DISSERTATION

IDENTIFICATION OF *CULEX TARSALIS* D7 SALIVARY PROTEIN AND ROLE OF SALIVARY PROTEIN VACCINE ON SUBSEQUENT WEST NILE VIRUS INFECTION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KRYSTLE LYNN REAGAN ENTITLED IDENTIFICATION OF *CULEX TARSALIS* D7 SALIVARY PROTEIN AND ROLE OF SALIVARY PROTEIN VACCINE IN WEST NILE VIRUS INFECTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

IDENTIFICATION OF *CULEX TARSALIS* D7 SALIVARY PROTEIN AND ROLE OF SALIVARY PROTEIN VACCINE ON SUBSEQUENT WEST NILE VIRUS INFECTION

Mosquito salivary proteins (MSPs) modulate the host immune response, leading to enhancement of arboviral infections. Identification of protein factors in saliva responsible for immunomodulation should lead to new strategies to prevent and protect against arboviral infection.

D7 salivary proteins are among the most abundant in mosquito saliva, and they function as both vasodilators and suppressors of local inflammation. Here we identify D7 salivary proteins in *Culex tarsalis*, an important disease vector in the western United States. Recombinant D7 proteins were used to analyze the systemic and local immunomodulatory properties of the host.

In this project, we immunized mice with recombinant D7 and tested for protection against subsequent challenge with West Nile virus (WNV) (NY99) delivered by mosquito bite. The vaccine was able to elicit a specific immune response. However, it enhanced WNV infection in the mouse model. We suggest that WNV enhancement is due to three factors. First, vaccinated mice had significant cellular infiltrates at the mosquito bite site, which included WNV permissive monocytes and dendritic cells.

Increases in these cell populations at the mosquito bite site leads to an increase in initial viral infection and dissemination. Others have shown that higher peripheral viral levels lead to a worse disease outcome from infection. Secondly, mortality curves in infected mice receiving passive transfer of serum containing antibodies from vaccinated mice mimicked those from vaccinated animals. Antibody neutralization of mosquito salivary proteins that are critical in completing a successful blood meal may lead to increased probing time by the mosquito. Increased probing by the mosquito results in an increase in amount of saliva being deposited, therefore an increase in initial viral dose. Lastly, the cytokine profile observed in vaccinated mice showed an increase in the Th2 cytokine IL-4 and regulatory cytokine IL-10 and a decrease in Th1 cytokines such as IL-12p70 and IFNγ. A protective immune response to WNV includes high levels of Th1 cytokines. Production of IL-4 by mice that received the rD7 vaccine directly inhibits the Th1 cytokines necessary for protection.

This work has increased our understanding of the complex nature of immunity to MSPs. Vector saliva vaccines have been successful in protecting against other blood feeding arthropods transmitted diseases. Nevertheless, differences in vector and pathogen physiology may preclude this approach from being successful for mosquito virus systems.

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CHAPTER I

LITERATURE REVIEW

Introduction

Arthropod-borne viruses (arboviruses) are of serious and growing concern to human and veterinary medicine. These pathogens, transmitted by the bite of an infected arthropod, enter the host together with a myriad of salivary proteins having a wide range of pharmacological properties. The effects of arthropod salivary proteins and immunity to these proteins have been an area of increased interest in arboviral research. Mosquito saliva contains compounds that promote more efficient blood uptake and alter the host's immune status. Immunomodulation by salvia creates an environment that is favorable for some pathogens in the vertebrate host. Indeed mosquito saliva has been shown to enhance the pathogenicity in the host of several viruses including vesicular stomatitis virus, Cache Valley fever virus, and West Nile virus (WNV) (Edwards et al. 1998, Limesand et al. 2000, Schneider et al. 2006). Immunization of the host using specific mosquito salivary proteins (MSPs) should alter the saliva induced immunomodulation and protect against arboviral infection.

The emergence of WNV into the Western hemisphere in 1999 spurred interest in therapeutics and vaccines to minimize WNV impact on human and equine populations. This epidemic became of widespread medical and veterinary concern in the united States and the Centers for Disease Control and Prevention confirmed 29,624 cumulative human cases and 1,161 deaths through the 2009 transmission season (CDC 2010). In addition the United States Department of Agriculture reports 25,729 equine cases from 1999 to 2010 (USDA 2010). WNV is maintained in a transmission cycle between birds and mosquitoes primarily of the genus *Culex (Cx.)*. In the United States *Cx. tarsalis, quinquefasciatus,* and *pipiens* have been identified as the primary vectors. Tangential

hosts, such as humans and equids, can become infected and in a small percentage of the cases develop into severe neurologic disease. Successful equine vaccines have been developed and are widely available, however there is no human vaccine commercially available.

The use of *Cx. tarsalis* salivary proteins as a vaccine would confer protection against not only WNV but also other viruses transmitted by the same species of mosquito including western equine encephalitis virus and St. Louis encephalitis virus. Investigation into salivary protein vaccines could yield new information about the role of saliva in arbovirus pathogenesis and lead to products that are protective against many important pathogens.

Mosquito Saliva

Hematophagous arthropods have evolved to have highly specialized salivary glands to aid in the uptake of blood from their hosts. The saliva that is secreted consists of a milieu of pharmacologically active substances to aid in blood uptake. Blood meals provide nutrients that are used as both an energy source and in reproductive development (Clements 2000). When a mosquito imbibes a blood meal she probes into the host with the proboscis and injects saliva to lubricate mouthparts and to aid in the uptake of blood. The host hemostatic and immune system act swiftly to counteract the injury and assault at the bite site. The pharmacologically active substances in the saliva function to offset the host hematopoetic system so the mosquito can successfully imbibe a blood meal.

The adult mosquito has two salivary glands located in the thorax of the body. Each salivary gland has three lobes (Clements 2000) consisting of two lateral lobes and a medial lobe, with the salivary glands in females being more larger and more developed

than those of the males or non-blood-sucking mosquito species (Wright 1969, Clements 2000). The lobes have been shown to produce distinct sets of salivary proteins with the lateral lobes producing proteins important in sugar feeding and the medial lobe producing proteins integral to blood feeding. The acinar cells that make up the salivary glands glandular structure produce the saliva and secrete the saliva into the lateral salivary ducts (Valenzuela 2004).

The evolution of blood feeding arthropods has required the saliva to aid in the uptake of blood by counteracting coagulation and inflammation in the host. Mosquito salivary proteins (MSP) include functional vasodilators, anti-coagulants, and platelet aggregation inhibitors (Valenzuela 2004).

D7 Salivary Proteins

The most abundant proteins found in the saliva of adult female mosquitoes belong to the D7 family of proteins (Malafronte Rdos et al. 2003, Almeras et al. 2009). D7 proteins make up between 5 and 20% of the total protein content of saliva (Calvo et al. 2006).

D7 proteins are specifically expressed in the salivary glands of adult Diptera (James et al. 1991), including Culicine and Anopheline mosquitoes, and some species in the families Psychodidae and Ceratopogonidae (Calvo et al. 2006). *In situ* hybridization experiments have localized D7 transcripts to the medial and distal lateral lobes of the female *Ae. aegypti* salivary glands (James et al. 1991). These areas of female salivary glands are highly specialized for blood feeding. This indicates that D7 plays a role in blood feeding activity. In *An. gambiae* the expression of D7 does not appear to fluctuate with blood feeding activity, but instead is constitutively expressed (Nirmala et al. 2005).

The proteins have been found to have a long and a short form with the long versions being in the 27 – 30 kDa range and the short versions 15-20 kDa (Valenzuela et al. 2002). Gene analysis indicates the short D7 is a result of deletion of the 5' half of the transcript coding for the aminoterminal end of the long D7 proteins (Valenzuela et al. 2002).

Comparative genome analyses in all mosquito species analyzed to date have shown duplication of the D7 encoding regions. Transcriptome and proteome analysis of cDNA from *Cx. quinquefasciatus* shows eleven genes that code for D7 proteins (Ribeiro et al. 2004). The *Anopheles gambiae* genome encodes eight D7 proteins (Calvo et al. 2006) while *Ae. aegypti* encodes five (Valenzuela et al. 2002). Recent analysis of the sialo-transcriptome of *Cx. tarsalis* showed expression of eight members of the D7 protein family (Calvo et al.).

Within the D7 family of proteins there are six clades represented (Calvo et al.). Figure 1.1 taken from Calvo et al (2006) shows that the D7 proteins found in one species of mosquito can belong to several clades. Phylogenetic analysis suggests that the D7 proteins may be a product of gene duplication in a common ancestor of mosquitoes leading to the multiple D7 proteins seen in a single species (Calvo et al. 2006). D7 proteins are related to the odorant binding proteins (OBP)(Arca et al. 2002). OBPs have been identified in many insect species and function by acting as carriers of small hydrophobic molecules, and D7 proteins may have a similar carrier type function (Arca et al. 2002). Short and long versions of D7 contain one or two OBP domains respectively, consisting of four conserved cysteine residues (Arca et al. 2002, Calvo et al. 2009).

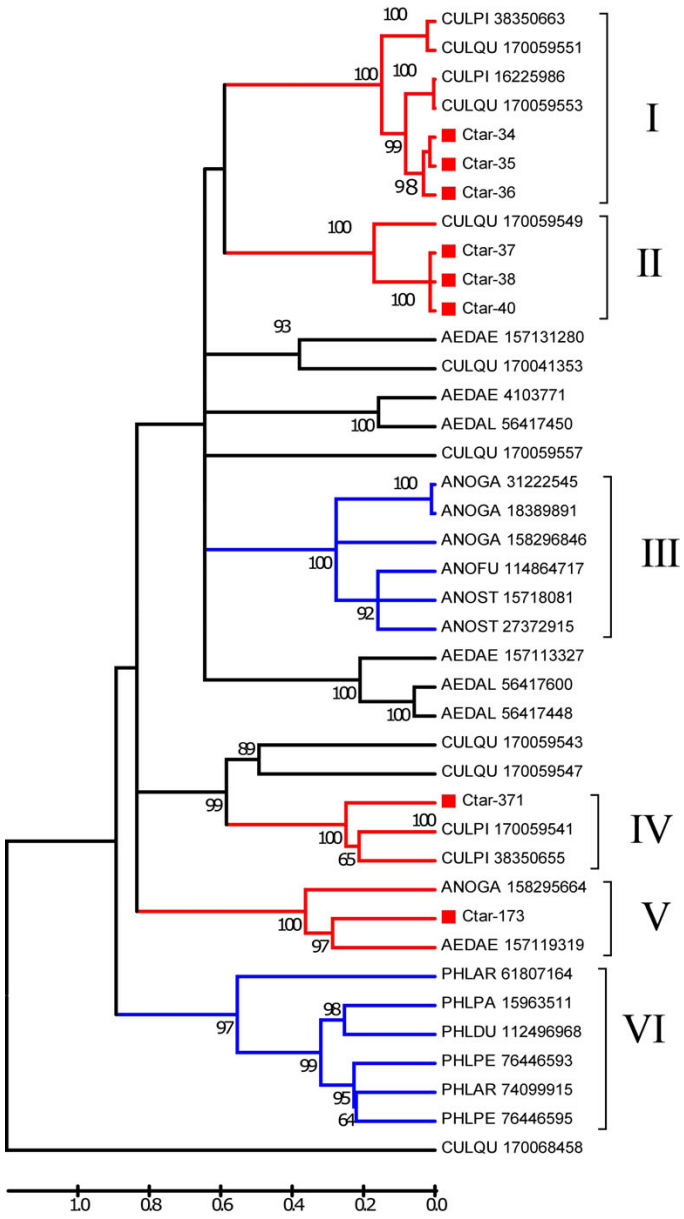


Figure 0.1.1 D7 Phylogenetic analysis of D7 genes encoding both long and short D7 proteins from *Culex pipiens, quinquefasciatus, tarsalis, Aedes aegypti, albopictus, Anopheles gambiae, funestus, stephensi, and Phlebotomine* sand flies. The first three letters represent the genus with the last two letters representing the species, and followed by the NCBI accession number. Strongly bootstrapped clades are numbered above.(Calvo et al. 2010a)

The crystal structure of a D7 protein from *An. gambiae* further strengthened the classification of D7 as an OBP. The structure of D7 was shown to contain 8 alpha-

helices that are stabilized by disulfide bonds (Mans et al. 2007). The arrangement leads to the formation of a hydrophobic binding pocket, similar to the OBPs (Mans et al. 2007). It was hypothesized based on the structure of the binding pocket that the D7 proteins would bind to biogenic amines (Mans et al. 2007). Indeed, recombinant D7 proteins from both *Ae. aegypti* and *An. gambiae* were expressed and shown to bind with high affinity to serotonin. In addition the D7 proteins were found to bind both histamine and norepinephrine (Calvo et al. 2006). Biogenic amines are released from platelets and mast cells in response to a tissue injury and result in both a pain response and vasoconstriction (Calvo et al. 2009).

In a separate study, the two OBP domains of *Ae. aegypti* D7 protein were shown to have additional functions. The N-terminal domain acted to bind biogenic amines as shown earlier, and the second, the C-terminal domain, binds to leukotrienes (Calvo et al. 2009). Leukotrienes are important immunological mediators that are released by mast cells. The cysteinal leukotrienes that are bound by D7 act to promote edema, erythema, pain and itching that may lead the host to take action to stop the mosquito from continuing the blood meal (Calvo et al. 2009). Sequestering biogenic amines and leukotrienes aids in the mosquito completing a blood meal by inhibiting vasoconstriction, inflammation, and the host pain response.

The D7 proteins have also been identified as allergens in people (Peng and Simons 2007). Humans who have been exposed to mosquito bites form both IgG and IgE antibodies to D7 proteins (Peng et al. 2006). In addition to antibody production people can have both an immediate type I reaction and a type IV cellular delayed type hypersensitivity in response to recombinant *Ae. aegypti* D7 (Peng et al. 2006). In mouse

models, injection of rD7 subqutaneously without an adjuvant was able to induce a strong Th2 type immune response characterized by high levels of IgE and IgG1 and low levels of IgG2a (Wang et al. 1999).

Vasodilators

Hematophagous arthropod saliva contains substances that prevent the host from constricting vessels, thus restricting the flow of blood to the bite site. Vasodilators have been identified in *Ae. aegypti* and *An. gambiae* saliva (Ribeiro and Francischetti 2003) and are likely present, but not yet identified in other mosquito species.

Sialokinins are highly potent vasodilatory peptides expressed in the medial lobes of female *Ae. aegypti* that mimic the activity of mammalian tachykinins (Champagne and Ribeiro 1994, Beerntsen et al. 1999). Two forms of this peptide, sialokinin I and II, were described originally that differed by one amino acid. However, it is now thought to be only one form and the second was an artifact of the isolation method (Ribeiro et al. 2007). Vasodilatory peptides similar to these have also been found in *Culex salinarius* (Meola et al. 1998)

Vasodilators have also been identified in the saliva of *Anopheles* mosquitoes (Clements 2000). The proteins have catechol oxidase activity and bind to and destroy norepinephrine and serotonin (Ribeiro and Valenzuela 1999, Ribeiro and Francischetti 2003).

Anti-coagulants

The blood coagulation cascade is a complex process and is a primary defense after injury. The extrinsic portion of the cascade is initiated when a mosquito takes a blood meal. A breach in the capillary endothelium leads to exposure of von Willebrand

factor and a release of high levels of ADP from cellular cytoplasm (Valenzuela 2004). This in turn leads to the activation of several clotting factors as diagramed in figure 2 that convert prothrombin to thrombin and fibrinogen to fibrin to crosslink platelets and create a blood clot (Davie et al. 1991). Fibrin cross-linking activates platelets to release granules rich in pro-clotting factors including ADP and thromboxane A_2 that further activate surrounding platelets (Sun et al. 2006).

Mosquito saliva has evolved to contain proteins that block coagulation at several different steps. There are members of the serpin family, serine protease inhibitors, in *Ae. aegypti* that block factor Xa in the coagulation cascade (Ribeiro and Francischetti 2003). In *Anopheles* mosquitoes a 6 kDa peptide has been identified that binds to the catalytic site of thrombin, preventing its enzymatic activity on fibrinogen (Francischetti et al. 1999).

In addition to the disruption of the clotting cascade, there are also enzymes in the saliva that act to prevent activation and recruitment of platelets to the site of vascular injury. Apyrase enzymes have been identified in several species of animals including mosquitoes. In mosquitoes, as members of the 5'-nucleotidase family, they act on ATP and ADP to cleave phosphates resulting in AMP (Champagne et al. 1995). These enzymes have been identified in *Ae. aegypti* and *Anopheles* species, but if they are present at all in *Culex* species, it is at very low levels (Ribeiro 2000, Calvo et al.). Because *Culex* mosquitoes evolved to obtain blood meals from avian species, which do not have a robust platelet response, it is hypothesized they did not evolve a salivary apyrase (Ribeiro 2000). In addition to the platelet activation disruption of apyrase, the protein aegyptin has also been found to inhibit platelet aggregation and activation (Calvo

et al. 2010b). Aegyptin binds to collagen and prevents it from binding to von Willebrand factor, an important factor in attracting platelets to the site of injury (Calvo et al. 2010b). It was originally described in *Ae. aegypti* mosquitoes, but since then similar proteins have been found in *Ae. albopictus, Anopheles* species and *Culex tarsalis* (Calvo et al. 2010a).

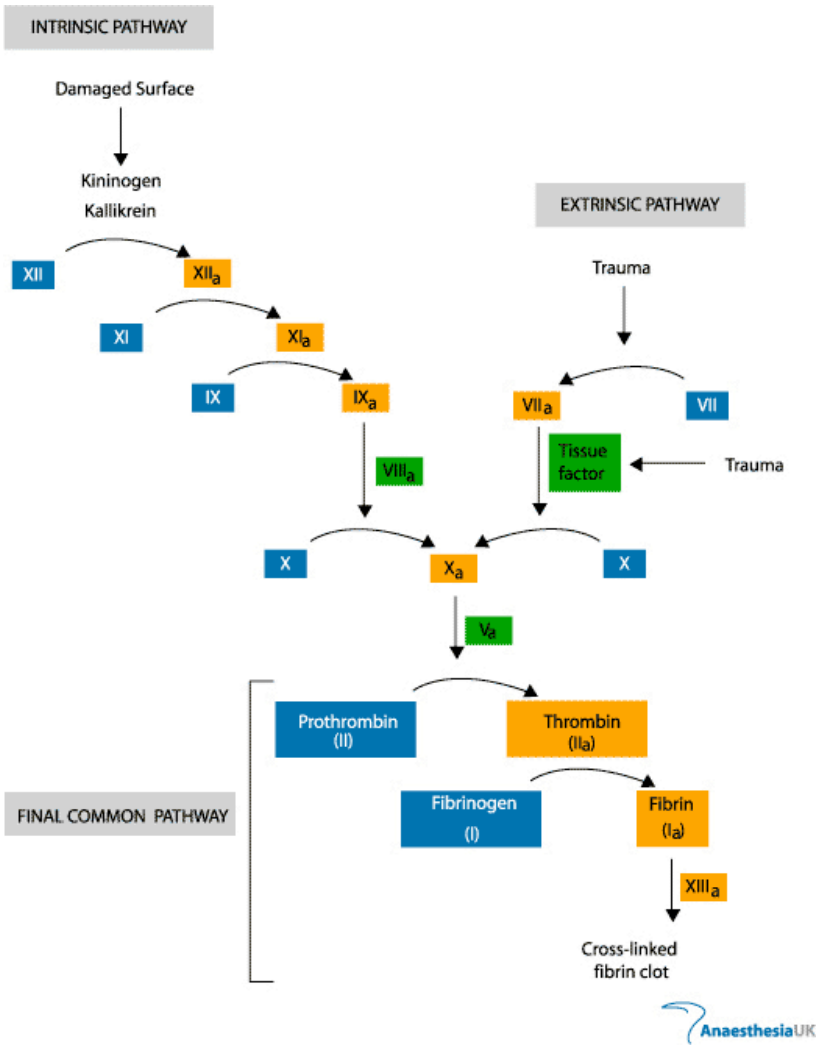


Figure 1.0.2 Blood coagulation cascade. Mosquito saliva blocks the coagulation cascade at Factor Xa and at the thrombin steps. (AnaesthesiaUK 2005)

Immunomodulators

Blood feeding arthropods must not only counteract hemostasis but also evade the host immune response. A strong immune response to vector saliva can inhibit blood feeding, so down regulation of inflammation is beneficial to arthropods. This area of

research has yielded extensive information about salivary immunomodulators in the Ixodid ticks that feed on their hosts for up to ten days and counteracting the vertebrate immune system is critical for a successful blood meal. However, similar research for mosquito saliva immunomodulators is rather limited.

Mosquito saliva contains cytokine modulators, neutrophil chemotactic factor, inflammation inhibitors, inhibitors of the plasma contact system and inhibitors of T and B lymphocytes (Titus et al. 2006). Splenocytes cultured with salivary gland extract from *Ae. aegypti* showed a significant decrease in the proliferation of both B and T lymphocytes as compared to controls (Wasserman et al. 2004) and a decrease in total splenocytes (Cross et al. 1994). Mosquito saliva also acts to attract to neutrophils, eosinophils, and mast cells at the bite site (Owhashi et al. 2001, Demeure et al. 2005, Owhashi et al. 2008).

In addition to the cellular immunomodulation, there is strong evidence for the altering of the cytokine milieu after exposure to mosquito saliva. Several reports have been made showing modulation of cytokines in cultured splenocytes with the addition of salivary gland extract. These studies have consistently shown a decrease in IL-2, IL-12, IFNγ and TNFα (Bissonnette et al. 1993, Champagne et al. 2004, Schneider et al. 2004, Wanasen et al. 2004) when the splenocytes are cultured with salivary glands from *Ae. aegypti*. There have been differing results with IL-4 and IL-10, where some found that when high levels of salivary gland extract were cultured with splenocytes the cytokine levels were decreased (Champagne et al. 2004, Wasserman et al. 2004). In a more natural exposure setting Zeidner et al (1999) was able to show an increase in the cytokines IL-4 and IL-10 and a decrease in IFNγ, after feeding on mice by either *Ae.*

aegypti or *Cx. pipiens* mosquitoes. Further supporting these findings, injection of salivary gland extract with Sindbis virus resulted in decreased IFNγ and IFNβ and an increase in IL-4 and IL-10 (Schneider et al. 2004).

Finding the components of the saliva that cause the immunomodulation is of particular interest. SAAG-4 is a protein found in the saliva of *Ae. aegypti* that stimulates $CD4^+$ T-cells to make IL-4 and simultaneously decreases expression of IFNγ (Boppana et al. 2009). Boppana et al (2009) were able to show that the bite of *Ae. aegypti* stimulated T helper cells to produce high levels of IL-4. Using a cDNA library of genes from transcripts originated from blood fed females several random gene products, including SAAG-4, were tested by intradermal injection of cDNA into mice. At the site of injection there was a significant decrease in IL-12, IFNγ, and TNFα and an increase in IL-4 and IL-10 (Boppana et al. 2009). In addition to SAAG-4, research has shown that sialokinin from *Ae. aegypti* can significantly decrease IFNγ and IL-2 while increasing IL-4 and IL-10 (Zeidner et al. 1999).

Saliva enhanced pathogen infection

The local and systemic immunomodulation that was described above leads to an environment that is more favorable for infection of intracellular pathogens. Indeed, many arthropod borne pathogens have enhanced replication or pathogenesis in the presence of MSPs. Most well studied is the enhancement of *Leishmania major* infection with addition of maxadilan, a salivary protein from the sand fly *Lutzomyia longipalpis* (Titus and Ribeiro 1988). Additionally, mosquito saliva induced-pathogen enhancement has been described and examples are presented in table 1.1.

IL-4, a cytokine that is upregulated by mosquito saliva, is an inhibitor of the cytotoxic T cell immune response. A cytotoxic T cell response is characterized by IL-12 and type I and II interferons. This type of immune response is critical for protection against intracellular pathogens such as viruses (Barton 2007). The down regulation of these pro-inflammatory cytokines enhances viral infection and can lead to an increase pathogenesis.

Immunity to Mosquito Salivary Proteins

There are many components in mosquito saliva that elicit an immune response in the vertebrate host. Several proteins have been identified as allergens. Three types of hypersensitivity have been demonstrated in response to mosquito salivary proteins; type I, III, and IV (Sandeman 1996). Patients with severe allergies to mosquito saliva have elevated levels of both IgE and IgG specific for mosquito saliva (Peng and Simons 1998). IgE and IgG1 are antibodies that are associated with the IL-4 dominated response to mosquito saliva and are implicated in both Type I and III hypersensitivities. The immediate wheal and flare reaction that takes place within the first 20 minutes after a mosquito bite are characteristic of a type I hypersensitivity and are mediated by IgE and mast cell degranulation (Peng and Simons 2004). Type III hypersensitivies consist of immune complexes between IgG antibodies and the allergen that can fall out of solution in blood (Peng and Simons 2004). The delayed reaction at the bite site is a type IV hypersensitivity that involves T cells that have migrated to the bite site and are releasing inflammatory

Table 1.1 Pathogens enhanced by mosquito saliva

Pathogen	**Mosquito**	**Host**	**Results**	**Ref**
Cache Valley virus	*Ae. triseriatus* *Ae. aegypti* *Cx. pipiens*	ICR mice	Only virus injected into mice that simultaneously fed upon by mosquitoes resulted in virema and seroconversion. Virus alone did not infect the mice.	(Edwards et al. 1998)
Vesicular stomatitis New Jersey virus	*Ae. triseriatus*	ICR mice	Juvenile and adult mice developed viremia and seroconverted when the virus was delivered via mosquito bite significantly more often than when the virus was injected alone.	(Limesand et al. 2000)
La Crosse virus	*Ae. triseriatus*	ICR mice White-tailed deer Chipmunks	Significantly higher rates of infection when virus is delivered via infected mosquito as compared to needle injection.	(Osorio et al. 1996) Beaty, unpub
Plasmodium berghei	*An. stephensi*	CD-1 Mice	Higher infection rates when pathogen was delivered via mosquito rather than IV inoculation.	(Vaughan et al. 1999)
West Nile virus	*Ae. aegypti*	Mice	When virus was delivered via mosquito bite there was higher viremia and faster onset of symptoms.	(Schneider et al. 2006)

cytokines (Peng and Simons 2004). Severe mosquito allergies can be debilitating, however experimental and natural desensitization has occurred with repeated exposure to mosquito bites (Peng and Simons 1998, 2004).

This natural immunity response to the bites from blood feeding arthropods has been shown in some models to alter pathogenesis of both West Nile virus and *Plasmodium* infections. In a mouse model of malaria, differing results have been obtained. In one study the exposure of mice to non-infected mosquito bites conferred resistance to later IV infection with *Plasmodium yoelii* (Donovan et al. 2007). The protection lasted through the liver stages and into the blood stages of infection (Donovan et al. 2007). However, in a second study the results could not be duplicated (Kebaier et al. 2010).

In a mouse model of West Nile virus, mice that were pre-exposed to the bites of *Ae. aegypti* were at a significantly higher risk of succumbing to illness (Schneider et al. 2007). The mice that were pre-exposed to mosquito bites had higher levels of $CD11c^{+}$ and $CD11b^{+}$ antigen presenting cell infiltrates to the bite site as compared to control mice. In addition, pre-exposed mice had significantly higher levels of IL-10 at the bite site and in the draining lymph node (Schneider et al. 2007). The increased mortality was duplicated when serum from pre-exposed mice was passively transferred to naïve mice (Schneider et al. 2007).

Salivary Protein Vaccines

Vaccines made from salivary proteins of arthropods can prevent the immunomodulation caused by arthropod saliva, and may be able to elicit an immune response that will aid in the host defense against pathogens present in the saliva (Morris

et al. 2001). This is a novel approach to the prevention of arthropod borne pathogens and could be used in conjunction with more conventional methods to enhance protection. Two types of anti-saliva vaccines have been proposed, those that elicit neutralization of immunomodulatory substances or those that contain highly immunogenic proteins to create a strong immune response (Valenzuela 2004).

For species of blood feeding arthropods in which the major immunomodulators in the saliva have been identified, the first approach is applicable. However, in some species, it appears that several different MSPs cause immunomodulation, for those it would be difficult to identify and target all of them. Now that the protein SAAG-4 in *Ae. aegypti* has been shown to cause immunomodulation, it could be used as a basis for this type of vaccine.

There is a successful example of this type of salivary protein vaccine. In the new world sand fly *Lutzomyia longipalpis* the salivary protein maxadilan (MAX) has been identified as not only a potent vasodilator but also the major cause of salivary immunmodulation. MAX can cause an increase in the Th2 type cytokines IL-6 and the regulatory cytokine IL-10 (Marcelo et al. 1998, Soares et al. 1998) and enhances infection with *Leishmania major* in mice (Titus et al. 2006). Vaccination with MAX yielded ELISA titers of MAX antibodies between 1/10000 and 1/20000 and $CD4^+$ T-cells specific for MAX (Morris et al. 2001). This response is protective against infection by *L. major* when delivered in the context of salivary gland homogenates (Morris et al. 2001). Vaccinated mice had significantly smaller lesions and a lower parasite load (Morris et al. 2001).

The second salivary vaccine approach is to isolate proteins from the saliva that are present in large quantities and are highly immunogenic. These proteins can be used in conjunction with an adjuvant to produce a strong cytotoxic T-cell memory response to make a complete vaccine. When the host is then exposed to mosquito saliva the memory response will be activated. A cytotoxic T-cell response would be characterized by high levels of Th1 type cytokines, IL-12 and type I and II interferons. These cytokines promote an immunological environment that is not favorable for infection by most arthropod borne intracellular pathogens.

The two vaccine types that have been illustrated here are not mutually exclusive. The MAX vaccine does have a component of the second type of vaccine as the adjuvant that was used creates a strong cytotoxic T-cell response and increased levels of Th1 type cytokines and memory $CD4^+$ T-cells (Morris et al. 2001).

The vaccine used in the research presented in this dissertation primarily falls into the second category. An adjuvant was used to create a memory cytotoxic T-cell response so that when a mosquito bites the animal there will be an overwhelming Th1 response, thus overriding the immunomodulation of other proteins in the mosquito saliva. However, because D7 may have some immunomodulatory properties that have not yet been identified, it may fall under the first category as well.

