DISSERTATION

THE ROLE OF IMMUNITY TO MOSQUITO SALIVARY PROTEINS IN THE PATHOGENESIS OF FLAVIVIRUSES (*FLAVIVIRUS:FLAVIVIRIDAE*)

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CARLOS IGNACIO MACHAIN WILLIAMS "THE ROLE OF IMMUNITY TO MOSQUITO SALIVARY PROTEINS IN THE PATHOGENESIS OF FLAVIVIRUSES (*FLAVIVIRUS:FLAVIVIRIDAE*)" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE ROLE OF MOSQUITO SALIVARY PROTEINS IN THE PATHOGENESIS OF FLAVIVIRUSES (*FLAVIVIRUS:FLAVIVIRIDAE*)

Mosquito salivary proteins (MSP) have been implicated in the enhancement of arthropod-borne virus pathogenesis. Dengue viruses (DENV) are emerging pathogens transmitted mainly by *Aedes aegypti* mosquitoes. West Nile virus (WNV) is also considered an emerging pathogen that recently has been introduced into the Western Hemisphere. WNV is transmitted by mosquitoes from *Culex* species.

Mosquito salivary proteins were obtained by a novel technique involving collection in a salivation buffer and concentration by trichloroacetic acid (TCA) precipitation. Immunoreactive proteins from *Cx. tarsalis* and *Ae.aegypti* were proposed to be related to viral pathogenesis.

In a retrospective study in Thai patients who had DENV2 secondary infections with disease severity ranging from dengue fever (DF) to dengue hemorrhagic fever (DHF), it was found that the proportion of patients with DF who had antibodies reacting against the 36 kDa *Ae. aegypti* MSP (71.4%) was significantly higher compared to those who had DHF (62%). In contrast, a higher proportion (36%) of DHF patients reacted to an *Ae. aegypti* protein identified as apyrase than those with anti-apyrase antibodies presenting with DF (14.3%).

To investigate the potential role of immune response to MSP in virus pathogenesis, NIH-Swiss mice were immunized with *Cx tarsalis* mosquito salivary gland homogenates followed by challenge by WNV-infected mosquitoes. In vaccinated mice, viremia was first detected by RT-PCR 48 hours after mosquito bite; in contrast, the unvaccinated control group presented viremia as early as 24 hours after mosquito challenge. WNV RNA was detected in brains of unvaccinated mice at day four post challenge; in contrast no viral RNA was detected by RT-PCR in the brains of vaccinated mice by day four. By day eight post challenge all the mice had viral RNA in their brains detectable by RT-PCR. The anti-WNV antibody titers were also higher in the vaccinated group than in unvaccinated mice ($P<0.0008$). When Th1/Th2 specific cytokines were analyzed by Q-RT-PCR, IL-2, IFNγ and TNFα were increased in the vaccinated group. In contrast, IL-4 was up-regulated in the unvaccinated group.

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LITERATURE REVIEW

INTRODUCTION

The study of mosquito salivary proteins (MSP) is a subject of great importance due to their implication in the enhancement of disease caused by pathogens transmitted by vectors. MSP are a cocktail of pharmacological compounds that help the mosquito to successfully acquire a blood meal; the main activities of these proteins include vasodilators, anticoagulants, platelet aggregation inhibitors, and immune regulators that help the mosquitoes to feed (280). At the same time the effects of MSP also help pathogens to infect the vertebrate host in which they can replicate (280). Pathogens that are transmitted by mosquito bite take advantage of the MSP components to help them to evade the immune response of the host in order to increase their chances to survive (245). This interaction not only helps the pathogens to infect and to evade the host immune response but also may increase the degree of virulence in the vertebrate host.

The viruses in the *Flavivirus* genus, *Flaviviridae* family are among those vector-borne pathogens that take advantage of the components of the mosquito saliva to infect their host (245, 264). Dengue viruses are considered to be the most important vector-borne viruses in tropical and subtropical areas; there are approximately 100 million cases of dengue fever (DF) and 500,000 cases of dengue hemorrhagic fever every year around the world (84). West Nile virus (WNV) is now becoming more important. Since it was first reported in the western hemisphere, WNV has spread through the continental U.S.A, Canada, Mexico, Central and South America (39, 72, 177, 178). Identifying the role that specific MSP play during flavivirus infection could be important in understanding virus transmission and pathogenesis and could lead to use of this knowledge to develop new

vaccine strategies that utilize not only pathogen's antigens but also components of the vector such as MSP (275)

Anatomy and physiology of mosquito salivary glands.

In mosquitoes the adult salivary glands are distinct from the larval glands. For these insects different groups of cells form the larval and adult glands. In the larval stages, the salivary glands remain relatively undifferentiated. During metamorphosis the cells of the salivary gland divide and undergo significant changes in shape to achieve the final form of the adult glands; particularly in mosquitoes the glands undergo a period of rapid synthesis of proteins before the adults are ready to feed on blood (259).

Mosquitoes have tubular glands that consist of a single layer of cells surrounding a duct, which can be partially or completely chitinized as shown in figure 1.1. In this structure each cell has a large extracellular acinus in which the products of secretions are stored.

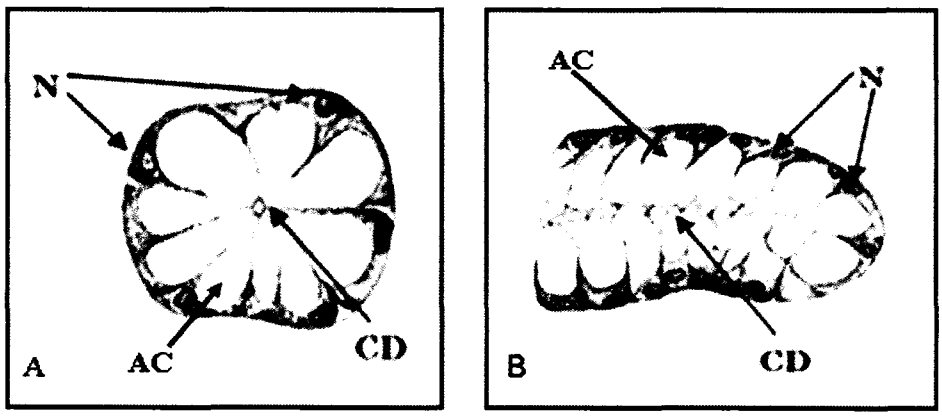

Figure1.1 Anatomy of salivary glands of mosquitoes. (A) cross section and, (B) longitudinal section. Cell nuclei (N), acinus (AC) and central duct (CD) (113, 259).

In mosquitoes, structural and functional dimorphisms exist between the sexes, reflecting the fact that only females feed on blood. In spite of structural differences, the salivary glands of both sexes are paired organs located in the thorax and each consists of

three lobes connected to a main salivary duct. The salivary ducts are lined with chitin and extend the full length of the gland in *Aedes* and *Culex* species (259) .

The female salivary glands in mosquitoes are morphologically different from those of the male; female salivary glands have two identical lateral lobes and a shorter and wider medial lobe as shown in figure 1.2. The lateral lobe can be divided into proximal and distal regions. Histochemical and molecular analyses of female *Aedes aegypti* salivary glands have revealed the secretion of different proteins. The proximal lateral lobe produces enzymes involved in sugar feeding; these parts of the female salivary glands are functionally and morphologically similar to the adult male salivary glands. Other secretory cell types located in the medial lobes and distal lateral lobes appear to be female specific. The adult female seems to have some control over the release of salivary products depending on whether she is feeding on sugar or blood (259).

Figure 1.2. Adult salivary gland from the mosquito *Aedes aegypti.* A) One pair of salivary glands from female, B) One pair of salivary glands from male. L=lateral lobes; M=medial lobes (259)

Mosquito salivary proteins, function and importance during blood feeding.

The saliva of blood feeding arthropods was believed for many years to be inert, utilized only to lubricate the insect mouth parts. This concept has changed with the demonstration that the saliva of the blood feeders is composed of potent pharmacologically active products that alter the host hemostatic, inflammatory and immune systems (259, 280) as well as cause allergic reactions (3). In mosquitoes, the saliva is a medium for acquiring a blood meal and also for providing enzymes that initiate digestion (259). Saliva of female mosquitoes contains α-amylase and α-glucosidase whose function is to digest sugars; saliva seems to be the primary source of these two enzymes for sugar digestion (259). When mosquitoes feed on blood, several important salivary proteins have been identified; among those are vasodilators, anticlotting factors, platelet inhibitors, as well as immune modulators (259, 280). More recently, bioamine binding proteins have been described in the mosquito saliva (33).

Vasodilators. These molecules increase blood flow by antagonism of vasoconstrictors produced by the hemostatic system following tissue injury by insect mouth parts (281). Vasodilator activities have been identified in the saliva of *Anopheles* and *Aedes* species (46, 217, 223, 224). The saliva of Anophelines, such as *Anopheles albimanus* mosquitoes, contains a salivary heme peroxidase thought to destroy hemostatically active vasoconstrictors such as biogenic amines that are released by the vertebrate host during tissue injury (223). The vasodilators of *Ae. aegypti* are sialokinin I and II. The gene encoding sialokinin I has been characterized; not surprisingly it was found to be expressed only in female *Ae. aegypti*, specifically in the medial lobe of the salivary gland (17). The activity of these two compounds was demonstrated because of their ability to

cause contraction of smooth muscle and endothelium dilation (46); similar vasodilators have also been described in *Culex* mosquitoes (169). Sialokinin I and II are neuropeptides that have been sequenced and demonstrated to have smooth muscle contracting activity similar to the mammalian tachykinin (46). Sialokinins act directly on the endothelium, activating production of nitric oxide, which activates guanylate cyclase in cells resulting in vasorelaxation (281). Sialokinins have also been implicated in modulating immune responses in flavivirus susceptible mice (309).

Anticoagulants. Hemostasis is the response that controls the loss of blood following the injury of the blood vessel. It consists of platelet aggregation, blood coagulation and vasoconstriction (222). Platelet aggregation is one of the first lines of defense against blood loss. Activated platelets release vasoconstrictor components and ADP to form the platelet plug that serves as a template for the blood coagulation cascade (280). The blood coagulation cascade involves the launch of proenzymes activating thrombin, which cleaves fibrinogen into fibrin; the polymerization of fibrin results in blood clot formation (280). In this regard, mosquitoes have evolved the ability to produce salivary proteins that counteract the effect of hemostasis, either by inhibiting platelet aggregation or by inhibiting steps in the blood coagulation cascade (259, 280). Salivary anticoagulants from blood feeders target specific proteases or complexes of the blood coagulation cascade (281). For the mosquito *Ae. aegypti* a specific protein secreted by females with a molecular weight of approximately 46 kDa has been implicated in the inhibition of the factor Xa of the coagulation cascade (258) as well as a serpin protein that might have anticlotting effects (284). In *Anopheles* mosquitoes the presence of factors that affect the coagulation cascade has also been reported. *An. albimanus* was shown to produce an anti-

thrombin factor (283), whereas in *An. stephensi* the responsible protein was shown to bind Factor XII and kininogen; this protein was reported to be a 16 kDa protein named hamadrin (108). Hamadrin affects activation of the plasma contact system by inhibition of the activation of factor XII and kalikrein (108). Another way found to interfere with hemostasis in the host by mosquito saliva is the release of proteins with antiplatelet aggregation properties (280). Platelets have a central role in hemostasis. When blood vessels are lacerated platelets come into contact with collagen present in subendothelial structures, the role of collagen consists in adhering to platelets in order for hemostasis to occur. The adhesion of platelets to the collagen exposed on endothelial cell surfaces is mediated by von Willebrand factor (vWF). The function of vWF is to act as a bridge between a specific glycoprotein on the surface of platelets and collagen fibrils. It is known that platelets are rich in ADP, ATP and serotonin (218). ADP induces platelet aggregation and serotonin induces vasoconstriction; blood contact with collagen therefore induces platelets to obstruct the injured vessel and also causes the vessel to constrict (218, 281). One of the main proteins identified so far in mosquitoes that can be related to platelet aggregation inhibition is apyrase (280). Apyrase is present in at least four mosquito genera, *Aedes*, *Anopheles*, *Culex* (216) and *Ochlerotatus* (210). The biological activity of apyrase has been reported to affect the platelet aggregation induced by ADP and thrombin (218, 265). Apyrase has also been related to the location of blood vessels during mosquito probing (225). The apyrase protein of the mosquito *Ae. aegypti* has been studied in depth; it belongs to the 5'-nucleotidase family (47), which are enzymes that catalyze the degradation of ADP and ATP to AMP (226, 259). The genes that code for the apyrase proteins of several mosquito species have been thoroughly studied and

described (34, 219, 284). A recombinant form of the *Ae. aegypti* apyrase (rAed 1) has been generated and studied (198, 199, 265). Apyrase is believed to be important in blood feeding because its activity is inversely proportional to probing time in mosquitoes (216, 259); nevertheless, mosquitoes can achieve a blood meal even in the presence of host high titered antibodies against apyrase (162). Interestingly in *Culex quinquefasciatus* a protein similar to apyrase that inhibits the platelet activating factor (PAF) has been reported (221).

Immunomodulators. Modification of the immune response by salivary components of blood feeding arthropods has been documented (79, 280). Inflammation, for instance, is the response to localized injury involving cells like neutrophils, monocytes and lymphocytes as well as various mediators such as chemokines, plasma enzymes, lipid inflammatory mediators and cytokines (81, 110). One of the main effects of mosquito feeding in the mammalian host is to redirect the immune response towards a Th2 immunity, downregulating important pro-inflammatory cytokines such as IFNγ (245, 309), increasing IL-4 production and, generating allergic reactions (51). It has also been demonstrated that mosquito feeding generates local infiltration of neutrophils, eosinophils and mast cells into the site of feeding (147). Mosquito saliva contains an extensive array of proteins that inhibit T and B cell activation (56, 294). The local immune response at the site of feeding has been shown to be affected by mosquito saliva (190) which also temporarily inhibits the inflammatory response (22, 56, 294). A systemic effect of mosquito saliva has also been reported, especially downregulation of cytokines (22, 56, 288, 294, 309) and antigen specific immune response (59). Mosquitoes also have the ability to modify local reactions by removing adenosine, which is associated with pain

perception and induction of mast cell degranulation in vertebrates. At the same time the presence of inosine in the mosquito saliva may potentially inhibit production of inflammatory cytokines (220). The proteins present in the mosquito saliva have been identified using novel genomic approaches such as cDNA generation and cDNA libraries have been used to identify possible secreted proteins from mosquito salivary glands (34, 219, 284). Some of these proteins have been reported to be immunomodulators that facilitate the survival of mosquitoes. New approaches are being investigated to see if these specific proteins are potential immunogens that can be useful for vector control by use as subunit vaccines that integrate the vector salivary proteins along with the pathogen's immunogenic proteins (275).

The D7 family of mosquito salivary proteins.

The D7 family of proteins that are expressed exclusively in the female mosquitoes that feed on blood was first described in *Ae. aegypti* (109). Since then D7 related proteins have been found in *Anopheles* (8, 267) and *Culex* mosquitoes (161, 219). The D7 family was first reported to be a large family of proteins related to the odorant binding proteins (9). There are two forms of the D7 family that have been described in mosquitoes, the D7 long (30-36 kDa) and the D7 short (15-20 kDa) proteins (282). The short form of the D7 proteins lacks a portion of the aminoterminal region compared to the long form, although both (short and long) forms of the protein share conserved cysteine residues (282). Exceptions to the long and short forms of D7 proteins are found in *An. gambiae* and *An. darlingi*. For instance *An. gambiae* lacks the long form and *An. darlingi* was found to possess only one short D7 protein (282). *An. stephensi* was found to have a long form of the D7 protein similar to that present in *Ae. aegypti* and at least one of the shorter forms

related to the one found in *An. gambiae* (267). In spite of these proteins being expressed exclusively in females and probably having a role in blood feeding (9, 32, 109, 161, 265, 282), the function of these proteins remains largely unknown. The use of recombinant proteins has revealed that some of the *Aedes* D7 proteins are previously identified allergens (51, 198, 200, 201). Although allergy is associated with glycosylated proteins (5, 20) there is no evidence that the D7 family of mosquito proteins have this characteristic (Dr. Jesus G. Valenzuela, personal communication). Nevertheless, *Aedes* proteins with similar molecular weight to those reported as D7 members have been shown to be highly immunogenic, especially in allergic patients (191, 197, 212). The D7 proteins have been classified as members of the odorant/pheromone binding proteins (82). This insight suggested a new function for these proteins and recently this group of abundant proteins from the mosquitoes *Ae. aegypti* (long form) and *An gambiae* (short form) have been demonstrated to bind biogenic amines such as serotonin, histamine, epinephrine and norepinephrine (33). Serotonin is a mediator released during platelet aggregation; it increases vascular permeability and induces vasoconstriction. Histamine is released from injured tissue and mast cells during inflammation; epinephrine and norepinephrin are vasoconstrictors (81). The sequestration of biogenic amines during mosquito feeding could serve an important function to inhibit platelet aggregation, vasoconstriction and inflammation (33).

Evidence of enhancement of viral pathogenicity by mosquito salivary proteins.

As described previously, mosquito salivary proteins can regulate the host immune response (280) both locally (147, 190) and systemically (22, 309). The down regulation of the immune system might help arthropod-borne pathogens to infect the vertebrate host

(280). The relationship between mosquito salivation and virus transmission has been reported (105, 259). Virus replication takes place in salivary glands of the mosquitoes and then viral particles are released with the saliva during feeding (269). This fact has been related to the high transmission rate of viruses even by a single mosquito (133). Mosquito salivary gland treatment of mouse fibroblast cells increased vesicular stomatitis virus growth kinetics by modulating the secretion of IFNα/β. This regulation of the type I interferons is critical in the transmission and pathogenesis of vesicular stomatitis virus (148). Flavivirus infection of mammals and birds by mosquito bite results in more aggressive infection and higher viremia titers than needle inoculation of the virus (244, 264). The enhancement of viral infection by mosquito bite has been documented in Bunyaviruses (68, 188), Rhabdoviruses (149), Flaviviruses (244, 264) and Alphaviruses (255). Surprisingly natural killer cells treated with salivary gland extracts have been linked to Epstein-Barr virus oncogenesis by a not well understood mechanism. It was observed that in patients infected with EBV, CD4+ T cell responded to certain mosquito salivary gland extract could induce reactivation of latent EBV infected NK cell oncogenesis (11, 12). Although there is not universal enhancement of viral pathogenesis by mosquito salivary proteins (1, 209, 255), most evidence indicates that vector-borne virus pathogenicity is increased when they are delivered by the natural route of infection.

Flaviviruses.

The Flaviviruses are divided into three genera; the genus Flavivirus, the genus Pestivirus and the genus Hepacivirus (263). The Flavivirus genus consists of nearly 80 viruses, many of which are arthropod-borne human pathogens (153). Flaviviruses cause a variety of diseases, characterized by fever, encephalitis and hemorrhagic fevers (153,

263). The Flavivirus genus has been divided into three major groups according to their ability to be recognized by specific antibodies; the three groups are the Japanese encephalitis serocomplex, which includes West Nile virus (WNV), Kunjin virus (KV), Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV) and Japanese encephalitis virus (JEV); the dengue serocomplex which includes dengue virus type 1 (DENV1), dengue virus 2 (DENV2), dengue virus 3 (DENV3), and dengue virus 4 (DENV4); and the yellow fever serocomplex which includes the yellow fever virus (YFV) (153, 263). Other members of the Flavivirus genus include those transmitted by ticks. The tick-borne Flaviviruses include louping ill virus, Powassan virus, the tick borne encephalitis viruses, including Central European encephalitis and far eastern encephalitis virus complex and Rio Bravo virus (153). The other genera of the Flavivirus family are the Pestiviruses, such as bovine viral diarrhea virus (BVDV), and the Hepaciviruses, such as hepatitis C virus (HCV). These have not been adapted to be transmitted by arthropods (153, 263).

Structure of the virion.

Flavivirus particles appear spherical in transmission electron microscopy, with a diameter of 40 to 60 nm. The main structural proteins associated with virions are the envelope protein (E), the membrane protein (M) and the capsid protein (C). The E protein is the major surface protein and probably mediates attachment and entry by virus-cell membrane fusion. The E protein is part of a lipid envelope and rather than forming a spike-like structure it is extended parallel to the viral surface (4, 213). The M protein is a small proteolytic fragment of prM, which is important for maturation of the virus into the infectious form (153).

It is thought that flaviviruses attach to the surface of the host cell through an interaction of the E protein with one or more receptors. E protein is also immunogenic and plays a role during host immune response to flaviviruses (260). Although no flavivirus cell-receptor has not been identified, many cell receptors have been proposed to be responsible for the virus-cell binding (50, 57, 77, 207, 214, 215). After binding viruses are taken up by receptor-mediated endocytosis. Fusion of the virion envelope with endocytic membranes follows conformational changes of the E protein to express fusiogenic domains. Then the nucleocapsid is dissembled, the RNA genome is liberated into the cytoplasm of the cell, and replication begins (153, 263).

Flavivirus genome and replication.

