once, in 1926 by Cotton,[2] with no other subsequent studies describing viral systemic spread. It is well established that serotypes Indiana (VSIV) and New Jersey (VSNJV) are potent inducers of interferon, and so these have been widely used in the laboratory for studies on interferon.[3,4] However, there are no reports assessing interferon or other cytokine responses in the natural host, especially livestock.

Previous *in situ* hybridization (ISH) studies in infected cattle have shown that after VSNJV inoculation via scarification (SC) of coronary bands (CB), virus replication occurs in the keratinocytes of the stratum spinosum of the thick non-haired skin, with peak of viral replication occurring between 24 and 48 hours post infection (HPI).[5] At 72 HPI there is a marked decrease in viral replication, which coincides with striking disruption (necrosis and loss) of the CB epidermis and peak of inflammatory cell infiltration occurring between 48 and 72 HPI. When VSNJV was inoculated via infected black fly (*Simulium vittatum*) bite (FB), lesion development was more prominent and extensive. Additionally, the same ISH study demonstrated that viral replication in the local draining lymph nodes (prescapular and popliteal) was limited to the first 24 HPI in both SC and FB animals. Similar to numerous other experiments, neither live virus nor viral RNA was detected in the blood or spleen. It is speculated that lack of viremia in

VSV infected livestock may be associated with a local acute innate immune response to the virus with marked cytokine production at the site of inoculation and draining lymph nodes preventing VSV from systemic spread.

Reports have shown that VSNJV is a great inducer of type I IFNs,[4,6,7] and it is well documented that IFNs exert a potent inhibitory effect on VSV replication and progeny virus production both *in vitro* and *in vivo* (mice).[8] In addition to IFNs, VSV is also a potent inducer of apoptosis in a variety of cells.[9,10] Neither cytokine response nor presence of apoptosis have been assessed in tissues of cattle inoculated with VSNJV by either SC or FB. Therefore we hypothesize that there is a strong and acute cytokine response and induction of apoptosis in cells susceptible to viral infection (keratinocytes), and that together cytokines and apoptosis limit viral replication in early infection preventing the virus from spreading systemically.

As we previously described,[5] inoculation via FB in the CB can be successful even with markedly decreased amounts of virus, compared to SC, which suggests that there may be a facilitating factor supplied by the insect inoculation. Besides the lower amount of inoculum needed to reproduce the disease, FB lesions were more severe and developed faster. Therefore a question that can be raised is whether the infected host has a different cytokine response when inoculated via FB as compared with SC. It has been shown that mosquito salivary gland extract or mosquito bites enhance transmission and infection of different arboviruses in various systems[11-13] including VSV *in vitro*[14] and *in vivo* (mice).[15] Intradermal *Aedes aegypti* salivary gland extract and Sindbis virus co-inoculation in mice downregulated T helper 1 (T_H1) cytokines IFN-γ and IFN-β at 24 and 72 HPI.[16] This same experiment demonstrated upregulation of the T helper 2 (T_H2)

cytokines IL-4 and IL-10. The ability of mosquito saliva to down-regulate the expression of T_H1 associated antiviral cytokines and to up-regulate T_H2 associated cytokines in many arboviral diseases suggests that it would be worthwhile to determine if biting insects may be exerting similar effects in VSV-infected livestock.

The aim of this study was to assess various cytokine production and presence of apoptosis during acute VSNJV infection in tissues from cattle inoculated via SC and FB.

MATERIALS AND METHODS

Samples and animals

For the current study, samples from one of our previous VSNJV experiment[5] to track viral *in situ* replication and distribution, and lesion development were used. All experimental infections and all methods utilizing non-fixed samples were conducted at the USDA-ARS Plum Island Animal Disease Center under biosafety level (BSL)-3Ag conditions. Briefly, the virus utilized was a VSNJV field strain (95COB) isolated from a cow during an outbreak in Colorado, USA, in 1995.[17] Eight of the Holstein steers were chosen from that experiment. As previously described,[5] six animals were inoculated on the right front and rear CB via SC and FB, and two mock animals were inoculated with the same methods on the left front and rear CB. Selected samples for processing in the current study were all four CB, and right and left pre-scapular and popliteal lymph nodes from the animals euthanized at 24, 48 and 96 HPI. In addition, samples of the same

tissues from two animals, each one of them mock inoculated with the same methodology on the right CB, and with no treatment on the left CB, were used as negative control samples. The aforementioned samples were collected immediately after euthanasia. Approximately 30mg of each of the collected tissues were quick-frozen in liquid nitrogen for later transfer to -70^{0}C for virus isolation (VI), and total RNA extraction for use in viral RNA real-time reverse transcriptase PCR (rRT-PCR), and cDNA synthesis (for cytokine mRNA analysis). Sections no thicker than 1cm of the remaining tissues were fixed in 10% buffered formalin and transferred to the Department of Pathology at the University of Georgia, for immunohistochemistry (IHC) studies to detect apoptosis.

Viral isolation (VI) and viral Real-Time Reverse Transcriptase PCR (rRT-PCR)

The materials and methods for the VI and rRT-PCR for VSNJV detection was already described in a previous study[5]. Viral isolation and quantification of viral RNA was determined prior to sample processing for cytokines assay in order to confirm viral infection.