Adjuvants

Adjuvants are added to inactivated vaccines along with antigen to help promote a strong, appropriate, and protective immune response to the given agent. In the United States there is only one adjuvant that is approved for use in human vaccines, alum (Marciani 2003). Alum acts to activate dendritic cells and prime a Th2 type memory

immune response (Lambrecht et al. 2009). Alum adjuvants have been used since 1926 in a variety of forms and vaccine preparations (Marciani 2003).

More recently adjuvants have been designed that will target innate immune pattern recognition receptors (PRR) such as toll like receptors (TLR). Pathogen-associated molecular patterns (PAMPs) are recognized by PRRs and initiate an innate immunity cascade. Cationic lipid DNA complexes (CLDCs) have been used as an adjuvant that stimulates a PRR. CpG DNA complexed with a liposome acts to stimulate TLR 9 to produce high levels of Th1 type cytokines such as interferons (U'Ren et al. 2006, Zaks et al. 2006).

In the laboratory complete Freund's adjuvant is widely used, however it is not licensed for human or veterinary use due to high incidence of reactions in immunized animals. It contains mycobacterial cell wall components in an oil solution. This adjuvant creates a strong antibody response and tends towards a memory response Th1 profile of cytokines (Stills 2005).

WNV overview

WNV was first isolated in 1937 from a febrile patient living in Uganda (Smithburn et al. 1940). This virus is a member of the family *Flaviviridae* genus *Flavivirus*, and is an enveloped positive sense single stranded RNA virus. It is included in the Japanese encephalitis sero-complex along with Japanese encephalitis, Murray Valley encephalitis, and St. Louis encephalitis viruses (Burke and Monath 2001). Most cases in humans are asymptomatic, however 20-30% of those infected will develop symptoms with about 1 in 150 infections resulting in serious neurologic disease (Mackenzie et al. 2004). West Nile virus fever can include a wide range of symptoms

including fever, myalgia, meningitis, encephalitis, and long term neurological sequelae (Klee et al. 2004, Mackenzie et al. 2004, Murray et al. 2007). Care for an infected patient consists only of supportive care. There are several commercially available equine WNV vaccines, however none are currently available for human use.

WNV epidemiology and transmission

WNV has a nearly worldwide distribution with the exception of Antarctica. Since the identification of WNV in 1937, there have been numerous large outbreaks in Africa, Australia, the Middle East, and Europe (Kramer et al. 2007). Historically the outbreaks had high infection rates with low levels of severe disease manifestations. Populations in Egypt and Sudan had seroconversion rates of over 50%, but rarely a case of neuroinvasive disease (Burke and Monath 2001). However, in the 1990's neurological disease began to increase and the case fatality rate approached 10% in a 1996 – 1997 Romanian outbreak (Kramer et al. 2007).

WNV was introduced into the Western hemisphere via New York City in 1999, with a single introduction event (Lanciotti et al. 1999). Four years following this introduction WNV had spread to the 48 contiguous United States, Canada, Mexico, and Central and South America (Reisen and Brault 2007). To date the epidemic in the United States alone has resulted in close to 30,000 cases of diagnosed West Nile virus infection and 1161 human deaths (CDC 2010). In addition to human cases, there have been 25000 equine cases reported in the United States (USDA 2010). Molecular analysis of the viral isolate introduced into New York in 1999 showed the virus had a 99.7% identity at the

nucleotide sequence level with an actively circulating virus in Israel, indicating that was the origin of the introduced virus (Lanciotti et al. 2002).

The natural enzootic transmission cycle of WNV occurs between avian species and mosquitoes, mainly in the genus *Culex*. In North America over 250 avian species have been shown to have a productive WNV infection (Komar et al. 2003, Hayes et al. 2005, Reisen and Brault 2007). Viremia in susceptible avian species can be between 8 and 10 $\log_{10}$ PFU/ml (Komar et al. 2003). This is thought to be the primary source of infection for mosquito species. However there have been a few experimental reports of mammalian species with viremias up to 10^7 pfu/ml which are levels sufficient to infect mosquitoes upon blood meal (Platt et al. 2007, Platt et al. 2008). Mosquitoes in the transmission cycle are primarily ornithophilic *Cx.* species. Even though the virus has been detected in more than 50 species in the United States, the primary vectors are those of the *Culex pipiens* complex and *Culex tarsalis* (Brault 2009). *Culex pipiens* is readily infected after a WNV artificial blood meal, with rates approaching 100%, and dissemination rates around 80% (Moudy et al. 2007). *Culex tarsalis* becomes infected at a lower rate of approximately 50% with a dissemination rate of 38% (Moudy et al. 2007). Once infected, these mosquitoes inoculate 4 – 6 logs of infectious virus when taking a blood meal (Styer et al. 2007).

Historically, the virus did not cause illness in birds; however, WNV isolates from the North American outbreak and the closely related Israeli viruses can cause morbidity and mortality in birds, especially corvids such as crows, blue jays, and magpies (Mackenzie et al. 2004). Humans and horses are tangential hosts that can become

infected and develop neurological disease, but do not develop a viremia titer high enough to re-infect mosquitoes.

WNV biology

WNV virions are spherical and enveloped and are approximately 40 to 60 nm in diameter (Burke and Monath 2001). The genome is approximately 11 kb long and consists of a single open reading frame that results in 10 mature viral proteins (Brinton 2002). The structural proteins consist of capsid (C), membrane (M), envelope (E) and non-structural proteins are NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. The viral genome has a 5' cap, however lacks a 3' poly A tail (Brinton 2002).

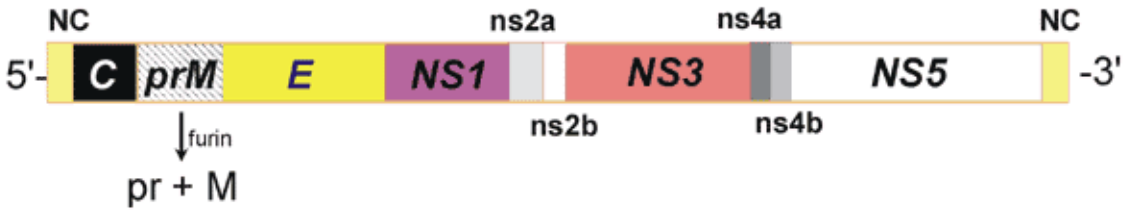


Figure 1.0.3 Genome arrangement of West Nile virus(Petersen and Roehrig 2001).

The virus gains entrance into cells by attachment of the envelope (E) protein to a plasma membrane receptor and using receptor mediated endocytosis in clathrin-coated pits (Chu and Ng 2004b). Though receptors have not been identified, there is evidence that DC-SIGN and cellular integrins are both important for cell entry (Chu and Ng 2004a, Davis et al. 2006). When domain three of the E protein is neutralized by binding antibodies, there is inhibition of virus entry into cells suggesting that this domain is important in receptor binding (Chu et al. 2005). Low pH in the endosomal compartments causes a conformational change in the E protein, a class II fusion protein, which leads to envelope-membrane fusion and release of the nucleocapsid (Brinton 2002). Once the viral RNA enters the cell the RNA is translated into a single polypeptide. The viral

proteases NS2b and NS3 along with cell proteases cleave the polypeptide into the mature proteins (Brinton 2002). The viral RNA dependent RNA polymerase, NS5, replicates the viral RNA in association with the endoplasmic reticulum (Brinton 2002, Samuel and Diamond 2006). The positive sense genomic RNA is used as a template to create negative strands of RNA. This is then used as the template to make viral genomic RNA for packaging into new virions and use for translation of viral proteins. Virus particles are assembled and bud into the endoplasmic reticulum to obtain an envelope (Samuel and Diamond 2006). At this point the membrane protein is in the immature form, prM. Once the virion has been transported through the trans-Golgi network there is furin-mediated cleavage of prM to M (Samuel and Diamond 2006). The virus is then released from infected cells via exocytosis (Samuel and Diamond 2006).

The non-structural proteins expressed by West Nile virus aid in host immune evasion and viral replication. NS1 is produced in both mammalian and insect cells, but is secreted from mammalian cells only (Brinton 2002). The NS1 protein plays a role in viral replication and evasion of the host immune response by blocking the complement cascade and TLR3 signaling (Chung et al. 2006, Samuel and Diamond 2006, Wilson et al. 2008). NS3 is a serine protease that forms a complex with NS2b to cleave portions of the viral polypeptide (Yusof et al. 2000). In addition NS3 has helicase and NTPase activity that may be important for RNA replication (Borowski et al. 2001). The proteins NS2a/b, NS3, and NS4a/b all were shown to interfere with interferon alpha and beta expression and signaling (Liu et al. 2005, Liu et al. 2006). The viral RNA dependent RNA polymerase and methyl transferase is the non-structural protein 5 (Egloff et al. 2002).

Immune Response to WNV infection

WNV infects Langerhans dendritic cells (lDC) that are resident within the dermis of the host (Johnston et al. 2000). lDCs are important players in the innate immune response as professional antigen presenting cells. When they are activated or infected with WNV they migrate to the local draining lymphnode, facilitating spread of the virus (Johnston et al. 2000).

Activation of DCs by infection or PRRs such as TLR3 or 7/8 leads to expression of type I interferons (IFN). Genetic knock out mice lacking type I IFN receptors were more susceptible to WNV infection, indicating Type I IFNs are important in limiting early WNV infection (Samuel and Diamond 2006). Type I IFNs are known to upregulate several hundred genes in the host immune response, including the anti-viral pathways protein kinase R, RNase L, and Mx and act to stimulate the adaptive immune response (Samuel and Diamond 2005).

IFNγ, a type II IFN, also plays an important role in the host immune response to WNV. IFNγ is produced by γδ T cells, CD 8^+ T cells, and NK cells (Samuel and Diamond 2006) and can polarize the adaptive immune response to a Th1 type environment. Th1 type immune responses have a very strong $CD8^+$ cytotoxic T-cell component, which is important in WNV infections (Samuel and Diamond 2006). In the mouse model of WNV γδ T cells are the major producers of IFNγ, and mice that are deficient in these cells succumb to infection at higher rates (Wang et al. 2003a).

In addition to the protective role of the innate immune system, the adaptive immune system is also important in the host defense against WNV. Mice deficient in B-cells, and thus a humoral response to the virus, are more susceptible to viral infection, however an

antibody response without a cellular response was not sufficient to protect the mice (Mehlhop and Diamond 2008). Antibodies made to WNV primarily bind envelope (E) protein (Samuel and Diamond 2006) and function by neutralization of the virus, complement activation, and Fc receptor mediated clearance of virions (Mehlhop and Diamond 2008).

A T-cell response is critical during a WNV infection. If CD 4^+ T helper cell populations are eliminated in the mouse model, the susceptibility to WNV increases (Sitati and Diamond 2006). This is due to the fact that the activated CD 4^+ T helper cells secrete Th1 type cytokines, such as IL-12p70, and drive other facets of the immune response into an anti-viral state. Additionally, CD8 $^+$ cytotoxic T-cell knock out mice when infected with a low dose viral challenge exhibit an increase in susceptibility, arguing that these cells play a role in limiting or clearing the viral infection (Wang and Fikrig 2004). Interestingly, when these same mice are infected with a high dose of WNV, they are more resistant to infection showing that the CD 8^+ T cells may also contribute to neurologic pathology (Wang and Fikrig 2004).

To have a protective immune response there must be induction of type I interferons and a productive Th1 type adaptive immune response characterized by cytotoxic T-cells, Th1 type T- helper cells, and neutralizing antibodies. However, without control on the immune system, immunopathology could lead to more severe disease symptoms.

WNV pathogenesis

Upon infection with WNV, the infected lDCs drain to the local lymph node resulting in viremia and infection of organs including the spleen and kidneys, with the peripheral infection ending after the first week of infection (Diamond et al. 2009). Increased serum

viral load corresponds to earlier viral entry to the central nervous system (CNS) (Samuel and Diamond 2005). The major hypothesis on how the virus crosses the blood brain barrier is that it is TLR3 mediated (Wang et al. 2004). TLR 3 is found in endosomal compartments within host cells and activation with double stranded RNA leads to up regulation of type I IFNs and thus pro-inflammatory cytokines such as TNFα (Kawai and Akira 2006). Increased TNFα levels in the blood can lead to vascular permeability and opening of the vascular endothelium of the blood vessels of the brain. It is thought that the virus in the blood can then enter the neural tissues and establish infection (Wang et al. 2004).

West Nile virus replicates primarily in neuronal cells and induces apoptosis (Diamond et al. 2009). Mice deficient in caspase-3, part of the apoptosis pathway, were shown to be resistant to severe disease due to WNV infection, but did not show a difference in viral burdens (Samuel et al. 2007).

Animal models of WNV Infection

Animal models including mice, hamsters, and crows have been used to characterize the immunological and pathological response to WNV-NY99 infection (Wang et al. 2001, Xiao et al. 2001). The mouse model is most widely used due to the availability of reagents used for analysis of the immune response. Mice are susceptible to WNV infection, develop viremia and neurological signs, and usually die within one to two weeks (Lustig et al. 2000, Wang et al. 2001). There are differences in susceptibility between mouse strains C3H and C57/BL6, with C3H mice exhibiting 80% mortality and C57/BL6 mice succumbing to illness at a rate of approximately 20% (Brown et al. 2007). However, viral load and tropisms did not differ (Brown et al. 2007). Studies using the

murine model have shown that both innate and adaptive immune responses are required for recovery and protection during WNV infection. Some components of the immune system that have been shown to be important in fighting WNV infection include type 1 and type 2 interferons, αβ and γδ T-cells, B-cells, and antigen presenting cells (Kulkarni et al. 1991, Anderson and Rahal 2002, Diamond et al. 2003a, Diamond et al. 2003b, Wang et al. 2003b) and these all have reagents for the murine model.

Project overview

Interactions between arthropods, hosts, and pathogens are complex, and not completely understood. The immunomodulation induced by MSPs causes an environment that is beneficial for arboviral infection. Immunity to MSPs, either through previous exposure or immunization, could lead to alterations in arboviral pathogenesis. Extensive work has been conducted in the sand fly-Leishmania system, but relatively little work with mosquitoes and arboviruses.

With the emergence of West Nile virus in the western hemisphere, there has been an increased need for novel prevention techniques. Here we investigated a MSP vaccine as a tool to prevent West Nile virus infection from a mosquito bite.

The role of *Cx. tarsalis* D7 salivary protein was examined during blood feeding and during WNV infection to determine immunomodulatory effects. The previously unidentified D7 proteins were identified and the cDNA was fully sequenced. Recombinant D7 protein was then expressed in an insect cell line using an alphavirus transducing system. The recombinant protein was incorporated into a vaccine using an adjuvant to produce a strong memory ctyotoxic T-cell response and administered to mice. The vaccine containing the *Cx. tarsalis* D7 protein was hypothesized to be protective

against a WNV infection from an infected mosquito bite, however the vaccine was found to increase susceptibility instead. A possible mechanism for enhancement is discussed in the following chapters.

CHAPTER II

IDENTIFICATION, CHARACTERIZATION AND PURIFICATION OF *CULEX TARSALIS* D7 SALIVARY PROTEIN

Introduction

Mosquito saliva contains proteins with pharmokinetic properties that can result in the enhancement of arboviral pathogenesis (Edwards et al. 1998, Limesand et al. 2000, Schneider et al. 2006, Limesand et al. 2009). Identifying the protein factors in the saliva that are responsible for this phenomenon could lead to ways to neutralize the enhancement and protect against many important diseases. Mosquito salivary proteins (MSPs) have been well described for the disease vectors *Aedes aeypti* and *Culex pipiens* (Ribeiro et al. 2004, Ribeiro et al. 2007); however until very recently there was very little information on the composition of saliva from *Cx. tarsalis*. *Culex tarsalis* is an important disease vector in the western United States, transmitting West Nile virus and western equine encephalitis virus (Turell et al. 2005, Mahmood et al. 2006).

The D7 salivary proteins are of particular interest in these studies as previous work has indicated that D7 can bind to the inflammatory cytokine interferon gamma (Machain-Williams unpublished data). D7 proteins are among the most abundant proteins found in mosquito saliva, with one estimate stating that between 5 and 20% of the total salivary proteins in *Ae. aegypti* consist of D7 (Calvo et al. 2006). D7 proteins have been identified in numerous dipteran species including mosquitoes in the genera *Anopheles, Culex,* and *Aedes* as well as Culicoides and sand flies in the family Psychodidae (Calvo et al. 2006). These proteins have four highly conserved cysteines that are consistent with members of the odorant binding proteins (Arca et al. 2002). Calvo *et al* (2006) have described biogenic amine binding properties of D7 proteins from *An. gambiae* and *Ae. aegypti*. More recently, the same research group found that D7 salivary proteins bind the immune molecules leukotrienes (Calvo et al. 2009).

Leukotrienes are important pro-inflammatory members of the innate immune system. They are important in recruiting leukocytes, up-regulation of phagocytosis and cell killing, and generation of cytokines (Peters-Golden et al. 2005). These taken together suggest that D7 salivary proteins are involved in modulating host vasoconstriction and local inflammation.

Mosquito salivary proteins induce a strong hypersensitivity reaction in naturally exposed individuals. D7 is a known allergen and is referred to as Aed a 2 and Aed a 3 in allergen literature (Peng and Simons 2007). These proteins elicit a strong IgE antibody response, characteristic of a type 1 hypersensitivity. Recombinant D7 protein can reproduce this effect, producing an immediate wheal and flare reaction in those with mosquito allergies (Peng and Simons 2004). In addition, IgE and IgG1 antibodies were produced in mice that were exposed to recombinant D7, again consistent with D7 being an allergen (Peng and Simons 2004).

Calvo *et al* (2010) analyzed the sialome of *Cx. tarsalis* and noted eight D7 genes in the mosquito's genome. Prior to this no sequences were available for *Cx. tarsalis* D7 genes. Here the identification of several salivary proteins from *Cx. tarsalis* is described using methods including immunoprecipitation, mass spectrophotometry, N-terminal protein sequencing, and 5' and 3' rapid amplification of cDNA ends. For future studies to analyze the role of D7 in the host immune response recombinant D7 protein (rD7) was produced, purified and compared to the native D7 protein.

Materials and Methods:

Mosquitoes and mosquito saliva preparation

Adult female *Cx. tarsalis* (Bakersfield, California) (Rasgon et al. 2006) mosquitoes were raised in the insectaries at the Arthropod Borne and Infectious Diseases Laboratory (AIDL), Colorado State University. Maintenance colonies were reared at 25°C and 80% humidity on a 16:8 light:dark cycle. Adult mosquitoes were offered restrained mice once per week for blood meals.

Saliva was collected from 5-7 day old female adult *Cx. tarsalis.* They were cold anesthetized at -20°C for approximately one minute and sorted on ice. Legs and wings were removed from each mosquito and discarded. The proboscis was then placed into a 10 µl glass capillary tube filled with immersion oil type B. Mosquitoes were left to salivate in insectaries conditions for 18 hours. Oil and saliva were collected from capillary tubes into a 1.7 ml tube with 500 µl of Dulbecco's modified Eagle's medium (MEM) (un-supplemented). The mixture was vortexed then centrifuged in a table top centrifuge at 12000 rpm for 10 min. Using a 28 gauge needle and a 1ml syringe, the aqueous layer was removed taking care not to include any oil.

Salivary protein precipitation

To precipitate mosquito salivary proteins the solution was combined 1:1 with 20% trichloroacetic acid (TCA) and mixed by vortexing. The mixture was incubated on ice for 30 min. Next the solution was centrifuged at 4°C and 15000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with 300 µl of cold acetone. Again, the sample was centrifuged at 4°C and 15000 rpm for 10 minutes. The supernatant was discarded and the protein pellet was allowed to dry at room temperature for 15

minutes. The proteins were resuspended in the desired amount of pure water or PBS with protease inhibitor cocktail (Roche).

Immunoprecipitation

Immunopreciptation was conducted to pull down salivary proteins from *Cx. tarsalis* using the Pierce Seize X Protein A Immunoprecipitation kit and antibodies made to *Ae. aegypti* D7 salivary proteins. The salivary glands from 100 adult female *Cx. tarsalis* mosquitoes were dissected and placed into 500 µl lysis buffer (10 mM Tris-HCl, pH7.5, 150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% Triton-X-100, 0.1% SDS and 1 mM PMSF). The tissue was ground using a disposable plastic pestle and a battery powered homogenizer. Next 250 µl PBS with protease inhibitor cocktail was added (Roche). To prepare spin columns a 50% slurry of immobilized protein A resin (Pierce) was placed into a column. 500 ng *Ae. aegypti* D7 antibody (provided by Dr. Jill Troyer) was diluted in 400 µl binding/wash buffer (Pierce). Diluted antibody was mixed with protein A resin and rocked for 15 minutes. The solution was then centrifuged at 8500 rpm for 1 minute and supernatant containing unbound antibodies was discarded. To cross-link the antibodies 25 µl of the provided disuccinimidyl suberate (DSS) was mixed with 80 µl DMSO and added to the resin. The mixture was then rocked for 60 minutes. The resin was washed 5 times as described by manufacturer. To immunoprecipitate antigen 750 µl of salivary gland homogenate was diluted 1:1 with the cross-linked antibody resin in a 1.7 ml tube. The mixture was rocked overnight at 4°C. The mixture was centrifuged at 12000 rpm for 5 min then was washed 4 times. Protein was eluted from the resin in five fractions. For each elution 190 µl of ImmunoPure elution

buffer(Pierece) were used. Proteins were then precipitated using TCA and fractionated using 10% SDS-PAGE and silver stained to visualize.

N-terminal sequencing

To determine the amino acid sequence of the amino terminus of the 37 kDa salivary protein, Edman degradation and N-terminal sequencing was performed. Saliva from 25 adult female *Culex tarsalis* mosquitoes was collected. The salivary proteins were precipitated using 10% TCA (final concentration). Next, the proteins were dissolved in PBS and fractionated by electrophoresis by non-denaturing PAGE. The proteins were then transferred onto a PVDF membrane. To visualize the proteins, the membrane was stained with Ponceau-S. The band that correlated with 37 kDa was excised from the membrane and stored in 15% methanol. The membrane section was submitted for Edman degradation and N-terminal sequencing of 10 amino acid residues at Macro Molecular Resources (MMR) at Colorado State University. The membrane strip was loaded into an Applied Biosystems Procise Sequencer. Briefly, phenylisothiocyanate (PITC, Edman reagent) was added to the protein under basic conditions. This led to the separation of the N-terminal amino acid. That amino acid was then subjected to high pressure liquid chromatography to determine retention time. Retention times for each subsequent amino acid are compared to control amino acids to determine identity.

Mass spectrometry analysis

Two hundred adult female *Cx. tarsalis* mosquitoes were salivated and proteins precipitated as previously described. The protein was resuspended in 10 µl PBS plus protease inhibitors. The salivary proteins were resolved on 10% SDS-PAGE and visualized using commassie blue. The four prominent bands were excised from the gel

and cut into approximately 1mm squares. The gel pieces were placed in 1% acetic acid. The gel slice was washed with 100 mM NH_4HCO_3/50% acetonitrile to completely remove residual stain. The dried gel was incubated in 10mM dithiothreitol and 100 mM NH_4HCO_3 at 60° C for 30 min. The sample was centrifuged and the supernatant was discarded. The gel fragments were incubated in 55 mM iodoacetic acid and 100 mM NH_4HCO_3 for 30 min. Next, the sample was washed in 100 mM NH_4HCO_3/50% acetonitrile. The proteins in the gel samples were digested with 600 ng trypsin in 100 mM NH_4HCO_3 at 37°C overnight. The protein was extracted in 50% acetonitrile with 0.1% trifluoroacetic acid and a C18 Zip-tip (Millipore). The protein sample was resuspended in 10µl of 0.1% trifluoracetic acid. One µl of sample was then mixed with an equal volume of a-cyano-4-hydroxycinnamic acid. Drops of the sample were applied to the MALDI target and allowed to dry. The mass spectrometric analysis was performed using an Ultra-Flex-Time of flight/time of flight spectrometer from Bruker Daltonics. The machine was in positive ion reflector mode using a 25kV acceleration voltage. Data was analyzed using the FlexAnalysis software (version 2.4, Bruker Daltonics). The MS and MS/MS spectra were searched using Mascot (v2.1) on December 6, 2006 against the NCBI nr database that is updated weekly. The parameters of the search were as follows: peptide mass tolerance 0.2 Da, fragment mass tolerance 0.8 Da, max missed cleavages 1.

Protein fractionation and detection by polyacrylamide gel electrophoresis

Protein samples were subjected to gel fractionation using the NuPage (Invitrogen) system. Samples were mixed with NuPage LDS Sample Buffer (4X) and were loaded into a 10% NuPage Bis-Tris polyacrylamide gel. NuPage MES Running buffer was used for running the gel at 200V for 35 minutes.

Both silver stain and Coomassie stains were used to visualize proteins in the gel. Silver stain was performed using Silver Stain Plus (BioRad) to detect lower quantities of protein. For higher concentrations of protein, gels were stained with 15% methanol, 7% acetic acid, and 0.1% Coomassie blue R-250 for 15 – 60 min at 55°C. The gel was destained with 40% methanol and 7% acetic acid overnight at 4°C. Gels were preserved by washing in a solution of 10% ethanol and 4% glycerol for 30 min. The gel was then placed between two sheets of cellophane and dried overnight.

Immunoblot

Once fractionated as described above, the proteins were transferred to a 0.2 μm nitrocellulose membrane. Transfer was conducted using NuPage Transfer Buffer (Invitrogen) with 20% methanol at 30 V for 60 minutes. The membrane was then submerged in blocking buffer (PBS, 0.05% Tween 20, 5% non-fat milk(w/v)) and rocked for 60 minutes. For immunodetection using serum antibodies, serum was diluted 1:20-1:100 in blocking buffer. Antibodies were incubated for 1 hour at room temperature or overnight at 4°C. The membrane was then washed 3 times for 5 minutes using PBS^{+}0.1% Tween 20. Horseradish peroxidase (HRP) conjugated secondary antibody was diluted 1:1000 in blocking buffer. Secondary antibodies included rabbit anti-mouse, goat anti-rabbit, or goat anti-human IgG (KPL Laboratory). The membrane was incubated at room temperature for 1 hour. For detection of rD7 a directly HRP conjugated six histidine tag antibody (Invitrogen) was used at 1:5000 and incubated for 1 hour at room temperature. Again the membrane was washed 3 times for 5 minutes with PBS Tween. The membrane was developed using the Vector VIP Substrate Kit (Vector Labs).

Trizol RNA extraction

To extract RNA from whole mosquitoes or from salivary glands, one mosquito or 50 pairs of dissected salivary glands were placed in 200 µl Trizol (Invitrogen). Tissue was disrupted using a disposable blue pestle (VWR) and battery-powered homoginizer. The disrupted tissue was left at room temperature for 5 minutes, 100 µl chloroform was added, vortexed, and the tube was left at room temperature for 3 min. The sample was centrifuged at 14000 rpm in tabletop centrifuge for 10 minutes at 4° C. The top (aqueous) layer was transferred to a clean RNase free 1.7 ml tube and 250 µl of isopropyl alcohol was added and vortexed. The mixture was left at room temperature for 10 minutes, and centrifuged at 8000 rpm for 5 minutes. The supernatant was removed and 1 ml 70% ethanol was added to the tube and vortexed. The sample was centrifuged at 8000 rpm for 5 min, the supernatant was removed and the RNA pellet dried at room temperature for 10-15minutes. RNA was resuspended in 30 -60 µl nuclease free dH_20.

3' Rapid Amplification of cDNA Ends (RACE)

To determine the nucleotide sequence of the *Cx. tarsalis* 37 kDa salivary protein gene, 3' RACE was utilized. A degenerate gene specific primer, Cxtar FWD Nterm, was designed using the N-terminal amino acid sequence. RNA from 5 adult female *Cx. tarsalis* thoraxes was isolated using Trizol reagent (Invitrogen). cDNA was made using a primer with a GC rich linker region and a poly T tail. The cDNA was constructed as follows: 1µl 3PrimeRaceRT primer (25ng/µl), 1µl dNTPs (2.5mM each), 5µl total RNA, 5µl nuclease free water were brought to a volume of 12µl. The sample was heated to 70°C for 10 minutes then placed on ice. To the sample 4µl (5X) first strand buffer, 2 µl (0.1M) DTT, 1.9 µl nuclease free H_2O, 0.1 µl Thermoscript reverse transcriptase were

added. The mixture was then incubated at 42°C for 50 minutes and 70°C for 15 minutes. Next, PCR amplification was set up using the gene specific primer and a primer that contained only the linker region of the 3'RACE primer. The reaction mixture was as follows: 5 µl(10X) PCR Buffer $^{+}$ $MgCl_2$, 4 µl (2.5mM) dNTPs, 2 µl each primer (25ng/ul), 24.6 µl nf H_2O, 0.4 µl Taq (Invitrogen). The samples were cycled as follows: 95°C for 5 min, 35 cycles (95°Cx30 sec, 50°Cx1min, 72°Cx2min), 72°C for 8 min. Products were electrophoresed on 1% agarose gel and visualized with ethidium bromide. The PCR products were cloned into the TOPO vector pCR4.1 (Invitrogen) and colonies were screened for insert with vector primers, M13. Positive clones were cultured and DNA isolated using Qiagen Spin Miniprep kit. The DNA was sent to MMR for sequencing using M13 primers.

Table 2.1 3'RACE primers

Primer name	Sequence (5' to 3')
Cxtar FWD Nterm	GAAACCGTTTAATCCGGAAG
3PrimeRaceRT	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTT
3PrimeRacePCR	GGCCACGCGTCGACTAGTAC
M13 Forward -20	GTTTTCCCAGTCACGAC
M13 Reverse	CAGGAAACAGCTATGAC

mRNA Purification

mRNA was purified from total RNA isolated from 5 adult female *Cx. tarsalis* using the Poly(A)Purist kit (Ambion). 2 ug of RNA extracted using the Trizol (Invitrogen) method was diluted in 500 ul nf dH_2O. The mixture was diluted with an equal volume of binding buffer (Ambion). The sample was then placed in a tube with provided cellulose beads adsorbed with oligo (dT). The sample was heated at 70° C for 5 min to denature the RNA. Next, it was rocked at room temperature for 60 min. The

beads were pelleted by centrifuging at 4000xg for 3 min. Supernatant was discarded. Next the RNA Storage Solution was heated to 80°C. Beads were washed by adding 0.5 mL of Wash Solution to the beads and placing them into a spin column. The beads were then centrifuged at 4000xg for 3 min. This step was repeated one additional time. Subsequently, the sample was washed again two times using 0.5 ml wash solution 2 each time. The bound mRNA was eluted using 200 ul of the warm RNA storage solution, centrifuging at 5000xg for 2 min twice.