The flavivirus genome consists of a single stranded positive sense RNA about 11 kilobases in length, containing a 5'terminal cap (m^7G5' ppp 5'A) but lacking a polyadenylic acid tail at the 3' nontranslated end (153). The 3' end of the flavivirus genome can form secondary structures that confer stability to the RNA (263). The cytoplasm of the host cell is used for translation of the viral genome to a single polyprotein, which is further processed by host and viral proteases. The processing of the polyprotein results in 10 viral proteins; these are known as C, prM/M, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. A host signal peptidase is responsible for the cleavages between C-prM-E and, E-NS1. The viral NS2B-NS3 serine protease is responsible for the cleavage between NS2A-NS2B, NS2B-NS3, NS3-NS4A, NS4A-NS4B and NS4B-NS5 (263). NS2A-NS3 also cleaves the signal sequence at C-terminus of capsid. The enzyme responsible for the cleavage between NS1-NS2A is still unknown (153), although evidence suggests that NS3 might have some role in this processing

(128). After translation, prM-E form heterodimers; these formations are important for the assembly and secretion of viral particles (152, 205). The flavivirus particles assemble in the ER and then pass through the host secretory pathway where maturation takes place (163, 193, 307). The nonstructural proteins assemble to form the viral replication complex, in which the positive-sense RNA serves as a template to generate the complementary negative-sense strands. During the first step of replication the positive strand viral genome binds to the viral replicase complex at the 3'non-translated region (NTR) to produce the negative sense intermediate, these intermediates are the templates to synthesize the positive sense viral genome. The 3' end of the positive sense RNA genome is different from the 3' end of the complementary minus strand; this characteristic is important to regulate transcription or translation interactions (153, 203, 204). In tick-borne viruses this phenomenon might be slightly different due to the position of the loops in the 3' NTR of tick-borne viruses (122). Early in viral infection negative and positive-strand RNA synthesis occur in equal rates but this ratio changes over time, favoring the positive-strand synthesis (131, 279). The genome of flaviviruses has conserved and essential motifs that are important for viral replication. Cis-acting elements in the 5' and 3' untranslated regions anneal, causing RNA cyclization and allowing the genome to replicate (87, 122, 273, 312); equally important are the short and long stem-loops at the 3' non coding region, which are critical for infectivity and replication of flaviviruses (122, 274, 311)

Dengue virus (DENV).

Dengue viruses belong to the genus *Flavivirus* in the family *Flaviviridae*; they are placed into the dengue serocomplex, which includes DENV 1, 2, 3 and 4 (153). Dengue

is the most significant mosquito-borne viral disease affecting humans. Up to one third of the world population is considered to be at risk to contract dengue infection (74). The main vector of dengue viruses is the mosquito *Ae. aegypti* whose distribution correlates with epidemic cases of dengue (figure 1.3) (263).

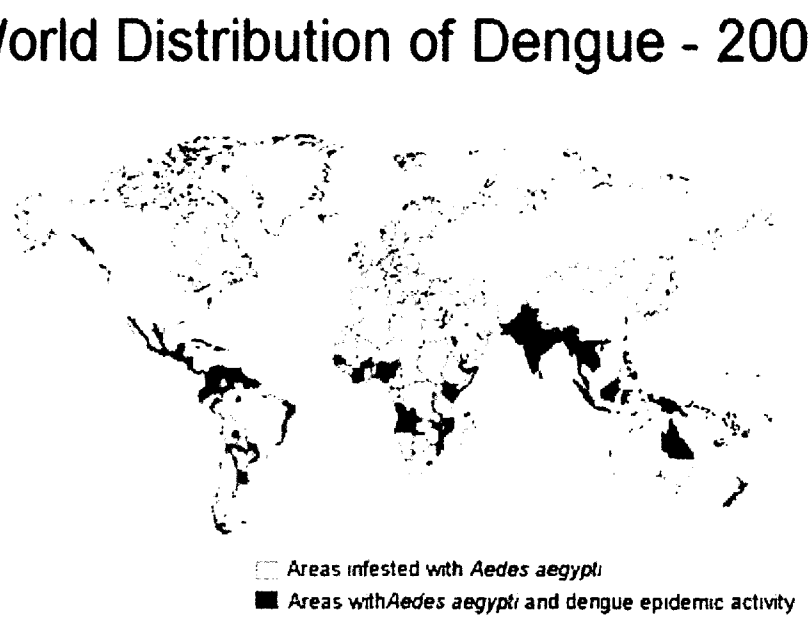

Figure 1.3. World distribution of dengue viruses and the main vector *Ae. aegypti*. The map shows the distribution of areas infested with *Ae. aegypti* (yellow) and areas where *Ae.aegypti* and dengue virus overlap (red). Places shown in yellow are at risk of DENV outbreaks (36)

In tropical Asia and West Africa a sylvatic cycle of DENV transmission involving non-human primates has been demonstrated. The implication of that sylvatic transmission of DENV on the epidemiology of DENV infection in human settings is not clear (173). After the infection of a susceptible person there is an intrinsic incubation period that ranges from 7 to 10 days before onset of symptoms. Since the viremic phase lasts around a week after disease onset, a feeding mosquito can be infected during this period. If the

mosquito is infected, the virus begins an extrinsic incubation period in the vector that lasts around 10 to 14 days, after which the virus can be transmitted to another human host as shown in figure 1.4 (129). DENV infection may result in a spectrum of clinical manifestations ranging from asymptomatic infection through dengue fever (DF) to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (296), which are severe life threatening complications typified by vascular leakage (228, 237). Dengue epidemic activity occurs throughout the tropical and subtropical regions of the world, with most cases occurring in South-East Asia and Central and South America (74, 237, 263). Increased travel, reinfestation of the American tropics by *Ae. aegypti*, the lack of proper mosquito control in dengue epidemic countries, lack of public health infrastructure required to deal with vector-borne infectious disease and wide-spread unplanned urbanization are some of the reasons to blame for the increase in the transmission of this mosquito-borne viral disease (84).

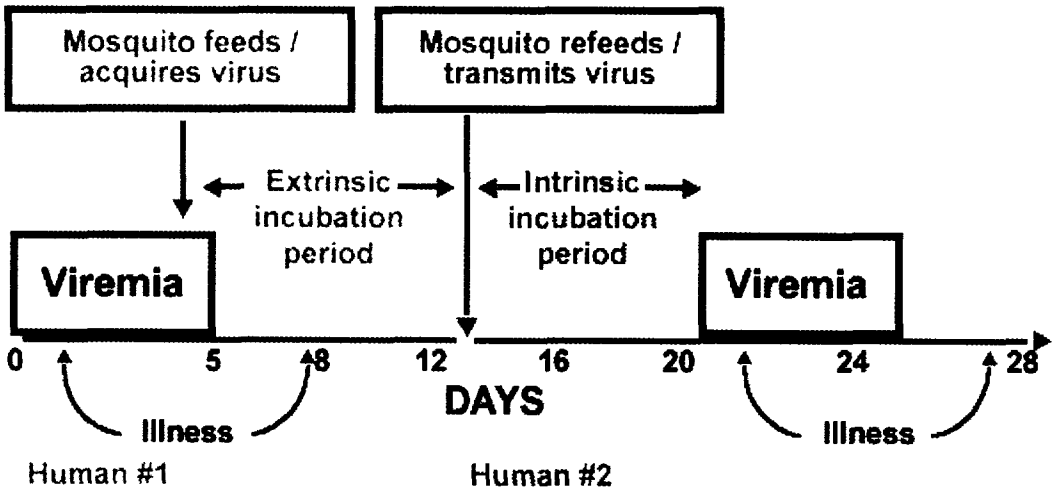

Figure 1.4.Dengue virus cycle in mosquitoes and humans showing the extrinsic incubation period (blue section) and the intrinsic incubation period (red section) (See text for details) (37)

Clinical signs and symptoms.

There are two main clinical manifestations of the disease. Dengue fever, which is a mild self limited disease is characterized by abrupt onset of high fever that lasts for 2-7 days, chills, headache with frontal and retro-orbital pain and rash. Clinical symptoms include severe myalgia and arthralgia (break-bone), retro-orbital pain, pruritis, and in some cases rash. Dengue fever is a severe, flu-like illness that affects infants, young children and adults, but seldom causes death. The clinical features of dengue fever vary according to the age of the patient. Infants and young children may have a non-specific febrile illness with rash. Older children and adults may have either a mild febrile syndrome or the classical incapacitating disease with abrupt onset and high fever, severe headache, pain behind the eyes, muscle and joint pains, and rash. A more severe form of the disease is dengue hemorrhagic fever (DHF). DHF is characterized by loss of intravascular fluids and hemorrhage; this can progress from mild hemorrhage to circulatory failure, weak pulse, hypotension, undetectable blood pressure, shock and death (84, 296). In DHF bleeding occurs in the skin (petechiae) and occasionally in the gums and nose. Increased vascular permeability can result in leakage of plasma into extravascular spaces, leading to plasma leakage. Dengue hemorrhagic fever is a potentially deadly complication that is characterized by high fever, hemorrhagic phenomena--often with enlargement of the liver--and in severe cases, circulatory failure. The illness commonly begins with a sudden rise in temperature accompanied by facial flush and other non-specific constitutional symptoms of dengue fever. The fever usually continues for two to seven days and can be as high as 40-41°C, possibly with febrile convulsions and hemorrhagic phenomena. In moderate DHF cases, all signs and

symptoms abate after the fever subsides. In severe cases, the patient's condition may suddenly deteriorate after a few days of fever; the temperature drops, followed by signs of circulatory failure, and the patient may rapidly go into a critical state of shock and die within 12-24 hours, or quickly recover following appropriate volume replacement therapy (298).

Dengue pathogenesis.

The pathogenesis of DF, DHF and DSS has not been clarified but it is likely a complex combination of events are determinants for the severe outcome of this disease (295).Once deposited in the host's skin by the mosquito vector, the virus is taken up by the Langerhans dendritic cells (DCs) (150, 158, 192, 299). After that, Langerhans cells migrate to regional lymph nodes where they stimulate T-cells (74). Viral infection converts DCs from immature into mature cells that migrate and present viral antigens to naïve T lymphocytes. Once in the lymph node DENV probably infects macrophages, B cells, and follicular DCs. Replication occurs in the lymphoid cells, inducing the entry of viral particles to the circulation through the efferent lymphatic and thoracic duct, resulting in the primary viremia; then, virus replicates and infects peripheral blood mononuclear cells (PBMC) and circulating macrophages. Virus replicating in these target cells is liberated into the blood for a secondary viremia (236, 237). A number of risk factors have been proposed for the progression of dengue infection to the most serious form of disease (73, 95, 116, 140, 155, 271); among others the virus strain and its dissemination capability are important for dengue infection and pathogenesis (6, 10, 54). Several hypotheses have been proposed to explain the occurrence of DHF and DSS. The most important of these hypotheses include: 1) Antibody-dependent enhancement (ADE),

which means that by preexisting immunity in the host to any of the four dengue serotypes is a risk factor to develop DHF and DSS in secondary infections with a different serotype. While infection with one dengue serotype confers immune protection to that specific serotype, the risk of being infected with another serotype remains; therefore the risk increases to contract DHF, which is thought to be the result of immune pathological events (92, 183) related to antibody-dependant enhancement of infection (ADE) (91). Evidence for ADE is the demonstration that in populations where pre-existing antibodies against the previous dengue serotype cross-react but do not neutralize the recent viral serotype, therefore, infection is enhanced (103, 304). ADE has been explained by formation of DENV-antibody complexes that bind via the Fc portion of the antibody to FcγRI and FcγRII bearing cells such as macrophages and B-cells, which take up the virus-antibody complexes. Inside the cells the virus escapes the phagocytic vacuole and infects the cells, allowing infection in greater numbers, which results in higher viral loads (27, 154). High viremia titers and antibody response patterns have been correlated with DHF manifestations (145, 286). Dengue virus-antibody complex formation has also been related to viral pathogenesis, specifically to bind receptors on mast cells (27) and an increase in hemorrhagic manifestations that correlates with presumably non-neutralizing IgE antibodies against dengue viruses (126, 171). In addition, it has been observed that cellular immune response can increase viral pathogenicity that has been associated with hemorrhagic manifestations (14, 83, 93, 236, 237). Besides ADE, another theory to explain severe disease during dengue infection is the more virulent strain hypothesis. The more pathogenic strain hypothesis is supported by the fact that epidemics associated with a particular virus genotype have been consistent with the presence of DHF in primary

infections (13, 179). Clearly some strains of dengue virus have been related to more severe disease. For example when the DENV2 Jamaica strain, considered an Asian genotype, was introduced into America, epidemics of DHF occurred in Cuba, Brazil and Venezuela, where DENV2 Puerto Rico strain had previously circulated with no evidence of DHF (227). When studies were conducted to identify the main factors associated with an increase in DHF in the Americas, it was suggested that dengue viruses classified in the Asian genotype were rapidly replacing the dengue viruses of American genotype. The Asian genotype viruses were associated with the increase in the severity of disease in humans (227) and Asian viruses were also shown to replicate better in the mosquito vector (54). Studies to identify main determinants of viral virulence revealed that viruses related to hemorrhagic disease had differences in nucleotide sequence in the viral genome. For instance, DENV2 (the serotype most associated with hemorrhagic epidemics) was found to have several structural differences when compared to those DENV2 isolated from patients with mild DF, these included six encoded amino acid differences in prM, E, NS4 and NS5 genes and sequence differences within the 5' and 3' NTR, which were predicted to modify viral RNA secondary structure (143). Amino acid substitution within the coding region of viruses may affect cell attachment and other virus functions and nucleotide changes in the NTR's affecting secondary structure of viral RNA could increase viral replication and ultimately increase severity of disease (143). This latter hypothesis was confirmed when higher peak titers of DENV were associated with more severe disease in humans (286).

DENV pathogenesis has been related to exacerbation of the host immune response. One mechanism proposed to increase pathogenicity is the release of cytokines that increase

vascular permeability, and dengue shock syndrome (DSS). DSS may result from overproduction of inflammatory cytokines such as IFNγ, TNFα and IL-2 (45, 74, 237), although there are reports that IFNγ and IL-2 cytokines are actually higher in patients with DF, whereas patients with more severe disease showed increases in IL-4, IL-6 and IL-10 with a lesser production of IL-2 and IFNγ (48). It has also been reported that, at least for primary dengue infection, IFNγ mediated immunity is crucial during early clearance of DENV infection in mice (248). The observation of cytokine over-production during DENV infection led to the assumption that endothelial permeability is not induced directly by the virus itself (142), but rather is induced by other mediators such as TNFα (53) or by the effect of cellular matrix rearrangement during migration of infected dendritic cells (159). Another effect of DENV infection that leads to increased vascular permeability is thrombocytopenia. This has been described as a result of immune related clearance of virus, initiated by antivirus antibodies that cross-react with platelets (151), which might activate the complement system. The complement system has also been related to DHF during DENV infection by the cross-reactive antibodies; these antibodies bound to platelets activate complement, which triggers the membrane attack complex (MAC) destroying platelets (151, 236, 237).

West Nile virus (WNV)

West Nile virus (WNV) belongs to the genus *Flavivirus* in the family *Flaviviridae*. The virus has been placed in the Japanese encephalitis serocomplex (153). WNV was first isolated in the West Nile district of Uganda in 1937, from the blood of a febrile woman (257). Since then, activity of WNV has been reported in Africa, Middle East, India, central Asia, Australia and Europe (89, 181). In 1999, WNV was isolated for the

first time in the Western hemisphere in New York City (39, 41). This virus was closely related to WNV circulating in Israel, but the means of its introduction into the U.S.A remains speculative (137). Based on genome sequence alignments there are four main lineages of WNV. These are WNV linage 1, which is considered to be the most pathogenic and is the one which contains the New York virus, and linage 2, which has been associated mainly with bird infections and causes only sporadic clinical infections in humans (135, 137). Recently lineages 3 and 4 have been reported as an evolutionary trend of the original virus based on amino acid substitution and increased pathogenesis in birds (25).

Since the first report in 1999, WNV has spread throughout the U.S, and by 2006 was affecting humans, birds horses, and other vertebrates in the continental U.S.A (figure 1.5) (42, 66, 231), Canada (55, 268), and Mexico (23, 69, 72, 157). Antibodies against WNV in horses have been reported recently in Guatemala (177), and horse viral infection was detected in Argentina (178).

WNV is maintained in nature by a mosquito-bird-mosquito transmission cycle (263). Humans and horses are incidental hosts of the virus and they are considered to be dead-end hosts, because neither humans nor horses are capable of sustaining high enough viremia titer to infect the mosquito vector (263). The most common risk factor for acquiring WNV infection is the exposure to infected mosquitoes (98). WNV is mainly transmitted by *Culex* species mosquitoes. In Europe, the main vector reported is *Cx. pipiens* (310). In North America, there are several vectors that have been implicated in the transmission of WNV; those include *Cx. pipiens* (northern house mosquito), *Cx. quinquefasciatus* (southern house mosquito), and *Cx. tarsalis* in the western U.S.A (276).

Other WNV-vector species include *Cx. salinarius* and *Cx. nigripalpus*, these last mosquito species seem to be important vectors, but their vectorial capacity has been related to their relative abundance (276). Less common means of WNV transmission have been reported, such as blood transfusion (176), organ transplant (132, 256), breast feeding (40, 298) and probably aerosolization (43).

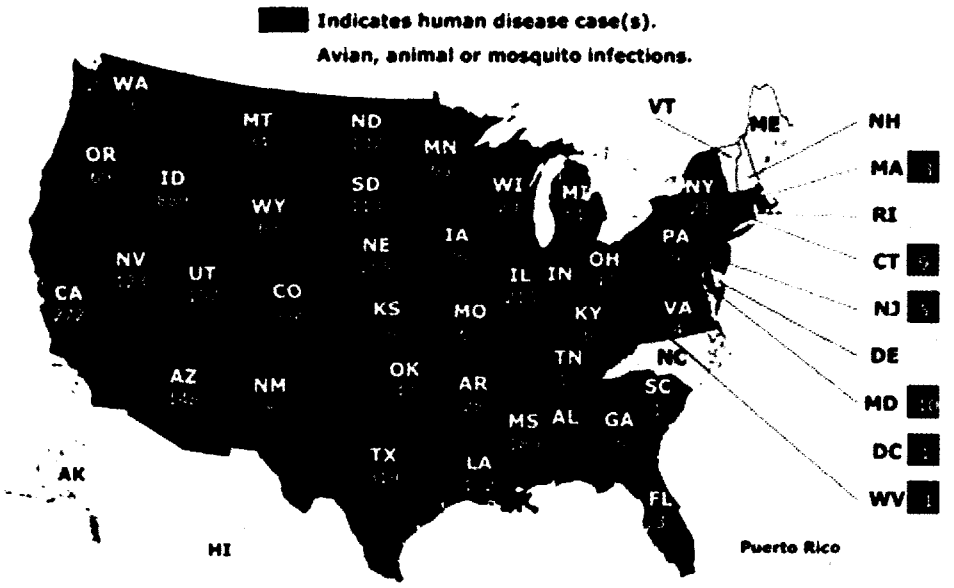

Figure 1.5. The WNV activity in the U.S.A during 2006 showing human disease, animal disease and mosquito transmission activity in the continental U.S.A as of December 11 2006 (38).

The intensity of WNV transmission is determined and conditioned primarily by the abundance of competent mosquitoes and the prevalence of infection in those mosquitoes (98). *Cx. tarsalis* is an important vector for WNV with an estimated transmission rate of 81% to 91% (278). Some *Aedes* and *Ochlerotatus* species have been described as vectors of WNV under controlled laboratory conditions (276). Among the *Aedes* species that have been described as poor or refractory vectors for WNV are *Ae. vexans*, *Ae. aegypti* and *Ae. taeniorhynchus* (277). *Ae. albopictus* has been suggested as a

potential vector for WNV in the USA (242). Other potential vectors of WNV are ticks. In laboratory conditions, *Argas arboreus* was shown to transmit WNV to chickens (139) as was the seabird soft tick *Carios capensis* (106). Soft and hard ticks can become infected, maintain, and transmit WNV under laboratory conditions, but their role as WNV vectors needs to be further studied, although they are unlikely to play a substantial role in WNV transmission (98).

Birds are the natural reservoirs and amplifying hosts of WNV (35, 98). A large number of bird species in America have been infected with WNV experimentally (125) and naturally (123, 124); some of the birds that have been found to be susceptible to infection by WNV include Passeriforms, Charadriiforms, Stringiformes, and Falconiforms (125). The most important bird species that develop viremia titers high enough to be infectious to mosquito vectors include certain passerines like grackles (*Quiscalus quiscula*), house finches (*Cardopacus mexicanus*), and common house sparrows (*Passer domesticus*), corvids like the American crow (*Corvus brachyrhynchos*) and blue jays (*Cianocitta cristata*). Corvids have been reported to be highly infectious to mosquitoes and also to have higher mortality rates (98, 125), however in a recent report corvids were found to have high seroprevalence to WNV and low mortality (19).

Clinical signs and symptoms

WNV seems to be particularly virulent in avian species belonging to the Corvidae family, including American crows and blue jays, which make these birds important for WNV surveillance activity in the dead-bird surveillance system (35). Recently WNV has been also reported to be highly infectious in waterfowl (164). In birds, WNV infection affects the heart, kidney, spleen, gut, adrenal glands, liver, and

brain (58). Infection of organs was detected in spleen, kidney, skin, heart, brain, and eye. The most common histopathologic lesions were subacute myocarditis and encephalitis. More severe disease conditions included arteritis associated with tissue degeneration and necrosis (185).