Total RNA extraction and cDNA synthesis

Tissue homogenization was performed using the aforementioned 30mg of frozen tissue (not allowed to thaw) placed in a 5.0ml snap-cap tube containing 600µl of RLT

lysis buffer (Qiagen RNeasy Kit #74106). Tissues were macerated directly in RLT buffer using a mechanical, hand-held homogenizer (Tissuemiser, Fisher Scientific), and placed back on ice. Tissue homogenate was transferred to a 2.0ml microcentrifuge tube and centrifuged at maximum speed for 2 min. to pellet debris. Approximately 600µl of homogenate was transferred to a Qiagen Qiashredder, and the protocol for tissue RNA extraction was carried out for the remainder of the procedure as recommended by manufacturer. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer. Then 1.0µg of RNA was treated with DNase I per manufacturer's instructions (Sigma catalog #AMP-D1). Then total RNA was reverse transcribed into cDNA using random hexamers (Thermoscientific #AB-1297). Briefly, the 25µl reactions contained 11.0µl of RNA, 5.0µl of 5x First-strand buffer (250mM Tris HCl, 375mM KCl, 15mM $MgCl_{2)}$, 2.5µl of 0.1M DTT, 2.5µl of random hexamers (125ng/µl), 1.25µl of RNAseOut Recombinant Ribonuclease Inhibitor (Invitrogen #10777-019), 1.0µl (200units) of Moloney murine leukemia virus reverse transcriptase (Invitrogen #28025-013), 0.125mM deoxynucleoside triphosphates (Applied Biosystems #N808-0007), and 0.5µl H_20. Samples were thermocycled at 25°C, for 10 min., 37°C for 60 min, and 95°C for 5 min. The cDNA was then diluted with H_20 1:8 in a final volume of 200µl.

Cytokine reverse Real time PCR (rRT-PCR)

Cytokine rRT-PCR was carried out on the ABI 7000 Sequence Detection System. Briefly, the 25.0µl reactions contained 12.5µl Taqman Universal PCR Master Mix

(Applied Biosystems #4304437), 300nM final concentration of each primer (Invitrogen), 150nM final concentration of Taqman 6FAM-labeled fluorogenic probe (Applied 0Biosystems), 4.5µl H_2O, and 2.0µl of cDNA template. Samples were thermocycled at 50°C for 2 min., 95°C for 10 min., and 40 cycles of 95°C for 15 sec. and 60°C for 60 sec. Primers/probes were designed to the following bovine cytokines: IFN-α (GenBank M10953), IFN-β (GenBank M15477), IFN-γ (GenBank M29867), TNF-α (GenBank Z48808), TGF-β1 (NCBI Ref. Seq. NM001166068), IL-4 (GenBank M77120), IL-5 (NCBI Ref. Seq. NM173922), IL-10 (Dr. Mark Estes - University of Missouri U00799), IL-12p40 (NCBI Ref. Seq. NM174356), IL-13 (NCBI Ref. Seq. NM174089), IL-15 (NCBI Ref. Seq. NM174090) and IL-18(NCBI Ref. Seq. NM174091).

Relative Quantification of Cytokine Expression Analysis

Including the GAPDH house-keeping gene, a relative expression of the listed cytokines was analyzed. Right side tissues (inoculated side) were compared to their respective left side tissue. The following samples were evaluated for cytokine up/down regulation: front coronary bands, rear coronary bands, prescapular lymph nodes and popliteal lymph nodes. These included both VSNJV-inoculated (right side) and mock-inoculated (left side) tissues. The cDNA from each sample was run in triplicate and the CT (cycle threshold) values from each sample averaged. The standard deviation and %CV (coefficient of variation) was calculated for each set of triplicates. No sample set exhibited a standard deviation higher than 1.5 or a %CV higher than 4.0%. The Pfaffl

method[18] was used to determine relative up/down regulation of the target gene in comparison to the reference gene (GAPDH). Ratios of the infected-side samples were then plotted in comparison to the uninfected side, with a ratio of "1" indicating no up or downregulation.

Immunohistochemistry (IHC)

Briefly, after deparaffinization, sections were subjected to antigen retrieval treatment under heat pressure (pressure cooker) for 2 min using antigen unmasking solution (Citrate-based, Vector Laboratories), followed by blocking of non-specific epitopes with ultra V block (LabVision Corporation). Samples were then incubated overnight at 4°C with primary antibody anti-caspase 3 antibody (rabbit anti-active caspase-3 antibody, Promega, cat. # G7481) at a dilution of 1:125, followed by incubation with an alkaline phosphatase-linked polymer system (LabVision Corporation). The reaction was revealed using Vector Red chromogen. The slides were lightly counterstained with Mayer's hematoxylin then cover-slipped using Permount for permanent record.

The right rear CB and the right popliteal lymph nodes from the mock-inoculated animals were used as negative control tissues samples. In addition, the left (mock-inoculated side) rear CB and the left popliteal lymph node from all VSNJV-inoculated animals were also used as negative control samples. A semi-quantitative score was

attributed according to the frequency of staining, with 0 = Negative; + = rare number of cells; ++ = Moderate number of cells, and; +++ = marked number of cells.

Results

Cytokine reverse Real time PCR (rRT-PCR)

The results of the cytokine gene expression are presented in figures 5.1 to 5.8. Graphic representation depicts a relative expression of bovine cytokine mRNAs on the side of VSNJV inoculation (right side) versus the mock-inoculated side (left) from each animal. In the SC (Fig. 5.1) and FB (Fig. 5.2) mock-inoculated animals there was cytokine gene expression variation, either down or upregulation, lower than 10-fold change on the right side tissue samples. The FB mock-inoculated steer had almost all cytokines upregulated compared to the left non-treated tissues, but similarly, none of the measured cytokines reached 10-fold change. Therefore, variations lower than 10-fold change were considered normal in gene expressions, and changes equal to or higher than 10 fold were attributed to be meaningful variations for up- (equal or higher than 10) or downregulation (equal or lower than 0.1).