5' Rapid Amplification of cDNA Ends (RACE)

The GeneRacer kit (Invitrogen) was used to perform 5'RACE. Total RNA was isolated from the thorax of five adult female *Cx. tarsalis* mosquitoes using a Trizol extraction method (Invitrogen). The mRNA was purified from the total RNA using Poly(A) Purist (Ambion) as above. The purified mRNA was then subjected to the GeneRacer protocol. RNA was dephosphorylated with 10 units of calf intestinal phosphatase to prevent non-messanger or truncated RNA from binding the GeneRacer oligo. The 5'Cap was then removed from mRNA using tobacco acid pyrophosphatase. A provided proprietary GeneRacer oligo was ligated to the 5' end of the RNA. A gene specific reverse primer, Ct D7 5'Race, was used to generate cDNA using SuperScript III reverse transcriptase. cDNA was amplified using a gene specific reverse primer and a forward primer binding to the GeneRacer oligo. Nested PCR using a second gene specific primer and a GeneRace primer generated a discrete band when visualized on 1 % agarose. This product was cloned into pCR4.1 TOPO vector (Invitrogen) for sequencing. Colonies were screened using vector primers and those with insert that was of the appropriate size were prepped to isolate cloned cDNA. The DNA was sequenced at

MacroMolecular Resources at Colorado State University and analyzed using CLC Free Workbench4.

Table 2.2 5' RACE primers

Primer name	Sequence (5' to 3')
Ct D7 5'RACE	GTFCACAGCTCTTCACTACCTTCCTTGAAATG
Ct D7 5'RACE nested	CGCTTCTTCACAGTAGAATCAGAGCTGTATATCTTG

Creation of full length D7 clone

A unique cloning approach was used to combine the sequence obtained from the 3' and 5' RACE protocols to create a full length D7 construct. Two segments of the D7 cDNA were amplified using PCR, a 3' product and a 5' product with overlap of 410 base pairs as seen in figure 2.1. Each product was amplified using 2.5 μl (10X) Buffer/$MgCl_2$, 1 μl of each primer (25 ng/μl), 0.5 μl dNTPs (10mM), 0.1 μl Taq polymerase, 100 ng of template DNA in a 25 μl reaction. The reactions were cycled as follows: 2' at 94°C; 35 cycles of 30" at 94° C, 30" at 60° C, 45" at 72° C; 5' at 72° C. The products were visualized on 1% agarose gels with ethidium bromide. The PCR products were purified as per manufacturers protocol with Qiagen PCR Purification kit. A second PCR reaction was used to amplify the full length product using the PCR products as primers; 5 μl 3'PCR product, 5 μl 5'PCR product, 0.5 μl dNTPs (0.1M), 2.5 μl buffer$^{+}MgCl_2$, and 0.1 μl Taq in a 25 μl reaction. It was cycled as given above for 10 cycles with a 55° C annealing temperature. To this mixture the following was added; 2 μl of primer 1 and 4 (25 ng/μl), 0.2 μl Taq, 20.8 μl H_2O. It was again cycled as before for an additional 30 cycles. The sample was applied to gel electrophoresis and the band corresponding with the correct size was excised and purified using Qiagen Gel Purification kit. The purified

DNA was cloned into the pCR4.1 TOPO vector (Invitrogen) for sequencing. Colonies were screened using the full-length gene primers (1 and 4) and analyzed using CLC Free Workbench4.

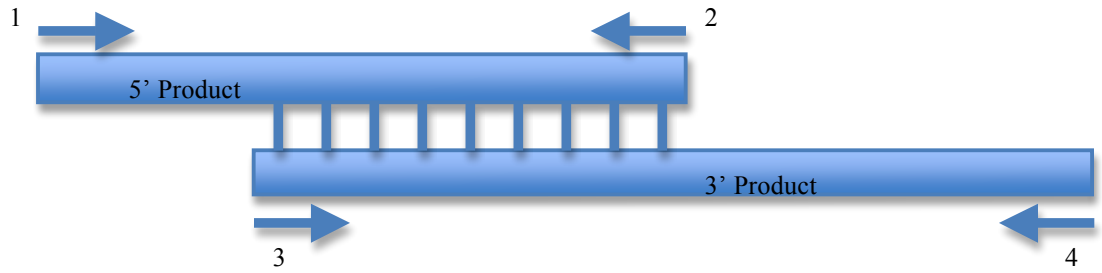


Figure 2.1 Schematic of the strategy used to amplify full length D7. The 3' product spans from base pair 2 – 580 while the 5' product encompasses bp 170 – 900. Primers are listed in table 2.3

Table 2.3 Full-length D7 primers

Number in fig 2.1	Primer name	Sequence (5' to 3')
1	Ct D7 Fwd bp2	GACACTGACATGGACTGAAGGA
2	Ct D7 Rvs bp 479	CGTGTACTGGGGCGTACTTT
3	Ct D7 Fwd exp	CCGTTTAACCCGGAAGAAACC
4	Ct D7 Rvs 3' end	GGGATAGGCTACCTTCAAGC

rD7 production in a bacterial system

The *Cx. tarsalis* 37 kDa salivary protein gene was cloned into pBAD/TOPO ThioFusion vector (Invitrogen) for expression in an *E. coli* based system. Primers were designed from the full-length sequence and PCR was performed. The fresh PCR product was cloned into the pBAD/TOPO ThioFusion vector and transformed into Top 10 *E. coli* cells. Colonies were screened by PCR to test for insertion of the D7 gene using gene specific primers. Positive clones were sent to MMR for sequencing using vector primers. When a clone without mutations was identified, that bacterial colony was grown in a 10

mL culture overnight. The next day 150 µl was inoculated into 50 ml of LB with ampicillin (75µg/ml). When the optical density reached 0.5, the bacteria were induced with 1% arabinose for 4 hours at 37°C. The bacteria were pelleted, and resuspened in bacterial lysis buffer (50mM potassium phosphate, 400 mM NaCl, 100mM KCl, 10% glycerol, 0.5% Triton X-100, 10mM imidazole). Lysed bacteria were further sonicated using a probe sonicator for 1 minute in 10 second bursts on ice. The sample was centrifuged at 14000 rpm for 5 minutes and the pellet and supernatant were stored separately at -20°C. The proteins from the lysed bacteria were fractionated by non-denaturing PAGE and visualized with a his tag-HRP antibody.

Inclusion Body Isolation

Inclusion bodies are made by bacteria to sequester proteins that may be harmful. Because the rD7 protein was only seen in the pelleted non-soluble portion in the previous protocol, inclusion bodies were a possibility. *E. coli* expressing a control protein or rD7 were grown in conditions described above. After a 4 hour induction the bacteria were pelleted and resuspended in 5 mL B-PER Bacterial Protein Extraction Reagent (Pierce). Soluble protein was separated by centrifugation at 15000 x g for 15 minutes. The supernatant, containing soluble proteins was transferred to a new tube. To purify the inclusion bodies 5 ml of B-PER Reagent was used to resuspend the remaining pellet. Next, 100 µl of a 10mg/ml solution of lysozyme was added to the solution and left to incubate at room temperature for 5 minutes. Fifteen ml of a 1:10 dilution of the B-PER reagent were added to the mixture and the sample was centrifuged at 15000 x g for 15 minutes. The supernatant was discarded and the pellet was resuspended in 20 ml of the diluted B-PER reagent. This process was repeated two more times. To isolate the

protein from the purified inclusion bodies, they were solubilized in Inclusion Body Solublization Reagent (Pierce). The inclusion body preparation was weighed and 8ml of the Inclusion Body Solubilization Reagent was added for one gram of wet inclusion body pellet. For rD7 the weight was 0.2g and 1.6ml of reagent was used, for control protein the weight was 0.6g and 4.8ml of reagent was used. The solution was shaken for 30 minutes. Cell debris was pelleted by centrifuging at 27000 x g for 15 minutes. The supernatant contains the solubilized protein from the inclusion bodies. The solubilized protein solution was then dialyzed against PBS in 10 kDa Slide-a-Lyzer dialysis cassette (Pierce) for 12 hours without changes of dialyzing buffer.

Alphavirus protein expression system

The full length D7 gene with a carboxy terminal six histidine tag and the native secretory signal sequence was inserted into the Sindbis virus TE 3'/2J infectious cDNA clone for expression from the 5' double sub-genomic promoter, termed pTE3'CtD7 His. An XbaI cloning site was located downstream of the 5' double sub-genomic promoter. The full length D7 cDNA was amplified using flanking gene specific primers with XbaI recognition sites and a his tag sequence, Ct D7 Fwd Xba and Ct D7 Rvs His Xba2. The amplified product was purified as per manufactures protocol with Qiagen QIaquick PCR Purification kit. The PCR product and vector were digested with XbaI, and purified again with the QIaquick PCR Purification kit. The vector and insert were ligated together using T_4 DNA ligase. The ligated plasmid, pTE3'CtD7 His, was then transformed into electrocompetent Top10 *E. coli* cells (Invitrogen) and plated on LB agar plates with 75 ng/μl of ampicilin. Colonies were screened with vector primers TE5' MCS Seq Fwd and Rvs and positive colonies were sent to MMR for sequencing. When a clone was

identified that did not contain any point mutations, large amounts of DNA were produced by culturing 100 ml of bacteria and isolating plasmid DNA using a Qiagen Plasmid Midi-Prep kit.

To produce protein, 20 μg of infectious clone DNA, pTE3'CtD7 His or control virus pTE5'GFP, was linearized using XhoI, purified using phenol:chloroform extraction and diluted to 500 ng/μl. RNA was transcribed *in vitro* with the following reaction: 4 μl linearized DNA (500 ng/μl), 2.5 μl of each ATP/CTP/UTP (10mM), 2.5 μl GTP (1mM), 2.5 μl Cap analog (10mM), 5 μl Sp6 transcription buffer (10X), 5 μl Sp6 RNA Pol (Ambion MegaScript), 23.5 μl nuclease free dH_2O. The reaction was incubated at 37°C for 1 hour. RNA from the infectious clone was electroporated into C6/36 *Aedes albopictus* mosquito cells. Approximately $8x10^6$ cells were shocked one time (250V, 25Ω, 550μF) with 25 μl of the transcription reaction. At 72 hours post transfection, medium from the cell culture was harvested and analyzed for the presence of recombinant D7 protein (rD7).

Purification of rD7 using ProBond

Purification of rD7 expressed in C6/36 cells was done using the Pro-Bond resin (Invitrogen). The purification was done under native conditions using a batch purification system. 4ml of Pro-Bond bead slurry was placed in a 50 ml tube. The resin was prepared for use by washing one time in water and two times in Native Binding Buffer. Fifteen ml of cell culture medium is added to the resin with 30 ml of Native Binding Buffer. The mixture was rocked for 60 minutes at room temperature. The beads were washed four times with 16 ml Native Wash Buffer, centrifuging at 800 x g between each wash to pellet the beads. Protein was eluted from the resin with 9 ml of Native

Elution Buffer. The sample was then concentrated using an iCon 9kDa 20ml concentrator (Pierce) to achieve a protein concentration of at least 100 ng/µl. Protein concentration was determined using BCA (bicinchoninic acid) Protein Assay Kit (Pierce) and measured using a spectrophotometer reading the samples at 562 nm.

Results

Mosquito salivary protein fractionation and immunoblot

Mosquito salivary proteins have been identified in some major vector species, however little investigation has taken place into the salivary proteins for the mosquito *Culex tarsalis*. Here we collected saliva from 50 adult female *Cx. tarsalis* mosquitoes, precipitated the protein and fractionated them on 10% PAGE gel. Upon silver staining of the gel four major salivary proteins were observed. The molecular weights of these proteins are approximately 14 kDa, 37 kDa, 40 kDa, and 63 kDa (Fig 2.2).

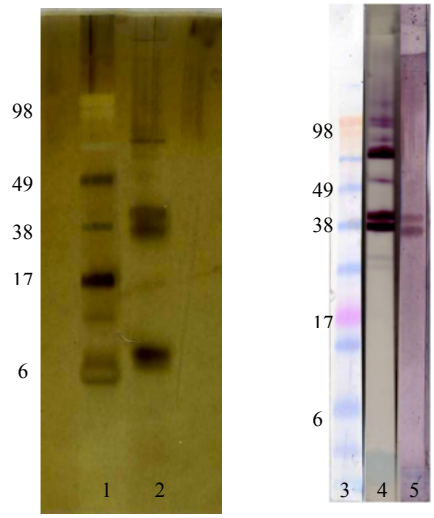


Figure 2.2 Mosquito salivary proteins were collected from *Culex tarsalis* mosquitoes, precipitated, and fractionated using SDS-PAGE. Lanes 1 and 3 are protein markers. Lane 2: Whole saliva silver stained. Lane 4: Whole saliva western blot with serum from mosquito bitten mice. Lane 5: Whole saliva western blot with human serum.

To further analyze the salivary proteins that were identified by silver stain, western blot analysis was performed. Serum from mice that were repeatedly exposed to the bites of *Cx. tarsalis* in a laboratory setting as well as serum from people living in the front range of Colorado within the range of *Cx. tarsalis* were used as the source for primary antibodies. These antibodies were detected with rabbit anti-mouse HRP conjugated antibodies or goat anti-human IgG HRP antibodies (Fig 2.2). These immunoblots revealed serum antibodies in mice recognizing mosquito salivary proteins with the molecular weights of 20, 37, 40, 45, 63 kDa. Human serum revealed antibodies that recognized proteins at 37 and 40 kDa.

Immunoprecipitation of D7 salivary protein

The two salivary proteins that induced the strongest immune response in humans and in mice fell into the size range of D7 salivary proteins. These proteins are found at very high levels in the saliva of other mosquito species including *Aedes aegypti*. Using antibodies made against *Ae. aegypti* D7 salivary proteins, an immunoprecipitation was conducted to isolate D7 salivary proteins from *Cx. tarsalis.* Proteins were eluted, precipitated, and visualized using silver stain on a 10% PAGE. Two proteins were found in the third elution fraction at 36 and 40 kDa (Fig 2.3).

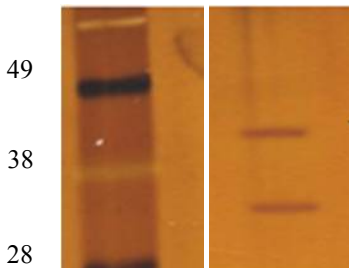


Figure 2.3 MSP from *Cx. tarsalis* were immuno precipitated with antibody to *Aedes aegypti* D7. The protein was eluted and fractionated with SDS-PAGE. Proteins were visualized using a sliver stain. 1) protein marks for the indicated sizes. 2) two discrete bands are visualized at 36 and 42 kDa.

Mass spectrophotometery of mosquito salivary proteins

The MSPs that were revealed to be present at high levels in *Cx. tarsalis* saliva were further characterized. The four that appeared to be present in the highest quantities were collected and subjected to mass spectrophotometric analysis. Each protein was digested with trypsin and submitted to Matrix-assisted laser desorption/ionization and time of flight (MALDI-TOF) analysis. The resulting masses were then analyzed using MS/MS and the resulting mass fingerprints were searched against nr NCBI database using MASCOT (Fig 2.4).

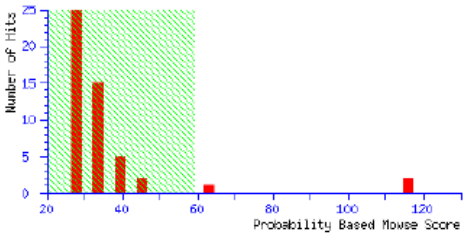
Database search results showed that both the 36 and 40 kDa proteins match significantly to two different D7 salivary proteins from *Cx. pipiens*. The 60 kDa protein that was submitted matched to a putative protein from *Anopheles gambiae*. The smallest protein analyzed at 14kDa showed a significant similarity to a RNA helicase from *Aedes aegypti*.

Mascot Search Results

User : venu
Email : vpujari@lamar.colostate.edu
Search title : 36
MS data file : DATA.TXT
Database : NCBInr 20061201 (4196452 sequences; 1444328266 residues)
Taxonomy : Mosquito (45625 sequences)
Timestamp : 6 Dec 2006 at 18:35:00 GMT
Warning : A Peptide summary report will usually give a much clearer picture of MS/MS search results.
Top Score : 116 for gi|16225986, long form D7clu12 salivary protein [Culex pipiens quinquefasciatus]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 59 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Mascot Search Results

User : venu
Email : vpujari@lamar.colostate.edu
Search title : 40
MS data file : DATA.TXT
Database : NCBInr 20061201 (4196452 sequences; 1444328266 residues)
Taxonomy : Mosquito (45625 sequences)
Timestamp : 6 Dec 2006 at 20:51:04 GMT
Warning : A Peptide summary report will usually give a much clearer picture of MS/MS search results.
Top Score : 61 for gi|16225983, long form D7clu1 salivary protein [Culex pipiens quinquefasciatus]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 59 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

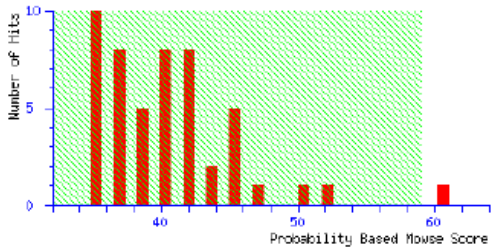


Figure 2.4 MSPs were subjected to MS/MS analysis using Mascot software. Here the significant matches for the 36 and 40 kDa proteins are shown. Each was matched to a D7 salivary protein found in *Culex pipiens.*

N-terminal sequencing

To further characterize the 36 kDa protein that was identified to be a D7 salivary protein, a sample was submitted to Edman degradation and N-terminal sequencing. Eleven residues were identified from the N-terminus, EWKPFNPEETL. Sequence analysis also indicated the second residue, a tryptophan, was oxidized. When compared to the *Cx. pipiens* D7 protein 9 of the 11 residues matched the N-terminal sequence after the putative secretory signal (Table 2.4).

Table 2.4 D7 N-terminal sequencing results. Retention time for each amino acid is compared to standards to determine amino acid. *Cx. pipiens* sequence shown as reference.

Residue number	*Cx. tarsalis*	Retention time (Min)	*Cx. pipiens*
1	Glu	6.36	Ala
2	Trp	16.27	Trp
3	Lys	17.75	Lys
4	Pro	12.83	Pro
5	Phe	16.95	Phe
6	Asn	4.43	Ser
7	Pro	12.83	Pro
8	Glu	6.38	Glu
9	Glu	6.37	Glu
10	Thr	5.63	Thr
11	Leu	17.96	Leu

Sequencing of the D7 gene and creation of full length clone

Using degenerate primers designed from the N-terminal amino acid sequence 3' RACE was conducted to determine the cDNA sequence of the *Cx. tarsalis* D7 protein. This resulted in the sequence minus the secretory signal. 5'RACE was used to obtain the

remaining signal sequence. An alignment of the *Cx. tarsalis* and the *Cx. pipiens* amino acid sequences shows 85% identity. The alignment of the two genes is shown in figure 2.5.

The *Cx. tarsalis* D7 cDNA is 942 base pairs long and the amino acid sequence is 314 amino acids long. The rD7 sequence shown in figure 2.5 is 320 amino acids with the included C-terminal six-histidine tag. The first 20 amino acids are cleaved in the mature protein and is thought to act as a signal peptide.

Figure 2.5 Amino acid alignment of *Cx. tarsalis* and *Cx. pipiens* D7 genes. The *Cx. tarsalis* sequence includes the six-histidine tag used for identification and purification of recombinant protein. The sequences have 85% identity at the amino acid levels.

The 3' and 5' RACE products resulted in two plasmids, each with a portion of the D7 gene. To create the full length D7 gene in one plasmid the PCR products from 3' and 5' ends that overlapped for 410 bp (nucleotides 170 to 580) were used as primers. The two products were incubated together in a PCR reaction with no additional primers. After 10 cycles of amplification, primers that flanked the full-length sequence were added and the reaction was cycled for 30 additional cycles. The band that corresponded with the full-length sequence, 900 bp, was visualized by gel electropohoresis (Fig 2.6).

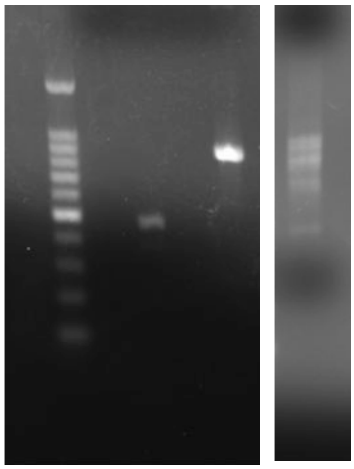


Figure 2.6 Agarose DNA electrophoresis showing PCR products in the generation of full length D7. Lane 1: 1 kb DNA ladder Lane 2: 3' D7 PCR product 570 bp Lane 3: 5' D7 PCR product 730 bp Lane 4: Product after PCR merging the two products. Distinct bands seen at 570, 730, and 900(full length product) bp.

Bacterial expression system

To further characterize the function of the *Cx. tarsalis* D7 protein a recombinant protein was needed as isolating large amounts of native protein was not feasible. An *E.coli* expression system was chosen for the simple cloning technique and possibility of very high levels of protein expression. The pBAD/TOPO Thio vector (Invitrogen) has topoisomerase enzymes for ease of cloning PCR products. The vector places the gene of interest under the control of an arabinose induced promoter and adds several tags convenient for identification and purification. The plasmid without the inserted gene of interest produces a 16 kDa control protein.

To find the optimal induction conditions bacteria were induced with a range of arbinose concentrations: 1%, 0.5%, 0.2%, and 0.002%. Each of these concentrations was tested at 4, 5, or 6 hours. The soluble protein fraction did not show any protein being

expressed at any of the conditions tested (lane 1, fig 2.7). The control protein was present at high levels in all samples (lanes 2, fig 2.7). The insoluble fraction did show low levels of D7 expression that did not appear to change with concentration of arabinose or hours of induction as seen in lanes 3-6 of fig 2.7.

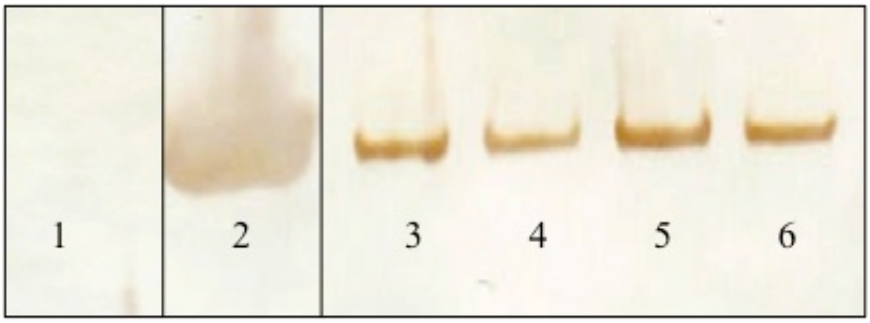


Figure 2.7 Western blot with his-tag-HRP antibody detecting rD7 and control protein. Lane 1: soluble fraction Lane 2: control protein soluble protein Lane 3-6: rD7 insoluble fraction with arabinose at 1%, 0.5%, 0.2%, and 0.002%.

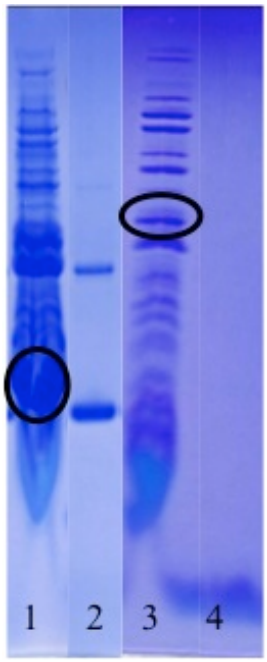


Figure 2.8 Coomassie stain of control protein and rD7 before and after purification with ProBond resin. The proteins of interested are circled. Lane 1: control protein Lane 2: purified control protein Lane 3: rD7 Lane 4: purified rD7

Purification of the rD7 and control protein was attempted using ProBond resin (Invitrogen) under native conditions. The control protein purified very well, however there was no recovery of rD7(Fig 2.8).

Although denaturing purification conditions may lead to misfolding of the recombinant protein, it was attempted to try and recover rD7 from the insoluble fraction. Again, no recombinant protein was recovered (data not shown).

Bacteria were grown in liquid culture and induced to produce the recombinant protein using a final concentration of 1% arabinose. There was a marked decrease in growth kinetics of the D7 protein producing cultures as compared to control bacterial cultures. Bacteria containing the plasmid containing D7 took 6 hours, 45 minutes to reach an optical density of 0.5, where *E. coli* with the control plasmid only took 3 hours and 30 minutes. Based on this reduction in growth kinetics and the protein being confined to the insoluble fraction, it was hypothesized that the protein was being sequestered in inclusion bodies. rD7 was isolated from the inclusion bodies as described in the methods. Figure 2.9 shows that rD7 was recovered from inclusion bodies, however it was only at very low levels.

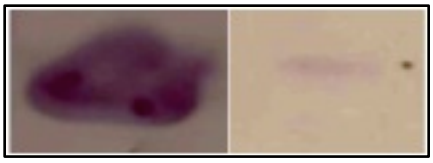


Figure 2.9 Western blot showing protein detected with his-tag-HRP antibody after inclusion body isolation and disruption. Lane 1: control protein Lane 2: rD7

Alphavirus transducing system protein expression

Sindbis virus infectious clones have been designed such that the subgenomic promoter is duplicated and placed either 5' or 3' to the structural proteins. This duplicated subgenomic promoter allows for the insertion of a gene of interest that will be expressed from the genome of viruses transcribed from the infectious clones. Here the D7 gene from *Cx. tarsalis* was cloned into the TE3'2J Sindbis virus infectious clone. The 3' location of the gene of interest leads to higher levels of protein production due to the nature of the viral replication and gene expression. The full length D7 gene with the native secretory signal and a C-terminal six histidine tag were inserted into the infectious clone.

C6/36 *Aedes albopictus* cell cultures were electroporated with *in vitro* transcribed RNA generated from the SINV TE3'Ct D7 His plasmid. As a control SINV TE5'GFP was transfected in parallel allow visual confirmation of virus replication (Fig 2.10). At 72 hours post transfection cells were visualized for GFP production and supernatant was tested for D7 presence using Western blot.

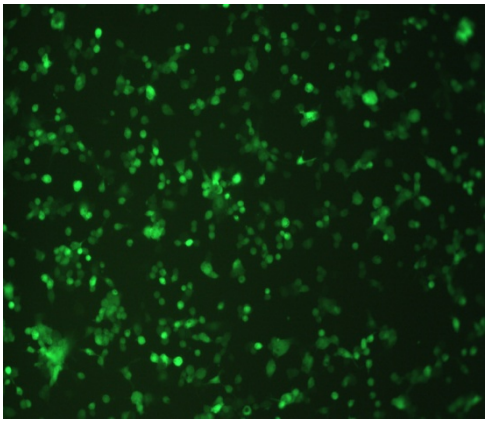


Figure 2.10 C6/36 cells infected with the SINV TE 5'GFP pictured using a UV filter showing the *in vitro* transcription and electroporation was successful.

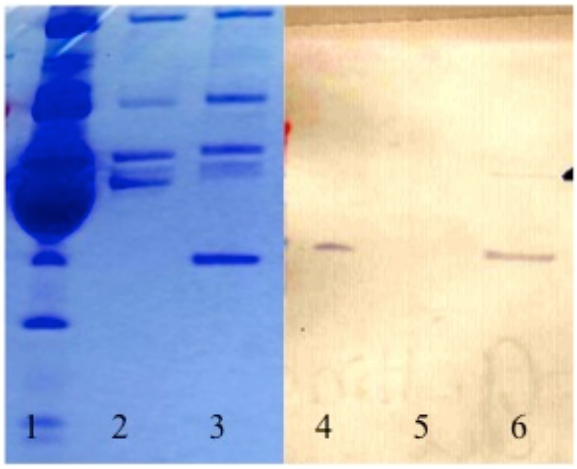


Figure 2.11 Commassie stain and Western blot visualization of rD7 protein in cell supernatant and after purification. Lane 1/4: Cell supernatant pre-purification Lane 2/5: Control purification of cell supernatant from SINV GFP Lane 3/6: SINV-rD7 purified supernatant

rD7 was found at high levels in the medium of cells infected with the SINV 3'Ct D7 His (Fig 2.11 lane 1). Cell supernatant was collected and a virus was passaged a second time at an MOI of 0.1. The medium was collected at 72 hours post infection. rD7 was purified from the collected medium using ProBond nickel agarose beads (Invitrogen) under native conditions. rD7 was visualized by Commassie stain and Western blot.

Characterization of rD7

To confirm the identity of the recombinant protein, it was subjected to mass spectrometric analysis at the Proteomics and Metabolomics facility at Colorado State University, which was carried out as described above. A theoretical trypsin digest was performed using the *Cx. tarsalis* rD7 amino acid sequence in Fig 2.5. These masses were then compared to the MALDI-time of flight mass data presented in Fig 2.12.

Comparison of the theoretical digest to the obtained data confirmed that the purified recombinant protein was the *Cx. tarsalis* D7 salivary protein. The masses that are highlighted in Fig 2.12 were observed in the theoretical trypsan digest. There was 54.7% coverage of the as shown in Fig 2.13 with the highlighted regions present in the mass spectral analysis.