In mice clinical signs are characterized by the appearance of a rough hair coat, lethargy, apathy, incoordination, segregation from the group, difficulty in swallowing, and prostration; histopathological analysis revealed glial-microglial nodules, satellitosis (defined as neurons surrounded by microglial cells and macrophages), and perivascular mononuclear infiltrates. Immunohistochemical staining demonstrated that macrophages and fibrocytes of the skin were positive for WNV. Few macrophages were detected as positive for WNV in spleen and some epithelial tubular cells of kidney contained viral antigen. In the brain, multifocal areas with some neurons from the olfactory bulb stained positive for WNV, virus was also detected in spinal cord, brainstem, and cerebral cortex. Findings included moderate multifocal mononuclear perivascular infiltrates as well as moderate multifocal gliosis. Inflammatory lesions were found in the cerebral cortex, hippocampus, brainstem and spinal cord. The inflammation was characterized by multifocal perivascular mononuclear infiltrates, composed mainly of macrophages and lymphocytes with scant neutrophil infiltration and neuronal necrosis (75).

Clinical signs and symptoms of WNV infection in humans are more severe in the elderly and immune compromised individuals. The mild disease includes fever, headache, and fatigue; skin rash on the trunk of the body, swollen lymph nodes and eye pain are also described. When the disease progresses to the more severe form, manifestations include gastrointestinal symptoms, ataxia and extrapyramidal signs, optic neuritis, seizures,

weakness, changes in mental status and myelitis (58). A minority of patients with severe disease develop a maculopapular rash involving the neck, trunk, arms, or legs. In severe affection of the CNS, flaccid paralysis is present and pancreatitis and fulminant hepatitis have been described (44, 97). CNS infection with WNV can cause brain damage with long term sequelae, including changes in cognitive status, confusion, loss of concentration, memory, understanding, and decision making were reported (86).

Pathogenesis of West Nile virus

Host and viral genetics influences WNV neuroinvasion and pathogenesis (15). After mosquitoes feed on a bird with high viremia titer (5.0 to 7.1 $\log_{10}$PFU/ml), the virus infects, replicates and after that begins an extrinsic incubation period of 7 to 10 days, after which the mosquito becomes infective (98, 263). Once the infectious mosquito feeds on a vertebrate host, WNV replicates in the skin Langerhans cells (240) which transport the virus to the lymph nodes. After replication there is a primary viremia which disseminates virus to peripherical organs. After a replication in the peripherical organs, WNV disseminates in the blood to the central nervous system (CNS), where it infects neurons and astrocytes (65, 240). Free virus travels in the blood and reaches the brain thorough blood-brain barrier. The mechanism by which WNV crosses the blood-brain barrier is still unknown, although a number of theories have been proposed. It is thought that tumor necrosis factor alpha (TNFα) facilitates changes in the permeability of endothelial cells leading to CNS infection (292). WNV also travels in blood in infected cells of the immune system. CNS infection could occur via the infection of immune cells that traffic to the brain (76), or by axonal retrograde transport from infected peripheral neurons (104, 114). Infection or passive transport through the endothelium or choroid

plexus epithelial cells has also been proposed (240). Other possibilities are that aerosolized viral particles might infect olfactory neurons and spread to brain through the olfactory bulbs (174).Another proposed mechanism is the induction of TNFα secretion by Toll-like receptor 3. Increased production of TNFα has been related to blood-brain barrier permeability leading to early WNV entry into mouse brain (292). Higher viremia titers correlate with infection of brain (64, 239). Once the virus reaches the CNS it produces a set of changes in the protein expression in the infected neurons (61). Some changes in the neurons induce cell-death by apoptotic mechanisms (250). Several mechanisms of WNV-induced apoptosis have been proposed. During WNV infection, the NS2B-NS3 proteolytic complex contributes to cell apoptosis by triggering the extrinsic apoptotic pathway involving caspases 8 and 3 (206). Apoptosis can also result from WNV replication by up-regulation of BAX gene expression (193). Although the nonstructural proteins have been proposed to induce apoptosis in cells of the CNS, the capsid protein (C protein) of WNV is also capable of inducing apoptosis via the mitochondrial pathway resulting in activation of caspase 9 and downstream caspase 3 (302). Introduction of the capsid gene in the skeletal muscle caused cell death and inflammation, leading to the conclusion that C protein of WNV might be responsible for aspects of viral pathogenicity through induction of the apoptotic cascade (302). The envelope protein (E protein) has also been associated with pathogenicity of WNV. The E protein appears to be important for neuroinvasion, specifically the N-linked glycosylation site plays a role in WNV pathogenesis since mutations in this region of the E protein affected viral replication and tropism (16, 247). The NS1 protein of WNV has also been linked to viral pathogenesis; mutations described in NS1 (residues 130, 175 and 203) are

associated with lower viremia and decreased lethality (240). WNV infection consistently causes diffuse inflammation of the thalamus, the medulla, other parts of the brain stem and the proximal spinal cord, where perivascular inflammation and microglial nodules predominate (35). CD8+ lymphocytes predominate within these nodules (35). Interestingly CD8+ cells have been related to both recovery and immunopathology of WNV (293) and WNV immunopathology was increased when high viral loads were inoculated into mice. This correlated with higher infiltration of CD8+ cells, and damage to the CNS could be due to the cytotolytic effect of the CD8+ cells (293). This effect is directly generated by CD8+ perforin activity, which is essential to clear WNV infected neurons (251).

On the other hand, humoral immune response has been shown, along with complement activation, to generate a protective immunity against WNV (165). The antibody response, especially IgM, is essential to control early viral dissemination to the brain (64). This fact has generated a new insight in the possible use of antibodies as treatment for WNV infection in order to prevent viral dissemination and encephalitis (230). Specific antibodies generated against the E protein of WNV may decrease viral replication by interfering with viral attachment to cell surface or by preventing intracellular structural rearrangement of the E glycoprotein that would normally allow endosomal fusion (60). The 56 kDa E protein binds cellular receptors, mediates membrane fusion and elicits neutralizing antibodies (100, 101, 194, 230).

Although antibodies and B cell response during WNV infection are critical in the control of viral dissemination, this response is not sufficient to eliminate the virus from the infected host (289); therefore, the immune system of the host has to confront viral

infection by using both humoral and cellular responses. In this regard, the importance of type I interferons during viral infection has been suggested. IFNα/β and also type II such as IFNγ participate in the control of viral infection (119, 144), (252). IFNγ appears to function as an antiviral agent, possibly limiting infection in dendritic cells; therefore, the early decrease of viral burden prevents higher viremia titers and early dissemination of the virus to the CNS (252). IFN can affect virus replication through the double-stranded RNA-dependent protein kinase (PKR) signaling or by the 2'-5' oligoadenylate synthetase (2'-5' OAS) antiviral pathways (289). The PKR-mediated signaling pathway affects viral translation; the 2'-5' OAS prevents viral propagation by activating RNAse L, which degrades viral RNA. Also IFN prevents infection by inhibiting translation of the infectious viral RNA through a novel, PKR-independent mechanism (62, 239, 289). IFNγ is produced by natural killer (NK) cells and CD8+ T cells to inhibit virus propagation (81, 110). Recently it was demonstrated that γδ T cells are also responsible for IFNγ production and these cells are important in controlling WNV infection in animal models (291). Furthermore γδ T cells are an essential key to facilitate adaptive immune response against WNV(290); it has been shown that γδ T cells not only provide early defense against WNV infection but also they contribute to cell memory response (290).

Vaccine strategy using MSP to control vector-borne viruses

The importance of understanding the effect of MSP in host immune response is necessary to elucidate strategies to control vector-borne pathogens. Host immune response to vectors and the pathogens they transmit is an array of dynamic interactions; cytokines, antibodies, and cell-mediated immunity are critical elements concerning establishment of infection, disease progression and host defense (297). Understanding

host response to salivary proteins is an important key to generate new strategies to prevent host immune modulation in order to improve immunity to control vectors and pathogens transmitted by them. As was discussed in the sections of DENV and WNV, these pathogens are released in the skin of the host along with the salivary components of the vector, therefore is important to understand immune mechanisms in the host skin during pathogen damage. The skin is composed of three distinct compartments relevant to immune function; first is the epidermis composed of keratinized epithelial cells that function as an early warning system for immune response (130). The main cells of the immune system found in the epidermis are specialized dendritic cells (DCs) known as Langerhans cells and intraepithelial lymphocytes. The second layer of skin is known as dermis and includes dermal DCs, mast cells, and lymphocyte positive memory T-cells (figure 1.6) (130). Epithelial cell injury or pathogen invasion leads to the release of primary cytokines such as IL-1α and TNF from keratinocytes, antimicrobial peptides such as β defensin and nitric oxide are also released; in early warnings keratinocytes can also release IL-18 (IFNγ inducing factor) (261).

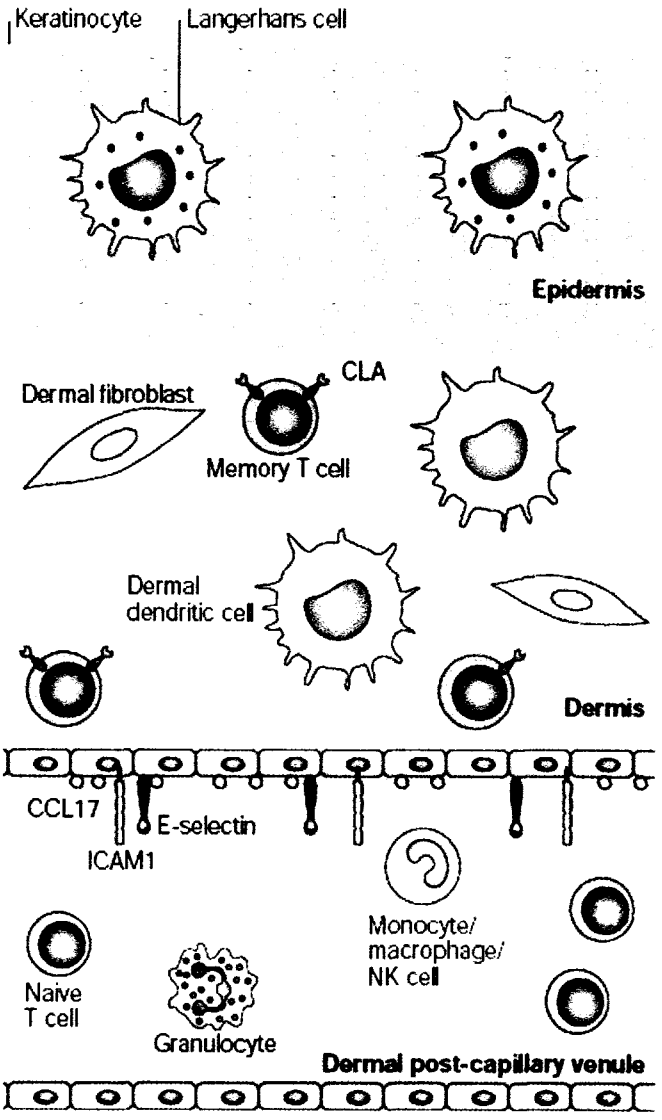

Figure 1.6 Immune-response in non-inflamed skin. First, the epidermis is composed of keratinized epithelial cells and functions as both a physical barrier and an early warning system. Immune cells resident in the epidermis include specialized dendritic cells (DCs) known as Langerhans cells and intraepithelial lymphocytes. Reproduced with permission (130)

The primary signaling of skin cells by pathogens stimulates a downstream activation cascade. Once the DCs are activated at site of pathogen invasion in the skin cells become activated by innate mechanisms including pattern recognition receptors and Toll-like receptors (TLRs). Langerhans cells and dermal DCs are stimulated to mature and emigrate from the tissue to a draining lymph node carrying antigen to present to naïve and memory T-cells (figure 1.7) (130). Presentation of antigen in regional draining lymph node results in production and distribution of antigen-specific effector memory T-cells expressing homing receptors that direct their migration to the tissue where the antigen was encountered (130),

Finally; immune surveillance triggers the long-term elements of the acquired immune response including the production of central memory and effector cells that are potentially directed to tissue other than the site of primary exposure (figure 1.8) (130).

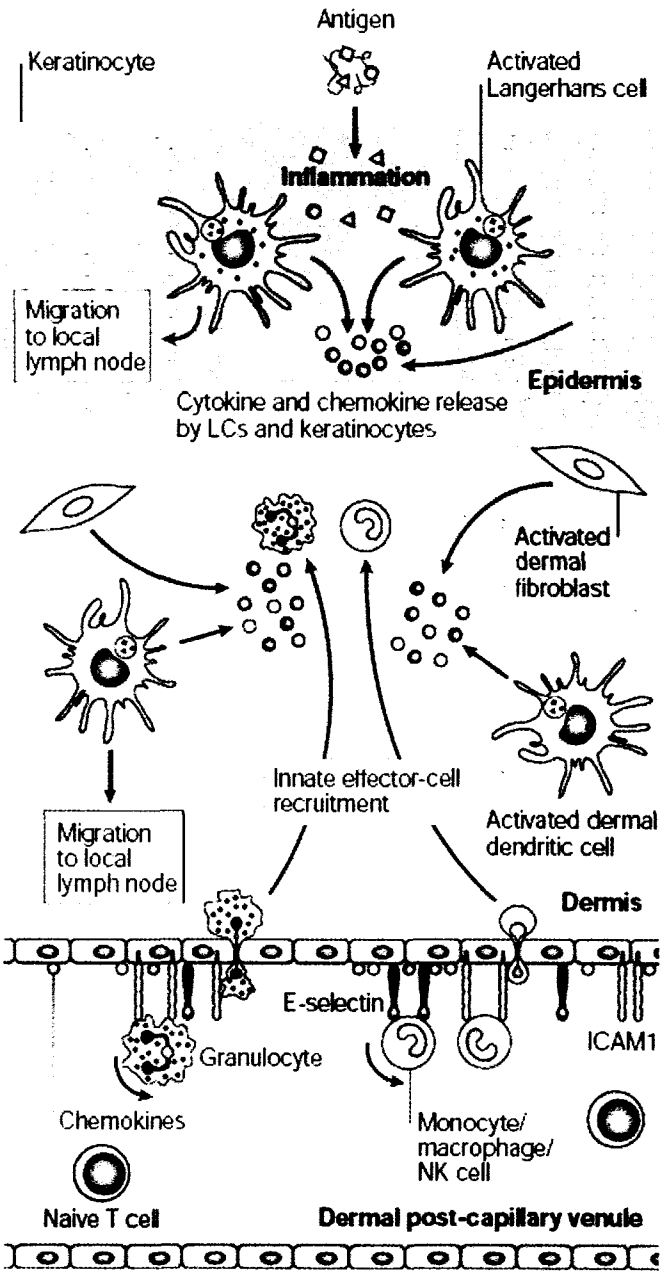

Figure 1.7 Innate immune mechanisms in the skin. Epithelial-cell injury or pathogen invasion leads to the release of primary cytokines and the activation of both skin cells (keratinocytes and fibroblasts) and resident innate immune cells (Langerhans cells (LCs), dermal dendritic cells (DCs) and mast cells), stimulating downstream activation cascades. Reproduced with permission (130)

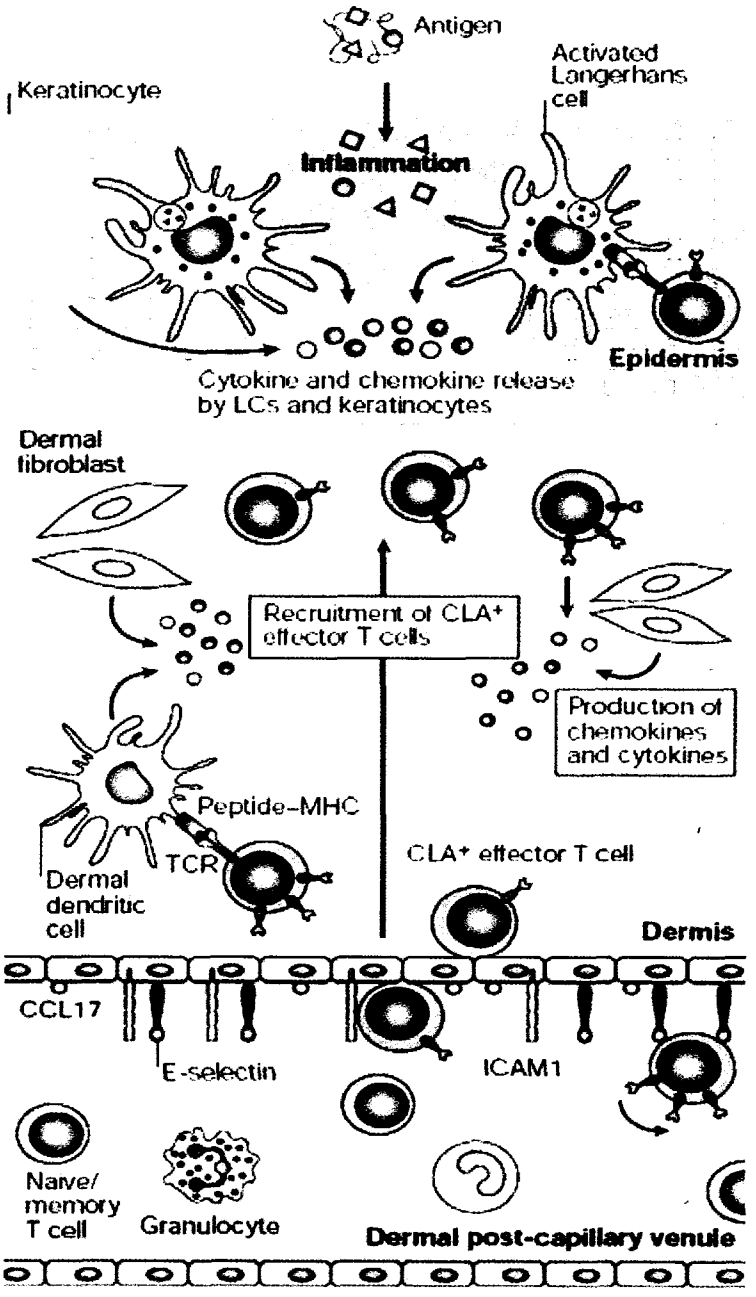

Figure 1.8 Adaptive immune responses in the skin. Circulating cutaneous lymphocyte antigen (CLA)-positive T cells represent a library of memory T cells with T-cell receptors (TCRs) specific for antigens previously encountered in the skin. Cytokines released by keratinocytes, fibroblasts and resident antigen-presenting cells stimulate the upregulation of expression of E-selectin and intercellular adhesion molecule 1 (ICAM1) through nuclear factor-κB (NF-κB)-mediated activation pathways. Reproduced with permission (130)

Immune responses to arthropod saliva stimulate a wide variety of host immune responses frequently characterized by allergies (195, 211). After mosquito feeding, histological sections of skin revealed infiltration of neutrophils, eosinophils, mast cells, lymphocytes and macrophages (147). In general, immune response to mosquito bites has been characterized in five stages; first is a period of sensitization with no observable skin reaction; second is a delayed skin reaction; third is the observation of immediate and delayed skin reaction, the fourth phase is characterized only by immediate reaction and finally the fifth phase is the loss of reactivity caused by possible desensitization (166).

It is known that mosquito salivary proteins are capable of eliciting immune response (195, 196) and also posses immunomodulators capable of affecting host immune response (79), which can be used a potential target for vaccine development in order to affect pathogen transmission (275). The two components previously mentioned are of great importance to generate strategies for vaccine development. Based in the previous observations two vaccine strategies have been proposed (280). The first strategy is the identificatin of immunosuppressors that affect the establishment of the parasite and prepare antibodies against these components to neutralize their activity; the second strategy includes targeting salivary proteins that generate strong immune responses and could indirectly kill the pathogen by improving host immune response.

Early studies have proven that using antigens derived from mosquito tissue are feasible to generate host immune response against those antigens and immunity against those antigens can have a great effect in mosquito survival and viability (2, 266). Recently it was demonstrated that pre-exposure of mice to non-infected *An. stephensi*

mosquitoes significantly decreased malaria parasite burden and improved immune response against the parasite and this could diminish parasite transmission (67).

It is clear that the use of immunogens based on MSP is an important field of study which must be addressed to find alternative methods to control vector-borne viruses.

Summary

Mosquito salivary proteins have been implicated in the enhancement of arbovirus infection. The hypothesis that led to the experiments summarized here was that immunity against salivary proteins of mosquitoes *Aedes aegypti* and *Culex tarsalis* affects pathogenesis of flaviviruses (dengue virus and West Nile virus).

Multiple attempts have been made to identify specific salivary proteins from arthropods in order to determine their function and role during blood feeding. Analysis of the interaction of specific mosquito salivary proteins and arbovirus infections has not been fully completed, including the effect of the host immune response to mosquito salivary proteins during flavivirus infection. Dengue viruses and West Nile virus are flaviviruses transmitted by *Aedes* and *Culex* species respectively, these viruses are also considered emerging pathogens in the Americas. In chapter 2 individual mosquito salivary proteins from *Aedes aegypti* and *Culex tarsalis* are identified and their possible roles in virus infection and the effects of salivary proteins on viral pathogenesis are discussed. A new technique using trichloroacetic acid to concentrate proteins is coupled with mass spectrometry analysis to identify proteins potentially implicated in viral pathogenesis. In chapter 3 the immune response against *Aedes aegypti* salivary proteins in a naturally exposed population of Thai children is investigated and differences in response to individual saliva proteins is associated with disease severity in DENV2

secondary infections. In chapter 4 the immune mechanisms by which vaccination of an animal model with *Culex tarsalis* salivary proteins modulates West Nile virus infection when animals were infected by natural route are investigated. Th1/Th2 cytokines expression was analyzed by Q-RT-PCR and flow cytometry, and viral disease was examined in order to understand the effect of anti-MSP antibodies during viral infection. Finally in chapter 5 the results of these experiments are summarized and a discussion of further work to be conducted is presented.