At 24 HPI, in the SC-inoculated animal (Fig. 5.3) there was upregulation (>10-fold change) of all 3 IFNs (-α, -β, -γ), TNF-α and IL-12 in the right front CB and right popliteal lymph node. The 24 HPI FB-inoculated steer (Fig. 5.4) had upregulation only of IFN-γ in the right prescapular and popliteal lymph nodes.

The 48 HPI SC-inoculated animal (Fig. 5.5) had intense upregulation of several of the cytokines measured, such as the three IFNs (-α, -β, -γ), TNF-α, IL-10, IL-15, IL-18 and IL-12 in the right front and rear CB. No cytokine upregulation was observed in the lymph nodes of the same steer. In fact, there was mild downregulation of TNF-α, IL-4, IL-18 and IL-12 in the right pre-scapular lymph node. Similarly, the 48 HPI FB-inoculated steer (Fig. 5.6) presented marked upregulation of α-, β-, γ-IFN, TNF-α, IL-10, IL-15, IL-18 and IL-12 in the right rear CB. In the right front CB of this animal, unfortunately the flies failed to feed. In this CB there was no up- or downregulation cytokine gene expression was observed in this foot or in the draining lymph node (right pre-scapular).

The 96 HPI SC-inoculated animal (Fig. 5.7) had marked drop in gene expression of several cytokines compared to the 48 HPI steers, with maintained upregulation of INF-γ and IL-12 in the right front CB, and α-, γ-INF and IL-18 in the right rear CB. Minimal downregulation (lower than 10-fold change) of several cytokines was present in the lymph nodes. Similarly there was marked drop in cytokine gene expression in the FB-inoculated steer (Fig. 5.8) at the same time point, with maintained upregulation of INF-γ, TNF-α and IL-10 in the right front CB, and INF-γ, IL-10 and IL-18 in the right rear CB. This steer had downregulation of IL-12 in the right pre-scapular and INF-α in the right popliteal lymph node.

Imunohistochemistry

Results of immunohistochemistry for anti-caspase 3 antibody are depicted in table 1. In the CB of the mock-inoculated animals, no signal for caspase 3 was detected. In the lymph node from the mock-inoculated animals, sparse signal (+) for caspase 3 was present, mostly in cells within the subcapsular sinus. Similarly, in the contralateral CB of the infected animals, sites which were harvested but were not inoculated, the level of caspase 3 was the same as the negative control animals. Also, the draining lymph node from this site remained with levels and distribution of caspase 3 comparable to the negative control lymph node.

In contrast, in the infected animals, active caspase 3 protein was detected in the CB of most animals, with increases over time. By 96 HPI, there was abundant (++ to +++) active caspase 3 evident in CB. Signal was seen within keratinocytes, especially those within and around the developing vesicle, as well as in inflammatory cells within both epidermis and dermis.

In the lymph nodes, there was an increase in amount of active caspase 3 in comparison to the negative control, and also an increase with time. Signal in lymph nodes began in cells within the subcapsular sinus but then by 48 and 96 HPI, had progressed to also include cells in the germinal centers and occasionally paracortex. By 96 HPI, signal was also present in cells within medullary sinuses and sometimes in the mantle area.

DISCUSSION

There was marked upregulation of various cytokines in the tissues of VSNJV-inoculated steers via either SC or FB. This response was more intense in the first 48 HPI and was characterized by upregulation of typical antiviral and proinflammatory cytokines, such as type I and II IFNs, IL-12, IL-15, IL-18 and TNF-α. The most dramatic upregulation was in the T helper 1 type cytokines. However, only IL-10 among the analyzed T_H2 cytokines (IL-4, IL-5, IL-10, IL-13 and TGF-β) was upregulated, and persisted at high levels of mRNA expression until 96 HPI in the FB-inoculated animal. The fact that only this single T helper 2 cytokine persisted upregulated for a longer period of time in one FB-inoculated steer is not sufficient to drawn any definitive conclusion and further investigations are warranted in order to determine whether or not insect transmission plays a role in driving host immunity into a T_H2 response.

It is of important note that the peak of cytokine expression reported in the current study coincides with peak of viral replication in the site of inoculation at 48 HPI, as we previously reported using *in situ* hybridization.[5]

No upregulation of the analyzed cytokines was observed in right (VSNJV-inoculated side) rear CB of the 24 HPI SC-inoculated steer, and neither in the right rear and front CB of the 24 HPI FB-inoculated animal. The peak time of viral replication, as envisioned by *in situ* hybridization is 24 HPI.[5] So it seems that the greatest amount of cytokines is just subsequent to the peak of viral replication. The most dramatic and prolonged cytokine upregulation, in both SC and FB steers, was IFN-γ. IL-12 was also upregulated in the majority of the animals and tended to correlate to but did not reach

such high level of expression when compared to the IFN-γ. It is widely known that these two cytokines act synergistically.[19] IFN-γ drives the immune response towards a T helper 1, directly by inducing differentiation of naive $CD4^+$ cells to T helper 1 lymphocytes or indirectly by activation of APCs to produce IL-12, which is the major T helper 1 cytokine. IL-12 contributes to T helper 1 lineage commitment by binding to receptors on antigen-stimulated $CD4^+$ T cells and activating the transcriptor factor STAT4, which further enhances IFN-γ production. IFN-γ is the principal macrophage-activating cytokine and serves critical functions in innate immunity and in adaptative cell-mediated immunity against intracellular microbes. Another important function of INF-γ is stimulation of expression of class I and II MHC molecules and co-stimulators on APCs. It is well established that IFN-γ is produced by activated NK cells, and T[20] and γδ T cells.[21,22] The activation of γδ T cells as well as other T lymphocytes and NK cells is also mediated by IL-12.[21] Therefore, the high levels of IFN-γ and IL-12 expression found in this study indicate that these cytokines likely play a key role in driving an effective T helper 1 immune response in cattle inoculated with VSNJV via either SC or FB inoculation.