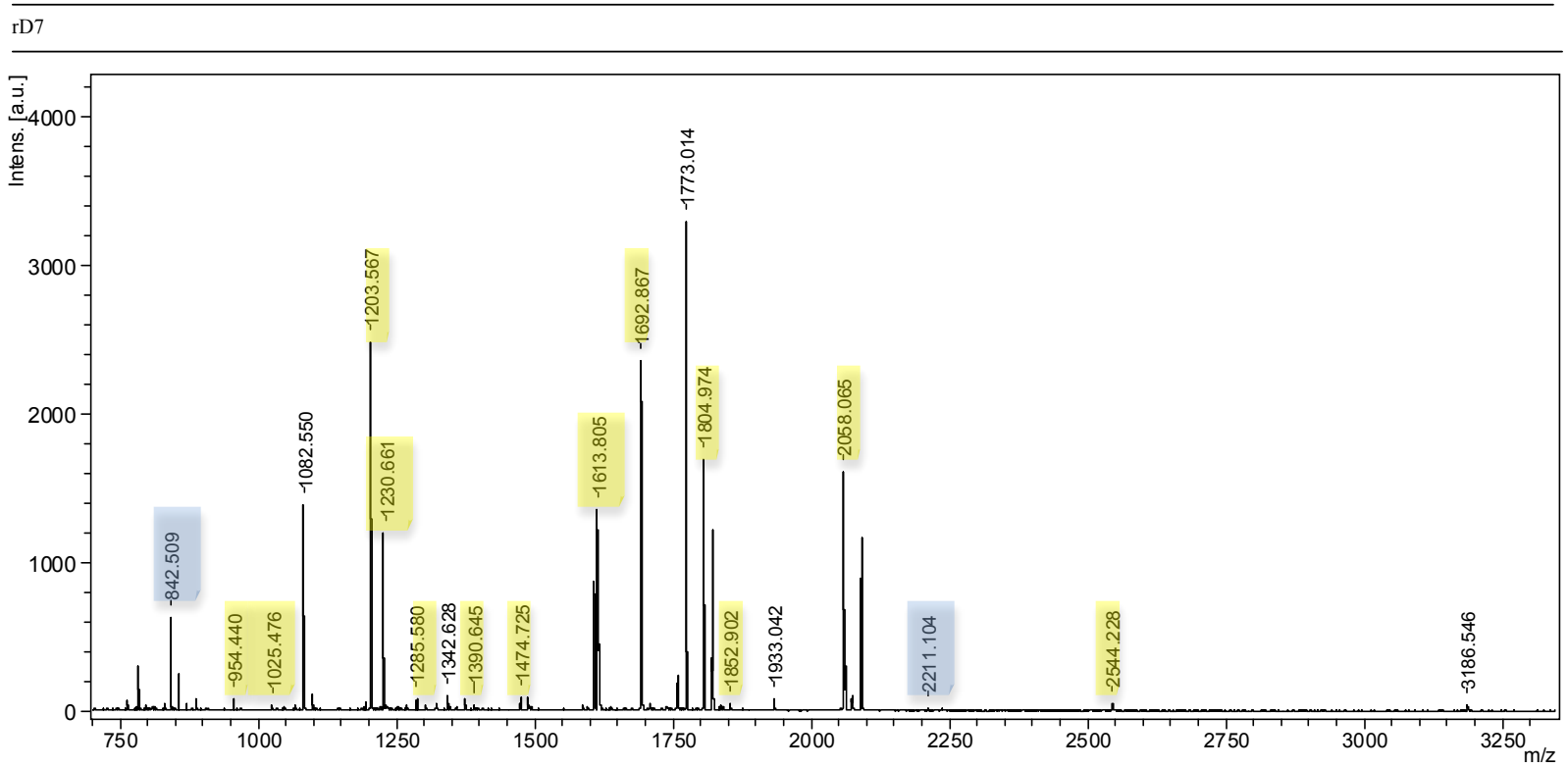


Figure 2.12 Mass spectral analysis of rD7 protein purified from SINV-Ct 3' D7 infected C6/36 cells. Peptide masses are noted above each of the major peaks. These were matched by hand to a theoretical digest of the rD7 protein with trypsin. Yellow highlighted peaks matched to rD7 peptides. Blue highlighted peaks match to trypsin autocleavage peptides and are not associated with rD7.

MNLLSITSLLIVAFFTGGNAEWKPFNPEETLFTYTRCMEDNAKGDMELAKKW
MNWKLEQDPKSAC**YAKCVLVGLE**LFDESSKTFKGDHILEQYQKYKDFTSQDEA
GVKEFQNAVQALGSVDSSD**CVKVLQK**YAPVHAKYTDVQRNV**YFGKKEITDKI**
YSSDSTVKKRDETMF**RFCERSHFKEGSEEL**CTLRKTGITTKNEHLDCL**FRGLRY**
LDRNGNINPAEIKRDLHFINVN**DKDDAVDNALNNCKV**NEATKATDYN**DCLWK**
DPNLKDIMMPVFDYREVRSESY**RYFIEHTEPYDATKV**KEKVRKYDQDAVAS

Figure 2.13 Mass spectrophotography coverage. Amnio acid sequence of rD7 protein with highlighted regions being represented in the mass spectral analysis. There is a 54.7% coverage which is well over the 30% accepted rate for identification of a protein.

To determine if the recombinant protein retained the linear epitopes of the natural D7 salivary protein, a Western blot was performed. In figure 2.14 serum from mice that had been repeatedly exposed to the bites of *Cx. tarsalis* show high levels of antibodies binding to the rD7 protein.

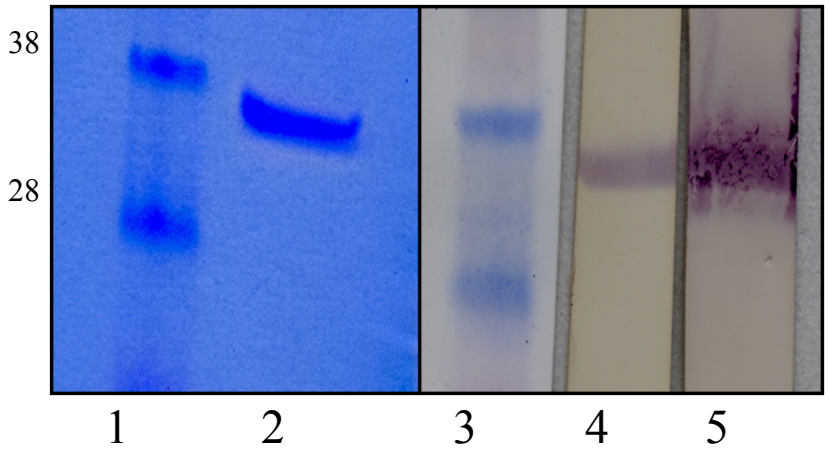


Figure 2.14 Western blot using his-tag-HRP antibody or serum from mosquito exposed mice to show antibodies made to native protein also bind rD7. Lane 1/3: Protein marker Lane 2: rD7 Lane 4: rD7 blotted with his-tag HRP antibody Lane 5: rD7 blotted with mouse serum.

Discussion

Culex tarsalis mosquitoes express D7 salivary proteins

Mosquito saliva plays an important role in the pathogenesis and immune response to many arboviruses. To better understand the role of MSPs during infection the major proteins must be characterized. *Culex tarsalis* is an important disease vector in the western United States, transmitting West Nile virus, western equine encephalitis virus, and St Louis encephalitis virus. Here the first investigation of the components of *Cx. tarsalis* saliva was performed.

Of primary interest in this study was identification of the D7 salivary proteins. D7 salivary proteins have been identified in several species of mosquitoes, with each species having multiple forms of the proteins. Antibodies have been generated to a D7 protein from *Ae. aegypti*. This antibody was used to immunoprecipitate proteins from *Cx. tarsalis* saliva. Two proteins were pulled down in this assay, one at 36 kDa and one at 40 kDa. These proteins fall within the size range of large D7 proteins; however further characterization using this method was not feasible due to low protein abundance.

Concentration of proteins from whole mosquito saliva followed by SDS-PAGE fractionation showed that four proteins were present in the saliva at very high levels. Two of these corresponded with the protein molecular weights found in the immuoprecipiation experiments. I hypothesized that these were D7 proteins. To confirm these findings, mass spectroscopy analysis was utilized. Comparison of the mass profiles to the NCBI nr database was conducted to find significant matches with previously reported mosquito protein sequences. The 36 and 40 kDa proteins matched significantly to two different D7 salivary proteins from *Culex pipiens*. With this information as well as the precipitation with *Ae. aegypti* D7 antibodies it can be concluded that we have

identified two D7 salivary proteins in *Cx. tarsalis*. Since these findings, transcriptome analysis has verified our results (Calvo et al. 2010a).

In addition to the two D7 proteins that were identified by mass spectrometric analysis, a 17 kDa and a 60 kDa protein were analyzed. The 17 kDa protein that was present at high levels in saliva significantly matched a DEAD box ATP-dependant RNA helicase from *Aedes aegypti*. Many RNA helicases have been identified in mosquitoes and some have been linked to the RNAi response (Campbell et al. 2008). The 60 kDa protein was most closely matched to a putative protein from *An. gambiae*, however the protein has not yet been identified.

Culex tarsalis D7 proteins are highly immunogenic

D7 proteins are highly immunogenic, and those found in *Cx. tarsalis* do not differ in that aspect. Western blots conducted with serum either from mice that were repeatedly exposed to mosquito bites or from humans that live in *Cx. tarsalis* endemic areas showed high reactivity to the two D7 proteins (Fig 2.1). Mice that have been repeatedly exposed to mosquito bites have high levels of both IgG1 and IgG2a antibodies, however mice exposed only once have primarily IgG1 antibodies (data shown Fig 3.2). IgG1 antibodies are associated with an interleukin-4 dominated response and creation of IgE antibodies observed after exposure to mosquito salivary proteins.

Culex tarsalis D7 gene has high level of identity to Culex pipiens D7

Sequencing of the 37 kDa D7 cDNA yielded a full length sequence including a putative signal sequence. Alignment of the full-length sequence to the *Cx. pipiens* D7 salivary protein gene showed 85% identity at both the amino acid nucleotide level. The

amino acid sequence contains 9 of the 10 conserved cysteine residues present in the *Cx. pipiens* D7 sequence.

Recombinant D7 protein is toxic to bacterial cells

To conduct further studies on D7 salivary proteins the gene needed to be expressed and the product purified. A bacterial expression system was chosen based on the relative ease of cloning and expression and potential for very high levels of protein to be produced. *E. coli* bacteria that were transformed with the pBAD-Ct D7 plasmid grew at a slower rate than bacteria containing the same plasmid with a control protein. Further investigation showed that the expressed protein was present at significantly lower concentrations than control protein and was being sequestered in inclusion bodies. Taken together this led to the conclusion that the D7 salivary protein is toxic to bacterial cells making expression and purification difficult.

Recombinant D7 protein expressed in insect cells closely resembles native protein

Introduction of the D7 gene including the native signal sequence under conrol of a second sub-genomic promoter in the Sindbis virus expression vector yielded protein at levels exceeding 200 ng/μl post purification. To confirm the identity of the recombinant D7 (rD7) Maldi-TOF analysis was conducted and the mass spectral data were compared to a theoretical trypsin digest of the rD7 gene sequence. Comparison of the theoretical peptide masses located in table 2.5 match very closely with the predominant peaks present in figure 2.12. Conclusions of this experiment were that the correct sequence was being expressed but could not ensure the correct folding pattern of the rD7. To ensure linear epitopes were conserved, antibodies generated against the native D7 protein were used to detect rD7. Indeed the antibodies readily bound to the rD7 (Figure 2.13).

Summary

In preparation for the characterization of a MSP vaccine, the major salivary proteins from *Cx. tarsalis* needed to be characterized. In this chapter we have shown that *Cx. tarsalis* has two D7 salivary proteins. Both proteins were demonstrated to be highly immunogenic as specific antibodies were found in sera of mice exposed to mosquito in a laboratory setting and in people living within the mosquito-transmitted region. The expression of 37 kDa D7 protein is toxic to bacteria, but can be - expressed in the mosquito cell line C6/36 using a Sindbis virus infectious clone. rD7 protein was shown to have the same contents as the native protein when analyzed by mass spectroscopy. The antibodies generated against the native D7 protein recognize the recombinant D7 protein.

CHAPTER III

IMMUNE RESPONSE TO D7 VACCINATION

Introduction

Mosquito salivary proteins alter the host immune status after a bite. There is an increase in the Th2 type cytokine IL-4 and the regulatory cytokine IL-10 after a mosquito bite both locally and systemically (Schneider and Higgs 2008). IL-4 directly inhibits expression of IL-12 and downstream Th1 type cytokines such as type I and type II interferons that are critical in the immune response against arboviruses. The protein SAAG-4 has been identified in the saliva of *Ae. aegypti* as the primary factor that causes saliva induced immunomodulation, however no such protein has been identified in *Culex* saliva (Boppana et al. 2009). Here we investigate the role of *Cx. tarsalis* D7 protein in the saliva-induced immunomodulation by exposing naïve mice to rD7 and analyzing inflammation at the injection site and systemic cytokine responses.

The immunomodulation seen after a mosquito bite leads to a phenomenon known as saliva-induced pathogen enhancement wherein arboviral infection is enhanced by the presence of mosquito saliva (Titus et al. 2006, Schneider and Higgs 2008). Vaccines targeted against the salivary proteins of blood feeding arthropods have been of great interest. If the immunomodulation caused by salivary proteins can be neutralized then the host should not be inhibited for production of a strong Th1 immune response to arboviruses delivered with the saliva. A D7 salivary protein vaccine was produced using rD7 as described in chapter 2. We investigated the immune response in vaccinated mice when exposed to a *Cx. tarsalis* bite or to rD7 injected intradermally. The humoral and cellular responses were measured, and the bite site was examined for histopathology. I hypothesized that vaccinated mice would have increased levels of Th1 type cytokines, IFNγ, TNFα, and IL-12 and decreased levels of Th2 cytokine IL-4 and the regulatory

cytokine IL-10, in response to exposure to salivary proteins. This immune status would be in contrast to the immune status seen in naïve animals after exposure to mosquito bites, and be beneficial in fighting an arboviral infection.

Methods

Mosquitoes

Culex tarsalis Bakersfield CA strain were raised as discussed in Chapter 2 under bio-safety level 2 conditions. Mosquitoes were offered water and sugar *ad libitum* until 24 hours before a mouse blood feed.

Mice

Animals used in these projects were purchased from Jackson Laboratories (Bar Harbor, Maine). 6 – 8 week old female C57/BL 6 mice were used for all experiments. The mice were housed in a BSL 2 facility and monitored daily by staff at CSU Laboratory Animal Resources. Mice were housed and handled in accordance to protocols approved by the Institutional Animal Care and Use Committee.

Mosquito feeding on mice

Mice pinna were exposed to the bite of *Cx. tarsalis* mosquitoes. Mice were anesthetized with 0.2 ml Ketamin/Xylazine (0.2mg/2mg) intraperitoneally and covered loosely with gauze with only the left ear being exposed. 1-5 mosquitoes imbibed a complete blood meal per mouse.

Immunization

Mice were immunized with 10 μg rD7 protein. Complete Freund's adjuvant (Imject, Pierce) was used for the primary inoculation, followed by boosters at 2 and 4

weeks with incomplete Freund's adjuvant (Sigma). 10 μg of rD7 was diluted in sterile PBS to a volume of 100 μl and placed in a 3cc syringe. Equal volume of adjuvant was placed in a second 3 cc syringe. The syringes were connected using a 3 way stop cock and the liquids were mixed by pushing into alternating syringes until the mixture turned a milky white color. Each mouse was anesthetized using 3% Attane isoflurane (Minrad Inc, Orchard Park, NY). Inoculations were made in four sites, one on each quadrant of the back, subcutaneously with 50 μl per site.

In vitro splenocyte stimulation

Mice were anethetized with isoflurane and blood was collected from the animals. 30 ml of PBS was then used to perfuse the animal. The spleens were removed and placed in RPMI medium^{+}10%FBS. The spleen was disrupted into a single cell suspension by passing through a 100 μm cell strainer with a 3cc syringe plunger and washed with 15 ml RPMI. The cells were pelleted at 500xg for 5 min. Red blood cells were lysed in 1ml of red blood cell lysis buffer at room temperature for 10 min and the remaining cells were washed two times with 10 ml PBS. Cells were counted in a 1:10 dilution in trypan blue(0.4% w/v) and plated in a 96 well flat bottom plate at 10^6 cells per well. For each spleen sample three wells were seeded; a non-stimulated control, a well stimulated with 0.5 μg of rD7, and a well stimulated with an equal volume of the control protein preparation. Control protein consists of purification of the media from cells infected with SINV-GFP. The cells were incubated at 37 °C and 5% CO_2 for 72 hours. At 48 hours post-stiumulation 20 μl RPMI medium was added to each well. At 72 hours post stimulation the cell supernatants were collected and stored at - 80°C for CBA analysis.

Cytometric bead array

Cytometric bead arrays (BD Pharminogen) were used to measure cytokine levels in serum and splenocyte supernatants. Standards were prepared by reconstituting one vial of lyophilized mouse standards (Kit Bottle C) in 2.0 ml Assay Diluent as described by manufacturer. 2-fold dilutions in 50 µl of Assay Diluent (Kit Bottle G) were prepared, creating 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 dilutions of the stock standards. Assay Diluent (Kit Bottle G) served as the 0 pg/ml negative control. Two and a half µl of each of the six capture beads for MCP-1, IFNγ, TNFα, IL-10, IL-6, and IL-12p70 were added to each assay well, for a total volume of 15 µl. The capture beads were added to all sample wells including standards, negative control and sample wells in a round bottom 96 well plate. Next, 50 µl of sample or standard cytokine dilutions were added to each well. Fifteen µl PE detection reagent (Kit Bottle B) were added to all wells and the plate was incubated at room temperature for 2 hours protected from light. The beads were washed with 200 µl 1X Wash Buffer (Kit Bottle F) per well. The plate was centrifuged at 200 x g for 5 minutes at room temperature, and the supernatant was removed. The beads were washed a second time by resuspending each well in 200 µl 1X Wash Buffer or FACS buffer (BD Pharminogen). Again, the plate was centrifuged at 200 x g for 5 minutes. The beads were resuspended in 150µl FACS buffer and transfered to flow tubes for analysis on a CyAn (Dako) flow cytometer.

Intracellular cytokine staining

Single cell suspensions of splenocytes were obtained as described above. The cells were washed once with 15 ml of RPMI^{+}10% FBS and centrifuged at 500 x g for 5

min. RBCs were lysed with 3 ml of cold RBC lysis buffer for 5 min at room temperature. Twenty seven ml of RPMI was added to dilute the buffer and the cells were centrifuged again. The cells were washed again in 30 ml of RPMI and were resuspended in 3 ml RPMI. The cells were counted with trypan blue (0.4% w/v) and 2 x 10^6 cells were plated in a 6 well plate. 10 µl of Golgi Plug/PMA/ianomyocin cocktail (BD Pharminogen) was added to each well and incubated at 37 °C for four hours for stimulation of the splenocytes. The cells were transferred to a 50 ml tube, pelleted, and washed in 15 ml of RPMI. The cells were resuspended in 1 ml of RPMI and 100 µl of the suspension was placed in 96-well round bottom plate, with 5 wells for each spleen sample. The plate was centrifuged at 1800 rpm for 1 min and the supernatant was removed. Ten µl of FcR Block (BD Pharminogen) was added to each well. Fifty µl of surface staining markers, CD4-FITC and CD8-APC, were added to each well at a 1:200 dilution. The cells were incubated at 4°C for 30 min. One hundred µl of staining buffer was added to each well, the plate was centrifuged, and supernatant was removed. One hundred µl of Fix/Perm buffer was added to each well and incubated for 20 minutes. One hundred µl of staining buffer was added to each well, centrifuged, and supernatant was removed. To each well 20µl of FcR Block and 30 µl of Perm/Wash was added. Each of the five wells from a single spleen sample were stained with a different PE labeled antibody to the following cytokines: IL-10, IL-4, IFNγ, TNFα, and IL-12. IFNγ, TNFα, and IL-12 antibodies were added at 20 µl per well as per BD Pharminogen Mouse ICS Starter kit, while the IL-10 and IL-4 (0.2 mg/mL, BD Pharminogen) antibodies were added at 1µl per well. The cells were incubated at 4°C for 20 minutes. Cells were washed with 100 µl of staining buffer. The stained cells were resuspended in 200 µl of staining buffer and placed in flow tubes

and held at 4°C until they could be read on a CyAn (Dako) flow cytometer. Data were analyzed using Summit software.

ELISA

Indirect ELISAs were used to measure D7 antibody levels. 96 well Immulon (VWR) plates were coated with 50 ng of rD7 in 50 µl of coating buffer (50mM sodium carbonate, 50mM sodium bicarbonate, pH 9.6) and placed at 4°C overnight up to 1 month. Plates were washed three times with PBS containing 0.1% Tween-20. Plates were blocked with PBS containing 0.1% Tween-20 and 0.5%FBS for 1 hour at room temperature. Serum samples were added at four fold dilutions from 1:20 to 1:5120 as noted. The plates were incubated at room temperature for 1 hour. The plates were then washed four times with PBS containing 0.1% Tween-20. Horseradish peroxidase conjugated secondary antibody, goat anti-mouse IgG, goat anti-mouse IgG1, or goat anti-mouse IgG2a (KPL Labs) were added to the samples at 1:1000 and incubated for 1 hour. The plates were again washed four times with PBS containing 0.1% Tween-20. The ELISAs were developed using 50µl of 3,3′,5,5′-tetramethylbenzidine (Sigma) per well until color appeared, approximately 5 minutes. The color reaction was stopped with 50µl of 1N HCl, and the plates were read on a plate reader at 450 nm. Results are presented as the optical density at 450 nm. The cut off for a positive reading was two standard deviations above the mean of negative control samples.

Histopathology

Histopathologic evaluation was conducted on mouse pinna exposed to intradermal rD7 (2µg/mouse in 50 µl PBS), control protein (media from cells infected with SINV-GFP that underwent the same purification procedure), or to the bites of *Cx. tarsalis*

mosquitoes. At two days post exposure the ears were removed and placed in 4% paraformaldehyde. The samples were then submitted to Colorado HistoPrep for sectioning and hematoxylin and eosin staining. Anatomic histopathology was interpreted and scored by a board certified veterinary pathologist at Colorado HistoPrep.

Results

Antibody response after rD7 vaccination or natural exposure

The humoral response to both vaccination and natural exposure to MSPs was measured using an indirect ELISA with rD7. Mice were vaccinated as described in the methods or exposed to 30-40 *Cx. tarsalis* mosquito bites every two weeks over the course of a year. At 10 days post final rD7 inoculation, blood samples were collected from the tail vein of five mice in each group. The serum was then tested for total IgG D7 antibodies. Mean optical density (MOD) of the samples is shown in Figure 3.1. The cutoff for a positive reading is two standard deviations above the mean of the negative control measurements. Both the rD7 vaccinated mice and mice used to feed the mosquitoes maintaining colonies were positive for D7 antibodies (MOD readings over 3.5) (Fig 3.1). Mice that were administered the control vaccine containing protein purified from SINV-GFP infected cells did not produce any detectable D7 antibodies.

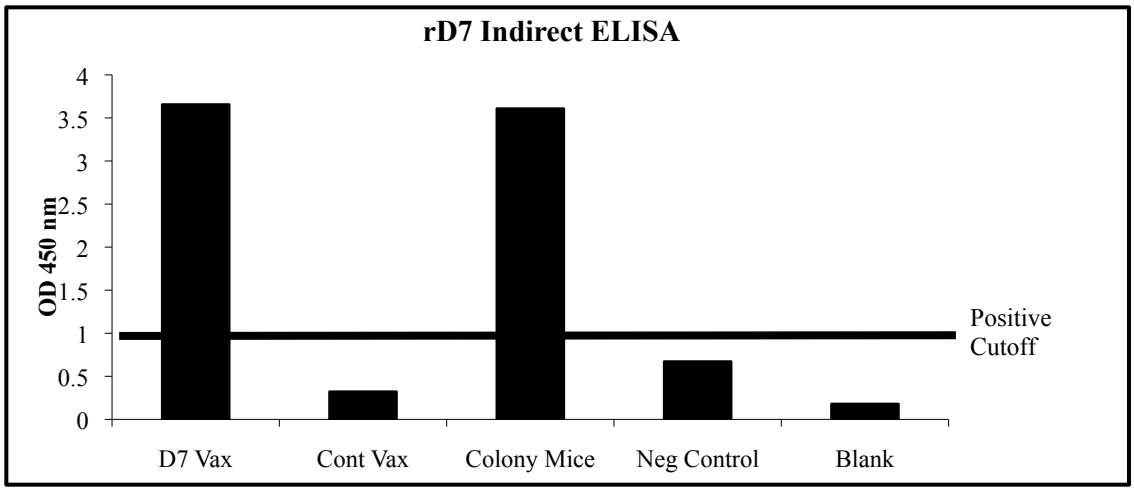
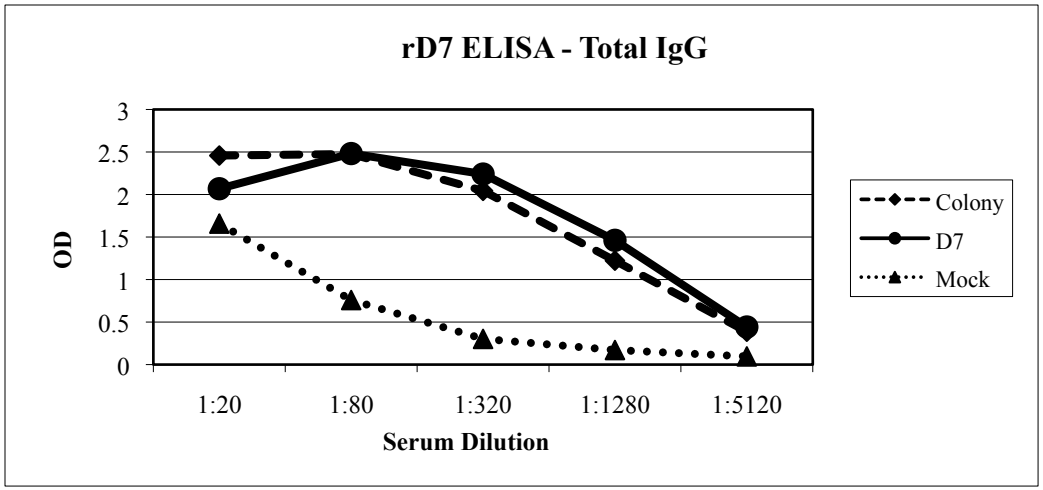
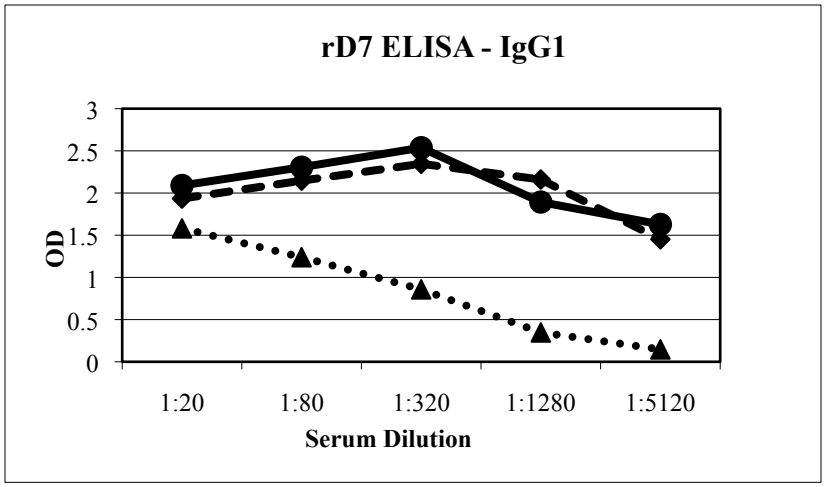


Figure 3.1 Indirect ELISA measuring total IgG reactive with rD7. D7 Vax: serum from mice 10 days post third D7 vaccination Cont Vax; mice inoculated three times with control protein. Colony Mice: mice repeatedly exposed to the bites of *Cx. tarsalisx* over the course of a year. Neg Control: commercially acquired mouse serum never exposed to mosquito saliva Blank: no serum added

To further characterize the antibodies made in response to the D7 vaccine IgG subtype ELISAs were conducted (Fig 3.2). Each sample was diluted 4-fold from 1:20 to 1:5120 and the secondary antibody detected total IgG, IgG1, or IgG2a. The mock vaccinated mice were fed upon one time by *Cx. tarsalis* after completion of the vaccination series. The results reiterate the high levels of total IgG antibodies shown in Fig 3.1 with colony and vaccinated mice having ELISA titers to 1280. The subtype ELISAs show that the colony mice had high levels of IgG1 subtype antibody titers higher than the highest dilution tested, and the IgG2a levels had a titer of 320. Vaccinated mice produced levels of IgG1 equivalent to mosquito colony-bitten mice, but did not produce any anti-D7 IgG2a. The mock-vaccinated mice that had been exposed to one mosquito bite had no detectable IgG2a but did show low levels of IgG1.





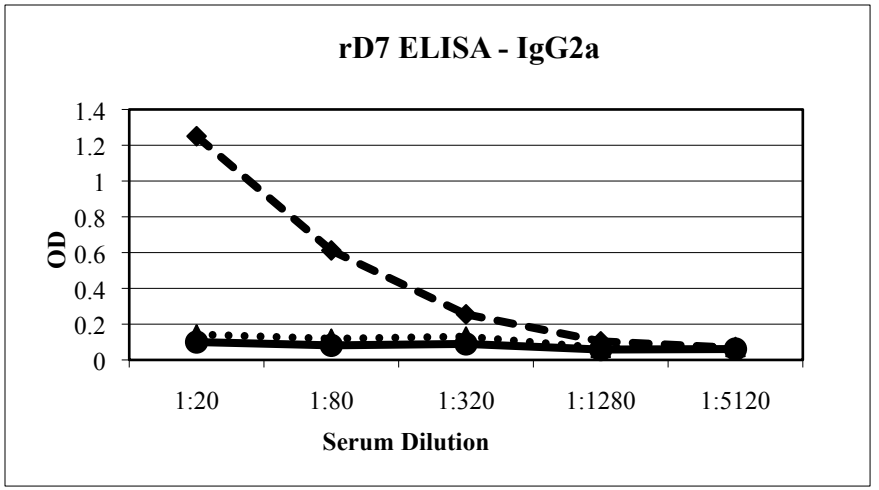


Figure 3.2 rD7 indirect ELISA showing titers of serum antibodies detecting total IgG, IgG1, or IgG2a. Colony mice: mice repeatedly exposed to the bites of *Cx. tarsalis* D7: mice vaccinated with rD7 vaccine Mock: mice administered vaccination containing mock protein then exposed to one mosquito bite. Sample is representative of pooled serum from four mice per group.