CHAPTER 2

IDENTIFICATION OF SPECIFIC IMMUNOGENIC PROTEINS FROM *CULEX TARSALIS* AND *AEDES AEGYPTI* SALIVA POTENTIALLY RELATED TO VIRAL PATHOGENESIS

INTRODUCTION

Mosquito saliva proteins (MSP) have been implicated in the enhanced pathogenesis of many viruses and other parasites transmitted by mosquito bites (68, 148, 229). MSP have the ability to regulate or alter the immune response of the host on which they feed (56, 147, 190, 288, 294, 309). This phenomenon may facilitate pathogen infection when injected along with the saliva of blood-feeding arthropods. Other functions of the MSP include vasodilation, anticoagulation, platelet inhibition as well as skewing Th1/Th2 responses (47, 108, 220, 258). To date, the components of mosquito saliva have been identified either by classical biochemical techniques involving purification from salivary gland extracts (9, 109) or by using complicated techniques to obtain specific components directly from the secreted saliva (24, 28, 196). Some research groups have identified several proteins from mosquito salivary glands by using a genetics/proteomics approach (7, 118, 156, 281, 301).

A straightforward method was developed to concentrate and identify specific proteins present in mosquito saliva. The precipitation, purification and analysis of specific proteins using trichloroacetic acid (TCA) have been broadly reported (49, 111, 182, 305). In this chapter TCA precipitation, and polyacrylamide gel electrophoretic fractionation and mass spectrometric analysis were used to identify a D7-related salivary protein from *Aedes aegypti* which is one of the most abundant salivary protein families secreted during mosquito feeding as well as one of the most immunogenic in populations naturally exposed to *Ae. aegypti* mosquito bite (see chapter 3) and *Culex tarsalis* tryptophan oxygenase, which is assosiated in mosquito with the metabolism of the amino acid tryptophan and also eye pigmentation. In mammals tryptophan oxygenase is related

to regulation of the immune response. Here I discuss potential implication in the pathogenesis of DENV and WNV of these two salivary proteins.

MATERIALS AND METHODS

Mosquitoes.

Two different mosquito species were used for these studies: *Aedes aegypti and Culex tarsalis.* The mosquitoes were reared in insectaries at the Arthropod-borne and Infectious Diseases Laboratory (AIDL), Colorado State University, Foothills campus with conditions of temperature, humidity and light/darkness periods presented in table 2.1.

Mosquito species	Temperature (°C)	Relative Humidity (%)	Photoperiod
Ae. aegypti	27-28	80	12:12
Cx. tarsalis	24-25	70	12:12

Table 2.1. Insectary conditions for rearing and maintaining two different mosquito species at AIDL . The *Aedes aegypti* strain used in these experiments was the Puerto Rico Rex-D. *Culex tarsalis* was the Bakersfield, California strain (208).

Mosquito eggs were hatched and allowed to develop in pans of water with food (approximately 25 grams of ground fish food in one liter of water) through the larval stages. At the pupal stage they were sexed by size. Approximately 250 female pupae were placed in individual containers, where sugar cubes and water were supplied *ad libitum.*

Salivation Induction.

At 4 to 5 days post emergence, sugar and water were removed for 24 hours. Mosquitoes were allowed to feed for 1 hour through a parafilm membrane on a water jacketed artificial feeder containing two milliliters of salivation buffer. The salivation buffer contained 164 mM NaCl and 10 mM $NaHCO_3$, according to the recipe provided by

Walter Reed Army Institute of Research (Jill Troyer, personal communication). The salivation buffer was warmed to 38°C and maintained at this temperature throughout the hour of feeding by a constant water flow.

Saliva collection and protein precipitation.

After one hour of salivation (mosquitoes probing the membrane), the buffer containing the saliva was placed in a 50 ml polypropylene sterile tube. Buffer containing MSP was mixed with 20% (w/v) trichloroacetic acid (TCA) in a 1:1(vol:vol) ratio by vortexing for 10 seconds. The solution was placed on ice and incubated for 30 minutes, and then aliquots of 1.0 ml were placed in 1.7 ml conical microcentrifuge tubes and centrifuged at 4° C for 15 minutes at 15000 rpm. The supernatant was discarded and the protein pellets were re-suspended in 0.3 ml of pure cold acetone (-20°C) and vortex mixed for 5 seconds. The suspension was centrifuged at 4°C for 10 minutes at 15000 rpm. After the second centrifugation the acetone was discarded and the protein pellets were dried in a vacuum centrifuge for 45 minutes at 30°C. If the pellets were not immediately re-suspended in 50µl of pure water they were stored at -20° C until use.

Protein quantification and fractionation by polyacrylamide gel electrophoresis (PAGE)

Protein pellets were re-suspended in 50µl pure distilled water with protease inhibitor cocktail (1milligram/ml) (Roche) and concentration was determined by use of the micro bicinchoninic acid (BCA) kit (Pierce), following the manufacturer's instructions. The concentration was determined with the Beckman model DV 640 spectrophotometer.

NuPAGE SDS 10% polyacrylamide pre-cast gels (Invitrogen) were used to fractionate the salivary proteins. Two hundred fifty nanograms (ng) of MSP were loaded per well, and run for 40 minutes at 200 volts. After de-casting the gel, it was briefly washed twice in de-ionized water and stained using the SilverQuest kit (Invitrogen), following instructions of the manufacturer.

Immunodetection of the MSP.

After MSP were fractionated by the method described above, they were transferred to a 0.2μm pore size nitrocellulose pre-cut membrane (Invitrogen) for 2 hours at 30 volts using the power apparatus EC 105 (ThermoEC). The membrane was briefly washed twice with de-ionized water and blocked overnight at 4°C with 10 ml of blocking buffer (5% skim milk (w/v) and 0.1% Tween 20 in PBS). The membrane was cut into strips for incubation with antisera. Antisera were obtained from mice used to feed one of the AIDL mosquito colonies of the species used for these experiments. From each mouse 0.1 ml of blood was drawn from the caudal vein and placed in 0.1 ml of minimal essential medium (MEM) without bovine fetal serum (BFS) nor phenol red; this was diluted in 5 ml blocking buffer and incubated overnight at 4°C with a nitrocellulose strip containing fractionated MSP from the homologous mosquito species. After overnight incubation the membrane was washed 3 times, 5 minutes each, with 0.1% Tween 20 in PBS at room temperature. Horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG (zymed) was diluted 1:1000 in blocking buffer (5 ml) and incubated with the strips for 3 hours at room temperature. The membrane was washed 3 times, 10 minutes each, with 0.1% Tween 20 in PBS at room temperature. The membrane strips were developed in 3,3' diaminobenzidine (DAB), (Sigma). Ten-milligrams DAB were dissolved in 10 mL PBS,

1.0% (v/v) hydrogen peroxide and 5 ml of the solution were applied directly to the membrane at room temperature. Presence of antibodies bound to the proteins was immediately demonstrated by a brown enzymatic product. The reaction was stopped by adding 5 ml of 0.1% Tween 20 in PBS.

Mass spectrometry analysis (MS/MS spectra)

The protein bands visualized by silver staining were excised in the gel and placed in a 1.7 ml microcentrifuge tube and destained with the A/B solutions from the SilverQuest staining kit (Invitrogen). After destaining, blocks containing bands were placed in clean tubes containing 1% acetic acid. MSP from *Cx. tarsalis* and *Ae. aegypti* proteins excised from the polyacrylamide gel were sent for MS/MS analysis at Macromolecular Resources, Colorado State University, (Dr. Jessica Prenni). Before analysis 1% acetic acid was discarded from samples and polyacrylamide gel slices containing MSP were finely diced, washed with 100 mM NH_4HCO_3/50% acetonitrile (ACN) to remove all dye, and dried. Dried gel fragments were incubated in 10 mM dithiothreitol, 100 mM NH_4HCO_3 at 60°C for 30 min, centrifuged and supernatant discarded. After washing in 55 mM iodoacetic acid in 100mM NH_4HCO_3 for 30 min, fragments were centrifuged and supernatant removed and discarded. Fragments were again washed in 100 mM NH_4HCO_3/50% ACN and dried. Each protein was digested in the gel by addition of 600 ng trypsin in 100 mM NH_4HCO_3 at 37° C overnight, then extracted in 50% ACN, 0.1% trifluoroacetic acid (TFA) and purified using a C18 Zip-tip (Millipore), following the manufacturer's protocol.

Each purified protein sample was dissolved in 10µl 0.1% TFA, and 1µl mixed with an equal volume of a-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% ACN, 0.1% TFA)

and spotted on the MALDI target and allowed to dry. External calibration used an 8 peptide mixture on a spot adjacent to the sample. Analysis was performed with an UltraFlex-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA) in positive ion, reflector mode with a 25 kV acceleration voltage. Data were processed using the SNAP algorithm in the FlexAnalysis software (version 2.4, Bruker Daltonics). A monoisotopic list was generated using a signal-to-noise threshold of 6 for MS spectra and 3 for MS/MS spectra. The combined MS and MS/MS spectra for each sample were searched using the Mascot (version 2.1) database search engine against the NCBInr database using a taxonomy filter for mosquito (Aedes, Taxonomy ID 7158) containing 16,885 sequence entries (updated weekly). Parameters used in the database search are as follows: peptide mass tolerance of 0.1 Da, fragment ion mass tolerance of 0.7 Da, trypsin peptides only allowing for 1 missed cleavage, variable modifications of cysteine carbamidomethylation and methionine oxidation.

Amplification of specific cDNA from *Culex tarsalis*.

Adult female *Cx. tarsalis* mosquitoes were cold anesthetized, briefly submerged in 70% ethanol, and salivary glands were dissected with fine forceps. The dissected salivary glands were placed in 500µl Trizol (Sigma) and total RNA was extracted as follows: salivary gland tissue was disrupted and homogenized by mortar and pestle in Trizol® followed by a room temperature incubation of 5 minutes. One hundred microliters of chloroform were added to the suspension and samples were vortexed at high speed for 10 seconds, after which samples were incubated once more at room temperature for 3 minutes and centrifuged at 12000 rpm for 10 minutes at 4°C; supernatant was collected and placed in a new microcentrifuge tube, 250µl of isopropyl

alcohol were added and incubated for 10 minutes at room temperature and centrifuged at 12000 rpm for 10 minutes at 4°C, supernatant was discarded and RNA pellet was washed once with 500 µl of 75% ethanol; ethanol was discarded and pellet dried in vacuum centrifuge for 30 minutes. The pellet was re-suspended in 50µl RNAse/DNAse free water and quantified using Beckman spectrophotometer (DU 640) at 260 nm.

Tryptophan oxygenase (TO) gene sequence was amplified by RT-PCR using degenerate primers based on *Ae. aegypti* (accession number gi:17225340) and *An. gambiae* (accession number gi:3342704) gene sequences. The 2 sequences were aligned using the NCBI BLASTN 2.2.10 version and regions that were highly conserved were used for primer design. The degenerate primers used are presented in table 2.2.

Primer name	5'→ 3' sequence
Try oxy 267-F	TACGARCTGTGGTTCAAGCAGAT
Try oxy 568-F	CGGARGTGTTYGCCAGCGATCCG
Try oxy 634-F	TGGCCGATCTGGTGCAGAARTGGCT
Try oxy 634-R	AGCCAYTTCTGCACCAGATCGGCCA
Try oxy 748-R	GCTTCCTGSTCAGCCAGCAGCTG
Try oxy 974-R	CATCAGTAGCATTAGCAGCYGGTGCG

Table 2.2. Degenerate primers used to amplify the tryptophan oxygenase gene of *Cx. tarsalis* mosquitoes The primer sequences are based on *Ae. aegypti* and *An. gambiae* genome sequences. (F) represents forward and (R) the reverse primers. Y, R or S represent the degenerate nucleotides in the primer according to Invitrogen recommendations where Y represents C and T, R is A and G and S represents C and G.

Several combinations of forward and reverse primers were used to amplify selected regions of the *Cx. tarsalis* TO mRNA; the reverse transcription (RT) reaction was performed using the superscript II RNase H reverse transcriptase (Invitrogen) and the reaction was as follows: The primers were diluted to a final concentration of 25ng/µl and 50ng of primers per reaction (2µl) were used. Total RNA in each reaction was 100ng; 1µl (10mM) dNTP mix and RNAse free water to bring the total volume to 12µl. The mixture was heated to 65°C for 5 minutes then chilled on ice for 10 minutes, and 4µl of 5x first-strand buffer, 2µl DTT (0.1 M), 1.9µl of RNAse free water and 0.1µl of Superscript RT were added per reaction. For the PCR reaction the following were used: 2.5µl of 10x buffer, 2µl of dNTP (2.5mM), 50ng of forward and reverse primers, 5µl of the cDNA from the RT reaction, 0.1µl of Taq DNA polymerase and RNAse, DNAse free water to a 25µl total volume per reaction. The PCR Reagent System (Invitrogen) was used for the

RT-PCR reactions. In order to ensure a better primer alignment during PCR and to ensure at least short amplification of DNA using degenerate primers, the touch-down PCR protocol was used. Touch-down PCR has annealing temperatures declining from 60°C to 41°C over 20 cycles, followed by 39 cycles annealing at 54°C. The complete protocol is presented in the appendix.

The PCR products were examined by electrophoresis in a 1% agarose gel. Products that were considered to be close to the expected size were excised from the gel and DNA was eluted using the Concert Gel Extraction System (Life Technologies). The product was re-amplified using the same primers and PCR kit with slight changes in the annealing temperature and amplification cycles. The PCR re-amplification protocol is presented in the appendix. The PCR products were TOPO cloned using the One Shot TOPO 10 plasmid in *E. coli* chemically competent cells (Invitrogen). After products were inserted into the vector and transformed into competent cells, the cells were plated in 2YT selective medium containing ampicillin/kanamycin. Plates were incubated overnight at 36°C and colonies were picked next day and screened by PCR using ZAP primers. The remaining 5µl RT-PCR products from positive screens were used for DNA sequencing.

Extension of *Cx. tarsalis* tryptophan oxygenase cDNA by 3' and 5' RACE.

Based on the region amplified and sequenced, a forward primer was designed for 3' random amplification of cDNA ends (3'RACE (F); table 2.3) to extend the cloned region. This forward primer was paired with the reverse primer named 3' RACE-screen (R) (table 2.3). In order to generate a DNA amplicon of *Cx. tarsalis* TO mRNA, a universal reverse primer that aligns to the poly A tail of mRNA was used. An RT-PCR reaction was performed to generate cDNA from total mRNA pairing the universal reverse primer that

aligns to the poly A tail and the forward 3' RACE-specific (F) primer. After RT was performed the universal 3' RACE-reverse without poly T tail for PCR was paired with the 5' RACE T.O Specific screening (F) as listed in table 2.3. In order to improve alignment of unspecific primers and DNA amplification the PCR was run using the touch down protocol. PCR products were confirmed by elecrophoresis in a 1% agarose gel. The products were re-amplified by non-touch down PCR and the product was TOPO cloned as previously described. DNA from PCR positive cells was sent to Macromolecular Resources for sequencing. In order to obtain a complete sequence of the TO gene, a 5' RACE was also performed. Primers for the 5' RACE were designed using the sequence generated by the 3'RACE (Table 2.3).

Primer Name	5'→ 3' Sequence
3' RACE (F)	CAAGAAGCTAGTGCCATGGAAGAG
3' RACE-screen (R)	TGAATCTACGATCTCCACGTGCAAG
3' RACE Universal RT (R)	*GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTT
3' RACE Universal PCR (R)	GGCCACGCGTCGACTAGTAC
5' RACE (F) Universal	*GACATCGAAAGGGGGGGGGG
5' RACE T.O Specific R1	CTTCACATTTTCGTGTTCCTCTTCC
5' RACE T.O Specific R2	TGGCACTAGCTTCTTGATCAGCCAG
5' RACE T.O Specific screening (F)	CGAATTCGCCCTTTGGCCGATCTGG
Cx. tarsalis T.O Specific (R) B	AAATGTTGAAATCAAGCTCGTGTTATC

Table 2.3. Primers used for the 3' and 5' RACE to amplify and sequence the tryptophan oxygenase gene of *Cx. tarsalis* female mosquitoes. The (F) represent forward primers, the (R) are reverse primers. The * represent anchor primers used for the 3' and 5' RACE. The primers named 3' RACE (F) and *Cx.tarsalis* T.O Specific (R) B are specific for the tryptophan oxygenase of *Cx. tarsalis.*

5' RACE was performed as described for the 3'RACE with slight modifications. The reverse transcription reaction used at least 2 specific primers to the TO cDNA generated from *Cx. tarsalis*. The cDNA product was purified using the QIAquick purification kit (QIAGEN) and C-tailing reaction was performed. The reagents used to add the oligo dC-tail for preparation of 5' RACE cDNA are as follows: 10μl (1μg) of cDNA, 1mM dCTP (1μl), 5x buffer (10μl), 1:10 BSA, terminal deoxynucleotide transferase (1μl) and nuclease free water to adjust volume to 200μl/reaction. The forward primer was an anchor primer for the 5' RACE and is shown with the reverse primer in table 2.3. The reaction was incubated at 37°C for 30 minutes, with a final incubation at 65°C for 10 minutes. The products from 5' RACE were reamplified and TOPO cloned as previously described.

Assay for expression of the 36 kDa D7 protein mRNA of *Aedes aegypti.*

Female *Ae. aegypti* pupae were placed in three 16 oz cartons, (100 per carton) and allowed to eclose to adults. Water and sugar were removed at day four after eclosion. One group of mosquitoes (fed group) was offered a non-infectious defibrinated sheep blood meal in an artificial blood feeder using a clean hog gut (pig intestine) as membrane. Another group was also allowed to probe through a hog gut membrane but nothing was added to the feeder (probed group), and a negative control group was neither fed nor stimulated by probing. After one hour of feeding or probing all three groups were cold anesthetized at 4°C and 50 to 60 salivary glands from fed, probed or negative control mosquitoes were dissected as previously described. The dissected salivary glands were placed in Trizol® and total RNA was extracted and measured by spectrophotometer. The production of mRNA encoding the 36 kDa protein was measured using a Q-RT-PCR assay based on protocols previously described (31, 292) with primers for *Ae. aegypti* 36 kDa protein and β actin. Primer sequences were selected from NCBI *Ae.aegypti* 36 kDa (D7 long) and β actin, accession numbers gi:16225991 and gi:94468485 respectively. Primers were designed using primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), with the parameters of 50:50 GC content for forward and reverse primers with amplification product size 200 to 300 bp maximum. The primers designed for D7 were 5'-CCACCTCTCAAAAGCTCCAG-3' forward, and 5'-ACGATTCCACTTCCCATCTG-3' reverse and for β actin primers were 5'-TGGTTACTCGTTCACCACCA-3' forward, and 5'-GAACGATGGCTGGAAGAGAG-3' reverse. First strand cDNA was generated from 100ng of total RNA using the ThermoScript RT-PCR system (Invitrogen) and Q-PCR was carried out using the Brilliant

SYBR Green QPCR Master Mix (Stratagene) in a Bio-Rad iQ5 system. Data were analyzed with the software provided by the manufacturer. Results were further analyzed by non-paired Student's *t-test* for a single mean comparison, (308)

Immune precipitation of a 36 kDa D7 protein from *Aedes aegypti* alivary glands.

The salivary glands from 100 female *Aedes aegypti* were dissected as described above. The dissected salivary glands were placed in 250µl of lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5mM EDTA, 1% sodium deoxycholate, 1% Triton-X-100, 0.1% SDS and 1 mM PMSF) and disrupted by mortar and pestle; the suspension was centrifuged at 15000 rpm for 10 minutes and the tissue pellet was mechanically disrupted once more. PBS containing protease inhibitor was added to a final volume of 1000µl. The suspension was then incubated with anti-*Ae. aegypti* 36 kDa protein specific antiserum (kindly provided by Dr. Jill Troyer). Attachment to protein A (Pierce) and immunoprecipitation were carried out using the Seize X Protein A Immunoprecipitation kit (Pierce). Briefly, 500 ng of anti-D7 antibody were diluted in 400 µl of binding/washing buffer (provided by manufacturer) and rocked in a 1.7 ml microcentrifuge tube at room temperature for 15 minutes with the gel-matrix attached to protein A. The mixture of antibodies-protein A were passed through a column coated with the resin trapping the antibodies bound to protein A by centrifuging at 8500 rpm for 1 minute, unbound antibodies were discarded. Next, dimethyl sulfoxide (DMSO) was added to the previous suspension mix and antibody cross-linking was performed by gently mixing slurry at room temperature for 60 minutes (cross-linking is carried out in order to fix the bound antibodies and avoid loss during the elution washes). Proteins obtained by salivary gland dissection and extraction were mixed

in equal volumes of 500µl with the antibodies bound to protein A. The 1ml mix was rocked overnight in a 1.7 ml microcentrifuge tube at 4°C in a rocking table; after incubation protein fractions were eluted through the column by centrifugation as described above to collect the specific five proteins fractions (washes). Five fractions were eluted using fresh acid elution buffer (Pierce) and then fractions were TCA precipitated. Pellets were resuspended in 50µl PBS with protease inhibitors and 1µl of each fraction was added to 9µl of PBS and run on an individual lane in a 10% SDS-PAGE, which was silver stained. Once the fraction from the immunoprecipitation containing the protein of interest was identified, the protein concentration was measured by BCA assay as described previously

Identification of *Ae. aegypti* salivary proteins that interact with human interferons.