This study shows upregulation of IL-18 in the later time points in the CB of both SC- and FB-inoculated animals. IL-18 has potent proinflammatory effects and belongs to the IL-1 family of cytokines as previously reviewed.[23] It is produced by macrophages and more recently it has been reported to be produced by keratinocytes and to play an important role in cutaneous inflammatory processes.[21,24,25] IL-18 is constitutively expressed by keratinocytes, and, as opposed to IL-1, needs only caspase 1, an enzyme which is part of the cytoplasmic inflammasome complex, to be activated.[24] It is known

that IL-18 as well as IL-12 function as inducers of cell-mediated immunity, and that these two cytokines have synergetic functions in stimulating IFN-γ production by T lymphocytes and NK cells.[26,27] Therefore, it seems that IL-18 in conjunction with IL-12 is playing an important role in the immune response in VSNJV infection in the CB of cattle during the first 48 HPI.

Similarly to IL-18, IL-15 was upregulated at 48 HPI in the CB of both SC- and FB-inoculated steers. Mononuclear phagocytes and other cells in response to viral infections produce IL-15.[19] It functions as a growth factor and activator of T lymphocytes and NK cells.

Levels of type I IFN expression were also elevated, but not as elevated as those of IFN-γ. Type I IFNs were upregulated especially in the early time points of infection. Reports have shown that VSNJV is a potent inducer of type I IFNs,[4] which are cytokines with potent antiviral, antiproliferative, and immunomodulatory functions.[20] It is well documented that type I IFNs exert a potent inhibitory effect on VSV replication and progeny virus production both *in vitro* and *in vivo*.[8] Our results agrees with the previous reports regarding the fact that VSV is a potent inducer of type 1 IFNs and may be the reason why viral spread has not yet been detected beyond draining lymph nodes.

Marked apoptosis, particularly at the later time points, was observed in this study in both SC- and FB-inoculated steers. This finding was expected since it is well known that VSV is a good inducer of cell death through apoptosis.[28,29] As previously reported VSV triggers apoptosis via a mitochondrial pathway through apaf-1 and caspase 9[28,29] via activation of Bak protein.[29] Type I IFNs also play a role in selectively eliminating virally infected cells by triggering cell death via apoptosis, as previously reported with VSV and

other viral infection *in vitro*.[30] Therefore, high level of apoptosis of lymphoid cells may help to explain why downregulation of certain cytokines was present in the lymph nodes draining inoculated CBs. It is likely that VSNJV is mediating cell host apoptosis via direct cytotoxic effects or via indirect mechanisms by inducing IFN production.

In summary VSNJV causes acute and intense T helper 1 cytokine response in the first 48 HPI in both SC- and FB-inoculated animals. Collectively, high expression of cytokines such as IFN-γ IL-12, IL-18, and type I IFNs, and marked apoptosis seems to play a central role in the early infection and may help to explain the puzzling lack of viremia noted consistently in this infection.

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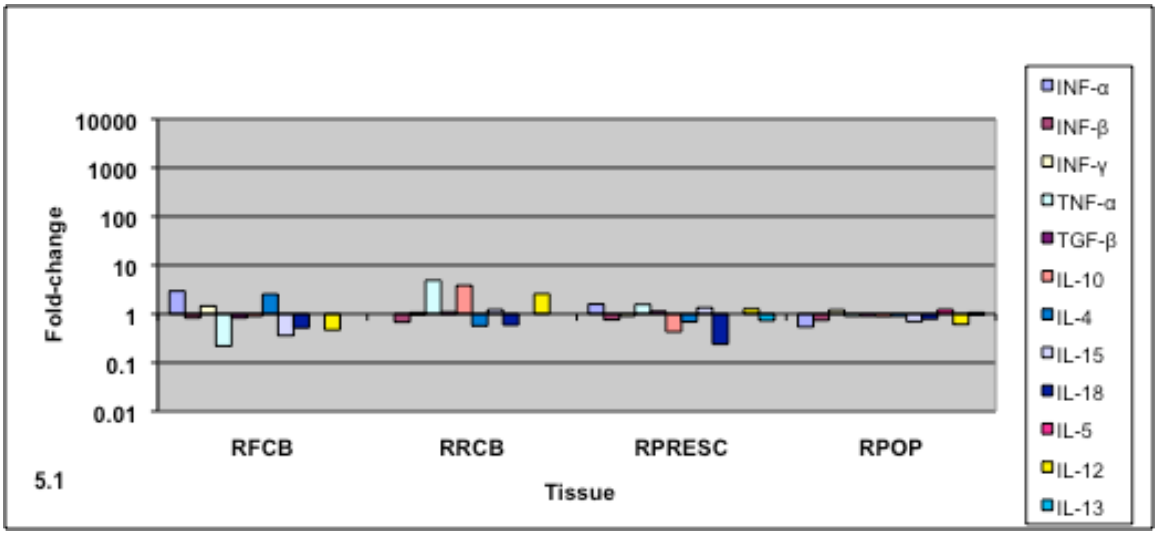

Fig. 5.1: 24 HPI, scarification, negative control steer. Relative expression of bovine cytokine mRNAs on side of inoculation (right) vs. mock inoculated side (left). RFCB = right front coronary band (CB); RRCB = right rear CB; RPRESC = right prescapular lymph node; and RPOP = right popliteal lymph node.