Cytokine analysis after exposure to rD7

Mice in three groups, naïve, rD7 vaccinated or mock vaccinated were administered rD7 or a control protein intradermally in the ear 10 days post final inoculation. Two days after intradermal injection, serum and spleens were harvested from three mice per experimental group. Serum levels of IFNγ, TNFα, MCP-1, IL-12p70, IL-6 and IL-10 were measured using CBA analysis. Mice that were exposed to intra-dermal rD7 after vaccination as compared to naive mice had a significant increase in MCP-1 and TNFα ($p<0.05$) at two days post exposure (Fig 3.3). In addition, intradermal D7 caused levels of MCP-1 to decrease in naïve mice that were exposed to rD7 as compared to naïve mice that received a mock protein injection (Fig 3.4). The other cytokines showed no differences between treatment groups.

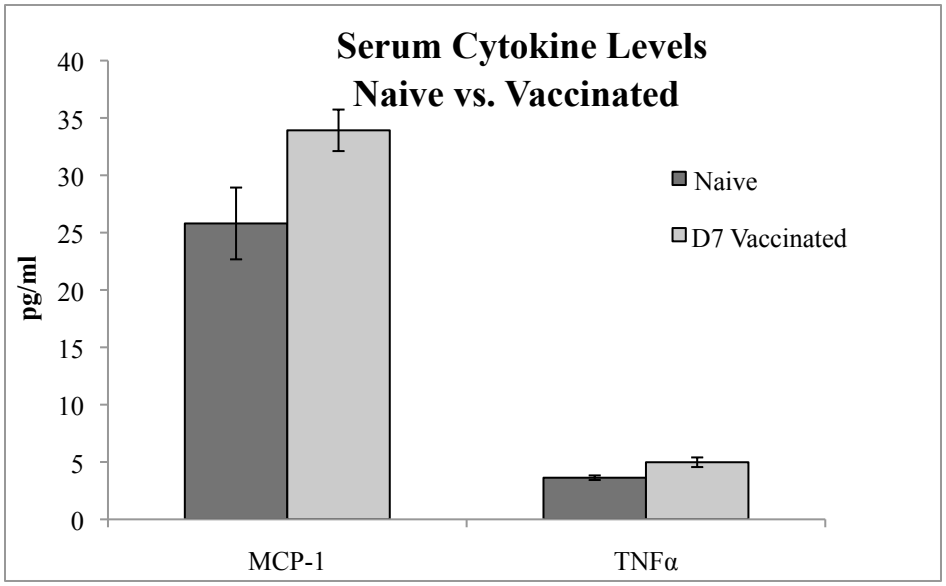


Figure 3.3 Serum cytokine levels were measured using cytometric bead array analysis. Mice were either vaccinated with rD7 or naïve then injected with rD7 ID. The D7 vaccinated mice that were exposed to rD7 had significantly higher levels of MCP-1 and TNFα two days post exposure (p=0.018, p=0.0072). Graph shows mean of three individuals with SEM error bars. Other measured cytokines showed no significant changes.

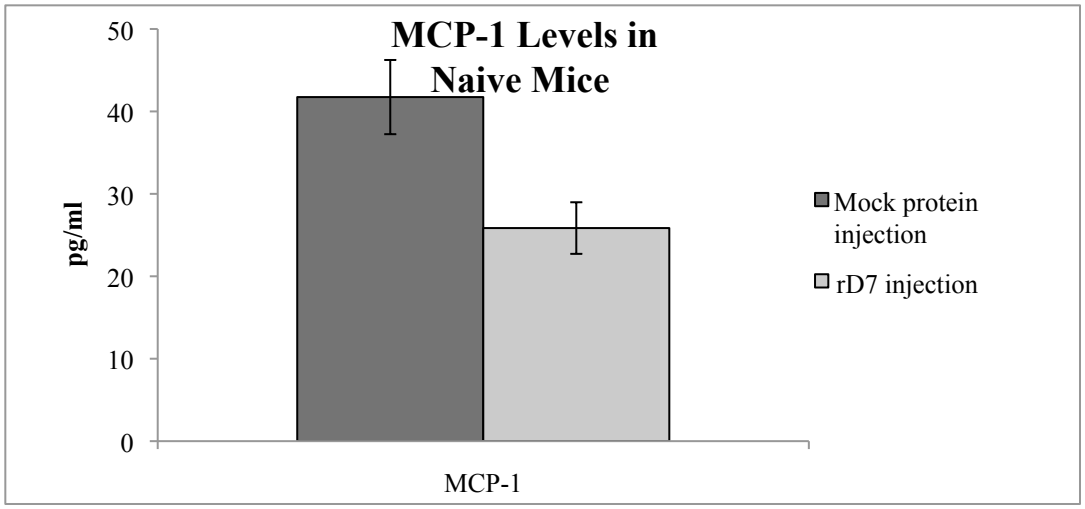


Figure 3.4 Serum cytokine levels were measured using cytometric bead array analysis. Naïve mice were injected with either rD7 or a mock protein intradermally. MCP-1 was significantly decreased in naïve mice injected with rD7 (p=0.0074). Graph shows mean of three individuals with SEM error bars. Other measured cytokines showed no significant changes.

Splenocytes from the same mice for which serum cytokines were measured were collected and were stimulated *ex vivo* with rD7 protein. The cell supernatants were analyzed by CBA for the same cytokines as above. There was a significant increase in

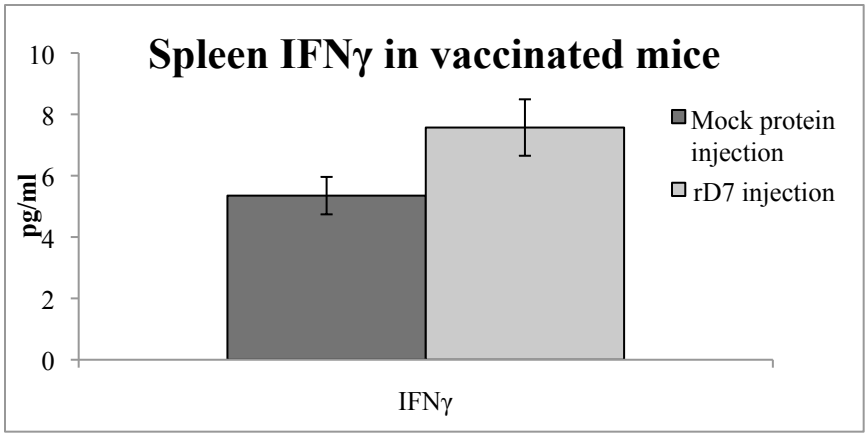


Figure 3.5 Cytokines from stimulated spleens were measured using cytometric bead array. Mice were vaccinated with rD7 then injected ID with either rD7 or a mock protein. Two days post injection there was a significant increase in spleen IFNγ in rD7 injected mice as compared to mock injected (p=0.025). Graph shows mean of three individuals with SEM error bars.

IFNγ levels in vaccinated mice that were exposed to rD7 versus those that were exposed to mock protein (p=0.025) at two days post injection (Fig 3.5). No other measured cytokines changed with exposure to rD7 regardless of vaccination status.

Cytokine analysis after exposure to mosquito bite

The proposed role of the D7 vaccine in protecting against WNV infection is to alter the host immune response after a mosquito bite, by switching the immune status away from a Th2 response and towards a Th1 response. To measure these changes cytokine analysis was conducted in D7 or mock vaccinated mice. Ten days after the final immunization, mice from each group were exposed to the bite of non-infected *Cx. tarsalis*. At two and four days post mosquito exposure, both serum and spleens were collected from 3 mice per group. IL-6, IL-10, MCP-1, IFNγ, TNFα, and IL-12p70 were measured using a CBA. There were no significant differences seen in serum cytokine levels at day 2, however on day 4 there was a significant increase in IFNγ in the D7 vaccinated mice as compared to mock vaccinated mice (p=0.02) (Fig 3.6).

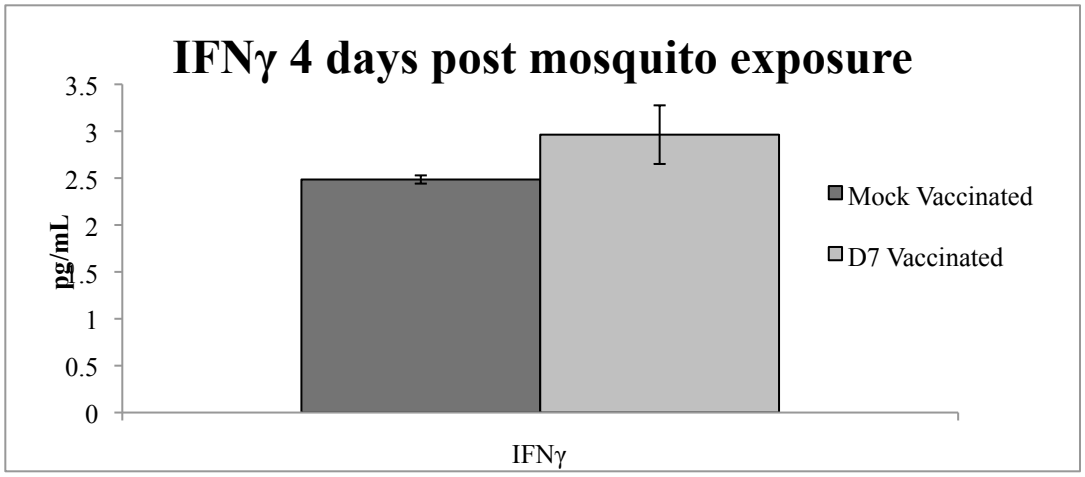


Figure 3.6 CBA analysis of mouse serum shows a significant increase in INFγ in mice that were vaccinated with rD7 then exposed to the bite of a *Cx. tarsalis*. (p=0.02) Graph shows mean of three individuals with SEM error bars. There were no significant changes in other measured cytokines

The spleen is an active immunological center with the white pulp being analogous to a lymph node. Here we conducted intracellular cytokine staining (ICS) as well as *ex vivo* splenocyte culture to determine cytokine activity after mosquito bite in vaccinated mice.

To prepare spleen cells for ICS they were stimulated *ex vivo* with PMA/ionomycin, a broadly active T-cell stimulator. The cells were then permeabilized and stained for the cytokines IL-10, IL-4, IFNγ, IL-2, and TNFα. At two days post exposure to mosquito saliva there was a significant increase in IL-10 and although it was not statistically significant, there was trend towards significance in IL-4 in the mock-vaccinated mice (Fig 3.7). There were no differences seen in IFNγ, IL-2, or TNFα.

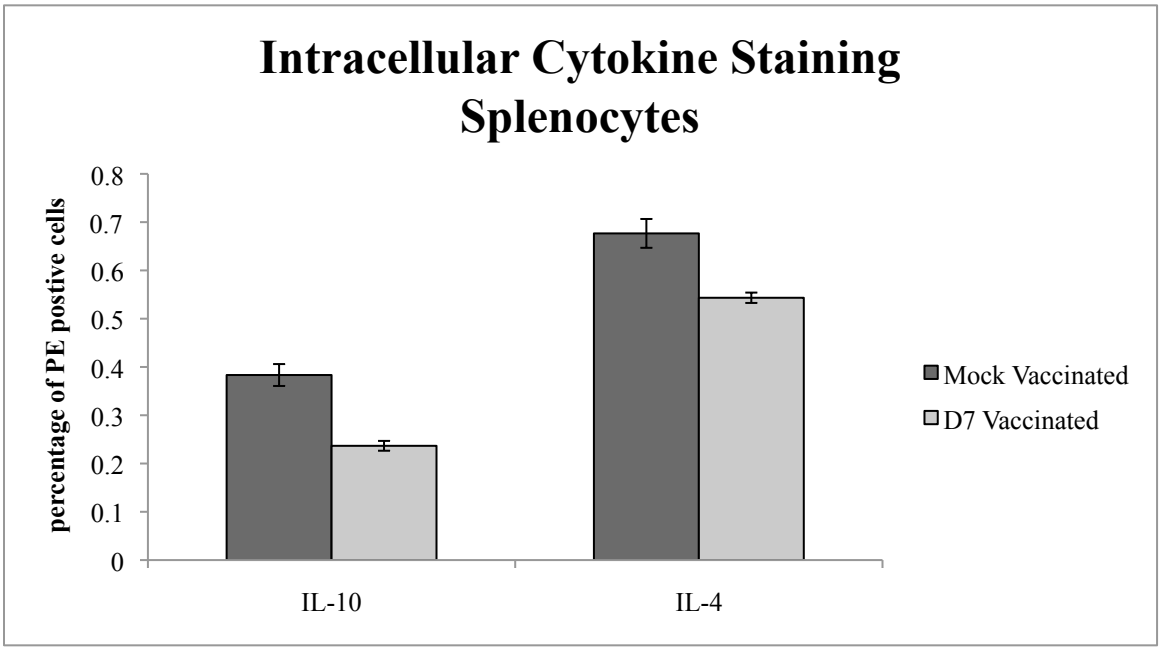


Figure 3.7 Intracellular cytokine staining shows a significant increase in IL-10 (p=0.056) and a trend towards significance IL-4 increase(p=0.072) in the mock vaccinated mice as compared to the D7 vaccinated mice. Graph shows mean of three individuals with SEM error bars. There were no significant changes in other measured cytokines

In addition to ICS, splenocytes were stimulated with rD7 protein or a control protein and cultured for 72 hours. Cell supernatant was collected and a CBA analysis was conducted as before. At two days post exposure the splenocytes from D7 vaccinated mice had a significant decrease in IL-10 as seen in ICS results. In addition there was an increase in IFNγ (Fig 3.8).

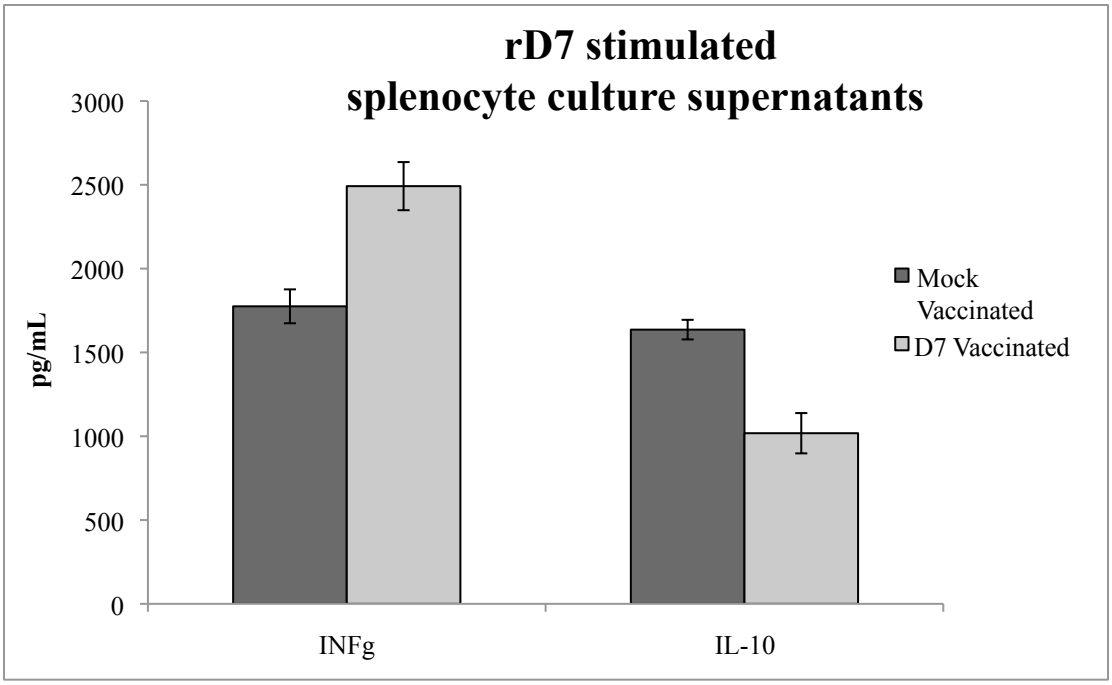


Figure 3.8 Supernatants from *ex vivo* stimulation of splenocytes were analyzed by cytometric bead array. There was a significant decrease in IFNγ and increase in IL-10 in the mock vaccinated mice as compared to D7 vaccinated mice (p=0.034, p=0.056). Graph shows mean of three individuals with SEM error bars.

Histopathologic analysis after exposure to mosquito salivary proteins

Histopathologic analysis was conducted on mouse ears from nine treatment groups as outlined in table 3.1. Each treatment group consisted of three individuals. Mouse ears were fixed, sectioned, and stained for hematoxylin and eosin to analyze the histopathology at the treatment site. Each sample was graded with severity of

inflammation from 1 – 4. The primary cellular infiltrate types are also noted: mononuclear cells, polymorphonuclear (PMN) cells, or mast cells. Some of the treatment groups showed no significant findings (NSF). Results of the histopathologic analysis conducted by a board certified veterinary pathologist are presented in table 3.1. Unless otherwise noted, the results describe all three mice in the group. Representative photomicrographs are presented in figures 3.9, 3.10, and 3.11 following the table.

Table 3.1 Histopathological results.

Treatment	Mouse Immune Status		
	D7 Vaccinated	Mock Vaccinated	Naive
rD7 Intra-dermal Fig 3.9	Chronic and acute inflammation, all mice grade 3, Primarily polymorphonuclear (PMN) cells, some mast cells and lymphocytes present	Mice with chronic and acute inflammation, grade 1, 3 and 4, primarily mononuclear cells	2 of 3 mice NSF 1 with grade 3 inflammation and edema, hemorrhage, and necrosis (shown in Fig 3.9 A)
Mock protein Intra-dermal Fig 3.10	Chronic and acute inflammation, grade 1, 3, and 3, primarily PMN cells	Chronic and acute inflammation, grade 2, 4, and 4, primarily mononuclear cells	Chronic and acute inflammation, all mice grade 3, mixture of PMN and mononuclear
Mosquito bite Fig 3.11	Chronic and acute inflammation, grade 1, 2, and 3, both PMNs and mononuclear cells	2 of 3 mice NSF 1 of 3 one with grade 2 inflammation, edema (shown in Fig 3.11 D)	2 of 3 mice NSF (shown in Fig 3.11 B) 1 with grade 2 inflammation, edema (shown in Fig 2.11 A)

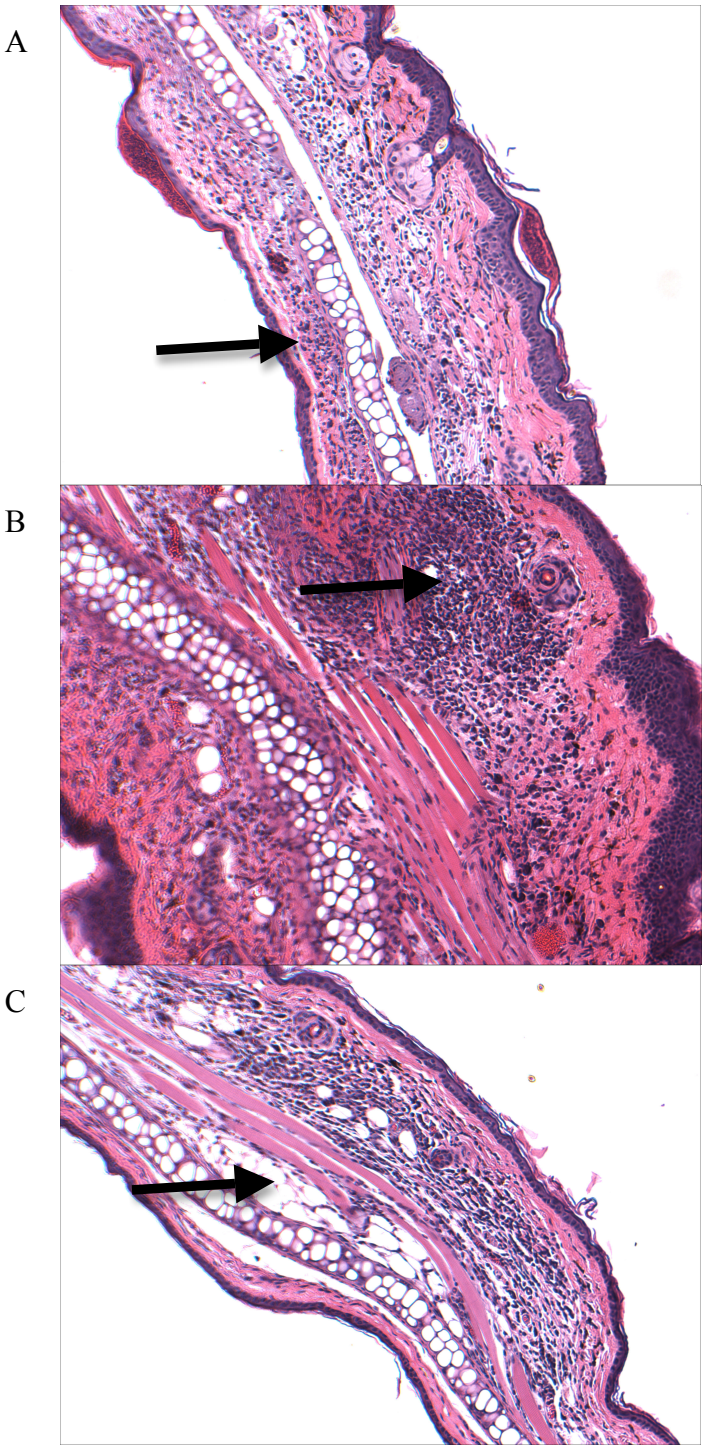


Figure 3.9 rD7 intradermal. 100X magnification of rD7 intradermal injection in mice that were A: Naïve B: rD7 vaccinated showing PMN infiltrations C: Mock vaccinated showing mononuclear cell infiltrates. Arrows indicate the following findings A: edema, hemorrhage and necrosis B: Grade 3 acute inflammation with PMN infiltration C: Grade 3 chronic inflammation with primary mononuclear cells.

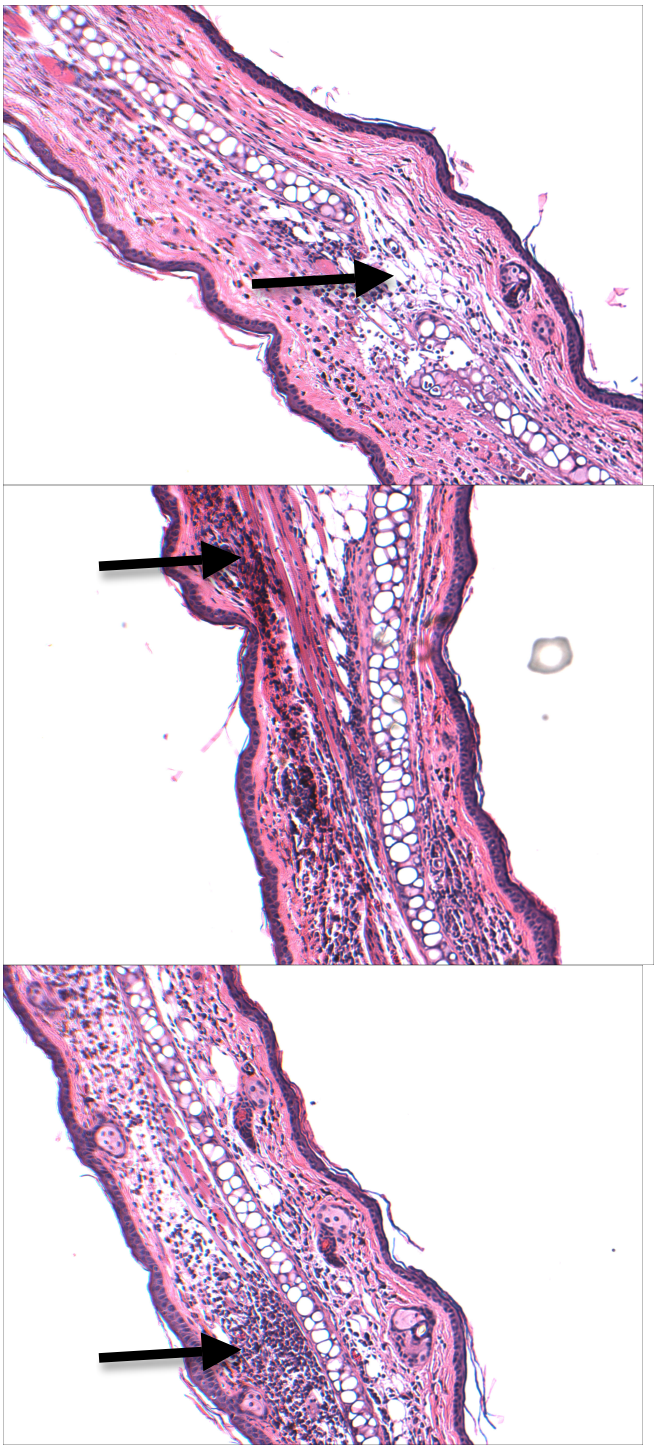


Figure 3.10 100 X magnification of mock protein intradermal injection in mice that were A: Naïve B: rD7 vaccinated C: Mock vaccinated. Arrows indicate the following findings A: Grade 3 inflammation with mixture of PMN and mononuclear cells B: Chronic inflammation grade 3 with primarily PMN cells C: Diffuse chronic inflammation grade 3 with primarily mononuclear cells.

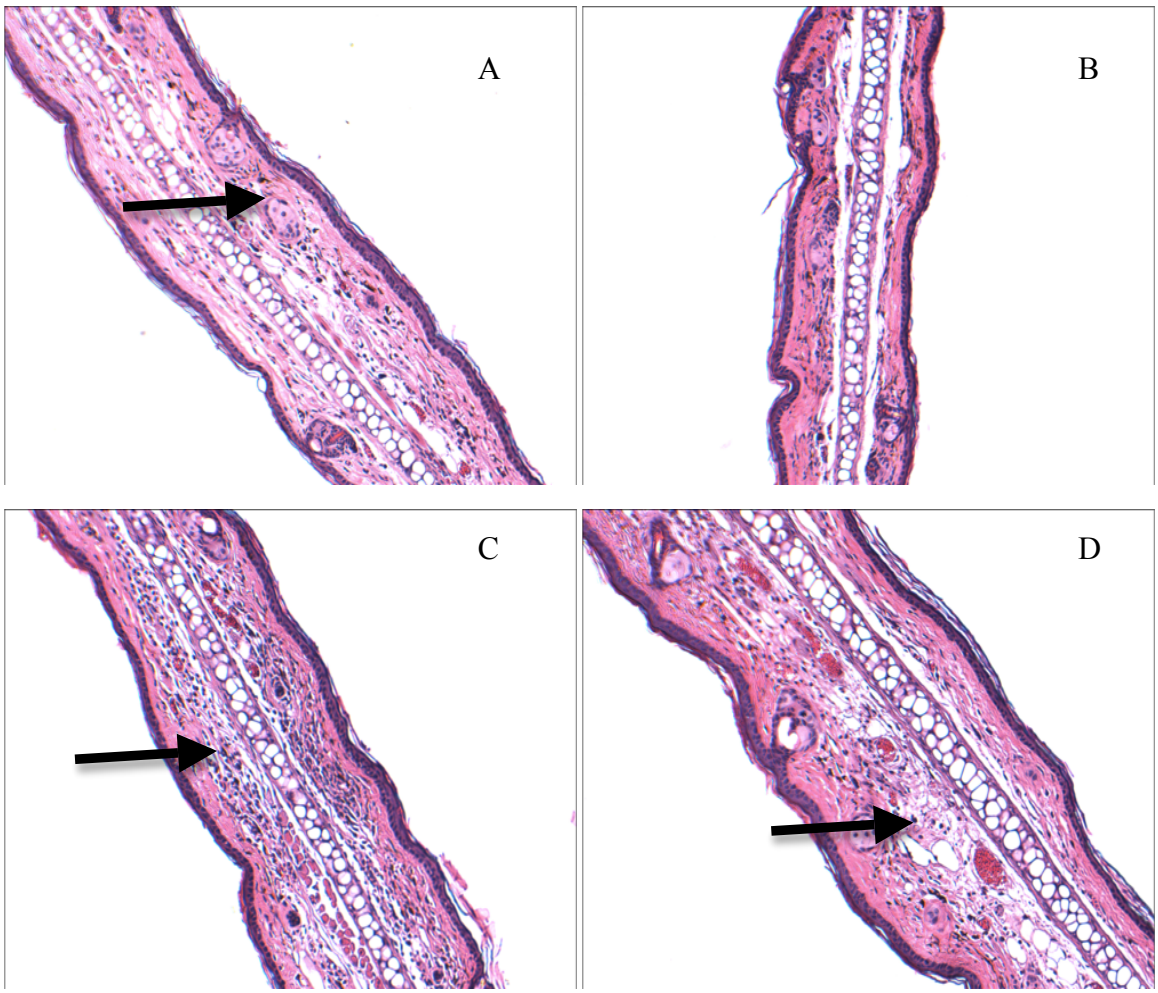


Figure 3.11 Mosquito bite sites at 100X magnification in mice that were A: Naïve B: Naïve with NSF C: rD7 vaccinated D: Mock vaccinated. Arrows indicate the following findings A: Chronic inflammation with edema B: No significant findings C: Chronic and acute inflammation with both PMN and mononuclear cells D: Grade 2 inflammation with edema.

Discussion

Mosquito salivary proteins alter the host immune system after a bite. The immunomodulation is systemic and can be measured as late as ten days post exposure (Zeidner et al. 1999). Here we analyzed the host immune response to the *Culex tarsalis* D7 salivary protein either via exposure to intradermally injection with rD7 or feeding *Cx. tarsalis* mosquitoes. Naïve mice were used throughout to assess immunomodulatory properties of D7. A vaccine was also created using complete Frend's adjuvant. Mice were then exposed to MSPs to determine if there was an alteration of host immunomodulation.