An overlay binding assay (OBA) was developed in order to identify MSP binding to the cytokines interferon alpha, beta or gamma (IFNα/β or γ). A similar binding assay has been previously used to identify viral receptors (112, 238, 303). *Ae. aegypti* adult females were starved for 24 hours, then allowed to salivate in the salivation buffer described above. Approximately 250 females were induced to salivate through a membrane for one hour. After salivation, the buffer containing the mosquito salivary proteins was collected and proteins were TCA precipitated. Proteins were quantified by BCA assay and then used to perform an overlay binding assay. Two hundred fifty nanograms of mosquito salivary proteins were fractionated in a 10% SDS-PAGE (pre-cast NuPAGE, Invitrogen) under non-denaturing conditions at 200 volts for 40 minutes, the gel was de-cast and proteins were transferred to a nitrocellulose membrane (Invitrogen) for 1 hour at 30 volts. After proteins were transferred, the membrane was cut into strips and incubated in blocking buffer at

room temperature for one hour. After blocking, 1μg of recombinant human IFNα (Abazyme), IFN β (Abazyme) or IFNγ (Sigma) diluted in RPMI medium was added to blocking buffer on individual nitrocellulose strips and incubated overnight at 4°C on a rocking table. After removing the first overlay, the membranes were washed three times with PBS, 0.1% Tween 20, 10 minutes each. After washing, a specific mouse anti human-IFNα (Abazyme), anti-IFNβ (Abazyme), or goat anti-IFNγ antibody (Sigma) diluted 1:250 in blocking buffer, was added to its corresponding strip and was incubated overnight at 4°C on a rocking table. The primary antibody was removed and the membrane was washed as previously described. Next, secondary HRP-conjugated anti-mouse (KPL) or anti goat antibody diluted 1:1000 in blocking buffer was added and incubated at room temperature for 1 hour. After the last incubation the membrane was again washed three times, 10 minutes each and developed with 10 mg of 3'3' diaminobenzidine in 10% hydrogen peroxide in PBS. When the bands developed the reaction was stopped by adding PBS and washing the nitrocellulose strips for 5 minutes.

Assay of IFNγ inhibition by immunoprecipitated 36 kDa D7 protein of *Ae. aegypti*.

The cells used in this experiment were A 549 human lung carcinoma cells kindly donated by Centers for Disease Control and Prevention (CDC), Fort Collins, Colorado. The cells were grown at 36°C with 5% CO_2 in DMEM with 10% FBS. The following mixtures in 500μl of RPMI 1640 medium were incubated overnight on a rotating table at 4°C: (1) 20ng (1.1m mole) of 36 kDa *Ae. aegypti* salivary protein incubated with 20 ng (2.3m mole) of human recombinant IFNγ (Sigma); (2) 20ng (1.1m mole) 36 kDa *Ae. aegypti* salivary protein incubated with 20ng (2.3m mole) of human recombinant IFNγ (Sigma) plus anti-D7 *Ae. aegypti* antibody diluted 1:100; (3) 20ng (2.3m mole) IFNγ, (4)

20ng (1.1m mole) 36 kDa *Ae. aegypti* salivary protein incubated with 20ng (2.3m mole) of human recombinant IFNγ (Sigma) plus anti-human IFNγ antibody diluted 1:250 (Sigma); and finally (5) an untreated control was added to compare cell viability. D7 protein was immunoprecipitated as described without TCA for this experiment. Mixtures 1-5 were added to triplicate wells of A549 cells in 6-well plates and the plates were rocked every 10 minutes for 1 hour. After last rocking 2.5 ml of DMEM (10% FBS) was added to each well and they were incubated overnight. Next day, the medium was aspirated from cells and they were washed once with PBS. All the treated and untreated cells were then infected with DENV2 (Jamaica 1409) at multiplicity of infection 0.01 in DMEM 2% FBS. Cells were infected by addition of virus for one hour, rocking the cells every 10 minutes, removing inoculum and adding 3 ml of DMEM 2% FBS. Cells were incubated at 36°C in 5% CO_2. Uninfected untreated cells were included as a control. Medium from each group was collected at 1 and 5 days post infection and frozen at -70°C to analyze viral titer by plaque assay.

Plaque assays of infectious virus

Plaque assays of infectious virus was performed in Vero cells using technique previously described (120) were grown to confluence in 6-well plates in MEM with Earle's salts and L-glutamine, 10% FBS (Gibco), 100 units/ml penicillin (Gibco), and 100 μg/ml streptomycin. Before infection, medium was removed, and cells were rinsed with sterile PBS. Serial ten-fold dilutions of 100μl of each sample were adsorbed for 1 hour by rocking. Next the cell monolayer was overlaid with a 50:50 mixture of 1% agarose (Sigma) and 2× MEM supplemented with 2% FBS (Gibco). Ten days following the first agar overlay, 3 ml of a mixture of 1% agarose and 2× MEM containing 3%

neutral red was added to each well. Plaques were visualized and counted the following days until no new plaques were visualized. Each virus dilution was assayed in triplicate and the average plaque number was used to calculate the viral titer. Each A549 cell treatment was done in triplicate and average titers were analyzed by a Students *t*-test comparing groups using the SAS program version 9.1.The comparison between groups was considered statistically significant if $P<0.05$.

RESULTS

MSP fractionation and immunoblot analysis.

In order to identify MSP that are secreted during blood feeding, proteins that were deposited during membrane probing were concentrated by TCA precipitation. After concentration, 250ng of salivary proteins from *Aedes aegypti* or *Culex tarsalis* mosquitoes were loaded in a 10% SDS-PAGE, fractionated, and silver stained as shown in figure 2.1.

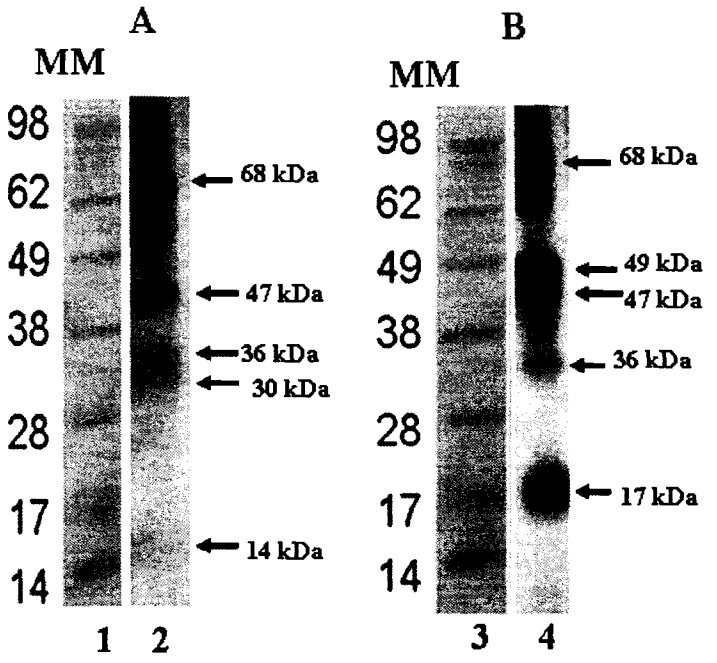

Figure 2.1. Mosquito salivary protein identified by silver stained 10% polycarylamide gel.Mosquito salivary proteins from A) *Ae. aegypti* and B) *Cx tarsalis* were obtained using salivation buffer and then were TCA precipitated. Proteins were fractionated in a 10% polyacrylamide gel and silver stained. Lanes 1 and 3, MM, molecular markers; lane 2, *Ae. aegypti* MSP; lane 4, *Cx. tarsalis* MSP.

Silver staining of the gel revealed five major salivary proteins from *Ae. aegypti* with approximate molecular sizes 14, 30, 36, 47and 68 kDa, (shown with arrows) and five proteins in *Cx. tarsalis* at approximately 17, 36, 47, 49 and 68 kDa (shown with arrows). Material near the top of the gel may be aggregates of proteins, since neither β-mercaptoethanol nor heat were used in preparing proteins for electrophoresis. Immunoblot analysis was performed, with the primary antibody being serum from mice that were regularly used for blood-feeding mosquito colonies at AIDL. The primary antibodies were detected by horseradish peroxidase conjugated goat anti-mouse antibody. Mouse serum antibodies recognized mosquito salivary proteins at 14, 17, 36, 46 and 68 kDa from *Ae. aegypti* and 17, 36, 47, 49, and 68 kDa fro *Cx. tarsalis* (figure. 2.2). Higher molecular weight bands observed in the immunoblot may be antibodies bound to

aggregates of proteins at top of gels although no protein identification analysis of these aggregates was performed to confirm this observation.

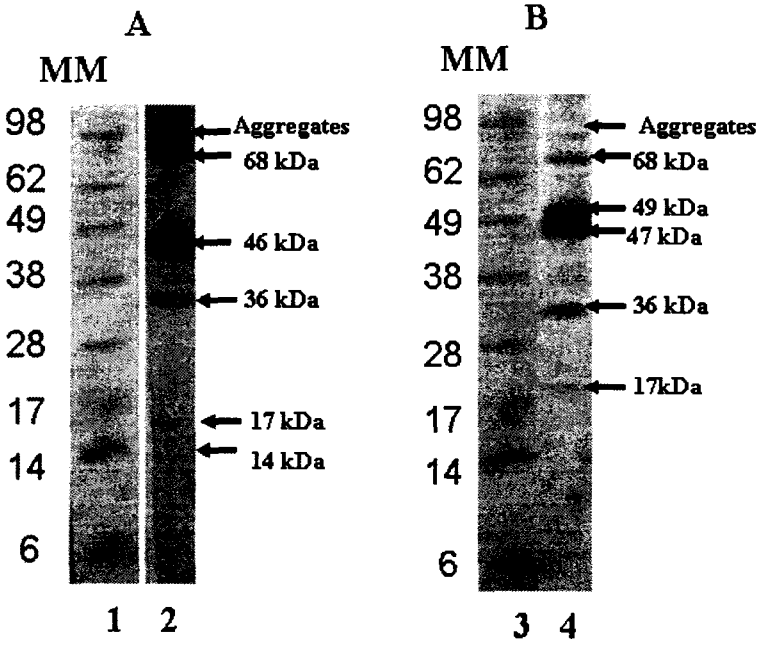

Figure 2.2 Detection of immunogenic proteins of Ae. aegypti and Cx. tarsalis mosquitoes by immunoblot assay. Immunoblot detection of MSP using sera from mice (1:200) that had been fed upon by the respective mosquito colony: A) *Ae. aegypti* MSP; B) *Cx. tarsalis* MSP. Left lanes 1 and 3 are molecular markers with sizes in kDa. Lanes 2 and 4 are reactivity of western blot. Sizes of immunogenic proteins are shown with arrows. Top of western labeled as aggregates.

Mass spectrometric analysis of MSP (MS/MS spectra).

In order to identify proteins in mosquito saliva, bands from the SDS-PAGE were excised, digested with trypsin, and analyzed by mass spectrometry. The peptides were compared to a database by a MASCOT search engine (figure 2.3).

Mascot Search Results

User	: Jessica
Email	: jprenni@colostate.edu
Search title	: p49
MS data file	: DATA.TXT
Database	: NCBInr 20050303 (2339066 sequences; 792779905 residues)
Taxonomy	: Mosquito (490 sequences)
Timestamp	: 11 Mar 2005 at 00:02:55 GMT
Top Score	: 48 for gi|17225341, tryptophan oxygenase [Aedes aegypti]

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 39 are significant (p<0.05).

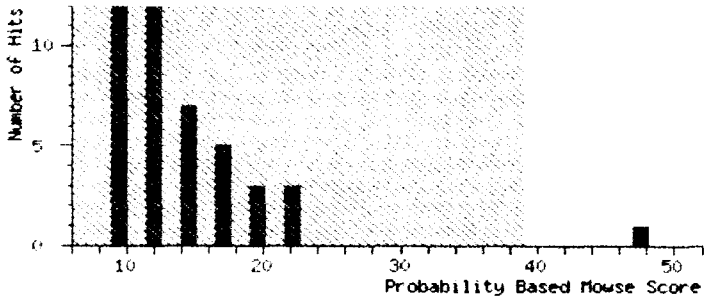

Figure 2.3. Mascot score for the 49 kDa protein from *Cx. tarsalis*. The mascot score for the identified peptide is 48(P<0.05). The red bars representing peptides in the green shaded area are not significant (<39). The red bar outside green area (48) represents maximum score and is significant and is considered a positive match to *Ae. aegypti* tryptophan oxygenase.

The 49 kDa protein from *Cx. tarsalis* had a mascot score identifying a relationship to tryptophan oxygenase (TO) of *Ae. aegypti,* as shown in figure 2.3.

Cx. tarsalis tryptophan oxygenase 3' and 5'RACE

In order to elucidate the specific gene sequence of the *Cx. tarsalis* TO, degenerate primers based on conserved sequences from *Ae. aegypti* and *An gambiae* TO genes were used, and are described (table 2.2). Total RNA was extracted from *Cx. tarsalis* salivary

glands and reverse transcriptase was used with reverse degenerate primers to generate the cDNA for PCR amplification. The primer pair that yielded the best amplification were Try oxy 267-F and Try oxy 634-R primers.

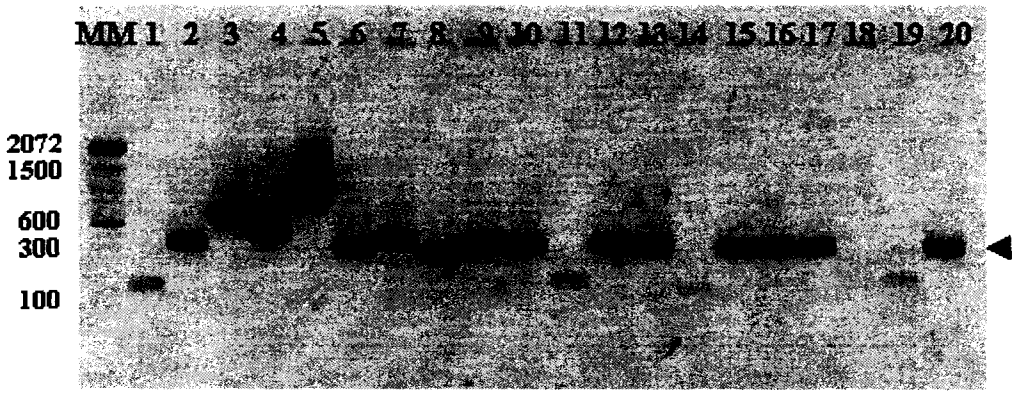

Figure 2.4. 1% Agarose gel showing amplicons from Cx. tarsalis T.O. Primer pair used to generate a 340 bp product were Try oxy 267-F and Try oxy 634-R. Lanes 1, 3, 5, 11, 14, 18 and 19 are considered negative. Lanes 2, 4, 6, 7, 8, 9, 10, 12, 13, 15, 16, 17 and 20 are from positive colonies (indicated by arrow)

The 340 bp amplicon was TOPO cloned and sequenced; the sequence was readable for 265 nucleotides (shown in red in figure 2.5) from which two specific primers for 3' RACE were generated. The sequence was analyzed by SeqMan (DNA star program) and a short consensus sequence was generated. The sequence was considered to be *Cx. tarsalis* specific.

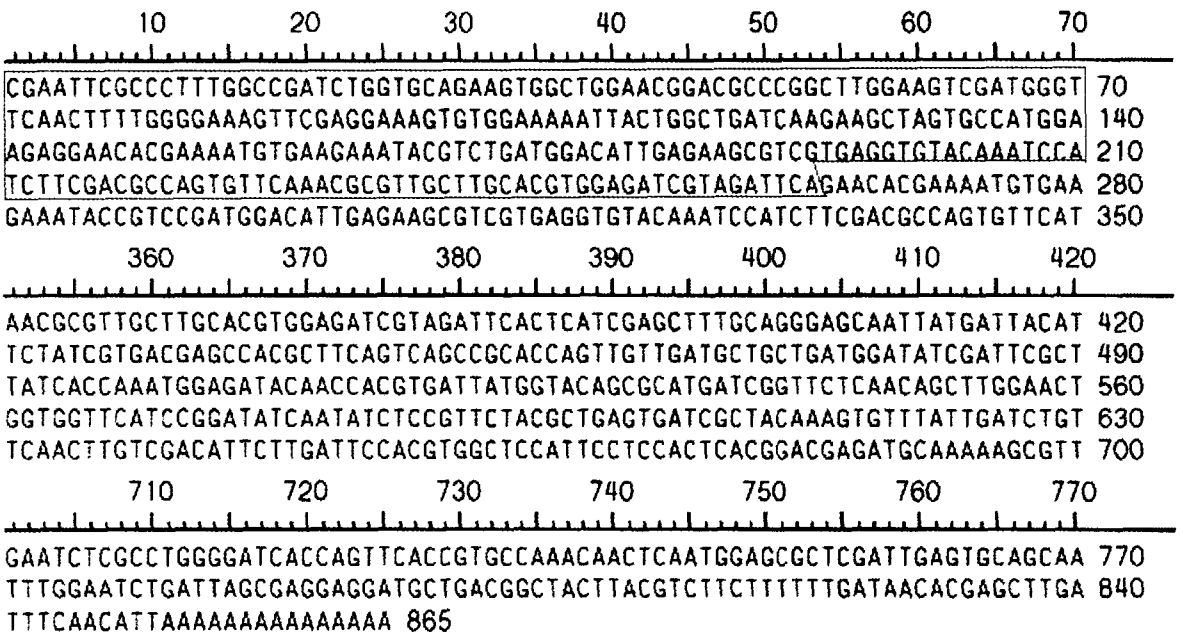

Figure 2.5. *Cx.tarsalis* tryptophan oxygenase sequence. The area shown in red was amplified using degenerate primers. The 3' RACE forward primer was generated from this sequence. The 3' RACE reverse primer (*Cx. tarsalis* T.O Specific (R) B) is located just upstream of the poly A tail.

The sequences obtained from clones were aligned using the DNAstar program and the unique consensus sequence is presented in figure 2.5. The sequences of cloned fragments were confirmed by re-amplifying the purified DNA fraction, TOPO cloning and re-sequencing. Sequences were extended by amplification of mRNA with 3' RACE Universal RT (R), using the forward 3' RACE (F) and the reverse 3' RACE Universal PCR (R) primers. The products of these amplifications were cloned in TOPO vectors. Sequences were confirmed by re-amplification by PCR with the same primers [(3' RACE (F) and reverse 3' RACE Universal PCR (R)] and TOPO cloned again. Colonies were screened with ZAP primers and products from positive colonies as shown in figure 2.6 were sent for sequencing.

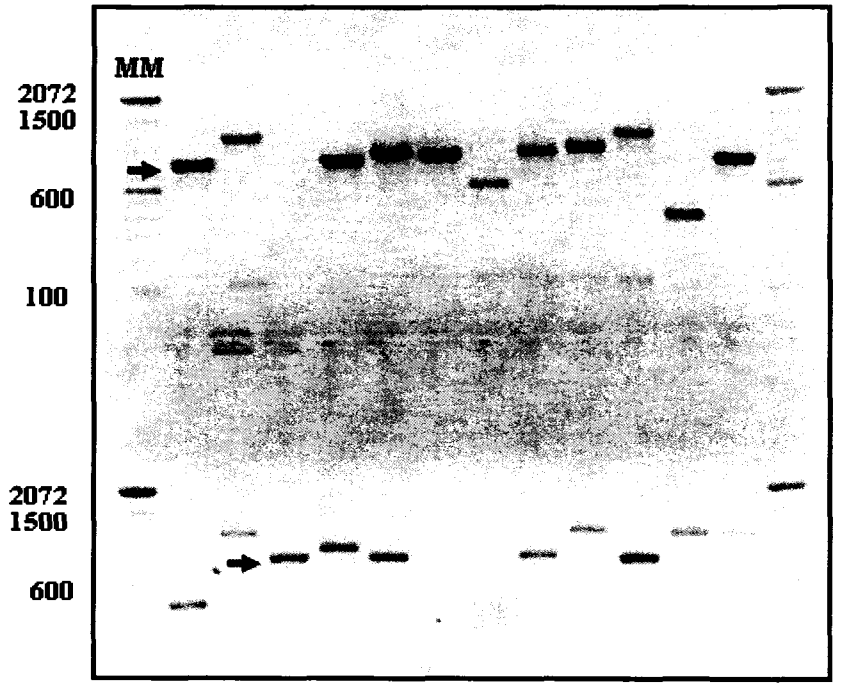

Figure 2.6. 1% Agarose gel showing DNA specific for Cx. *tarsalis* T.O. Arrows show the expected size (~ 633 base pairs) of the amplicon. Products were sequenced by Macromolecular Resources at Colorado State University. MM, molecular markers.

The consensus sequence was created from cloned fragments generated from 3' RACE. The consensus sequence was confirmed by using a specific *Cx. tarsalis* reverse primer that binds just upstream of the poly A tail to generate products shown in figure 2.5. from RNA extracted from *Cx. tarsalis* salivary glands. A PCR product around 600 bp was confirmed in a 1% agarose gel stained with ethidium bromide as seen in figure 2.7.