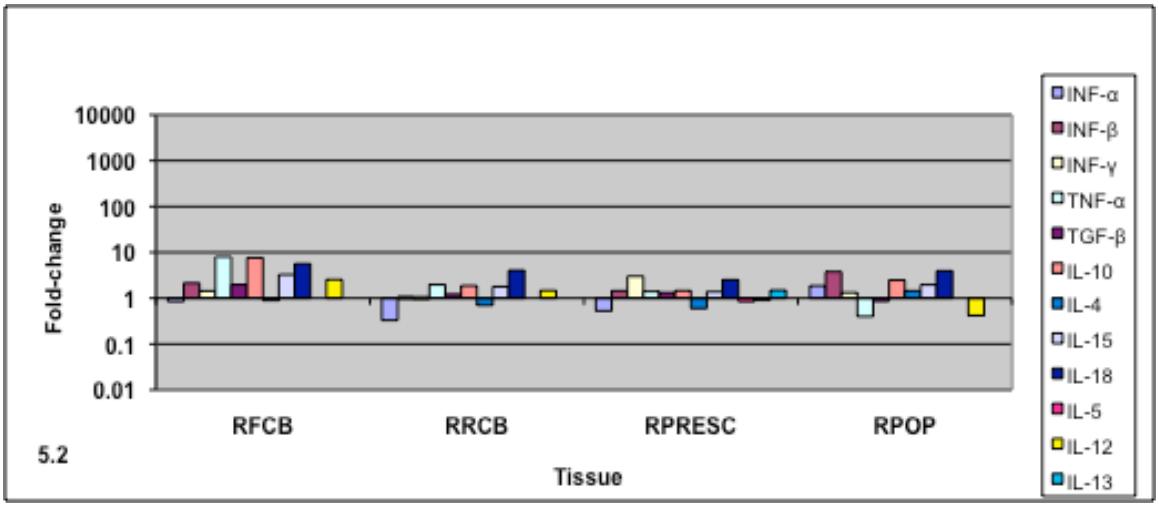

Fig. 5.2: 24 HPI, fly bite, negative control. Relative expression of bovine cytokine mRNAs on side of inoculation (right) vs. mock inoculated side (left). RFCB = right front coronary band (CB); RRCB = right rear CB; RPRESC = right prescapular lymph node; and RPOP = right popliteal lymph node.

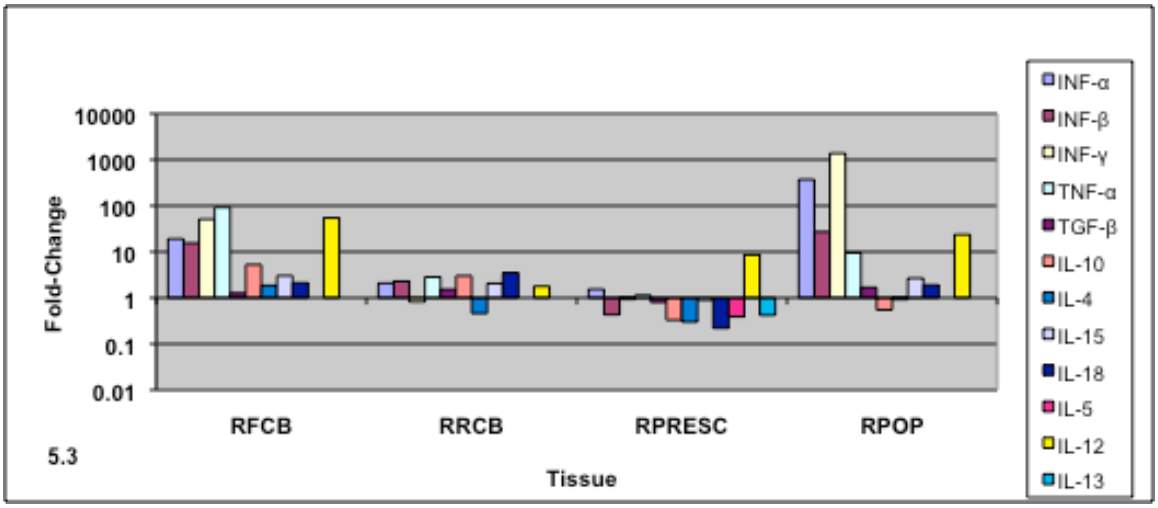

Fig. 5.3: 24HPI, scarification. Relative expression of bovine cytokine mRNAs on side of incoculation (right) vs. mock inoculated side (left). RFCB = right front coronary band (CB); RRCB = right rear CB; RPRESC = right prescapular lymph node; and RPOP = right popliteal lymph node.