Antibody response after rD7 vaccination or natural exposure

Antibodies produced to MSPs, including D7, have been well documented in humans and other animals (Chen et al. 1998, Peng et al. 2006). We showed that mice repeatedly exposed to the bites of *Cx. tarsalis* or those that were vaccinated with rD7 produced rD7 specific IgG antibodies at levels significantly higher than mice vaccinated with mock protein or those that were not exposed to mosquito bites. The rD7 vaccine elicited an antibody response that was as robust as repeated natural exposure to MSPs.

IgG subtype ELISAs were conducted to further characterize the antibodies. The subtypes IgG1 and IgG2a are made during a Th2 or Th1 type immune response, respectively. $CD4^+$ T-cells can be activated to a Th1 or Th2 status, among others. Th1 type T-cells have been shown to produce IL-12p70 and IFNγ, which promote the class switching of antibodies from IgM to IgG2a. In contrast, Th2 type T-cells characteristically produce IL-4 that directly inhibits the Th1 response. IL-4 induces class

switching to IgG1. A Th2 dominated response is usually seen after mosquito bite; however, complete Freund's adjuvant should cause a Th1 type memory response.

In each group, mice that were repeatedly exposed to *Cx. tarsalis*, those that were exposed only once to a mosquito bite, and D7 vaccinated mice had detectable levels of IgG1 antibodies. The repeatedly exposed and vaccinated mice had the highest levels, with the mice only exposed once to a bite having lower levels. The only group to show detectable levels of IgG2a was the mice that were repeatedly exposed to mosquito bites.

Surprisingly the mice that were vaccinated with rD7 produced IgG1 subtype antibodies. Again, complete Freund's adjuvant is a strong Th1 type adjuvant and usually elicits IgG2a antibodies. However, vaccination with D7 protein from *Ae. aegypti* and CFA yielded similar results (Chen et al. 1998). The D7 protein may be driving the development of IgG2a subtype antibodies regardless of the adjuvant properties.

Immunomodulation by D7 protein

D7 proteins are known to bind leukotrienes and biogenic amines that are important parts of the host immune system (Calvo et al. 2006, Mans et al. 2007, Calvo et al. 2009). Here we analyzed immunomodulatory properties of rD7 when injected intradermally into naïve mice. The cytokines and chemokines IL-10, IL-6, IL-12p70, TNFα, IFNγ, and MCP-1 were measured in the serum and from stimulated splenocytes two days after exposure to the recombinant protein. In mice that were given rD7 there was a significant decrease in MCP-1 as compared to mice that received a mock protein. MCP-1, monocyte chemotactic protein, is a chemokine that is released after tissue injury to recruit monocytes. Down-regulation of MCP-1 by D7 could play a role in the

prevention of inflammation after a mosquito bite by preventing monocyte and macrophage migration to the bite site.

Histopathology at the site of injection of rD7 or after the bite of a *Cx. tarsalis* did not show significant levels of inflammation in naïve mice two days after exposure. 1 out of 3 mice in each of those groups did show minor inflammation, with the other mice having no significant findings. These results were as expected, as mosquito saliva does not create a strong inflammatory response in naïve individuals (Schneider et al. 2007).

Immune response to rD7 protein vaccine

rD7 vaccinated mice that were exposed to *Cx. tarsalis* mosquitoes had significantly higher levels of IFNγ in the serum. Intracellular cytokine staining of splenocytes showed that vaccinated mice also had lower levels of both IL-10 and IL-4. Additionally, *in vitro* splenocyte stimulation with rD7 revealed that vaccinated mice again had significantly lower levels of IL-10 and higher levels of IFNγ. These results taken together illustrate that exposure to the bite of a *Cx. tarsalis* after rD7 vaccination leads to an immunologic environment in the host that is predominately a Th1 based immune response, as opposed to unvaccinated mice that have a strong Th2 response (Zeidner et al. 1999). This switch in the arm of the immune system that is being triggered after exposure to *Cx. tarsalis* should be favorable in fighting arboviral infections such as WNV. A strong cellular immune response and type two interferons that were increased by vaccination are critical in fighting these infections (Wang and Fikrig 2004).

Studies were conducted to determine if intradermal injection of rD7 could produce the same results as a mosquito bite to show that exposure to D7 in the saliva was

responsible for the cytokine switch discussed above. Indeed, rD7 injected intradermally caused an increase in the Th1 cytokines TNFα, IFNγ, and MCP-1 as compared to mice that were inoculated with a mock vaccine. It can be assumed that the memory immune response created by the D7 vaccine is specific for D7 when a mosquito probes for a blood meal and injects whole saliva.

Histopathology in rD7 vaccinated mice

Histopathologic examination conducted on tissue samples from mice that were rD7 vaccinated or mock vaccinated yielded distinct results for each of the treatment groups. Both vaccinated and mock-vaccinated animals had inflammation graded from mild to severe in response to intradermal rD7 or mock protein injection, but the cell populations differed. Vaccinated animals experienced inflammation characterized by primarily PMN cells. PMN cells are comprised of neutrophils and are present during acute inflammation. In contrast, mice that received the mock vaccination had predominantly mononuclear cellular infiltrates. Mononuclear cells can include lymphocytes, monocytes, macrophages, and plasma cells and are present in more chronic inflammation reactions.

The major differences seen in the histopathology, were in the mice that were exposed to *Cx. tarsalis* bites. D7 vaccinated mice had severe inflammation in all three samples, while the mock-vaccinated mice only had slight inflammation in one sample. These results show that vaccination leads to severe inflammation and mixed cellular infiltration at the bite site. The cells were classified as both PMN and mononuclear cells. Monocytes and dendritic cells are susceptible to WNV infection (Johnston et al. 2000, Rios et al. 2006) and other cell types have yet to be investigated as to their susceptibility

to WNV infection. The cell population seen in our findings need to be further characterized to determine if they are WNV susceptible populations. If this is the case, it could be detrimental to protection from WNV infection, as there are more cells available for immediate infection and migration to regional lymphnodes.

Summary

I demonstrated that *Cx. tarsalis* D7 protein is able to cause a decrease in the chemokine MCP-1. This is the first observation of a D7 protein altering the host immune response. This could play an important role in the down-regulation of pro-inflammatory cytokines after mosquito bite. That may in turn be part of the saliva enhancement of viral pathogens, however that has not been shown experimentally.

The cytokine profiles that were obtained after vaccination with rD7 showed promise for protection against a subsequent WNV infection. It is hypothesized that if a vaccine could switch the host's immune response from Th2 to a Th1 environment after exposure to a mosquito bite this would be protective against arboviral infection. Here we showed success in the switching of the immunological reaction to a mosquito bite. However, the large amount of cellular infiltrates found at the bite site in rD7-vaccinated mice could be detrimental to the vertebrate host. Monocytes are susceptible to WNV infection (Rios et al. 2006) and recruitment to the bite site may allow for more efficient initial viral infection. The efficacy of the rD7 vaccine against WNV infection will be discussed in chapter 4.

CHAPTER IV

THE EFFECT OF D7 VACCINATION ON A SUBSEQUENT A WNV INFECTION

Introduction

Mosquito saliva induces increases in IL-4 and IL-10 and decreases in IL-12, IFNγ, and TNFα in spleen cytokines (Zeidner et al. 1999). Mosquito saliva is also known to increase the pathogenesis of arboviruses transmitted while the mosquito is taking a blood meal (Titus et al. 2006, Schneider and Higgs 2008). As a result, the idea of a mosquito salivary protein (MSP) vaccine is very attractive. Neutralization of MSP-induced immunomodulation was hypothesized to be protective against arboviral infection delivered by a mosquito bite. The cross reactivity in orthologous MSPs from different genera and species suggests that a well designed and effective vaccine would be protective against a wide range of arboviral infections.

The maxidilin protein from sand fly saliva has been used as a successful vaccine to prevent the transmission of Leishmania parasites. Although no MSP vaccines have been as widely studied, preliminary results from Dr. Carlos Machain-Williams (unpublished data) showed that mice vaccinated with whole saliva from *Cx. tarsalis* had decreased viral levels in the brain at four days post-infection and an increase in transcript levels for IFNγ, TNFα, and IL-10. The vaccine did not yield measurable differences in spleen cytokines when measured by intracellular cytokine staining and it was not ultimately protective against WNV induced neurological disease. However, decreased viral replication was a promising first step in the development of a *Cx. tarsalis* salivary protein vaccine.

Exposure to MSPs leads to antibody development in humans and animals (Peng and Simons 1998, Schneider et al. 2007). Work conducted with sand flies and malaria-transmitting *Anopheline* mosquitoes have shown pre-exposure to salivary proteins can

decrease the pathogenesis of the transmitted parasites (Donovan et al. 2007). This may be due to neutralization of immunomodulatory proteins found within the saliva. However, more recent publications were not able to replicate the malaria transmission work (Kebaier et al. 2010). On the other hand, pre-exposure to the bites of *Ae. aegypti* led to an enhancement of West Nile virus infection (Schneider et al. 2007). These findings illustrate the complex nature of immunity to salivary proteins and effects this may have on pathogen transmission and infection.

Here we investigate the role of *Cx. tarsalis* rD7 vaccine in mice infected with WNV via the bite of an infected *Cx. tarsalis* mosquito. D7 proteins are highly immunogenic and found at very high levels in mosquito saliva making them an ideal vaccine target. Results presented in chapter 3 show that vaccinated mice that are exposed to a non-infected mosquito bite yield higher levels of Th1 cytokines that are known to be important for fighting viral infections (Wang and Fikrig 2004). This led to the hypothesis that mice vaccinated with rD7 would be refractory to WNV infection delivered via an infected mosquito bite.

Methods

Mosquito rearing

Culex tarsalis Bakersfield CA strain were raised as discussed in Chapter 2 under bio-safety level 2 conditions. For work to be conducted in bio-safety level 3 conditions 1-3 day old adult mosquitoes were transferred to the Regional Biocontainment Laboratory, Colorado State University. The mosquitoes were double contained in an environmental chamber at 24°C, 80% humidity, and a 16:8 light:dark cycle. Mosquitoes were offered water and sugar *ad libitum.*

WNV stocks

WNV- NY99 was kindly provided by Dr. Bradley Blitvich. Virus stocks were generated by infection of Vero cells at an MOI of 0.1. A T-75 flask with cells at 95% confluency had the medium removed. The seed virus in 5 ml of MEM-2%FBS was applied and the flask was rocked at room temperature for 1 hour. The medium was then removed and 15 ml of MEM-7%FBS was added to the flask. Cells were incubated at 37°C and 5% CO_2. At 3 dpi supernatant was removed from the flask and stored in 1 ml aliquots at -80°C. Virus stocks were titrated by plaque assay.

WNV plaque titrations

12 well plates were seeded with $5x10^4$ Vero cells per well with 1 ml MEM-10% FBS. At 3-4 days post plating the cells were 100% confluent. WNV samples were diluted from 10^{-1} to 10^{-8} in serial 1:10 dilutions with a total volume of 200µl in DMEM medium with HEPES and 7% FBS. Each well was infected with 150 µl of diluted virus. The cells were then rocked at room temperature for 1 hour and 1ml of nutrient overlay (1% agarose, 1x Medium-199, 7% FBS, 4% w/v sodium bicarbonate (Cellgro), 1x Eagle's vitamins (Cellgro), 1x Eagle's aminoacids (Cellgro)) was added to each well. Plates were incubated at 37°C and 5% CO_2. On 4 dpi 150 µl of 3mg/ml thiazolyl blue tetrazolium bromide (MTT) in PBS was added to each well and incubated overnight at 37° C. Plaques were read and virus titer was calculated using the following calculation: pfu/ml = # of plaques / (dilution factor x mL of inoculum)

WNV mosquito infection

Five day old *Cx. tarsalis* were given an artificial blood meal using a Hemotek feeder (Discovery Workshops). The blood meal contained defibrinated sheep blood

(Colorado Serum Company) containing WNV-NY99 at a concentration of $1x10^7$ pfu/ml. Mosquitoes were allowed to feed for one and a half hours with the blood meal maintained at 37 ºC. Blood fed females were separated under cold anesthesia and stored in one gallon plastic cages for ten days with sugar and water *ad libitum*. At ten days post infection, six to ten mosquitoes were removed and tested via reverse transcriptase PCR for the presence of disseminated WNV infection.

WNV reverse transcriptase-PCR

Mosquitoes were tested for WNV infection using reverse transcriptase polymerase chain reaction (RT-PCR). RNA from mosquitoes was isolated using Trizol (Invitrogen) extraction method (Chapter 2). cDNA was made using WNV E-protein reverse primer (WNV Rev 619) and Thermoscript reverse transcriptase (Invitrogen). The reaction mix consisted of 1 µl WNV E reverse primer (25ng/µl), 1 µl dNTPs(10mM), and 1 µl RNA (100ng/ µl). The primer and RNA mixture was incubated at 70°C for 10 minutes then placed on ice. To that mixture 4 µl first strand buffer (5x), 2 µl DTT (0.1M), 1.9 µl nf dH2O, and 0.1 µl Thermoscript reverse transcriptase were added. The mixture was incubated at 42°C for 50 min, 70°C for 15 min. cDNA was amplified using primers to the WNV E-protein. PCR reaction mix contained 2.5 µl PCR buffer $^{+}MgCl_2$, 1 µl WNV 619 Rev (25 ng/ µl), 1 µl WNV 212 Fwd (25 ng/ µl), 0.1 µl Taq polymerase and 1 µl of the cDNA reaction. The reaction was incubated for 2 min at 94°C, then cycled 35 times for 30 sec at 94°C, 30 sec at 60°C, 45 sec at 72°C, and finally 5 min at 72°C. The products were electrophoresed on a 1% agarose gel and visualized with ethidium bromide.

Table 4.1 Primers for WNV RNA amplification

Primer name	Sequence (5' to 3')
WNV 212 Fwd	TTGTGTTGGCTCTCTTGGCGTTCTT
WNV 619 Rvs	CAGCCGACAGCACTGGACATTCATA
WNV Env Forward	TCAGCGATCTCTCCACCAAAG
WNV Env Reverse	GGGTCAGCACGTTTGTCATTG

WNV quantitative PCR

Primers designed to amplify a 70 bp portion of the WNV envelope gene were designed by Lanciotti *et al* (2000). Sequences for WNV Env Forward and WNV Env Reverse are given in table 4.1. To quantify RNA, qPCR reactions were conducted using Quantitect SYBR Green RT-PCR kit (Qiagen) and a Bio-Rad iCycler iQ5 real-time PCR detection System (Bio-Rad). PCR reactions contained 12.5 μl Quantitect SYBR Green RT-PCR Master Mix, 0.2 μl Quantitect RT Mix, 1 μl of each primer (10μM), 9.3μl nuclease free H_2O, and 1μl of template RNA (100 ng/μl). Each reaction was set up in duplicate, and no template controls were included. Standard quantities of WNV cDNA were amplified simultaneously to predict original concentration of viral RNA. PCR fragments were cloned into the pCR 2.1 plasmid (Invitrogen), used to transform *E. coli*, and purified using QIAprep Spin Miniprep kits (Qiagen). Plasmids were diluted to a concentration of 10^{10} plasmids/μl and used to construct standards containing 10 to 10^8 copies. The samples and standards were cycled as follows: 50°C for 30 min, 95°C for 15 min, then 40 cycles of 94°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec, 77.5°C for 8 sec. Melting curve analysis was also conducted to ensure no primer dimers or non-

specific products were being generated. Fluorescence generated by standards was used to calculate viral genome copy numbers in the samples tested.

Mice

Animals used in these projects were purchased from Jackson Laboratories (Bar Harbor, Maine). Six to eight week old female C57/Black 6 mice were used for all experiments. They were housed in BSL 3 animal facilities and monitored daily by Laboratory Animal Resources personnel. Mice were housed and handled in accordance to protocols approved by the Institutional Animal Care and Use Committee.

Immunization

Mice were immunized with 10 μg rD7. Complete Freund's adjuvant (Imject, Pierce) was used for the primary inoculation, followed by boosters at 2 and 4 weeks with incomplete Freund's adjuvant (Sigma). To mix the vaccine rD7 was diluted in sterile PBS and placed in a 3cc syringe. Equal volume of adjuvant was placed in a second 3 cc syringe. The syringes were connected using a 3 way stop cock and the liquid was mixed by pushing into alternating syringes until the mixture turned a milky white color. Each mouse was anesthetized using 3% Attane isoflurane (Minrad Inc, Orchard Park, NY). Inoculations are made in four sites, one on each quadrant of the back, subcutaneously with 50 μl per site.

WNV infection in mice

Fifteen mosquitoes infected with WNV via blood meal 10 days previously were placed into a 1 gallon carton. Mice were anesthetized with 0.2 ml Ketamin/Xylazine (0.2mg/2mg) and one mouse was placed into each carton. Once 1-10 of the mosquitoes

completed a blood meal, the mouse was removed from the carton. Blood fed female mosquitoes were then separated and tested for WNV by RT-PCR, as described above. Mice were monitored twice daily for development of symptoms.

In vitro splenocyte stimulation

Mice were anesthetized with isoflurane and blood was collected from the animals. Thirty ml of PBS was then used to perfuse the animal. The spleens were removed and placed in RPMI medium^{+}10%FBS. The spleen was disrupted into a single cell suspension by passing through at 100 µm cell strainer with a 3cc syringe plunger and washed with 15 ml RPMI. The cells were pelleted at 500 x g for 5 min. Red blood cells were lysed by addition of 1ml of red blood cell lysis buffer at room temperature for 10 min and cells were washed twice with 10 ml PBS. Cells were counted in a 1:10 dilution in trypan blue (0.4% w/v) and plated in a 96 well flat bottom plate at 10^6 cells per well. For each spleen sample three wells were seeded; a non-stimulated control, a well stimulated with 0.5 µg of rD7, and a well stimulated with an equal volume of the control protein preparation. The cells were incubated at 37 °C and 5% CO_2 for 72 hours. At 48 hours post-stimulation 20 µl RPMI was added to each well. At 72 hours post stimulation the cell supernatants were collected and stored at - 80°C for CBA analysis.

Cytometric bead array

Cytometric bead arrays (BD Pharminogen) were used to measure cytokine levels in serum and splenocyte supernatants. Standards were prepared by reconstituting one vial of lyophilized mouse standards (Kit Bottle C) in 2.0 ml Assay Diluent as described by manufacturer. Two-fold dilutions in 50 µl of Assay Diluent (Kit Bottle G) were prepared creating 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 dilutions of the stock

standards. Assay Diluent (Kit Bottle G) served as the 0 pg/ml negative control. Two and a half µl of each of the six capture beads for MCP-1, IFNγ, TNFα, IL-10, IL-6, and IL-12p70 were added to each assay well, for a total volume of 15 µl. The capture beads were added to all wells including standards, negative control and sample wells in a round bottom 96 well plate. Next, 50 µl of sample or standard cytokine dilutions were added to each well. Fifteen µl PE detection reagent (Kit Bottle B) were added to all wells and the plate was incubated at room temperature for 2 hours protected from light. The beads were washed with 200 µl 1X Wash Buffer (Kit Bottle F) per well. The plate was centrifuged at 200 x g for 5 minutes at room temperature, and the supernatant was removed. The beads were washed a second time by resuspending each well in 200 µl 1X Wash Buffer or FACS buffer (BD Pharminogen). Again, the plate was centrifuged at 200 x g for 5 minutes. The samples were resuspended in 150µl FACS buffer and transfer to flow tubes for analysis on a CyAn flow cytometer (Dako).

Intracellular cytokine staining

Single cell suspensions of splenocytes were obtained as described above. The cells were washed once with 15 ml of RPMI^{+}10% FBS and centrifuged at 500xg for 5 min. RBCs were lysed with 3 ml of cold RBC lysis buffer for 5 min at room temperature. Twenty seven ml of RPMI was added to dilute the buffer and the cells were centrifuged again. The cells were washed again in 30 ml of RPMI and were resuspended in 3 ml RPMI. The cells were counted with trypan blue (0.4% w/v) and 2 x 10^6 cells were plated in a 6 well plate. Cells were stimulated with WNV specific CD4 E (amino acid (aa) 641-655) and NS3 (aa 1616-1630) or CD8 E (aa 347-354) peptides at 10 µg/ml and incubated at 37 °C for six hours that were previously identified as the MHC class I and class II

immunodominant peptides (Purtha et al. 2007, Brien et al. 2008). The cells were transferred to a 50 ml tube, pelleted, and washed in 15 ml of RPMI. The cells were resuspended in 1 ml of RPMI and 100 µl of the suspension was placed in 96-well round bottom plate, with 5 wells for each spleen sample. The plate was centrifuged at 1800 rpm for 1 min and the supernatant was removed. Ten µl of FcR Block (BD Pharminogen) was added to each well. Fifty µl of surface staining markers, CD4-FITC and CD8-APC, were added to each well at a 1:200 dilution. The cells were incubated at 4°C for 30 min. One hundred µl of staining buffer was added to each well, the plate was centrifuged, and supernatant was removed. One hundred µl of Fix/Perm buffer was added to each well and incubated for 20 minutes. One hundred µl of staining buffer was added to each well, centrifuged, and supernatant was removed. To each well 20µl of FcR Block and 30 µl of Perm/Wash was added. Each of the five wells from a single spleen sample were stained with a different PE labeled antibody to the following cytokines, IL-10, IL-4, IFNγ, TNFα, and IL-12. IFNγ, TNFα, and IL-12 antibodies were added at 20 µl per well, while the IL-10 and IL-4 antibodies were added at 1µl per well. The cells were incubated at 4°C for 20 minutes. Cells were washed with 100 µl of staining buffer. The stained cells were resuspended in 200 µl of staining buffer and placed in flow tubes and held at 4°C until they could be read on a CyAn flow cytometer (Dako). Data were analyzed using Summit software.

Passive immunization

Serum was collected from mice that were vaccinated with rD7 at 10 days post final immunization. Serum from five individuals was pooled and recipient naive mice were given 200 µl of D7 serum, mock vaccinated serum, or control serum

intraperitoneally. Thirty hours post serum transfer mice were infected with WNV via the bite of infected *Cx. tarsalis.*

WNV indirect ELISAs

Indirect ELISAs were used to measure serum antibody levels reactive against WNV. Plates were coated with 5 x 10^6 pfu WNV per well in 50 µl of coating buffer (50mM sodium carbonate, 50mM sodium bicarbonate, pH 9.6) and placed at 4°C overnight up to 1 month. Plates were washed three times with PBS^+0.1% Tween-20. The serum was heat inactivated at 55°C for 30 min and was added at dilution of 1:100. The plates were incubated at room temperature for 1 hour then washed four times with PBS^+0.1% Tween. Horseradish peroxidase conjugated secondary antibody goat anti-mouse IgG was added to the samples at 1:1000 and incubated for 1 hour. The plate was again wased four times with PBS^+0.1% Tween. The ELISAs were developed using 50µl of 3,3′,5,5′-Tetramethylbenzidine (Sigma) per well until color developed, approximately 5 minutes. The color reaction was stopped with 50µl of 1N HCl. The plates were read on a plate reader at 450 nm. Results are presented as a ratio of samples to blank wells. A positive control sample was used from a mouse that was infected with WNV intraperitoneally, and negative control serum was obtained from a commercial source (Innovative Research).

Results

WNV infection in rD7-vaccinated mice

To determine the efficacy of the rD7 vaccine to prevent WNV infection a mouse model was used. After immunization mice were infected with WNV via the bite of an infected *Cx. tarsalis*. The animals were monitored twice daily for 21 days for signs of

illness and at the onset of neurological signs the animals were humanely euthanized. Neurologic signs included paralysis, spinning, loss of balance, and hunched rodent posture. The results of four independent experiments show that mice vaccinated with the rD7 developed severe neurologic symptoms including hunched posture, paralysis, whirling, and repetitive behaviors at a significantly higher rate than the mock vaccinated animals (Fig. 4.1). The mortality curves were compared using Mann-Whitney test (n=13, p=0.0347).

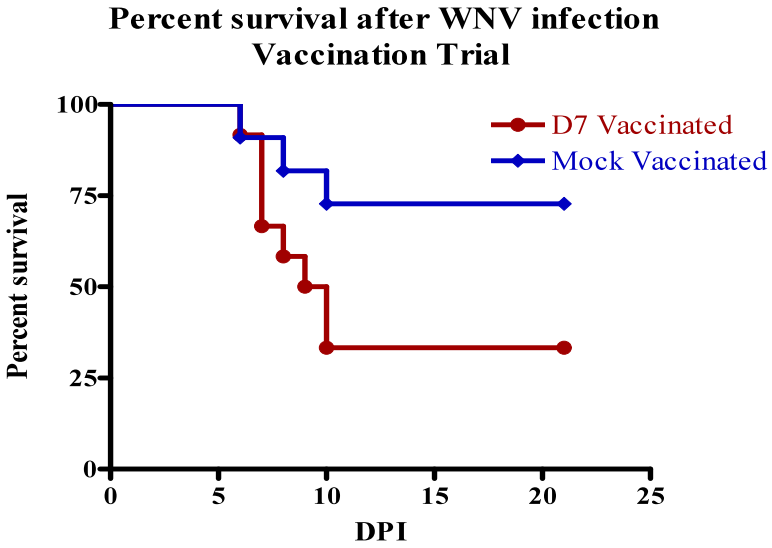


Figure 4.1 Vaccination trial results showing mice vaccinated with rD7 succumb to illness at a significantly higher rate than those administered a mock vaccination. Results are combined from four experiments. (n=13, p=0.0347)

Passive transfer of serum

Naïve mice were injected intraperitoneally (IP) with serum from mice that had been vaccinated with rD7, control serum or serum obtained from mice that had been repeatedly bitten by *Cx. tarsalis* over the course of one year (colony mice). Thirty hours

post injection of serum the mice were infected with WNV via the bite of an infected *Cx. tarsalis* mosquito. Mice were then monitored twice a day for 21 days for signs of illness. There was no difference between mice receiving the control serum or colony mice serum, however there was a significant difference between the control mice and the mice that were given serum from the D7 vaccinated mice (Fig 4.2) (control and colony mouse serum n=5, D7 serum n=4, p=0.002). One hundred precent of the animals receiving serum from D7 vaccinated mice succumbed to the illness by 7 dpi. The illness was characterized by rapid onset and progression to neurologic signs that are not usually observed in WNV infected mice.

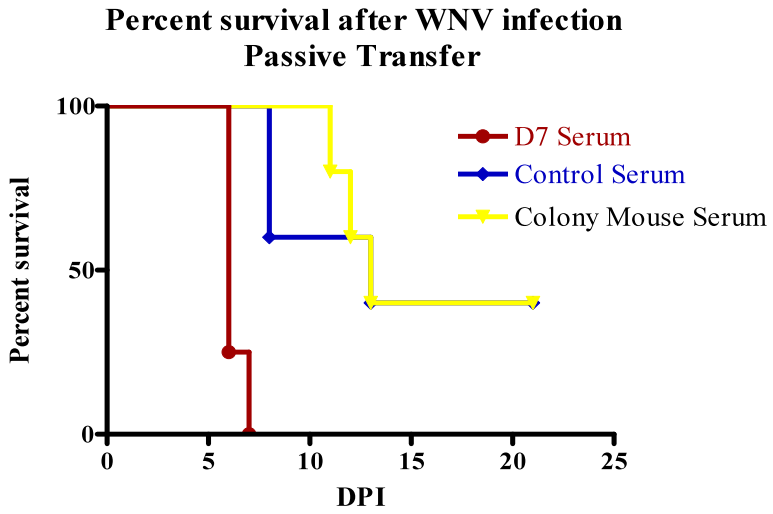


Figure 4.2 Mice were injected IP with serum from D7 vaccinated mice, naïve mice, or mice that were repeatedly bitten by *Cx. tarsalis* (colony mouse serum). 30 hours post serum treatment mice were infected with WNV via the bite of an infected *Cx. tarsalis.* D7 serum injected mice succumbed to illness at a significantly higher rate than control mice. (p=0.002, n=5,4) No difference was noted in the colony mouse serum and naïve mouse serum treated mice.

WNV indirect ELISA

ELISAs were conducted to determine levels of WNV antibodies in mice that were exposed to WNV 10 days after vaccination. Serum samples were collected 21 days after

exposure to WNV. The ELISA was designed to detect total IgG antibodies. Results are shown as a ratio to control wells. Mice that were vaccinated or mock vaccinated both showed seroconversion to WNV. Positive control serum was collected from mice that were infected with WNV intraperitoneally. Mock vaccinated mice showed slightly higher levels of WNV antibodies as compared to D7 vaccinated animals, but the difference was not significantly different (Fig 4.3).

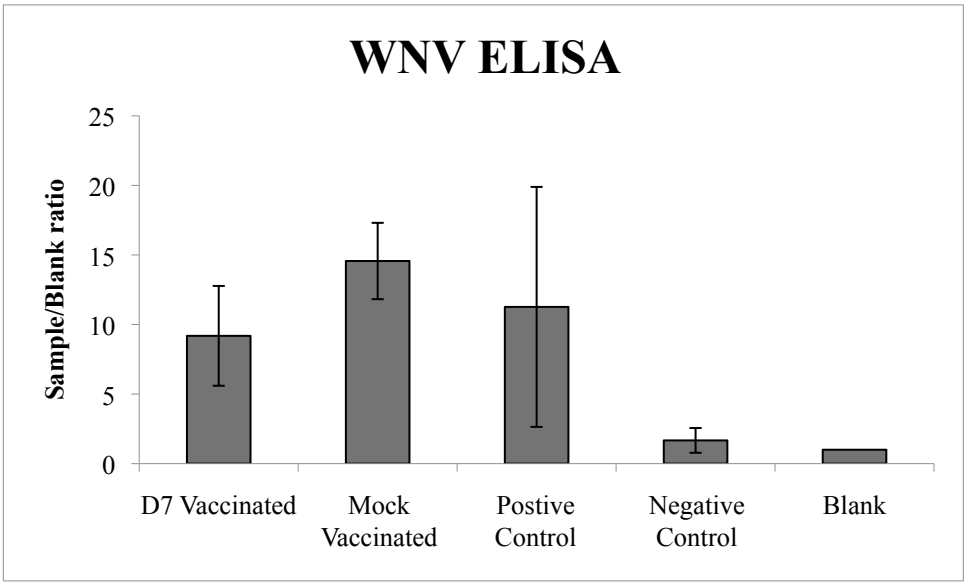


Figure 4.3 Detection of WNV antibodies. WNV indirect ELISA showing sample to blank ratio levels for D7 vaccinated, mock vaccinated, positive and negative control serum. There were no significant differences between D7 vaccinated and mock vaccinated.