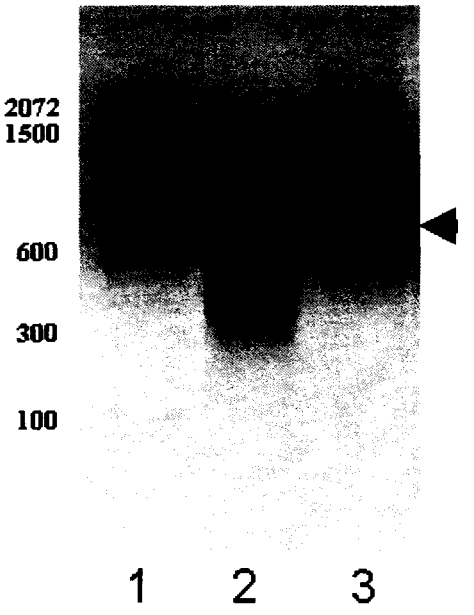

Figure 2.7. 3' RACE amplification of Cx. tarsalis tryptophan oxygenase in 1% agarose gel stained with ethidium bromide. 1) Molecular markers in base pairs, 2) cDNA generated using 3' RACE (F) and 3' RACE universal PCR (R), 3)cDNA generated using 3' RACE (F) + *Cx. tarsalis* T.O Specific (R)..

The product shown in lane 3 demonstrates the ability of the primers to amplify 633 bp of the tryptophan oxygenase mRNA from *Cx. tarsalis* mosquitoes. The cDNA from lane 3 was excised, purified, and reamplified with the same set of primers (3' RACE (F) and 3' RACE universal PCR (R)) The product of these sequences were aligned with the previously generated sequence to confirm the consensus sequence. In an attempt to determine the full sequence of the tryptophan oxygenase gene of *Cx. tarsalis,* additional primers were created from a 265 bp region of *Cx. tarsalis* TO shown in red, figure 2.5. From this region three different primers to amplify the 5' end of *Cx. tarsalis* TO mRNA were generated and are described in table 2.3 as 5' RACE T.O Specific R1, 5' RACE T.O Specific R2, and 5'RACE T.O Specific screening (F). In order to test the primers as 5' RACE anchor primers, total RNA was extracted from *Cx. tarsalis* salivary glands and

cDNA generated by reverse transcription, using each of 5' RACE primers. When using the 5' RACE universal anchor primer (5'RACE (F)) it was not possible to achieve any amplification. Although different primers were tested under several conditions none of the attempts was successful. The GenBank accession number for this sequence is EU155179.

Identification of the 36 kDa protein (D7) from *Ae. aegypti.*

Based on previous observations the 36 kDa (D7) protein was recognized as a potential candidate to be related to viral pathogenicity. The data obtained from this protein by MS/MS spectra and its identification is explained in detail in chapter 3.

Expression of *Aedes aegypti* 36 kDa D7 mRNA during blood feeding and immune precipitation of D7 protein from salivary glands.

To characterize the importance of the D7 protein during blood feeding, 4-5 day old Ae. *aegypti* mosquitoes were starved for 24 hours, after which they were either blood fed, salivation stimulated by allowing to probe a membrane, or placed in a container without feeding or stimulation (see material and methods). Total RNA was extracted from salivary glands and RT-PCR was used to amplify a short region of D7 mRNA or β-actin mRNA. Q-PCR was performed to measure cDNA. A 1% agarose gel was used to confirm amplification and functionality of the primers from both β actin and D7.
The results of quantitative RT-PCR showed that blood feeding stimulated transcription of mRNA from the D7 gene. Blood fed mosquitoes had a significant increase ($P<0.05$) in production of the D7 mRNA (D7/βactin ratio) compared to the non-fed, non stimulated mosquitoes (figure 2.8). The stimulation of salivation also triggered D7 mRNA transcription significantly compared to control ($P<0.03$). When mRNA from

stimulated or blood-fed mosquitoes were compared, no significant difference was observed (P=0.40). The results are summarized below (figure 2.8).

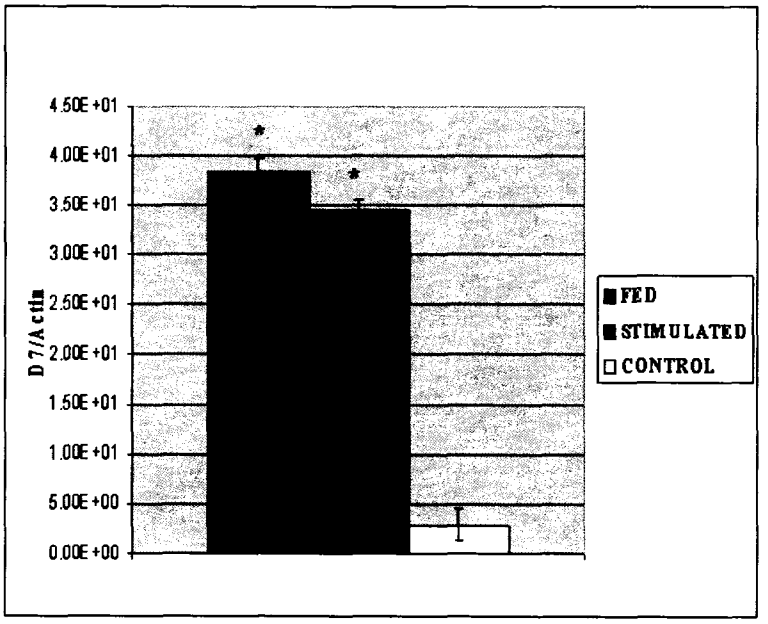

Figure 2.8. Transcription of D7 mRNA during mosquito blood feeding or stimulation by probing. The data are unitless and expressed in the ratio of D7 mRNA/ β actin mRNA. (* shows significant difference between fed and stimulated compared to non-fed non-stimulated)

In order to obtain the D7 protein from *Aedes aegypti*, salivary glands were dissected, disrupted mechanically in lysis buffer and immunoprecipitated with specific antibodies against the D7 protein. The protein eluted from the column is shown in a 10% SDS-PAGE in figure 2.9.

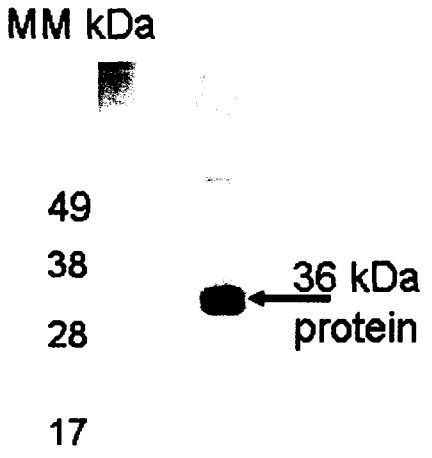

Figure 2.9. Immunoprecipitation of the D7 (36 kDa) protein from *Ae. aegypti* salivary gland homogenate. The protein was eluted from the antibodies bound to an affinity column, TCA precipitated, resuspended in 50 µl of PBS. 1 µl of the suspension was fractionated in a 10% SDS-PAGE and silver stained.

Binding of *Ae. aegypti* salivary proteins to human IFN.

Overlay binding assay was performed as described in materials and methods in order to identify MSP binding to interferon alpha, beta or gamma (IFNα/β or γ). Development of the blot with specific anti-interferon antibodies showed a band corresponding to a 36 kDa protein in the strip that was overlaid with IFNγ and reacted with specific anti-IFNγ antibodies. There was not a visible reaction when the overlay was performed either with IFNα, IFNβ or anti-IFNγ alone or anti-interferon antibody pool with the strip that had not been previously overlaid with IFN's. The specific reaction of anti-*Ae. aegypti* MSP can be observed in figure 2.10.

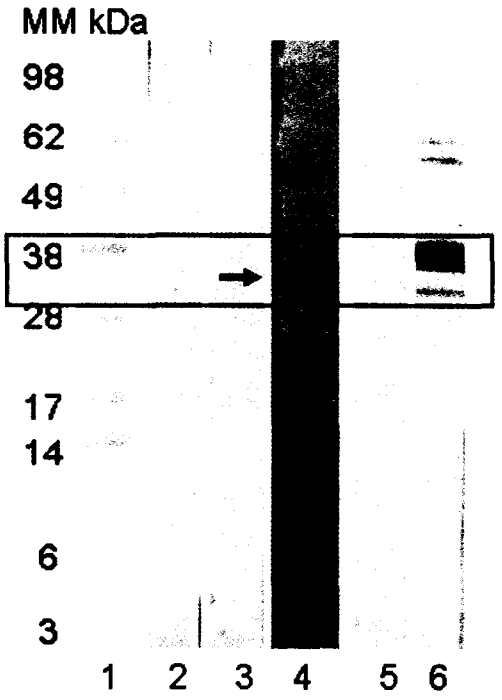

Figure 2.10. Overlay binding assay of MSP with recombinant human IFNα, (lane 2), IFNβ (lane 3) IFNγ (lane 4),or with pooled anti IFN antibodies (lane 5) and detected with homologous antibody. Lane 6 shows MSP reacted with anti MSP antibodies from mouse serum.

Effect of 36 kDa D7 protein from *Aedes aegypti* salivary glands in IFNγ protection assay.

The overlay assay suggested that human IFNγ bound to a 36 kDa MSP. In order to further elucidate the role that MSP might have during viral infection we concentrated the 36 kDa protein from *Ae. aegypti* salivary gland homogenate by immunoprecipitation for use in an interferon protection assay. Protection assay was performed by treating A549 cells with preincubated mixtures of IFNγ and the *Ae. aegypti* 36 kDa (D7) protein and then A549 cells were infected with DENV 2 as described in material and methods. Plaque assay showed that DENV titer was significantly increased when IFNγ and D7 protein were mixed compared to when IFNγ was used alone to treat the cells before infection (figure 2.11 $P<0.021$). DENV titers were lowest when cells were pretreated with only IFNγ, but viral titer was significantly increased when using IFNγ an anti-IFNγ antibody compared with IFNγ solely ($P<0.01$). When the mixture using an anti-D7 protein antibody was added, there was a significant decrease in viral titer ($P<0.01$) compared with mixture 1 (IFNγ and D7 only). By day five post infection there was no statistical difference among mixtures and all of them had reached higher DENV titers compared to day 1 post infection (figure 2.11

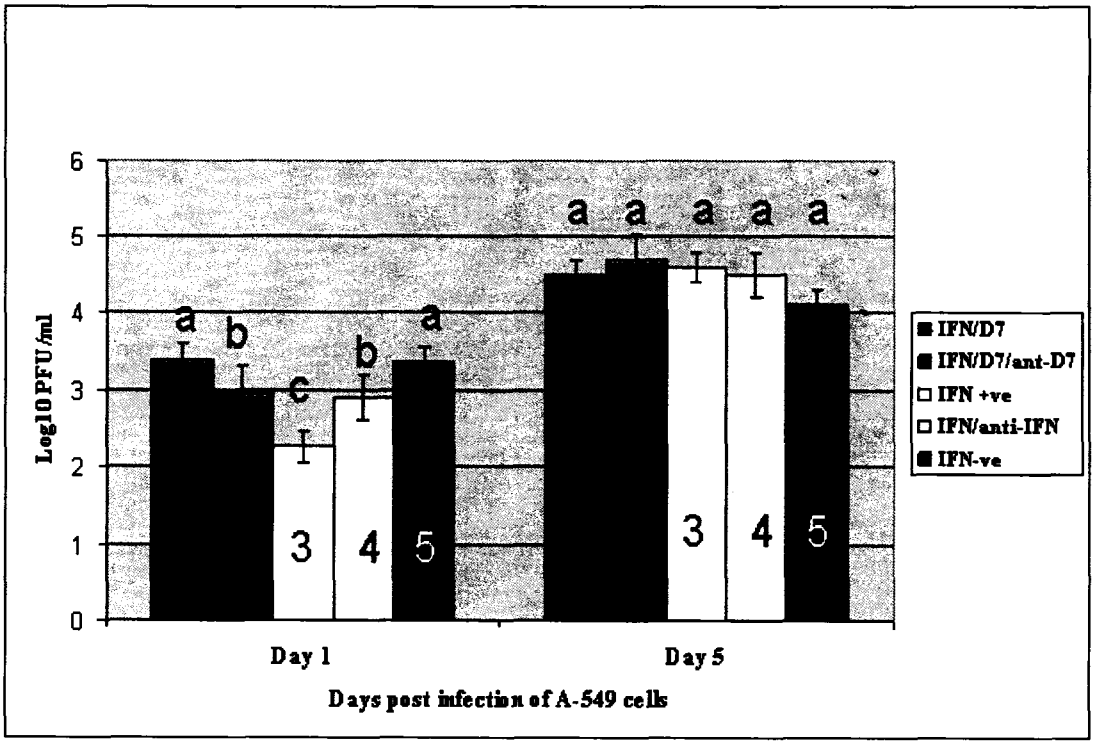

Figure 2.11 Effect of incubation of *Ae. aegypti* D7 protein with IFNγ on DENV replication in A-549 cells. The numbers represent the different mixtures described in material an methods. Viral titers are statistically different ($P<0.05$) if letters at top of bars are different, same letters on top of bars show no statistically significant difference. Data are from days 1 and 5 post infection. Results in table 2.4 are pairwise comparisons by student's *t* test at day 1 post infection.

Mixture comparison	Mixture content	*P* Value
1 and 2	IFNγ/D7 (mixture 1) and IFNγ/D7/anti-D7 (mixture 2)	*P*<0.021
1 and 3	IFNγ/D7 (mixture 1) and IFNγ + control (mixture 3)	*P*< .003
1 and 4	IFNγ/D7 (mixture 1) and IFNγ/ anti- IFNγ (mixture 4)	*P*< .035
1 and 5	IFNγ/D7 (mixture 1) and IFNγ - control (mixture 5)	*P*<0.31
2 and 3	IFNγ/D7/anti-D7 (mixture 2) IFNγ + control (mixture 3)	*P*<0.01
2 and 4	IFNγ/D7/anti-D7 (mixture 2) and IFNγ/ anti- IFNγ (mixture 4)	*P*<0.5
2 and 5	IFNγ/D7/anti-D7 (mixture 2) and IFNγ - control (mixture 5)	*P*<0.02
3 and 4	IFNγ + control (mixture 3) and IFNγ/ anti- IFNγ (mixture 4)	*P*<0.01
3 and 5	IFNγ + control (mixture 3) and IFNγ - control (mixture 5)	*P*<0.003
4 and 5	IFNγ/ anti- IFNγ (mixture 4) and IFNγ - control (mixture 5)	*P*<0.05

Table 2.4 Statistical analysis of DENV2 protection assay by Stdent's *t*-test comparing individual mixtures of DENV 2 and *Ae. aegypti* 36 kDa (D7) protein. The results are comparisons of day one post infection, day five did not showed any statistical difference among mixtures (for details see material and methods).

DISCUSSION

Use of trichloroacetic acid (TCA) to concentrate mosquito salivary proteins (MSP) for analysis

Trichloroacetic acid has been used to precipitate and concentrate a wide array of proteins (49, 111, 305), but its use for concentration, precipitation and analysis of MSP has not been previously described. A novel method was developed to obtain mosquito salivary proteins without dissecting salivary glands. Mosquitoes were allowed to probe through a membrane and deposit saliva into a buffer. By using TCA we could concentrate approximately 375μg of protein from approximately 250 mosquitoes. Considering that only 0.15 to 1.5μg of protein is released by each mosquito during probing (282) TCA is an efficient method to concentrate and analyze MSP. This concentration allowed for polyacrylamide gel electrophoretic fractionation of the proteins and their analysis by western blot (immune detection) or by silver staining the gel. Proteins were also extracted from the glands and analyzed by mass spectrometry (figure 2.3). The TCA precipitation method produced high quality proteins for analysis (111). TCA precipitation is a good method for sample preparation for protein analysis because it is simple yet highly efficient and increases the ability to identify less abundant proteins (52, 121). Although TCA has been shown to modify the structure of some proteins, it also conserves most proteins in native state, including preservation of disulfide bonds (52, 121). In our study, the modification of the protein (if any) did not affect either MS/MS analysis or immune detection by antibodies. MSP contain high numbers of cysteine residues, we attempted to conserve disulfide bonds by avoiding heating or reduction by β-mercaptoethanol (232).

Identification of MSP putatively related to pathogenesis.

In this study MSP from two different mosquito species were identified. One of the proteins identified by MS/MS spectrometry was the tryptophan oxygenase in *Cx. tarsalis* saliva. Tryptophan oxygenase (TO), also known as indolamine dioxygenase (IDO), 2,3-dioxygenase or tryptophan pyrrolase (141), is a heme enzyme that catalyzes a step in the oxidative degradation of tryptophan (141). Tryptophan is an essential amino acid with two aromatic rings and without a polar (hydrophilic) group. (141). In insects, TO plays a role in the synthesis of ommochrome eye pigments (262). TO converts tryptophan into *N*-formyl kynurenine, which is hydrolyzed to formic acid and kynurenine. This last metabolite results in deposition of the eye pigment of *Drosophila melanogaster* (172). In addition to pigment synthesis, the primary function of TO is the metabolism of excess tryptophan that occurs during high protein turnover (70) such as blood meal digestion. The TO enzyme is encoded by the *vermilion* gene in *Drosophila*, *Tribolium*, *Anopheles* (70) and recently has also been described in *Ae. aegypti* (96). Metabolites from tryptophan degradation have been found in the salivary glands of *Anopheles* mosquitoes (187) and these metabolites are important during *Plasmodium* development (187, 233). In humans, TO has been related to immune regulation by inducing a toleragenic state in dendritic cells (168), and it also affects T-cell proliferation (300). It has been observed, for instance, that tumor cells prevent their rejection by constitutively expressing TO (285). Injection of TO during mosquito bite could regulate the immune response at the site of feeding, since TO suppresses T-cell proliferation and affects antigen presenting cells such as skin Langerhans cells by blocking the cell cycle (167). The presence of TO in salivary glands of *Cx. tarsalis* is a finding that could account for modulation of the

host immune response during mosquito bite. It is not surprising that *Cx. tarsalis* have a tryptophan-degrading enzyme in the salivary glands (219), that might function as an immune regulator and degrade tryptophan during the metabolism of amino acids.

Production of the 36 kDa protein D7 during blood meal

The 36 kDa protein in *Ae. aegypti* saliva also was identified by genomic sequencing and phylogeny alignment as a member of the D7 family of proteins (282), which is one of the most abundant proteins of the mosquito saliva; nevertheless the function of this family of proteins remains largely unknown (259, 282, 284). The D7 protein family of mosquitoes is related to the odorant/pheromone binding proteins (109, 282). It was found that the mRNA of the D7 protein is actively transcribed while mosquitoes are taking a blood meal or probing compared to the inactive (not feeding/not probing) negative control (figure 2.8). This observation suggests that production of this protein might be important during blood feeding. One of the activities of this protein has been shown to be the sequestration of bioamines (33) and the high quantity produced during blood meal could be important to inactivate mediators that are related to coagulation, vasoconstriction and pain perception. Expression of this protein might also function in odorant binding to locate a source of blood or to detect pheromones during mating.

Identification of *Aedes aegypti* salivary proteins related to viral pathogenesis

In this study, I demonstrated by overlay binding assay (OBA) that *Ae. aegypti* 36 kDa protein binds to IFNγ. The OBA has been reported elsewhere as an assay to identify putative receptors to which viral particles might bind (112, 238, 303). The same approach was used to identify MSP that could be used by mosquitoes to down-regulate the immune

response of the host on which they feed by binding and sequestering immune mediators. Mosquito bites direct the immune response of the host towards the Th2 immune response (humoral) even when a delayed type hypersensitivity (DTH) is observed (51). This phenomenon has been observed in DENV infected patients, who switched towards Th2 response approximately three days into the course of infection, with significant increase of IL-4 and IgE production (160). IgE production has been associated with disease severity (126). DTH is characterized by an increase in IFNγ production and down regulation of IL-4 (81). IFNγ is a cytokine that stimulates the Th1 type immune response, stimulates uninfected cells and macrophages, inhibits viral replication and increases expression of MHC class I and II as well as induces immunoglobulin class switching to IgG1 and IgG2 (81, 110). Based on previous experiments were D7 protein sequestered bioamines, it was important to determine the potential role of the D7 protein of *Ae. egypti* in IFNγ sequestering, A549 cells were treated with IFNγ that had been preincubated with D7 protein 24 hours before infecting them with DENV2. Preincubation of IFNγ with the 36 kDa protein resulted in increased DENV2 titers in A549 cells, compared to IFNγ alone which resulted in significantly decreased DENV titer 24 hours post infection (figure 2.11). Addition of anti D7 antibodies resulted in decreased viral titer compared to IFNγ combined with the 36 kDa protein alone, indicating that removal of D7 protein prevented the sequestering effect. IFNγ is important during early stages of DENV infection by restricting virus dissemination and promoting viral clearance (248). The data indicate that IFNγ seems to restrict DENV infection during the first 24 hours. A549 cells have been previously used in IFN protection assays with several viruses (18, 306) including DENV (180). Pre- treatment of dendritic cells also protects them during early

DENV infection (102); the same effect has been observed in monocytes pretretated with IFNγ (254). In our study, at day 5 post infection viral production had reached high titers in all the treatment groups and there were no significant differences between groups. The early effect of INFγ may limit virus replication, but once the infection becomes established, IFNγ mediated protection appears to be less important in limiting expansion of viral load (102, 248). The microenvironment in which the mosquito deposits the virus in the skin is the first step during infection. The presence or absence of IFNγ in the local microenvironment surrounding the DENV-infected dendritic cell may be a key determinant of disease manifestation and severity (146).Taking these observations, one can postulate that, when IFNγ is released to promote an antiviral state, local sequestration of IFNγ by the D7 protein may occur. This prevents the IFNγ signaling in the neighboring cells and therefore DENV infection takes place, overcoming one of the first cell-defense mechanisms.