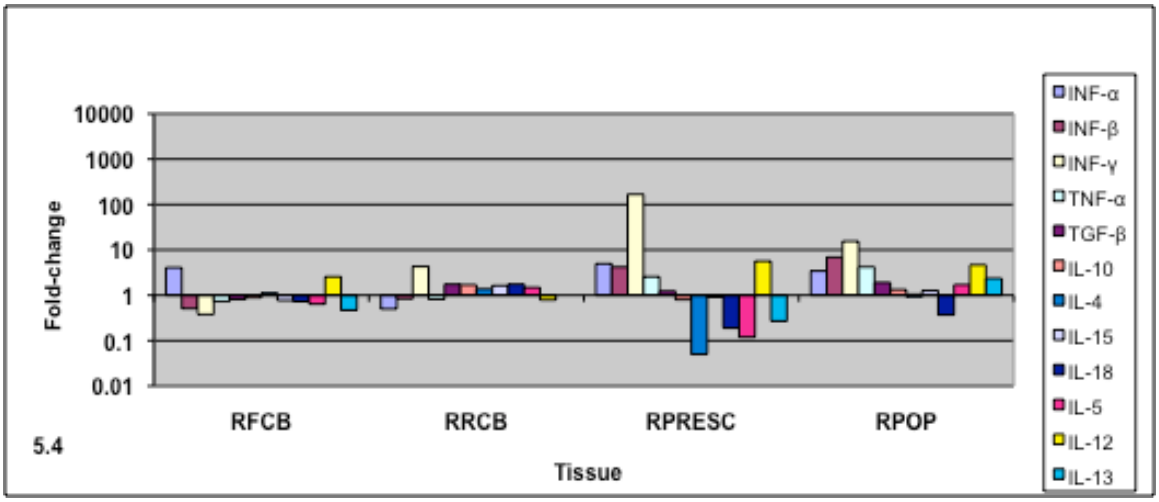

Fig. 5.4: 24 HPI, fly bite. Relative expression of bovine cytokine mRNAs of inoculation (right) vs. mock inoculation side (left). RFCB = right front coronary band (CB); RRCB = right rear CB; RPRESC = right prescapular lymph node; and RPOP = right popliteal lymph node.

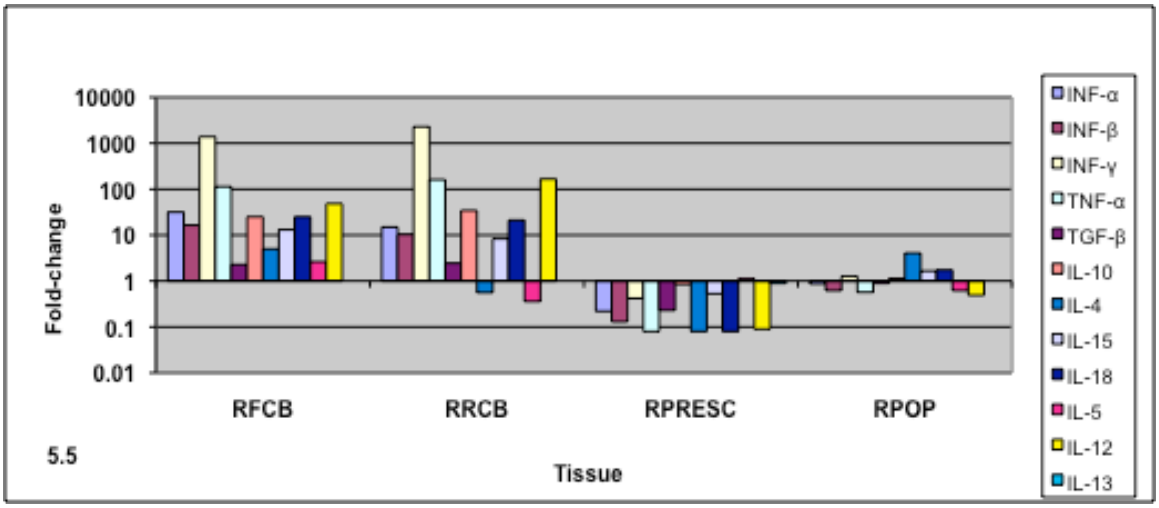

Fig. 5.5: 48 HPI, scarification. Relative expression of bovine cytokine mRNAs on side of inoculation (right) vs. mock inculated side (left). RFCB = right front coronary band (CB); RRCB = right rear CB; RPRESC = right prescapular lymph node; and RPOP = right popliteal lymph node.