Serum cytokine levels

Serum from mice infected with WNV was collected at two, four, six, and twenty-one days post infection. IL-6, IL-10, IL-12p70, IFNγ, TNFα, and MCP-1 were measured using a cytometric bead array. No significant differences were found in any measured

cytokines between D7- and mock-vaccinated mice at any measured time point. Figure 4.4 shows data from days 2 and 4 post infection. Days 6 and 21, not shown, had similar results.

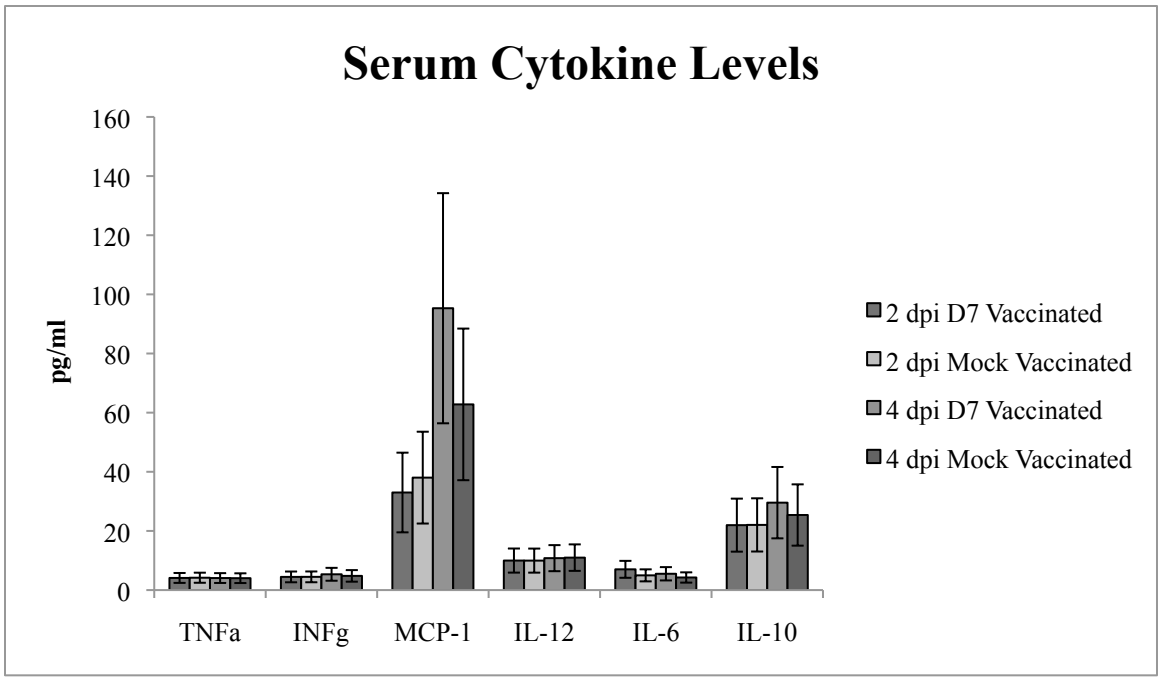


Figure 4.4 Serum cytokine levels measured at 2 or 4 dpi with WNV in D7- or Mock-vaccinated mice. No significant differences were observed. (n=6)

Spleen cytokine levels

Spleens were harvested from mice two, four and six days post WNV infection to monitor cytokine levels. Splenocytes were stimulated with rD7 and cytometric bead array analysis was used to determine cytokine expression levels for IL-10, TNFα, IFNγ, MCP-1, IL-12, and IL-6. At two days post infection all measured cytokines showed a significant decrease in expression ($p<0.05$) (Fig 4.5) in D7-vaccinated mice, but at 4 and 6 dpi there were no significant differences seen in the cytokines that were measured (data not shown).

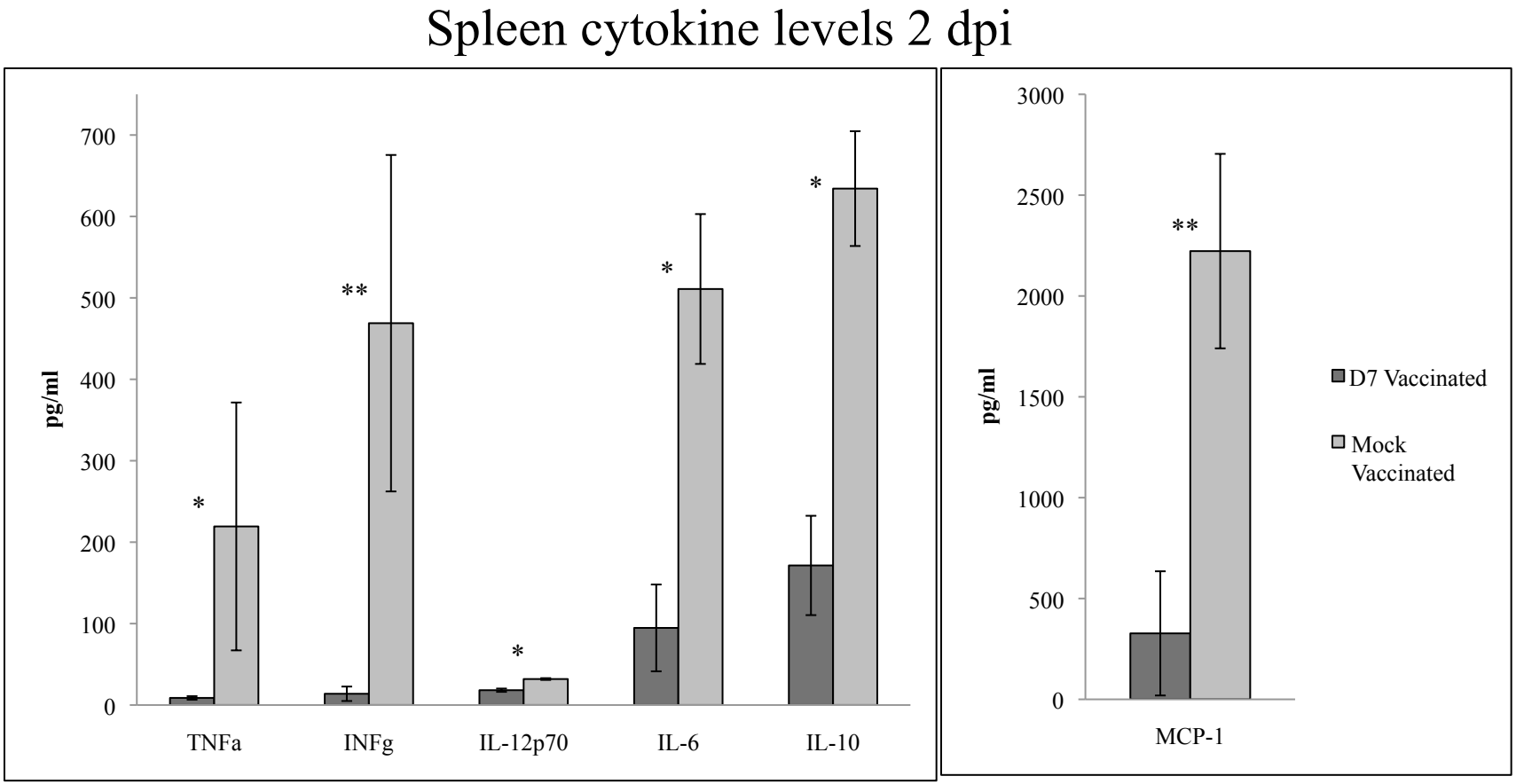


Figure 4.5 Cytokines measured in the supernatants of rD7 stimulated splenocytes from either D7 or mock vaccinated mice 2 dpi with WNV. All cytokines were significantly higher in mock vaccinated animals(n=3). *p<0.05, **p<0.001

Intracellular cytokine staining with WNV specific T-cell stimulation

On 2 and 4 dpi cytokines in WNV specific T-cells were measured. The splenocytes were stimulated with either a MHC Class I or MHC Class II WNV specific peptide to determine if there was a deficiency in the adaptive cellular response to WNV. Cells were surface stained with CD8 and CD4 antibodies and permeabilized to stain for IFNγ, IL-10, IL-4, IL-2, or TNFα. At 2 dpi there were a significantly higher percentage of $CD4^+$ T cells producing IL-10 in D7-vaccinated mice as compared to mock-vaccinated ($p=0.016$) (Fig 4.6). No significant differences were seen in other cytokines produced by $CD4^+$ WNV stimulated T cells.

At 4 dpi, splenocytes from D7 vaccinated mice stimulated with the MHC class I/CD 8^+ peptide had an increase in the percentage of cells producing IL-4 ($p=0.01$) (Fig 4.7). There were no significant differences at 2 dpi or in other cytokines at 4 dpi in CD8 peptide stimulated cells.

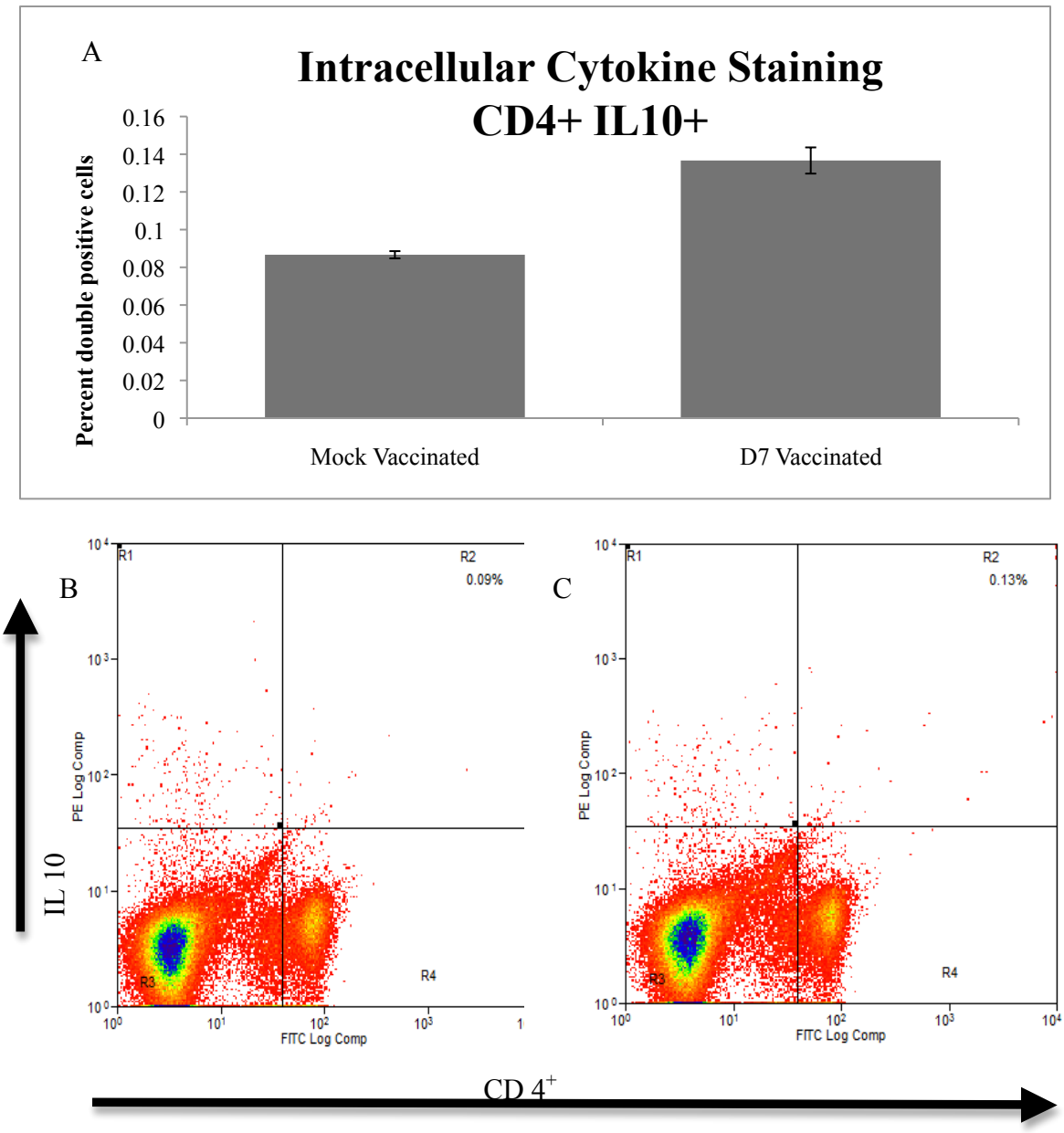


Figure 4.6 Intracellular cytokine staining on splenocytes from Mock-or D7-vaccinated mice and infected with WNV. The spleens were stimulated using WNV $CD4^+$ specific peptide to analyze cytokines from WNV specific T-cells. A. There is a significant increase in IL-10 in D7 vaccinated mice (n=3, p=0.016) on day 2 post infection B. Mock vaccinated histogram with 0.09% double positive cells C. D7 vaccinated histogram with 0.13% double positive cells.

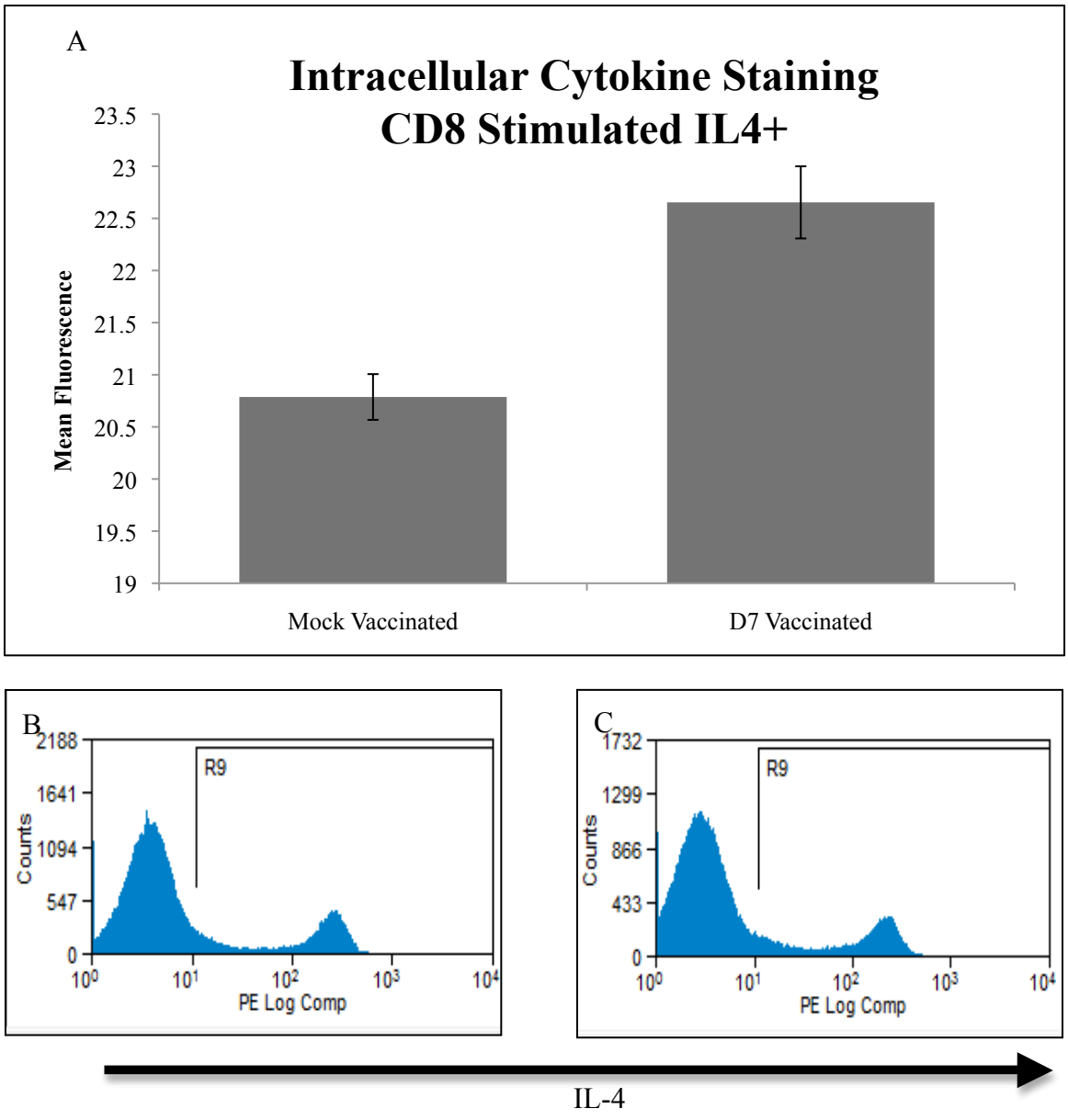


Figure 4.7 Intracellular cytokine staining on splenocytes from mock or D7 vaccinated mice and infected with WNV on day four post infection. The spleens were stimulated using WNV $CD8^+$ specific peptide to analyze cytokines from WNV specific T-cells. A. There is a significant increase in IL-4 in D7 vaccinated mice (n=3, p=0.010) B. Mock vaccinated histogram showing 20.63% positive cells C. D7 vaccinated histogram showing 23.33% positive cells.

Quantification of WNV in brain and blood

WNV RNA was measured via quantitative PCR in the brain and blood of D7 or mock vaccinated mice 2 and 4 dpi. Though no significant differences were seen between the groups, there was a trend towards higher WNV RNA levels in the D7 vaccinated animals in both blood and brain samples.

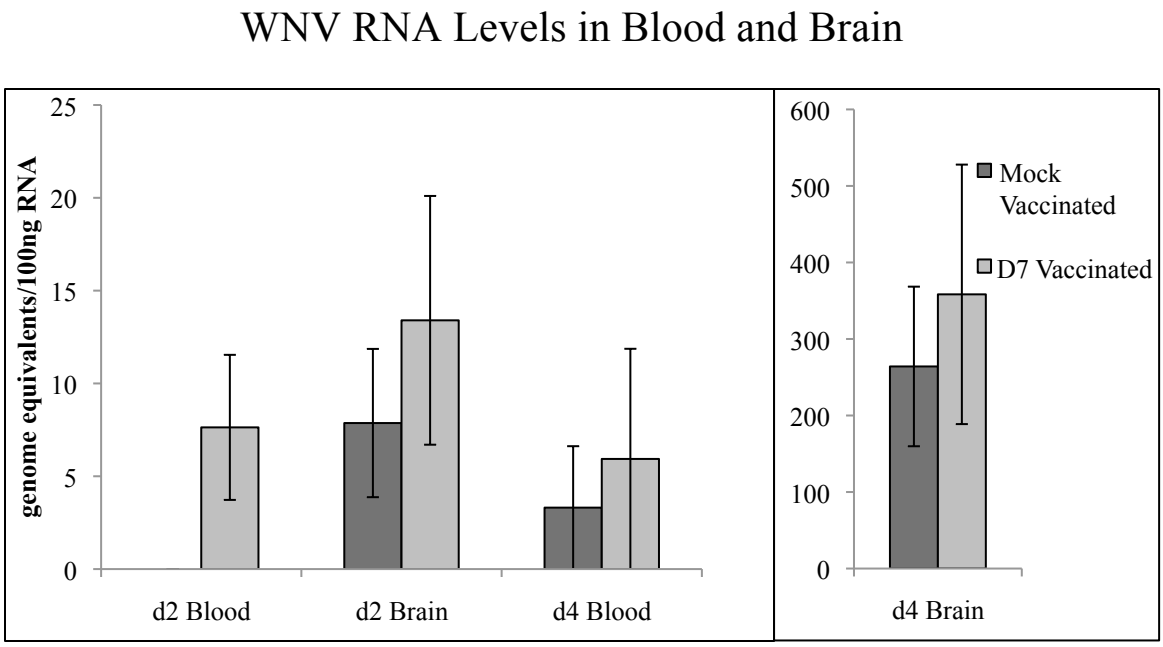


Figure 4.8 WNV genome equivalents per 100 ng of total RNA from the blood or brain of WNV infected mice. At 2 and 4 dpi in the blood and brain, there was a trend toward more viral RNA in the D7 vaccinated mice as compared to mock-vaccinated mice, however none of these differences were statistically significant. (n=3)

Discussion

Efficacy of rD7 vaccine and immune response after WNV infection

Multiple tests of the effect of rD7 vaccination on mice infected with WNV by mosquito inoculation revealed that the salivary protein vaccine enhanced the pathogenesis of WNV infection. These results refuted to the hypothesis formed before experimentation; a salivary protein vaccine will protect against WNV transmitted by *Cx.*

tarsalis. Experiments conducted following the finding of enhanced pathogenesis were designed to elucidate a mechanism of WNV enhancement.

Passive transfer of serum (containing antibodies) from D7-vaccinated or naturally mosquito-exposed mice were conducted. The results show that transfer of serum from rD7-vaccinated mice significantly increased the chances of succumbing to WNV infection. Interestingly, the serum from mice that were naturally exposed to mosquito saliva did not alter the outcome of WNV infection.

Work conducted by Schneider *et al* (Schneider et al. 2007) demonstrated that prior exposure to *Ae. aegypti* mosquito bites or passive transfer of serum from prior mosquito exposed mice increased the percentage of animals succumbing to WNV infection from the bite of an infected *Ae. aegypti.* This suggests that antibodies to MSPs, or another serum component such as cytokines, enhanced viral pathogenesis. Again, in our work, antibodies to rD7 enhanced WNV pathogenesis; however, the prior exposed mouse serum did not alter the outcome as seen by (Schneider et al. 2007). Discrepancies between these findings may lie in the mosquito species and salivary protein differences or concentrations of the antibodies that were present in transferred serum. Antibody subtype differences may also account for the different outcomes. rD7-vaccinated mice have primarily IgG1 subtype antibodies and the naturally mosquito-exposed mice have a combination of IgG1 and IgG2a. IgG2a antibodies are more effective at complement activation, can bind with higher affinity Fc receptors, and are efficient at antibody dependant cell mediated cytotoxity, whereas IgG1 antibodies are less effective at each of those functions (Markine-Goriaynoff et al. 2000).

The rD7 vaccine produced a strong immunity to the salivary protein, however it was unknown how this would affect the immune response to WNV infection. Here we measured the humoral response after mosquito transmission of WNV to vaccinated mice. ELISAs were used to measure WNV IgG antibodies. Vaccinated mice seroconverted when exposed to WNV, and levels of antibodies to WNV did not differ significantly between the D7 and mock vaccinated mice. The humoral response to WNV remained intact in D7 vaccinated mice.

Spleens and serum from D7- or mock-vaccinated mice were used to measure cytokines by CBA or intracellular cytokine staining. The results seen in these experiments showed a dramatic decrease in inflammatory spleen cytokines produced by D7-vaccinated mice and an increase in Th2 cytokines in WNV specific T-cells. These results were not expected and varied from the experiments performed with non-infected mosquito injected salivary proteins presented in chapter 3.

There were decreases in TNFα, IFNγ, MCP-1, IL-10, and IL-12p70 produced by rD7 stimulated splenocytes isolated from D7 vaccinated mice. All cytokines measured here are pro-inflammatory cytokines that are important in the Th1 type environment needed to effectively fight WNV infection. Spleens were also harvested at 2 and 4 days post infection to assess the WNV specific effector T-cell functions. The splenocytes were stimulated with MHC class I or class II restricted peptides that have been identified for WNV (Purtha et al. 2007, Brien et al. 2008). These results showed an increase in IL-10 and IL-4 in mock-vaccinated mice. IL-10 is a regulatory cytokine and IL-4 is a Th2 promoting cytokine. An increase in IL-4 would explain the decrease in inflammatory cytokines seen above.

Together, the cytokine data show that the splenocyte population when stimulated with both D7 and WNV have a significant reduction in the production of pro-inflammatory cytokines, while WNV specific T-cells have an increase in the Th2 cytokine IL-4 and regulatory cytokine IL-10. The adjuvant chosen, complete Freund's adjuvant, is a potent Th1 inducer, so the results seen after WNV infection were not expected. This appears to be a protein specific response, as vaccination with CFA and *Ae. aegypti* D7 yielded results that were Th2 skewed (Chen et al. 1998). A mechanism for this is unknown.

D7 protein mediated immunopathogenesis during WNV infection

There are now two reports showing that immunity to mosquito salivary proteins, either from vaccination or prior exposure, leads to an unfavorable outcome in subsequent WNV infection. This leads to the theory that immunity to mosquito salivary proteins, specifically D7 protein, enhances WNV pathogenesis when virus is delivered from an infected mosquito bite. Here innate, humoral, and cell mediated immune responses were analyzed after vaccination. The results led to the following three possibilities that we suggest are important in the enhancement of WNV infection.

As noted in chapter three, D7 vaccinated animals when bitten by a mosquito have a significant amount of PMNs and mononuclear cells in infiltrates as compared to naïve mice. Although the cells were not characterized to cell type beyond mononuclear cells, it can be assumed that a portion of the cells are monocytes or dendritic cells. These two cell types are susceptible to WNV infection (Johnston et al. 2000, Rios et al. 2006). This could lead to a greater initial viral infection. The infected monoctyes or dendritic cells

could become activated and drain to the local lymph node, further disseminating the infection.

Secondly, D7 antibody passive transfer mimicked the results of D7 vaccinated mice. This implicates antibodies, or another serum component, in the enhancement. High levels of circulating antibodies to MSPs leads to difficulty completing a blood meal, as critical vasodilators and platelet inhibitors are having their functions neutralized. It is hypothesized this could lead to increased probing and therefore more saliva containing virus being deposited. This would lead to an increased initial dose of virus. However, observations during blood feeding did not indicate that mosquitoes had difficulty imbibing a blood meal, though *Culex* mosquitoes are known to have prolonged probing activity when taking a meal from a mammal (Ribeiro 2000). In vaccinated mice there may be slightly more probing taking place to located a blood source due to neutralization of a critical MSP.

Lastly, the cytokine profiles seen after exposure to mosquito bites show an increase in IL-10 and IL-4 which can decrease the type I and II interferons that are critical in a productive defense against WNV.

CHAPTER V

SUMMARY

Arboviruses are of global medical and veterinary importance, leading to significant public health concern. With repeated emergence of mosquito borne viruses in naïve populations, such as the outbreak of WNV in the United States, novel control strategies are of increased interest to researchers. Vaccination against hematophagous arthropod saliva has been investigated as a strategy to protect against multiple viral pathogens or intracellular parasites. Vaccines are attractive as they can be an easily distributed and cost effective way of controlling disease. Salivary proteins can be targeted that will cross-react with several species of mosquitoes, thus conferring protection against both known pathogens, as well as those that have not yet been identified.

Research into the immunomodulatory properties of blood feeding arthropods has yielded large amounts of information for hard ticks and sand flies where several individual immunomodulatory factors have been identified. This has in turn led to the development of salivary protein vaccines targeted against the sand fly salivary protein, maxidilan. This vaccine is efficacious in laboratory models, and additionally, natural sensitization to sand fly saliva in reservoir animals results in a favorable outcome after Leishmania infection.

Mosquito saliva has shown to have similar immunomodulatory activities as sand fly and tick saliva, however until very recently no single protein in their saliva has been identified to be responsible for this. The protein SAAG-4 was shown in *Aedes* mosquitoes as a potent inducer of IL-4. Hypersensitivity reactions are induced in the presence of IL-4, and may be a protective mechanism that evolved in hosts to encourage evasive maneuvers from blood feeding arthropods. Research into the sialome of *Culex*

species has not yielded a similar protein. Induction of IL-4 leads to a down regulation of Th1 cytokines. This environment allows for the mosquito to complete a blood meal and creates an immunologically advantageous environment for viral pathogens.

In this dissertation, saliva from the primarily ornithophilic mosquito *Cx. tarsalis* was investigated. This species is responsible for transmission of WNV, St. Louise encephalitis virus, and western equine encephalitis virus in the western United States. The main goal was to identify and characterize the immune response to D7 salivary proteins from *Cx. tarsalis* mosquitoes, and then incorporate that protein into a salivary protein vaccine to protect against WNV infection. D7 protein was of particular interest because it was shown to have IFNγ binding activity in an overlay-biding assay by Dr. Machain-Williams (unpublished data). D7 sequestering of IFNγ leads to an increase in viral infection (Machain-Williams unpublished data).

Here we have identified for the first time D7 salivary proteins from *Cx. tarsalis*. We have investigated the role of the D7 protein in host immunity to WNV infection in mice, and were able to show that administration of the D7 protein caused a decrease in the systemic expression of the chemokine MCP-1. MCP-1 is produced by monocytes, vascular endothelium, and smooth muscle cells and is responsible for recruitment of monocytes, T-cells, and dendritic cells to a site of tissue injury (Ibelgaufts 2010). MCP-1 also induces CD11b, CD11C, IL-1 and IL-6. A decrease in recruitment of these cells types reinforces the down regulation in inflammation associated with mosquito saliva. It did not appear that D7 protein was responsible for the dramatic increase in IL-4 as SAAG-4 does in *Aedes aegypti*, and we were not able to show modulation in IFNγ

production. The cytokines measured here were systemic. Investigation into local cytokine expression may reveal further immunomodulation due to D7 protein.

In addition to identification and characterization of the *Cx. tarsalis* D7 protein, we utilized it as a vaccine candidate to determine its ability to protect the host against WNV infection. This research shows that the D7 vaccine was highly immunogenic, and vaccinated mice exhibited a strong immune response after exposure to mosquito bites. Modulations in CD 8^+ and CD 4^+ T-cells, serum and spleen cytokine levels, and local inflammation were seen in vaccinated animals. Unexpectedly, the immunized mice were more susceptible to WNV infection delivered by mosquito bite. Further experiments revealed vaccination with D7 led to an immune status that was not protective against WNV, with high levels of the Th2 cytokine IL-4. In addition there were significant amounts of cellular infiltrates at the bites site that are known to be WNV susceptible populations. These cells can become infected and activated, draining to the local lymph node and promoting the dissemination of the virus. Lastly, results from WNV infected animals that received a passive transfer of antibodies from vaccinated animals mimicked the results seen in vaccinated mice. This suggests that antibodies to rD7, or another serum component, enhance WNV infection. Previously published research has shown similar results. This is not a finding that should be overlooked. Neutralization of salivary proteins that are critical to blood feeding activity could lead to difficulty in mosquitoes completing a blood meal, therefore increase probing time and virus deposition in the host. Increased dose could lead to a detrimental outcome in vaccinated animals.