CHAPTER 3

CHARACTERIZATION OF IMMUNE RESPONSE TO *AEDES AEGYPTI* SALIVARY PROTEINS WITH DENGUE DISEASE SEVERITY

INTRODUCTION

DF and DHF are caused by infection with DENV (85), which are transmitted principally by *Aedes aegypti* mosquitoes. After an incubation period of 3 to 14 days, a person who is bitten by an infected *Ae. aegypti* mosquito may experience acute fever accompanied by a variety of nonspecific signs and symptoms (85). In the last 30 years, the incidence of DF epidemics in the Americas has increased and hyperendemic transmission of DENV has been established. This has resulted in a dramatic increase in the most severe form of the disease, DHF, which was first described in Southeast Asia. It is widely accepted that a risk factor for acquiring DHF is reinfection with a virus serotype different from the first infection, especially when the secondary infection is with DENV2 (90, 173, 241, 272), due to the phenomena known as "original antigenic sin"(94, 175) or antibody-dependent enhancement of infection (91). Epidemiologic evidence also has shown that there is an increased risk of developing DHF when the infection is caused by a virulent virus genotype (143), especially DENV2 Asian genotypes. Additional factors also have been implicated in disease severity (116, 140, 271).

Mosquito salivary proteins (MSP) have been associated with enhanced virulence of arboviruses and other infectious agents transmitted by mosquito bites (68, 148, 229). It is known that female MSP modulate the immune response of the hosts on which they feed; this effect occurs both locally (147, 190, 229) and systemically (56, 288, 294, 309). Co-infection with salivary proteins or salivary gland extracts also can facilitate the dissemination of pathogens, (78, 88, 245, 288) possibly due to functions of the proteins such as vasodilation, anticoagulation, platelet aggregation inhibition, and anti-inflammatory activity (46, 47, 220, 258). The retrospective study reported here was

conducted with well-characterized serum specimens from children who were hospitalized in Bangkok, Thailand to investigate a possible relationship between the immune response to MSP and the pathogenesis of DENV infections.

MATERIALS AND METHODS

Patients.

A total of 99 pairs of coded serum specimens were obtained from Thai children who had been admitted to a Bangkok hospital. Paired (admission and convalescent), de-personalized serum specimens from pediatric patients had been subjected to laboratory diagnosis as part of service efforts of the U.S Armed Forces Research Institute of Medical Sciences (AFRIMS) to the Bangkok community. The sera were collected in compliance with Thai and U.S. regulations on human use research. Because they had no personal identifiers, their use in this study was considered exempt from the need for informed consent by the AFRIMS Institutional Review Board. Diagnosis of acute DENV infections was made and Japanese encephalitis virus infection was excluded by IgM and IgG capture ELISA for both viruses. Dengue infection status (i.e., acute primary versus acute secondary) was assigned to all patients based on established criteria for AFRIMS IgM and IgG capture ELISA results (107, 108, 220, 287). Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to determine DEN serotype using the method previously described(134) modified by the use of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), 12.5 pmol primers, and a nested PCR amplification of 25 cycles. Disease severity was assigned using World Health Organization (WHO) criteria (296). Results reported in this study were limited to those of serum samples from DENV2-positive secondary infections and non-DENV non-

JEVinfections, determined after the code was broken. All sera were collected between July and December 2001. Patient ages ranged between 3 months and 17 years.

Mosquito saliva collection.

Ae. aegypti, (Rex-D strain, Puerto Rico) were reared at 28°C, 80% relative humidity, and a 12 h light:12 h dark photocycle at the Arthropod-borne and Infectious Diseases Laboratory (AIDL), Colorado State University. After eclosion, approximately 250 female mosquitoes were placed into each individual container, where sugar cubes and water were supplied *ad libitum*. Four to five days after mosquitoes eclosed, sugar and water were removed for 24 hr. Salivation induction followed the technique previously described (190) with slight modifications. Mosquitoes were allowed to feed for 1 hr through a parafilm membrane on an artificial feeder containing 2 mL sterile salivation buffer, which consisted of 164mM NaCl and 10mM $NaHCO_3$; neither ATP nor sucrose was added to the buffer. The salivation buffer was warmed to 38° C and temperature was maintained during feeding. This procedure yielded ~500 μg protein from 250 mosquitoes and was repeated as necessary.

Mosquito salivary protein concentration and fractionation.

After mosquitoes had probed the feeder for 1 hr, MSP were precipitated from the buffer by addition of trichloroacetic acid (TCA) to a final concentration of 10% (w/v) and vortex mixed for 10 sec. The mixture was incubated on ice for 30 minutes and centrifuged at 4° C for 15 minutes at 15000 rpm. The supernatant was discarded and protein pellets were resuspended in 0.3 ml acetone (-20°C) and vortexed for 5 sec followed by a second centrifugation at 4° C for 10 minutes at 15000 rpm. The acetone was discarded, the protein pellets were dried in a vacuum centrifuge and resuspended in

50µl distilled water or stored at -20° C until use. Protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce) according to manufacturer's instructions. NuPAGE Bis-Tris 10% polyacrylamide pre-cast gels (Invitrogen) were used to fractionate the saliva proteins. Two hundred fifty ng of MSP were loaded per well using non-denaturing loading buffer (Invitrogen) and electrophoresed for 40 minutes at 200 volts. After de-casting, the gel was silver stained using the SilverQuest staining kit (Invitrogen) following manufacturer's instructions. The number of stained protein bands, mobility, and relative intensity of staining were reproducible (figure 3.1A).

Immunodetection of the MSP.

After PAGE separation, unstained MSP were transferred to a nitrocellulose membrane at 30 volts for 2 hr. The membrane was blocked with 5% non-fat milk in PBS/0.1% Tween 20. The membrane was cut into strips and each strip was incubated overnight at 4° C on a rocking table with an individual Thai patient serum sample diluted 1:200 in blocking buffer. The strips of nitrocellulose were washed with PBS/0.1% Tween 20. Subsequent to washing, the samples were incubated at room temperature for 3 hr with a pooled mixture of peroxidase-labeled anti-human IgE, IgG, IgM, IgA (KPL) diluted 1:500 in blocking buffer. The small amounts of patient sera available necessitated testing for all immunoglobulin isotypes simultaneously. After incubation with the secondary antibodies, the nitrocellulose strips were washed and developed with 3,3'-diaminobenzidine (Sigma) in 0.1% hydrogen peroxide in PBS. The reaction was stopped by adding PBS/0.1% Tween 20. Reactivity with any of the MSP shown in figure 3.1 B was scored as positive for that protein. Of the 20 non-DENV infected patient sera tested, 18 contained antibodies to at least one MSP; of the 28 DF patients' sera tested, all had

antibodies to at least one MSP; and of the 51 DHF patients' sera, 50 had antibodies to at least one MSP.

Detection of human antibodies against MSP by indirect ELISA.

An indirect ELISA was used to assay reactivity of antibodies in Thai serum samples to total MSP. Briefly, MSP were diluted to a concentration of 0.1ng/µl in coating buffer (50mM sodium carbonate and 5mM sodium bicarbonate, pH 9.6), and 100µl were added to each well of a 96-well plate and incubated overnight at 4° C. The plate was washed, blocked with blocking buffer, washed again, and serum samples diluted 1:100 were added and incubated for 2 hr at 37°C. After washing, pooled HRP-labeled anti-human IgG, IgM and IgE diluted 1:1000 were added. The reaction was developed with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), (KPL) and plates were read at 405 nm. Negative controls were the human serum samples that showed no reaction in the immunoblot analysis. The cut-off for positive samples was the mean negative control value (OD = 0.0004) plus three standard deviations

Preparation of proteins and mass spectrometric analysis.

All proteins detected in immunoblot analysis of mosquito saliva also can be detected in salivary gland extracts. In order to obtain sufficient protein to identify the major proteins detected in MSP immunoblots, 100 pairs of female *Ae. aegypti* salivary glands were dissected and placed in 200 µl of lysis buffer containing 10mM Tris-HCl (pH 7.5), 150mM NaCl, 5mM EDTA, 1% sodium deoxycholate, 1% Triton-X-100, 0.1% SDS and 1 mM PMSF. The salivary glands were mechanically disrupted using a pestle and proteins were concentrated by TCA precipitation and fractionated by PAGE as described above. Proteins were stained with Coomassie blue, bands were marked, and the

gel was destained. Marked proteins were excised from the gel and half of each band was submitted to the Macromolecular Resource Facility, Colorado State University, for analysis by mass spectrometry. The other half of each band was eluted from the gel and proteins were concentrated with PAGE Prep Advance Kit (Pierce Laboratories) and TCA precipitated. Proteins were resuspended in 20μl of pure water, separated by PAGE and blotted to a nitrocellulose membrane as previously described. A pool of patient serum samples known to react with all major MSP was used in an immunoblot to verify the reactivity of the protein in each band. Mascot ion scores and cutoffs for each identified protein are presented in table 3.2.

Statistical analysis.

ELISA results were tested for differences between patient groups using one-way analysis of variance (ANOVA). Group means were compared using a *t*-test. A chi-square test was used to analyze differences in percentage of sera from each group reacting with each band of the immunoblot. Analyses were carried out using the SAS program version 9.1 (243).

RESULTS

PAGE protein fractionation and determination of immunoreactive MSP.

Ae. aegypti MSP were concentrated and quantified as described in Materials and Methods. After 250 ng of the proteins were loaded in each lane, they were fractionated by PAGE and a gel strip was silver stained. Seven prominent bands were seen with approximate molecular masses of 68, 46, 36, 30, 19, 17, and 14 kDa (figure 3.1A). Because the proteins were separated under nondenaturing conditions, high molecular weight material, assumed to be aggregates, was seen at the top of both stained gels and

immunoblots. Unstained proteins were transferred to a nitrocellulose membrane, which was cut into strips, and each was incubated with serum from an individual patient. Antibodies in individual sera were scored as reacting with one to seven MSP, as shown in the examples in (figure 3.1B). The immunonblots shown are representative from one patient classified as not infected (figure 3.1B lane 2) and one patient classified as DHF grade one (figure 3.1B lane 4)

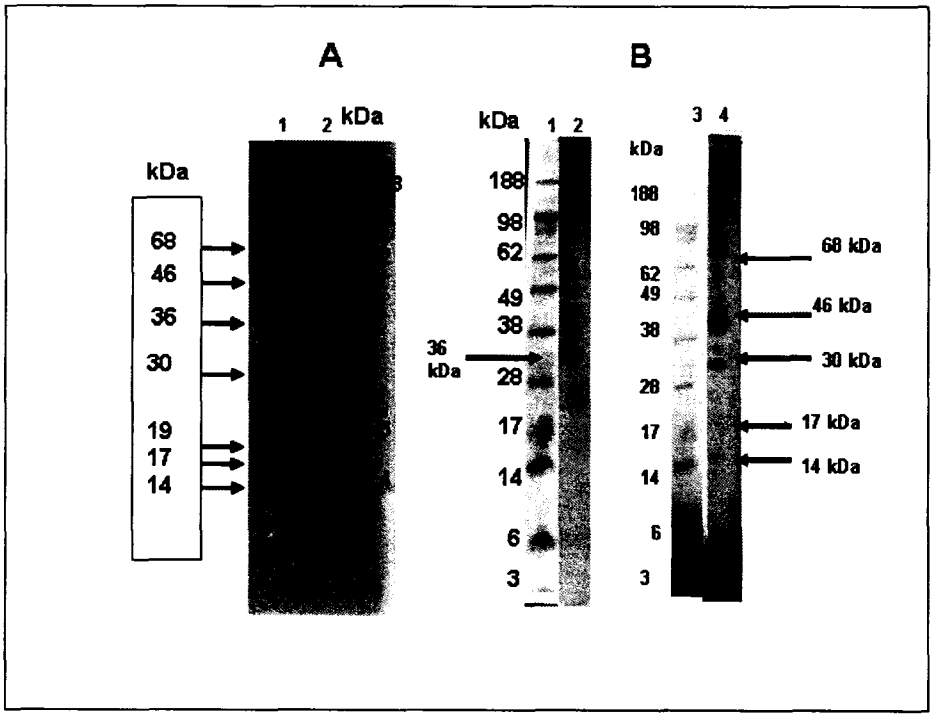

Figure 3.1. Fractionation of MSP by PAGE and examples of immunoblots with patients' sera. A) *Aedes aegypti* MSP concentrated with TCA, fractionated by nondenaturing 10% PAGE and silver stained; Lane 1, *Ae. aegypti* MSP; Lane 2, molecular markers; sizes and positions of major MSP in kDa are indicated at left. B) Nitrocellulose membranes showing the MSP that reacted with 2 patients' sera as detected by immunoblot (lane 2 and 4). Molecular markers shown in Lanes 1 and 3, approximate sizes in kDa of immunoreactive proteins shown with arrows

Reactivity in immunoblots and ELISA of sera from each patient group.

After completion of immunoblots with each of the 99 patients' sera, the key to the disease status of each patient was broken. The proportion of non-DENV infected (NI), uncomplicated DEN fever (DF), and DEN hemorrhagic fever (DHF) patients' sera that had antibodies reactive with each of the seven major MSP are shown in figure 3.2A. Indirect ELISA was used to determine reactivity of serum samples against total MSP and results are shown in figure 3.2B.

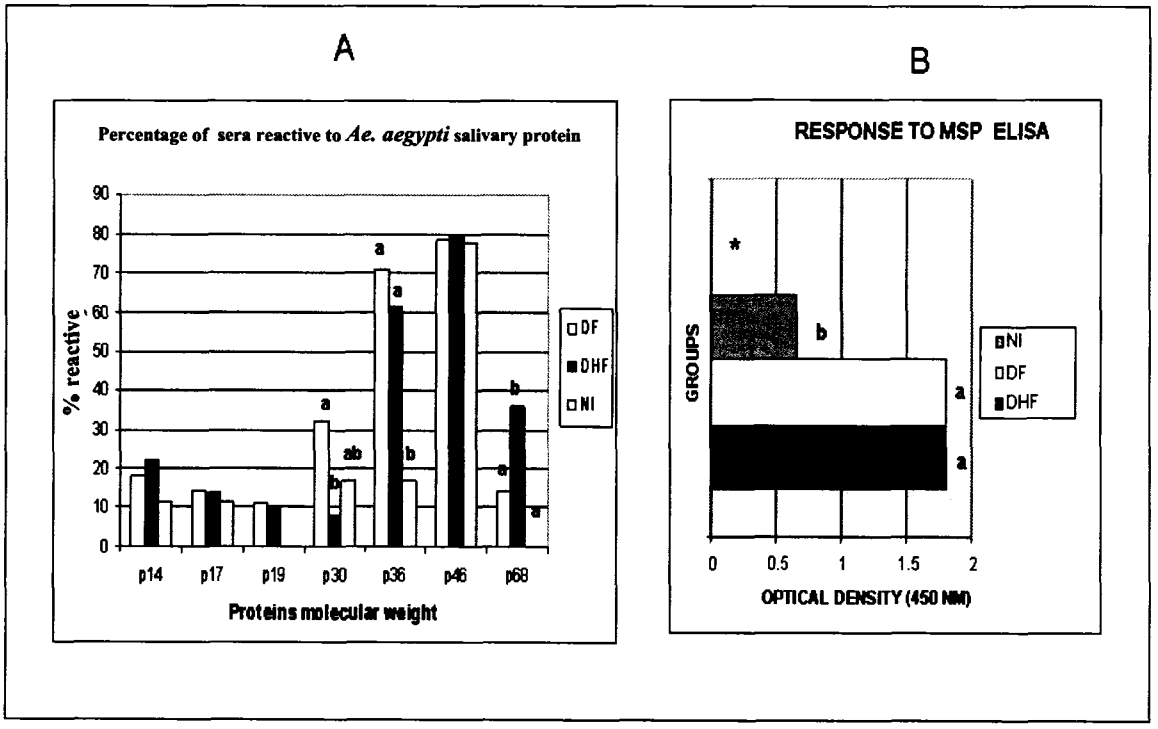

Figure 3.2. Reactivity of patients' sera with MSP in immunoblots and ELISA. A) Percentage sera from each patient group reacting by immunoblot to each mosquito salivary protein (MSP). DF = dengue fever, DHF = dengue hemorrhagic fever, NI = non-DENV infected. B) Mean reactivity of patient sera from each group to total MSP determined by indirect ELISA. In analysis of differences between groups by *t*-test, means with common superscripts [a, b] are not significantly different ($P>0.05$).

The number of patients in each group and percent with reactivity to each protein are given in Table 3.1A. Percent reactivity with each protein was compared between the three groups (DHF, DF or NI) and then between pairs of groups using a chi-square test. Significantly more DF patients' than DHF patients' sera reacted with p30. Conversely, significantly more DHF than DF patients' sera reacted with p68. Sera from both infected groups (DF and DHF) reacted with p36 in significantly higher percentages than sera from non

A

sup

	No. of patients positive/No. tested (%)						
	P14	P17	P19	P30	P36	P46	P68
DF	5/28(17.9)[a]	4/28(14.3)[a]	3/28(10.7)[a]	9/28(32.1)[a]	20/28(71.4)[a]	22/28(78.5)[a]	4/28(14.3)[a]
DHF	11/50(22)[a]	7/50(14.0)[a]	5/50(10)[a]	4/50(8)[b]	31/50(62)[a]	40/50(80)[a]	18/50(36)[b]
NI	2/18(11.1)[a]	2/18(11.1)[a]	0/18(0)[a]	3/18(16.7)[ab]	3/18(16.7)[b]	14/18(77.7)[a]	0/18(0)[a]

B

	ELISA mean	Standard Error
DF (28)	1.800[a]	0.00059
DHF (50)	1.8005[a]	0.00044
NI (18)	0.6595[b]	0.00073

Table 3.1. Reactivity of sera from each patient group to MSP in immunoblots and ELISA. A) Number patients' sera reactive with each protein in immunoblots/ total patients in group (percent reactive). Percentages with common superscripts [a], [b], are not significantly different ($P>0.05$).
B) Mean ELISA O.D for sera from each patient group measured at 450 nm. In analysis of differences between groups by *t*-test, means with common superscripts are not significantly different ($P>0.05$).

For ELISA, mean reactivity was lowest for non-DENV infected patients. Higher mean values were obtained for DF and DHF groups, although no significant difference was observed between these two groups. The differences between groups were tested by one-way ANOVA. The overall F-test was significant ($P<0.0001$) so group means were compared using a *t*-test as shown in Table 3.1B.

Identification of mosquito salivary gland proteins by mass spectrometry (MS).

Three MSP (p68, p36, and p30) showed significantly different reactivity in immunoblots between patient groups' sera. Proteins with these molecular masses previously have been shown to be allergens and designated Aed a 1, Aed a 2, and Aed a 3 (28, 201).To prepare sufficient protein for identification by mass spectrometric analysis of tryptic peptides, *Ae. aegypti* salivary glands were removed by dissection, homogenized in lysis buffer, and fractionated by PAGE as described in Materials and Methods. After trypsin digestion and elution from the gel, the three proteins were identified by MS/MS analysis and search of results against NCBI mosquito protein databases, as follows: a 30 kDa salivary gland allergen of unknown function; a 36 kDa protein related to the D7 family, which bind host amines such as serotonin, histamine, and norepinephrine (34); and a 68 kDa protein known as apyrase, which functions as an anticoagulant. The MS/MS results are presented in figures 3.3, 3.4 and 3.5 respectively, and the significant mass spectra ion scores that indicate probable identities ($P<0.05$) and peptide sequences used in protein identification are shown in Table 3.2 (47, 265).

Protein designation (Accession number)	Mascot protein ion score (cutoff score)	Sequence coverage (No. of matched peaks)	Peptide sequences identified by MS/MS spectra	Mascot MS/MS score (cutoff score)
30 kDa (gi|14423642)	101(55)	12 %(3)	K.VDHIQSEYLR.S R.SALNNDLQSEVR.V	57(28) 32(28)
36 kDa (gi|118216)	124(55)	37%(14)	R.SQIYAFNLPK.K K.FDASVIQEQFK.A	32(27) 37(27)
68 kDa (gi|1703351)	311(55)	44%(16)	K.LFPLTLIHINDLHAR.F K.GADIWDVAEHSFALDDEGR.T K.NPIYLNAGDNFQGTLWYNLLR.W	49(24) 122(24) 23(24)

Table 3.2. Protein identification from MS/MS spectra by MASCOT ion score. Data from MS and MS/MS analysis of tryptic peptides were processed using the SNAP algorithm in the FlexAnalysis software as described in Materials and Methods (chapter 2) and a monoisotopic list was generated for each sample. Combined MS and MS/MS results for each sample were searched using the Mascot (version 2.1) search engine against the NCBInr mosquito protein database. Protein ion scores that exceeded the cutoff scores for each sample were used in identification and are shown here.