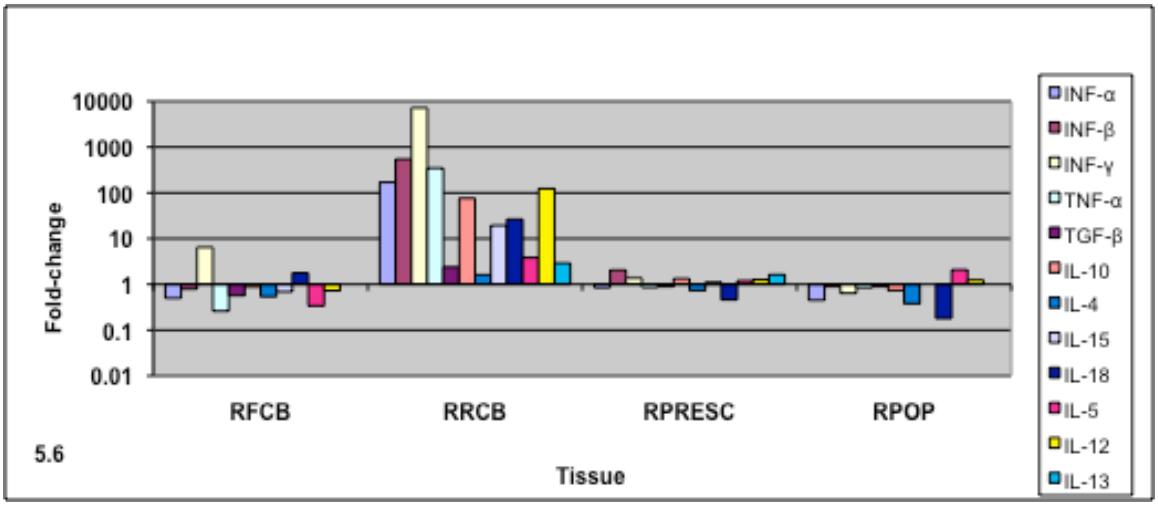

Fig. 5.6: 48 HPI Fly-bite (#8180) relative expression of bovine cytokine mRNAs on side of inoculation (right) vs. mock inoculation side (left). RFCB = right front coronary band (CB); RRCB = right rear CB; RPRESC = right prescapular lymph node; and RPOP = right popliteal lymph node.

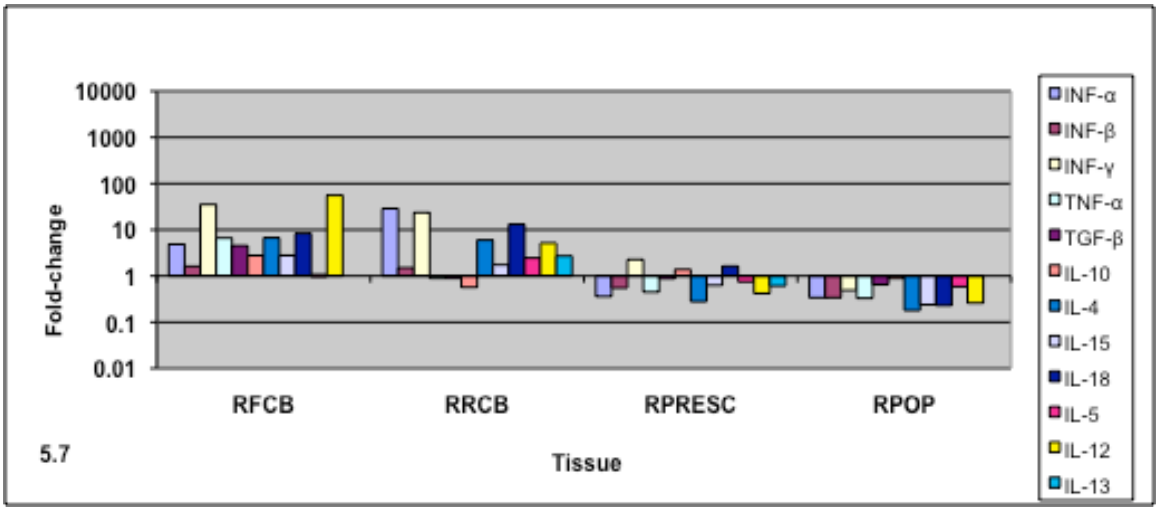

Fig. 5.7: 96 HPI Scarification. Relative expression of bovine cytokine mRNAs on side of inoculation (right) vs. mock inoculation (left). RFCB = right front coronary band (CB); RRCB = right rear CB; RPRESC = right prescapular lymph node; and RPOP = right popliteal lymph node.

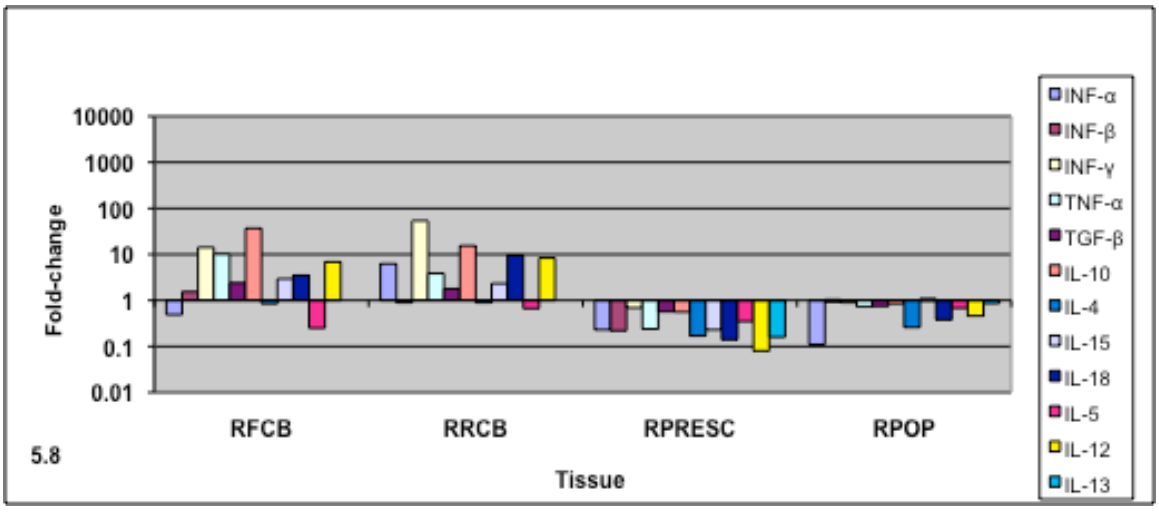

Fig. 5.8: 96 HPI, fly bite. Relative expression of bovine cytokine mRNAs on side of inoculation (right) vs. mock inoculated side (left). RFCB = right front coronary band (CB); RRCB = right rear CB; RPRESC = right prescapular lymph node; and RPOP = right popliteal lymph node.