Research conducted again by Dr. Machain-Williams (unpublished data) into mosquito salivary protein antibody levels in dengue patients showed a significant association with D7 antibodies and development of disease. Conclusions in that work suggested that neutralization of the D7 protein could lead to increased probing due to inhibited blood uptake, therefore increased deposition of virus at the bite site. Large scale studies on the association of D7 antibodies and increase in disease progression need to be undertaken to fully understand this finding.

Although this was at first a discouraging step towards the original goal of preventing WNV infection, it does broaden our understanding of the complex interaction between hosts and vectors. This is now a second report of immunity to mosquito saliva enhancing arboviral infection, previously by Schneider et al. (2007) by natural sensitization, and here by immunization. Along with an association with increased D7 antibody levels in dengue patients, this indicates immune status to not only the virus, but also the vector plays an important role in infection, warranting further investigation.

More research into the pharmacokinetic properties of mosquito saliva needs to be conducted to identify other MSPs that could be targets for neutralization. Similarly, adjuvant development could aid in the creation of strong Th1 memory producing immune responses that would be necessary in a protective vaccine. With these efforts together, this may still be a valid approach, as it has been in other arthropod-pathogen systems.

REFERENCES

Almeras, L., A. Fontaine, M. Belghazi, S. Bourdon, E. Boucomont-Chapeaublanc, E. Orlandi-Pradines, M. Baragatti, N. Corre-Catelin, P. Reiter, B. Pradines, T. Fusai, and C. Rogier. 2009. Salivary Gland Protein Repertoire from Aedes aegypti Mosquitoes. Vector Borne Zoonotic Dis.

AnaesthesiaUK. 2005. Coagulation-Classical model. *In* L. Koupparis and S. Das [eds.], Anaesthesia UK.

Anderson, J. F., and J. J. Rahal. 2002. Efficacy of interferon alpha-2b and ribavirin against West Nile virus in vitro. Emerg Infect Dis 8: 107-8.

Arca, B., F. Lombardo, A. Lanfrancotti, L. Spanos, M. Veneri, C. Louis, and M. Coluzzi. 2002. A cluster of four D7-related genes is expressed in the salivary glands of the African malaria vector Anopheles gambiae. Insect Mol Biol 11: 47-55.

Barton, G. M. 2007. Viral recognition by Toll-like receptors. Semin Immunol 19: 33-40.

Beerntsen, B. T., D. E. Champagne, J. L. Coleman, Y. A. Campos, and A. A. James. 1999. Characterization of the Sialokinin I gene encoding the salivary vasodilator of the yellow fever mosquito, Aedes aegypti. Insect Molecular Biology 8: 459-467.

Bissonnette, E. Y., P. A. Rossignol, and A. D. Befus. 1993. Extracts of mosquito salivary gland inhibit tumour necrosis factor alpha release from mast cells. Parasite Immunol 15: 27-33.

Boppana, V. D., S. Thangamani, A. J. Adler, and S. K. Wikel. 2009. SAAG-4 is a novel mosquito salivary protein that programmes host CD4 T cells to express IL-4. Parasite Immunol 31: 287-95.

Borowski, P., A. Niebuhr, O. Mueller, M. Bretner, K. Felczak, T. Kulikowski, and H. Schmitz. 2001. Purification and Characterization of West Nile Virus Nucleoside Triphosphatase (NTPase)/Helicase: Evidence for Dissociation of the NTPase and Helicase Activities of the Enzyme. J. Virol. 75: 3220-3229.

Brault, A. C. 2009. Changing patterns of West Nile virus transmission: altered vector competence and host susceptibility. Vet Res 40: 43.

Brien, J. D., J. L. Uhrlaub, and J. Nikolich-Zugich. 2008. West Nile Virus-Specific CD4 T Cells Exhibit Direct Antiviral Cytokine Secretion and Cytotoxicity and Are Sufficient for Antiviral Protection. J Immunol 181: 8568-8575.

Brinton, M. 2002. The molecular biology of West Nile Virus: a new invader of the western hemisphere. Annu Rev Microbiol 56: 371-402.

Brown, A., K. Kent, C. Bennett, and K. Bernard. 2007. Tissue tropism and neuroinvasion of West Nile virus do not differ for two mouse strains with different survival rates. Virology 368: 422-30.

Burke, D. S., and T. P. Monath. 2001. Flaviviruses, pp. 1043 - 1125. *In* B. N. Fields, D. M. Knipe, P. M. Howley and D. E. Griffin [eds.], Fields' virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia.

Calvo, E., B. J. Mans, J. F. Andersen, and J. M. Ribeiro. 2006. Function and evolution of a mosquito salivary protein family. J Biol Chem 281: 1935-42.

Calvo, E., B. J. Mans, J. M. Ribeiro, and J. F. Andersen. 2009. Multifunctionality and mechanism of ligand binding in a mosquito antiinflammatory protein. Proc Natl Acad Sci U S A 106: 3728-33.

Calvo, E., I. Sanchez-Vargas, A. Favreau, K. Barbian, V. Pham, K. Olson, and J. Ribeiro. 2010a. An insight into the sialotranscriptome of the West Nile mosquito vector, Culex tarsalis. BMC Genomics 11: 51.

Calvo, E., F. Tokumasu, D. M. Mizurini, P. McPhie, D. L. Narum, J. Ribeiro, R. Q. Monteiro, and I. M. Francischetti. 2010b. Aegyptin displays high-affinity for the von Willebrand factor binding site (RGQOGVMGF) in collagen and inhibits carotid thrombus formation in vivo. FEBS Journal 277: 413-427.

Campbell, C., W. Black, A. Hess, and B. Foy. 2008. Comparative genomics of small RNA regulatory pathway components in vector mosquitoes. BMC Genomics 9: 425.

CDC. 2010. Statistics, Surveillance, and Control. http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm.

Champagne, D. E., and J. M. Ribeiro. 1994. Sialokinin I and II: vasodilatory tachykinins from the yellow fever mosquito Aedes aegypti. Proceedings of the National Academy of Sciences of the United States of America 91: 138-142.

Champagne, D. E., C. T. Smartt, J. M. Ribeiro, and A. A. James. 1995. The salivary gland-specific apyrase of the mosquito Aedes aegypti is a member of the 5'-nucleotidase family. Proceedings of the National Academy of Sciences of the United States of America 92: 694-698.

Champagne, D. E., H. A. Wasserman, S. Kumar, and S. Singh. 2004. Pharmacological and immunological properties of saliva of the blood-feeding insects Rhodnius prolixus and Aedes aegypti. Physiological Entomology 29: 269-277.

Chen, Y. L., F. E. Simons, and Z. Peng. 1998. A mouse model of mosquito allergy for study of antigen-specific IgE and IgG subclass responses, lymphocyte proliferation, and IL-4 and IFN-gamma production. Int Arch Allergy Immunol 116: 269-77.

Chu, J. J., and M. L. Ng. 2004a. Interaction of West Nile virus with alpha v beta 3 integrin mediates virus entry into cells. J Biol Chem 279: 54533-41.

Chu, J. J. H., and M. L. Ng. 2004b. Infectious Entry of West Nile Virus Occurs through a Clathrin-Mediated Endocytic Pathway. J. Virol. 78: 10543-10555.

Chu, J. J. H., R. Rajamanonmani, J. Li, R. Bhuvanakantham, J. Lescar, and M.-L. Ng. 2005. Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein. J Gen Virol 86: 405-412.

Chung, K. M., M. K. Liszewski, G. Nybakken, A. E. Davis, R. R. Townsend, D. H. Fremont, J. P. Atkinson, and M. S. Diamond. 2006. West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. Proceedings of the National Academy of Sciences 103: 19111-19116.

Clements, A. 2000. The Biology of Mosquitoes: Development, Nutrition and Reproduction. CABI Publishing, New York, Ny.

Cross, M. L., E. W. Cupp, and F. J. Enriquez. 1994. Differential Modulation of Murine Cellular Immune Responses by Salivary Gland Extract of Aedes aegypti. Am J Trop Med Hyg 51: 690-696.

Davie, E. W., K. Fujikawa, and W. Kisiel. 1991. The coagulation cascade: initiation, maintenance, and regulation. Biochemistry 30: 10363-10370.

Davis, C. W., H.-Y. Nguyen, S. L. Hanna, M. D. Sanchez, R. W. Doms, and T. C. Pierson. 2006. West Nile Virus Discriminates between DC-SIGN and DC-SIGNR for Cellular Attachment and Infection. J. Virol. 80: 1290-1301.

Demeure, C. E., K. Brahimi, F. Hacini, F. Marchand, R. Peronet, M. Huerre, P. St-Mezard, J. F. Nicolas, P. Brey, G. Delespesse, and S. Mecheri. 2005. Anopheles mosquito bites activate cutaneous mast cells leading to a local inflammatory response and lymph node hyperplasia. J Immunol 174: 3932-40.

Diamond, M. S., E. Mehlhop, T. Oliphant, and M. A. Samuel. 2009. The host immunologic response to West Nile encephalitis virus. Front Biosci 14: 3024-34.

Diamond, M. S., B. Shrestha, E. Mehlhop, E. Sitati, and M. Engle. 2003a. Innate and adaptive immune responses determine protection against disseminated infection by West Nile encephalitis virus. Viral Immunol 16: 259-78.

Diamond, M. S., E. M. Sitati, L. D. Friend, S. Higgs, B. Shrestha, and M. Engle. 2003b. A critical role for induced IgM in the protection against West Nile virus infection. J Exp Med 198: 1853-62.

Donovan, M. J., A. S. Messmore, D. A. Scrafford, D. L. Sacks, S. Kamhawi, and M. A. McDowell. 2007. Uninfected mosquito bites confer protection against infection with malaria parasites. Infect Immun 75: 2523-30.

Edwards, J. F., S. Higgs, and B. J. Beaty. 1998. Mosquito feeding-induced enhancement of Cache Valley Virus (Bunyaviridae) infection in mice. J Med Entomol 35: 261-5.

Egloff, M.-P., D. Benarroch, B. Selisko, J.-L. Romette, and B. Canard. 2002. An RNA cap (nucleoside-2[prime]-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. EMBO J 21: 2757-2768.

Francischetti, I. M. B., J. G. Valenzuela, and J. M. C. Ribeiro. 1999. Anophelin: Kinetics and Mechanism of Thrombin Inhibition. Biochemistry 38: 16678-16685.

Hayes, E. B., N. Komar, R. S. Nasci, S. P. Montgomery, D. R. O'Leary, and G. L. Campbell. 2005. Epidemiology and transmission dynamics of West Nile virus disease. Emerg Infect Dis 11: 1167-73.

Ibelgaufts, H. 2010. Cytokines and Cells Online Pathfinder Encyclopedia. Ibelgaufts, H.

James, A. A., K. Blackmer, O. Marinotti, C. R. Ghosn, and J. V. Racioppi. 1991. Isolation and characterization of the gene expressing the major salivary gland

protein of the female mosquito, Aedes aegypti. Molecular and Biochemical Parasitology 44: 245-253.

Johnston, L. J., G. M. Halliday, and N. J. King. 2000. Langerhans cells migrate to local lymph nodes following cutaneous infection with an arbovirus. J Invest Dermatol 114: 560-8.

Kawai, T., and S. Akira. 2006. Innate immune recognition of viral infection. Nat Immunol 7: 131-7.

Kebaier, C., T. Voza, and J. Vanderberg. 2010. Neither mosquito saliva nor immunity to saliva has a detectable effect on the infectivity of Plasmodium sporozoites injected into mice. Infect Immun 78: 545-51.

Klee, A., B. Maidin, B. Edwin, I. Poshni, F. Mostashari, A. Fine, M. Layton, and D. Nash. 2004. Long-term prognosis for clinical West Nile virus infection. Emerg Infect Dis 10: 1405-11.

Komar, N., S. Langevin, S. Hinten, N. Nemeth, E. Edwards, D. Hettler, B. Davis, R. Bowen, and M. Bunning. 2003. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg Infect Dis 9: 311-22.

Kramer, L., L. Styer, and G. Ebel. 2007. A Global Perspective on the Epidemiology of West Nile Virus. Annu Rev Entomol.

Kulkarni, A. B., A. Mullbacher, and R. V. Blanden. 1991. Functional analysis of macrophages, B cells and splenic dendritic cells as antigen-presenting cells in West Nile virus-specific murine T lymphocyte proliferation. Immunol Cell Biol 69 (Pt 2): 71-80.

Lambrecht, B. N., M. Kool, M. A. M. Willart, and H. Hammad. 2009. Mechanism of action of clinically approved adjuvants. Current Opinion in Immunology 21: 23-29.

Lanciotti, R., J. Roehrig, V. Deubel, J. Smith, M. Parker, K. Steele, B. Crise, K. Volpe, M. Crabtree, J. Scherret, R. M. Hall, JS. , C. Cropp, B. Panigrahy, E. Ostlund, B. Schmitt, M. Malkinson, C. Banet, J. Weissman, N. Komar, H. Savage, W. Stone, T. McNamara, and D. Gubler. 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 286: 2333-7.

Lanciotti, R. S., A. J. Kerst, R. S. Nasci, M. S. Godsey, C. J. Mitchell, H. M. Savage, N. Komar, N. A. Panella, B. C. Allen, K. E. Volpe, B. S. Davis, and J. T. Roehrig. 2000. Rapid Detection of West Nile Virus from Human Clinical Specimens, Field-Collected Mosquitoes, and Avian Samples by a TaqMan Reverse Transcriptase-PCR Assay. J. Clin. Microbiol. 38: 4066-4071.

Lanciotti, R. S., G. D. Ebel, V. Deubel, A. J. Kerst, S. Murri, R. Meyer, M. Bowen, N. McKinney, W. E. Morrill, M. B. Crabtree, L. D. Kramer, and J. T. Roehrig. 2002. Complete Genome Sequences and Phylogenetic Analysis of West Nile Virus Strains Isolated from the United States, Europe, and the Middle East. Virology 298: 96-105.

Limesand, Higgs, Pearson, and Beaty. 2000. Potentiation of vesicular stomatitis New Jersey virus infection in mice by mosquito saliva. Parasite Immunology 22: 461-467.

Limesand, K. H., S. Higgs, L. D. Pearson, and B. J. Beaty. 2009. Effect of Mosquito Salivary Gland Treatment on Vesicular Stomatitis New Jersey Virus Replication

and Interferon Î±/Î² Expression In Vitro. Journal of Medical Entomology 40: 199-205.

Liu, W. J., X. J. Wang, V. V. Mokhonov, P.-Y. Shi, R. Randall, and A. A. Khromykh. 2005. Inhibition of Interferon Signaling by the New York 99 Strain and Kunjin Subtype of West Nile Virus Involves Blockage of STAT1 and STAT2 Activation by Nonstructural Proteins. J. Virol. 79: 1934-1942.

Liu, W. J., X. J. Wang, D. C. Clark, M. Lobigs, R. A. Hall, and A. A. Khromykh. 2006. A Single Amino Acid Substitution in the West Nile Virus Nonstructural Protein NS2A Disables Its Ability To Inhibit Alpha/Beta Interferon Induction and Attenuates Virus Virulence in Mice. J. Virol. 80: 2396-2404.

Lustig, S., U. Olshevsky, D. Ben-Nathan, B. E. Lachmi, M. Malkinson, D. Kobiler, and M. Halevy. 2000. A live attenuated West Nile virus strain as a potential veterinary vaccine. Viral Immunol 13: 401-10.

Mackenzie, J., D. Gubler, and L. Petersen. 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 10: S98-109.

Mahmood, F., R. E. Chiles, Y. Fang, E. N. Green, and W. K. Reisen. 2006. Effects of time after infection, mosquito genotype, and infectious viral dose on the dynamics of Culex tarsalis vector competence for western equine encephalomyelitis virus. J Am Mosq Control Assoc 22: 272-81.

Malafronte Rdos, S., E. Calvo, A. A. James, and O. Marinotti. 2003. The major salivary gland antigens of Culex quinquefasciatus are D7-related proteins. Insect Biochem Mol Biol 33: 63-71.

Mans, B. J., E. Calvo, J. M. Ribeiro, and J. F. Andersen. 2007. The crystal structure of D7r4, a salivary biogenic amine-binding protein from the malaria mosquito Anopheles gambiae. J Biol Chem 282: 36626-33.

Marcelo, B., B. P. S. Milena, T. B. Patricia, R. S. Abhay, G. D. Thomas, B. Frank, G. T. Richard, B. S. Charles, and R. D. John. 1998. The PACAP-type I receptor agonist maxadilan from sand fly saliva protects mice against lethal endotoxemia by a mechanism partially dependent on IL-10. European Journal of Immunology 28: 3120-3127.

Marciani, D. J. 2003. Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. Drug Discovery Today 8: 934-943.

Markine-Goriaynoff, D., J. T. M. van der Logt, C. Truyens, T. D. Nguyen, F. W. A. Heessen, G. Bigaignon, Y. Carlier, and J.-P. Coutelier. 2000. IFN-{gamma}-independent IgG2a production in mice infected with viruses and parasites. Int. Immunol. 12: 223-230.

Mehlhop, E., and M. Diamond. 2008. The molecular basis of antibody protection against West Nile virus. Curr Top Microbiol Immunol 317: 125-53.

Meola, S. M., F. L. Clottens, G. M. Holman, R. J. Nachman, R. Nichols, L. Schoofs, M. S. Wright, J. K. Olson, T. K. Hayes, and M. W. Pendleton. 1998. Isolation and immunocytochemical characterization of three tachykinin-related peptides from the mosquito, Culex salinarius. Neurochem Res 23: 189-202.

Morris, R. V., C. B. Shoemaker, J. R. David, G. C. Lanzaro, and R. G. Titus. 2001. Sandfly maxadilan exacerbates infection with Leishmania major and vaccinating against it protects against L. major infection. J Immunol 167: 5226-30.

Moudy, R. M., M. A. Meola, L. L. Morin, G. D. Ebel, and L. D. Kramer. 2007. A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by Culex mosquitoes. Am J Trop Med Hyg 77: 365-70.

Murray, K., M. Resnick, and V. Miller. 2007. Depression after infection with West Nile virus. Emerg Infect Dis 13: 479-81.

Nirmala, X., O. Marinotti, and A. A. James. 2005. The accumulation of specific mRNAs following multiple blood meals in Anopheles gambiae. Insect Mol Biol 14: 95-103.

Owhashi, M., M. Harada, S. Suguri, H. Ohmae, and A. Ishii. 2001. The role of saliva of Anopheles stephensi in inflammatory response: identification of a high molecular weight neutrophil chemotactic factor. Parasitol Res 87: 376-82.

Owhashi, M., M. Harada, S. Suguri, H. Omae, and A. Ishii. 2008. Identification of an eosinophil chemotactic factor from anopheline mosquitoes as a chitinase family protein. Parasitol Res 102: 357-63.

Peng, Z., and F. E. Simons. 1998. A prospective study of naturally acquired sensitization and subsequent desensitization to mosquito bites and concurrent antibody responses. J Allergy Clin Immunol 101: 284-6.

Peng, Z., and F. E. Simons. 2004. Mosquito allergy: immune mechanisms and recombinant salivary allergens. Int Arch Allergy Immunol 133: 198-209.

Peng, Z., and F. E. Simons. 2007. Advances in mosquito allergy. Curr Opin Allergy Clin Immunol 7: 350-4.

Peng, Z., W. Xu, H. Lam, L. Cheng, A. A. James, and F. E. Simons. 2006. A new recombinant mosquito salivary allergen, rAed a 2: allergenicity, clinical relevance, and cross-reactivity. Allergy 61: 485-90.

Peters-Golden, M., C. Canetti, P. Mancuso, and M. J. Coffey. 2005. Leukotrienes: Underappreciated Mediators of Innate Immune Responses. J Immunol 174: 589-594.

Petersen, L., and J. Roehrig. 2001. West Nile virus: a reemerging global pathogen. Emerg Infect Dis 7: 611-4.

Platt, K. B., B. J. Tucker, P. G. Halbur, S. Tiawsirisup, B. J. Blitvich, F. G. Fabiosa, L. C. Bartholomay, and W. A. Rowley. 2007. West Nile virus viremia in eastern chipmunks (Tamias striatus) sufficient for infecting different mosquitoes. Emerg Infect Dis 13: 831-7.

Platt, K. B., B. J. Tucker, P. G. Halbur, B. J. Blitvich, F. G. Fabiosa, K. Mullin, G. R. Parikh, P. Kitikoon, L. C. Bartholomay, and W. A. Rowley. 2008. Fox Squirrels (Sciurus niger) Develop West Nile Virus Viremias Sufficient for Infecting Select Mosquito Species. Vector-Borne and Zoonotic Diseases 8: 225-234.

Purtha, W. E., N. Myers, V. Mitaksov, E. Sitati, J. Connolly, D. H. Fremont, T. H. Hansen, and M. S. Diamond. 2007. Antigen-specific cytotoxic T†lymphocytes protect against lethal West Nile virus encephalitis. Eur J Immunol 37: 1845-1854.

Rasgon, J. L., M. Venkatesan, C. J. Westbrook, and M. C. Hauer. 2006. Polymorphic microsatellite loci from the West Nile virus vector Culex tarsalis. Molecular Ecology Notes 6: 680-682.

Reisen, W., and A. C. Brault. 2007. West Nile virus in North America: perspectives on epidemiology and intervention. Pest Manag Sci 63: 641-6.

Ribeiro, J., and J. Valenzuela. 1999. Purification and cloning of the salivary peroxidase/catechol oxidase of the mosquito Anopheles albimanus. J Exp Biol 202: 809-816.

Ribeiro, J. M. 2000. Blood-feeding in mosquitoes: probing time and salivary gland anti-haemostatic activities in representatives of three genera (Aedes, Anopheles, Culex). Med Vet Entomol 14: 142-8.

Ribeiro, J. M., and I. M. Francischetti. 2003. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. Annu Rev Entomol 48: 73-88.

Ribeiro, J. M., R. Charlab, V. M. Pham, M. Garfield, and J. G. Valenzuela. 2004. An insight into the salivary transcriptome and proteome of the adult female mosquito Culex pipiens quinquefasciatus. Insect Biochem Mol Biol 34: 543-63.

Ribeiro, J. M., B. Arca, F. Lombardo, E. Calvo, V. M. Phan, P. K. Chandra, and S. K. Wikel. 2007. An annotated catalogue of salivary gland transcripts in the adult female mosquito, Aedes aegypti. BMC Genomics 8: 6.

Rios, M., M. J. Zhang, A. Grinev, K. Srinivasan, S. Daniel, O. Wood, I. K. Hewlett, and A. I. Dayton. 2006. Monocytes-macrophages are a potential target in human infection with West Nile virus through blood transfusion. Transfusion 46: 659-667.

Samuel, M., and M. Diamond. 2005. Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. J Virol 79: 13350-61.

Samuel, M., and M. Diamond. 2006. Pathogenesis of West Nile Virus infection: a balance between virulence, innate and adaptive immunity, and viral evasion. J Virol 80: 9349-60.

Samuel, M. A., J. D. Morrey, and M. S. Diamond. 2007. Caspase 3-Dependent Cell Death of Neurons Contributes to the Pathogenesis of West Nile Virus Encephalitis. J. Virol. 81: 2614-2623.

Sandeman, R. M. 1996. Immune Responses to Mosquitoes and Flies. *In* S. K. Wikel [ed.], The Immunology of Host-Ectoparasitic Arthropod Relationships. CAB International, Wallingford, UK.

Schneider, B. S., and S. Higgs. 2008. The enhancement of arbovirus transmission and disease by mosquito saliva is associated with modulation of the host immune response. Trans R Soc Trop Med Hyg 102: 400-8.

Schneider, B. S., L. Soong, N. S. Zeidner, and S. Higgs. 2004. Aedes aegypti salivary gland extracts modulate anti-viral and TH1/TH2 cytokine responses to sindbis virus infection. Viral Immunol 17: 565-73.

Schneider, B. S., L. Soong, Y. A. Girard, G. Campbell, P. Mason, and S. Higgs. 2006. Potentiation of West Nile encephalitis by mosquito feeding. Viral Immunol 19: 74-82.

Schneider, B. S., C. E. McGee, J. M. Jordan, H. L. Stevenson, L. Soong, and S. Higgs. 2007. Prior exposure to uninfected mosquitoes enhances mortality in naturally-transmitted west nile virus infection. PLoS ONE 2: e1171.

Sitati, E., and M. Diamond. 2006. CD4+ T-cell responses are required for clearance of West Nile virus from the central nervous system. J Virol 80: 12060-9.

Smithburn, K. C., T. P. Hughes, A. W. Burke, and J. H. Paul. 1940. A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med Hyg 20: 471 - 492.

Soares, M. B. P., R. G. Titus, C. B. Shoemaker, J. R. David, and M. Bozza. 1998. The Vasoactive Peptide Maxadilan from Sand Fly Saliva Inhibits TNF-{alpha} and Induces IL-6 by Mouse Macrophages Through Interaction with the Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Receptor. J Immunol 160: 1811-1816.

Stills, H. F., Jr. 2005. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR J 46: 280-93.

Styer, L. M., K. A. Kent, R. G. Albright, C. J. Bennett, L. D. Kramer, and K. A. Bernard. 2007. Mosquitoes inoculate high doses of West Nile virus as they probe and feed on live hosts. PLoS Pathog 3: 1262-70.

Sun, D., A. McNicol, A. A. James, and Z. Peng. 2006. Expression of functional recombinant mosquito salivary apyrase: a potential therapeutic platelet aggregation inhibitor. Platelets 17: 178-84.

Titus, R. G., and J. M. Ribeiro. 1988. Salivary gland lysates from the sand fly Lutzomyia longipalpis enhance Leishmania infectivity. Science 239: 1306-8.

Titus, R. G., J. V. Bishop, and J. S. Mejia. 2006. The immunomodulatory factors of arthropod saliva and the potential for these factors to serve as vaccine targets to prevent pathogen transmission. Parasite Immunol 28: 131-41.

Turell, M. J., D. J. Dohm, M. R. Sardelis, M. L. Oguinn, T. G. Andreadis, and J. A. Blow. 2005. An update on the potential of north American mosquitoes (Diptera: Culicidae) to transmit West Nile Virus. J Med Entomol 42: 57-62.

U'Ren, L., R. Kedl, and S. Dow. 2006. Vaccination with liposome--DNA complexes elicits enhanced antitumor immunity. Cancer Gene Ther 13: 1033-44.

USDA. 2010. West Nile Virus Surveillance. *In* U. S. D. o. Agriculture [ed.].

Valenzuela, J. G. 2004. Blood-feeding Arthropod Salivary Glands and Saliva, pp. 377 - 386. *In* W. C. Marquardt [ed.], Biology of Disease Vectors. Elsevier Academic Press, Amersterdam.

Valenzuela, J. G., R. Charlab, E. C. Gonzalez, I. K. de Miranda-Santos, O. Marinotti, I. M. Francischetti, and J. M. Ribeiro. 2002. The D7 family of salivary proteins in blood sucking diptera. Insect Mol Biol 11: 149-55.

Wanasen, N., R. H. Nussenzveig, D. E. Champagne, L. Soong, and S. Higgs. 2004. Differential modulation of murine host immune response by salivary gland extracts from the mosquitoes Aedes aegypti and Culex quinquefasciatus. Med Vet Entomol 18: 191-9.

Wang, H., X. Mao, F. E. Simons, and Z. Peng. 1999. Induction of IgE responses using a recombinant mosquito salivary allergen rAed a 2 without adjuvant in mice. Int Arch Allergy Immunol 120: 135-40.

Wang, T., and E. Fikrig. 2004. Immunity to West Nile virus. Curr Opin Immunol 16: 519-23.

Wang, T., J. F. Anderson, L. A. Magnarelli, S. J. Wong, R. A. Koski, and E. Fikrig. 2001. Immunization of Mice Against West Nile Virus with Recombinant Envelope Protein, pp. 5273-5277.

Wang, T., T. Town, L. Alexopoulou, J. Anderson, E. Fikrig, and R. Flavell. 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med 10: 1366-73.

Wang, T., E. Scully, Z. Yin, J. Kim, S. Wang, J. Yan, M. Mamula, J. Anderson, J. Craft, and E. Fikrig. 2003a. IFN-gamma-producing gamma delta T cells help control murine West Nile virus infection. J Immunol 171: 2524-31.

Wang, Y., M. Lobigs, E. Lee, and A. Mullbacher. 2003b. CD8+ T cells mediate recovery and immunopathology in West Nile virus encephalitis. J Virol 77: 13323-34.

Wasserman, H. A., S. Singh, and D. E. Champagne. 2004. Saliva of the Yellow Fever mosquito, Aedes aegypti, modulates murine lymphocyte function. Parasite Immunol 26: 295-306.

Wilson, J. R., P. F. de Sessions, M. A. Leon, and F. Scholle. 2008. West Nile Virus Nonstructural Protein 1 Inhibits TLR3 Signal Transduction. J. Virol. 82: 8262-8271.

Wright, K. A. 1969. The anatomy of salivary glands of Anopheles stephehsi Liston. Canadian Journal of Zoology 47: 579-587.

Xiao, S.-Y., H. Guzman, H. Zhang, A. P. A. Travassos da Rosa, and R. B. Tesh. 2001. West Nile Virus Infection in the Golden Hamster (Mesocricetus auratus): A Model for West Nile Encephalitis. Emerging Infectious Diseases 7: 714.

Yusof, R., S. Clum, M. Wetzel, H. M. Murthy, and R. Padmanabhan. 2000. Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids in vitro. J Biol Chem 275: 9963-9.

Zaks, K., M. Jordan, A. Guth, K. Sellins, R. Kedl, A. Izzo, C. Bosio, and S. Dow. 2006. Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. J Immunol 176: 7335-45.

Zeidner, N. S., S. Higgs, C. M. Happ, B. J. Beaty, and B. R. Miller. 1999. Mosquito feeding modulates Th1 and Th2 cytokines in flavivirus susceptible mice: an effect mimicked by injection of sialokinins, but not demonstrated in flavivirus resistant mice. Parasite Immunol 21: 35-44.