Table 5.1: Immunohistochemistry for anti-caspase 3 in coronary bands and lymph nodes of steers inoculated with VSVNJ via scarification or fly bite.

Treatment	Tissue	SC-Steer	FB-Steer
24 HPI Mock-inoculated	RRCB	0	0
	RPOP	+	+
24 HPI VSNJV-inoculated	RFCB	+	0
	RRCB	+++	+
	LRCB (mock)	0	0
	RPRE	++	+
	RPOP	++	+
	LPOP (mock)	+	+
48 HPI VSNJV-inoculated	RFCB	+	0[#]
	RRCB	++	++
	LRCB (mock)	0	0
	RPRE	++	+
	RPOP	++	++
	LPOP (mock)	+	+
96 HPI VSNJV-inoculated	RFCB	+++	++
	RRCB	+++	+++
	LRCB (mock)	0	0
	RPRE	++	+
	RPOP	+++	++
	LPOP (mock)	0	+

Right samples were the VSNJV-inoculated side tissues, and left samples were mock-inoculated side tissues from each inoculated animal; HPI = Hours post-inoculation; SC = Scarification; FB = Fly bite; RFCB = Right front coronary band (CB); RRCB = Right rear CB; LRCB = Left rear CB; RPRE = Right pre-scapular lymph node; RPOP = Right popliteal lymph node; LPOP = Left popliteal lymph node; [#]No flies fed on the RFCB at 48 HP; 0 = Negative; + = rare number of cells; ++ = Moderate number of cells, and; +++ = marked number of cells.

CHAPTER 6

CONCLUSIONS

This study describes several events in the acute infection of vesicular stomatitis virus (VSV) in cattle, with animals followed from 12 to 120 HPI. There was successful transmission of vesicular stomatitis New Jersey virus (VSNJV) with viral replication in keratinocytes and vesicle formation when the virus was inoculated in the coronary bands (CB) of steers via both scarification (SC) and fly bite (FB). In general, lesions in the CB were more extensive in the FB animals, despite the lower inoculating dose when using FB. Inoculation into neck skin failed to produce any evidence of viral replication or any lesions. Other studies have described lesions only in CB, oral mucosa, teats, prepuce, and vulva, all of which are areas containing very thick, multi-layered, non-haired epidermis. Using *in situ* hybridization to detect viral mRNA and therefore viral replication, the peak of viral replication in the CBs in our study occurs between 24 and 48 HPI, in the keratinocytes within the thick non-haired epidermis. Then with immunohistochemistry to detect viral protein, there is persistence beyond these periods. All of the lesion foci described in various VSV experiments have in common thick stratum spinosum, lack hair and are subjected to mechanical trauma. It is reasonable to speculate that the keratinocytes of those areas present either a membrane receptor probably an intercellular adhesion molecule or suitable intracellular molecular conditions for viral replication.

In examining the lymph nodes draining the sites of inoculation, replicating virus was limited to 24 HPI, with viral antigen detected until the end of the experiment. No virus was detected beyond draining lymph nodes, confirming lack of viremia as seen in many other VSV studies.

A cell marker study helped to clarify the inflammatory cells and their markers. Inflammatory infiltrates in the CB of both SC and FB-inoculated animals are mainly composed of $MAC387^+$ cells, in which most of cells are neutrophils early in infection, and towards the end of the experiment most are macrophages. The second most common inflammatory cell population is composed of $CD3^+$ cells in both SC- and FB-inoculated animals throughout the study. It appears that the majority of the $CD3^+$ cells are $WC1^+$, which indicates the majority of the T cells are γδ T lymphocytes. These cells are likely playing a key role in VSNJV infection and contributing to the high levels of IFN-γ present.

In the lymph nodes an increase, according to the time of inoculation, in detection of $CD1b^+$ dendritic cells was observed in subcapsular and paracortical areas. These same areas correspond to the distribution of replicating virus and viral antigen in early stages of the infection and suggest that these cells, likely dermal or interdigitating dendritic cells, contain replicating virus and/or are presenting viral antigen to T helper lymphocytes. In the same manner, increased detection of follicular dendritic cells was observed in late time points and their distribution coincides with viral antigen localization in mantle areas. This likely represents follicular dendritic cells presenting viral antigen to B lymphocytes.

Comparing SC and FB, we demonstrated that gross and microscopic lesion development is more prominent and happens faster with FB. Upregulation of MHC II

was present only in the SC animals, and actually decreased in the FB-inoculated animals. It may be that some substance inoculated from the fly interferes with MHC II expression and this may explain why the lesions with FB are more severe than with SC inoculation.

Coinciding with the time of maximal viral replication, levels of T helper I cytokines peak at 48 HPI in both SC and FB-inoculated animals. In particular, IFN-γ and IL-12, as well as type I IFNs were markedly upregulated, especially at the site of inoculation. There was a marked increase in the number of cells undergoing apoptosis, as determined by caspase 3 expression, at the site of inoculation as well as the lymph nodes, most notable at 48 and 96 HPI. It may be this tremendous outpouring of T helper I cytokines and the apoptosis activity that prevents more extensive viral replication and the lack of viremia.

Collectively, this study presents new data to better understand the pathogenesis of the VSNJV infection in cattle and these findings may be instrumental for future pathogenesis studies, and for implementing measures of disease control and prevention.