MATRIX SCIENCE Mascot Search Results

```
User            : Jessica
Email           : jprenni@colostate.edu
Search title    : aed 30
MS data file    : DATA.TXT
Database        : NCBInr 20050618 (2521256 sequences; 854568500 residues)
Taxonomy        : Mosquito (24893 sequences)
Timestamp       : 26 Oct 2005 at 22:11:10 GMT
Significant hits: gi|2114497  30 kDa salivary gland allergen Aed a 3 [Aedes aegypti]
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 27 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

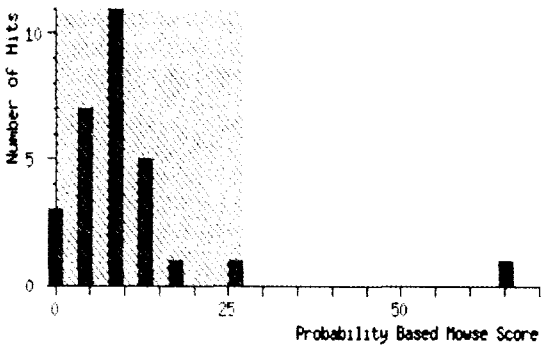

Figure 3.3 Example of mascot identification for the 30 kDa protein from *Aedes aegypti*. The cutoff mascot score is 27. The red bars in the green shaded area (<27) are not statistically significant, the red bar outside green area (65) represents maximum score, is significant and is considered a positive match to *Ae. aegypti* 30 kDa allergen.

Mascot Search Results

```
User              : Jessica
Email             : jprenni@colostate.edu
Search title      : aed 36
MS data file      : DATA.TXT
Database          : NCBInr 20050618 (2521256 sequences; 854568500 residues)
Taxonomy          : Mosquito (24893 sequences)
Timestamp         : 26 Oct 2005 at 22:51:59 GMT
Significant hits: gi|118216  D7 protein precursor (Allergen Aed a 2)
```

Probability Based Mowse Score

Ions score is -10*Log(P). where P is the probability that the observed match is a random event.
Individual ions scores > 28 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

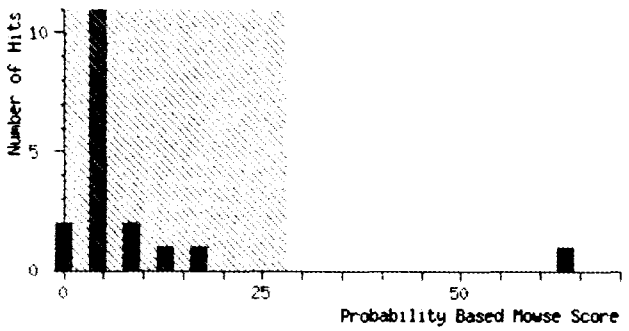

Figure 3.4 Example of mascot identification for the 36 kDa protein from *Aedes aegypti*. The cutoff mascot score is 28. The red bars in the green shaded area (<28) are not significant, the red bar outside green area (64) represents maximum score, is significant and is considered a positive match to *Ae. aegypti* 36kDa allergen (D7).

MATRIX SCIENCE Mascot Search Results

User	: Jessica
Email	: jprenni@colostate.edu
Search title	: p68B
MS data file	: DATA.TXT
Database	: NCBInr 20050618 (2521256 sequences; 854568500 residues)
Taxonomy	: Mosquito (8812 sequences)
Timestamp	: 14 Dec 2005 at 21:38:14 GMT
Warning	: A Peptide summary report will usually give a much clearer picture of MS/MS se
Top Score	: 319 for gi|1703351, Apyrase precursor (ATP-diphosphatase) (Adenosine diphosph

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 52 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

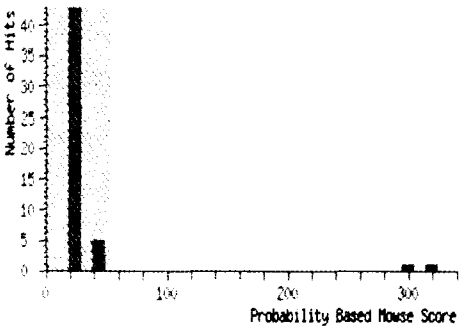

Figure 3.5 Example of mascot identification for the 68 kDa protein from *Aedes aegypti*. The cutoff mascot score is 52. The red bars in the green shaded area (<52) are not significant, the red bars outside green area (300 and 319) represents maximum scores, are significant and are considered positive match to *Ae. aegypti* 68 kDa apyrase.

In order to ensure that the proteins detected by immunoblot were the same as the ones that were analyzed by MS, half of the protein in each band of the three salivary gland extract proteins was refractionated and blotted as described above. Each of the three proteins reacted with pooled patients' sera (figure 3.6 B).

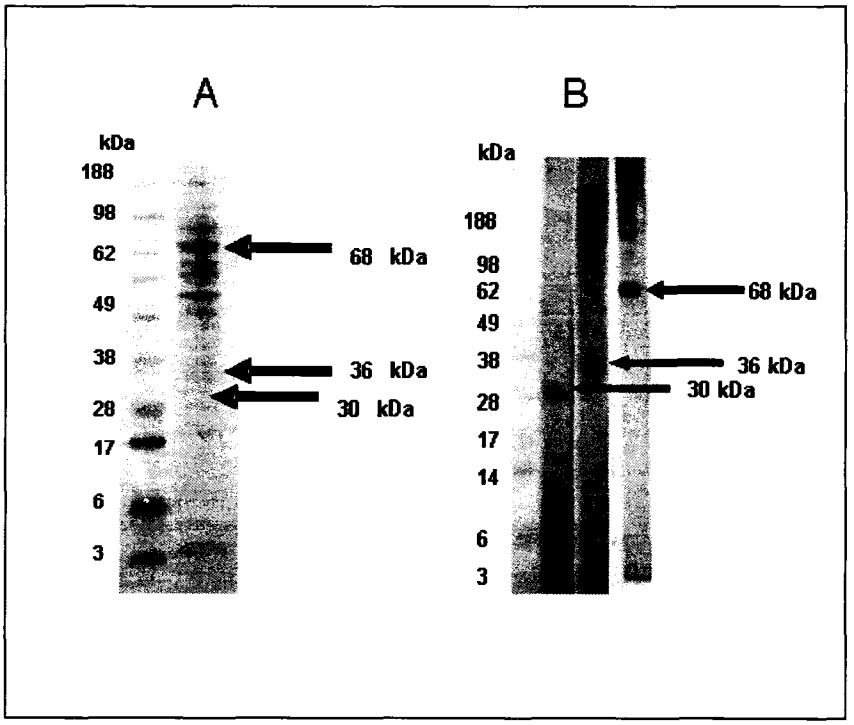

Figure 3.6. Identity of salivary gland homogenate proteins and immunoreactive MSP. A) Salivary gland homogenate fractionated by 10% PAGE and Coomassie blue stained. Bands indicated by arrows were excised from the gel and half was used for MS analysis. B) Immunoblot analysis of individual proteins excised and eluted from gel and fractionated by PAGE.

DISCUSSION

In this study, we showed that proteins collected in *Ae. aegypti* saliva could be concentrated by TCA precipitation and fractionated by non-denaturing PAGE, reproducibly demonstrating seven protein bands with molecular sizes ranging from 14

kDa to 68 kDa. Almost all serum samples from individual Thai pediatric hospital patients, whether or not they had a DENV infection, were shown in immunoblots to contain antibodies that bound to at least one and as many as seven of these proteins. The proportion of patients' sera in each of three groups, secondary DENV2 infection with DHF (DHF), secondary DENV2 infection with DF (DF), or non-DENV infected (NI), that contained antibodies to each of the protein bands was determined by immunoblot. Comparison between groups showed significant differences ($p<0.05$ by chi-square analysis) in immunoreactivity for p30, p36, and p68 (Table 3.1A). Each of these proteins was identified by mass spectrometric analysis. The results we have reported are based on MS and MS/MS spectra so they are of high confidence. The proteins we identified were previously described as allergens designated Aed a 1 (p68), Aed a 2 (p36), and Aed a 3 (p30) (253).

When reactivity with total MSP was assayed by ELISA, we observed that the groups of individuals infected with DENV2, independent of disease severity (hemorrhagic and non-hemorrhagic), had higher antibody titers to total MSP than non-DENV infected patients (Table 3.1 B). It is known that greater exposure to vectors correlates to higher risk to become infected (138, 246), thus the lower overall reactivity in NI patients may correlate with lower exposure rates.

As further evidence that the NI patients had less exposure to MSP, significantly fewer NI patients had antibodies to p36, one of the most abundant MSP (figure 3.2 A) and the second most immunoreactive protein in DENV infected patients (71% of DF and 62% of DHF as compared to 16.7% of NI patients; Table 3.2 A). The 36 kDa MSP was identified as a member of the D7 protein family (195, 211, 253). The long D7 protein of *Ae. aegypti*

has recently been shown to bind biogenic amines such as serotonin, histamine, norepinephrine and epinephrine (34). These biogenic amines have important functions in host defense including increasing vascular permeability, vasoconstriction, platelet aggregation, and pain induction, and their inhibition by D7 protein sequestration could affect immune response, promoting increase in viral pathogenesis. However, possession of antibodies against this protein did not appear to protect from infection or to differentially affect disease severity in DENV infections.

It was found that a significantly higher proportion of sera from patients with secondary DENV2 infection and no hemorrhagic manifestations than from patients with DHF had antibodies against a 30 kDa protein of *Ae. aegypti* saliva with unknown function (figure 3.2 A). The 30 kDa protein has been reported as an allergen (29, 211). Allergic reactions to mosquito proteins are proposed to occur as early immune response to mosquito bites (195, 211). During an allergic reaction, some individuals experience a delayed type hypersensitivity (DTH) response, whereas others experience immediate cutaneous reactions and finally tolerance (211, 222). Allergy mediated by MSP involves the presence of IgE and IgG4 immunoglobulins (211); however, since it was not possible to discriminate between patients' immunoglobulin isotypes, it is not known if the antibodies detected were IgE, and this is a question that should be pursued in future studies. Furthermore, although some individuals experience a DTH response, in experimental models, a mosquito bite generally triggers a Th2 immune response, which is directed toward humoral immunity (51, 245, 309).

A significantly higher proportion of patients experiencing hemorrhagic manifestations had antibodies to a 68 kDa protein (Table 3.1 A), which was identified as apyrase. This

protein has been widely reported in the saliva of blood sucking arthropods (47, 222). Apyrases act as anti-coagulants that facilitate the location of capillary vessels during blood feeding (8, 225). One might expect that encountering antibodies against this protein would make it difficult for mosquitoes to feed; however, it has been reported that mosquitoes can achieve a blood meal even in the presence of high titers of antibodies against apyrase (162). Further, it has been shown that if the production or effect of apyrase is inhibited, the behavioral probing of the mosquito is prolonged (186, 225, 235). Thus, the presence of antibodies to apyrase might increase the risk of inoculation of DENV2 in each feeding attempt (234).

It was observed in this study that presence of antibodies against certain MSP is a potential indicator of contracting DF or DHF upon secondary infection. This is a provocative finding, but larger studies, including patients who did not require hospitalization, will be necessary to fully understand the role of specific mosquito saliva proteins and specific antibody isotypes that could modulate DENV transmission and pathogenesis. Lack of an animal model for DENV infections precludes controlled studies of the role of antibodies to MSP in modulating infection. However, prospective studies of human responses in DENV endemic regions could give further insight into these observations.

CHAPTER 4

IMMUNIZATION WITH *CULEX TARSALIS* MOSQUITO SALIVARY PROTEINS MODULATES WEST NILE VIRUS INFECTION IN MICE

INTRODUCTION

Mosquito salivary proteins have been shown to increase the pathogenesis of viruses transmitted by mosquito bite during the acquisition of blood (244). Regulation of the host immune response during arthropod feeding has been widely reported (1, 21, 56, 245, 275, 288). However, most studies showing immune regulation by arthropod salivary proteins have been conducted in naïve animal models that have no known previous exposure to vector saliva (56, 149, 264, 288, 294). It has been reported that previous exposure to vector saliva decreased the pathogenicty of parasites transmitted by sand flies (117, 270).

West Nile virus (WNV) is a member of the Japanese encephalitis virus serological complex, which includes Japanese encephalitis virus (JEV), Saint Louis encephalitis virus (SLEV), and Murray Valley encephalitis virus (MVEV) (99). WNV belongs to the family *Flaviviridae*, genus *Flavivirus,* which have a single-stranded positive-sense RNA genome (30). WNV is naturally transmitted by *Culex* spp. mosquitoes, including *Culex tarsalis,* which is a major vector of WNV in the western U.S (276, 278). WNV is naturally maintained in cycles between mosquitoes and birds; humans and other mammals are usually incidental hosts (202). Based on previous observation the hypothesis for this study was that immunization with *Cx. tarsalis* salivary proteins will affect pathogenicity of WNV mosquito-bite infection in mice.

MATERIALS AND METHODS

Mosquito rearing and infection

Cx. tarsalis from Bakersfield California (26, 208) were obtained from Dr. Nordin Zeidner from Centers for Disease Control and Prevention (CDC) Fort Collins, Colorado.

The Bakersfield, CA colony was established by William Reeves in 1953. It was maintained at the Arbovirus Field Station in Bakersfield since its original founding. Bakersfield mosquitoes were maintained autogenously for 10 years and had several infusions of wild material from the local area over the years. None of this was carefully recorded prior to 1980 (William Reisen, personal communication). Mosquitoes were reared at 24-25°C with 70% relative humidity in a 12h/12h cycle of light/darkness at the Arthropod-Borne and Infectious Diseases Laboratory (AIDL). Female mosquitoes were selected at the pupal stage and around 200 were placed in each pint carton. When they reached adult stage, water and sugar were supplied *ad libitum* for 4 days. At day five, sugar and water were removed for 24 hours after which they were offered an infectious artificial blood meal using defibrinated sheep blood containing 7.3 $\log_{10}$ pfu/ml of WNV (strain NY99). Briefly, equal volumes of WNV NY99 strain and defibrinated sheep blood (Colorado Serum Company) were mixed and warmed to 40°C. Two milliliters of this mixture were placed in the artificial glass feeder and mosquitoes were allowed to probe through the hog gut membrane. Temperature was maintained during the blood meal by 40°C water flowing through the jacket. Mosquitoes were also infected by intrathoracic (IT) inoculation with approximately 1µl of the same viral strain (4.3 $\log_{10}$ pfu). After infectious blood meal or IT inoculation, 50 mosquitoes were placed in plastic cages supplied with water and sugar *ad libitum* for 7 days.

Salivary gland antigen preparation.

Mosquitoes were reared as described above. When mosquitoes emerged as adults they were cold-anesthetized and briefly submerged in 70% ethanol. Salivary glands were dissected using fine forceps, then placed into 200 µl of cold lysis buffer (10 mM Tris-

HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% Triton-X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride). One hundred salivary glands were mechanically disrupted in a 1.5 ml microcentrifuge tube using a pestle,the homogenate was centrifuged at 3500 rpm for 5 minutes at 4°C, and the supernatant was collected and proteins were concentrated by 10% trichloroacetic acid (TCA) precipitation. Proteins were resuspended in 100 µl of sterile phosphate-buffered saline (PBS, pH 7.4) containing a protease inhibitor cocktail (Roche Laboratories). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. Protein lysates were diluted to a concentration of 10ng/µl and mixed with an equal volume of 10ng/µl of synthetic peptide adjuvant, N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) (Sigma).

Animals.

NIH Swiss mice were used for these experiments. Two week-old mice were purchased from Charles River Laboratories. Mice were handled and housed in a BSL 3 facility according to the protocol approved by the Animal Care and Use Committee at Colorado State University.

Immunization.

Mice were given a week for adaptation after which ten mice per group (in two experiments) were immunized intramuscularly with 500ng protein from Cx. *tarsalis* salivary gland homogenate plus 500ng of MDP (100µl total volume). Two intramuscular booster immunizations were carried out at days 7 and 21 after the first immunization. The control group was mock immunized with PBS plus 500ng of MDP, and booster immunizations were given at days 7 and 21.

Immunoblot analysis.

Confirmation of antibody generation against mosquito salivary proteins in vaccinated mice was determined by immunoblot analysis. Salivary gland homogenate (SGH) was prepared from ten salivary glands from female *Cx. tarsalis* as described above and resuspended in 30µl of PBS containing protease inhibitor cocktail, mixed with 7.5µl of lithium dodecyl sulfate (LDS) 4x buffer, and 12 µl were loaded in each lane of a SDS 10% PAGE and fractionated. Proteins were transferred to a nitrocellulose membrane as described in chapter 2. Vaccinated and unvaccinated control mice were bled from the caudal vein one week after the last booster immunization at approximately 5 weeks of age. Blood samples of each group were pooled in 100µl of MEM, samples were then centrifuged at 3500 rpm for 10 minutes and the supernatant was collected. The 100µl of MEM containing pooled mouse serum was diluted in 1000µl of blocking buffer and incubated with the nitrocellulose strips containing the SGH overnight. The next day mouse serum was removed and strips were washed 3 times with 0.01%Tween 20 in PBS. Strips were incubated with secondary peroxidase-labeled rabbit anti-mouse IgG in blocking buffer at room temperature for 1 hour, washed 3 times with 0.01%Tween 20 in PBS, and developed using a solution containing 3,3' diamenobenzidine (DAB)(Sigma). The reaction was stopped by adding 1ml of 0.1% Tween 20 in PBS directly to the nitrocellulose membrane.

Mouse infection.

Seven days after the last booster immunization, mice were anesthetized by intraperitoneal injection of 0.2ml ketamine/xylazine (0.2mg/2mg) and placed into cages containing infected mosquitoes. Mosquitoes were allowed to probe the mice and take a

blood meal. Engorged mosquitoes were aspirated out of the cage using a manual entomological aspirator and immediately frozen in dry ice for virus titration.

Organ harvesting and RNA extraction.

Brains and spleens were removed from mice and 0.05 gram of each organ was placed in 150µl MEM supplemented with 2% fetal bovine serum (FBS) and antibiotics (Gibco). Tissues were mechanically disrupted by grinding with a pestle in a 1.7 ml microcentrifuge tube. Homogenates were centrifuged at 14000 rpm for 5 minutes and supernatants were placed into new 1.7 ml microcentrifuge tubes. Aliquots were frozen immediately at -70°C and used later for virus titration by plaque assay. The tissue pellet was used to extract total RNA by Trizol (Invitrogen) following the manufacturer's instructions. RT-PCR was used to detect viral RNA in tissue.

Mosquito Processing.

Once fed, the mosquitoes were collected from the cage and triturated by pestle in a 1.7 ml microcentrifuge tube in 300 µl of MEM containing 2% FBS and antibiotics. The homogenate was centrifuged at 14000 rpm for 10 minutes and total RNA was extracted from 140µl of supernatant using an RNA extraction kit (Qiagen). The remaining160µl of supernatant was transferred to a new tube, frozen, and used to titrate infectious virus by plaque assay.

Flow cytometry analysis.

Mice were euthanized at days 4 and 8 post infection. To study cellular immune response, spleens from control and immunized mice were harvested (4 mice/time point/group). Splenocytes were isolated to analyze cell populations and Th1/Th2 cytokine profiles. Spleens were placed in a sterile Petri dish with RPMI 1640 medium (Invitrogen)

containing penicillin/streptomycin and L-glutamine, and 10% fetal bovine serum. Spleens were disrupted by rubbing them between two frosted ends of glass slides and filtered through a 70μm nylon cell strainer (Falcon). Cells were washed twice with RPMI 1640 medium. The cells were resuspended in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum and 0.5mM 2-mercaptoethanol and stimulated at 2.5 x 10^6 cells/well with 50 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 5 h at 37°C. Golgi-plug (BD Pharmingen) was added during the final 3.5 h. The cells were harvested, stained with antibodies (BD Pharmingen) for CD3 and CD8 and fixed in 2% paraformaldehyde. Cells were permeabilized with 0.5% saponin before adding PE-conjugated anti-IFN-γ (clone XMG 1.2), anti-TNF-α, anti-IL-2, anti-IL-4, anti-IL-10 or rat IgG1 as control (BD Pharmingen). Samples were examined using a Coulter XL instrument (Beckman Coulter, Fullerton, CA). A total of 25,000 events were analyzed for each sample. Data were analyzed using FCS express 2 (De Novo Software Ontario, Canada). Dead cells were excluded on the basis of forward and side light scatter. $CD3^+$ $CD8^-$ T cells were gated as $CD4^+$ T cells.

RT-PCR.

Detection of WNV RNA in mouse tissues and mosquitoes was done by reverse transcription-PCR assay. Total RNA was used as template to generate the first strand cDNA. One hundred nanograms of RNA were mixed with 25 nanograms of WNV reverse primer, 10mM dNTPs in a total volume of 12 μl. The mix was heated to 70°C for 10 minutes and immediately cooled in ice for 5 minutes. The rest of the reagents, including 5x first strand buffer, 0.1M DTT and Superscript II (Invitrogen) reverse

transcriptase were added to the mix according to manufacturer recomendations. The RT was carried out at 42°C for 50 minutes and denaturation at 70°C for 15 minutes. Five microliters of the above reaction were used for amplification of the desired region of the WNV cDNA. The enzymes used were the PCR reagent system (Invitrogen) following the manufacturer's instructions; the program used was 95°C for 5 minutes, 35 cycles of 95°C for one minute, 50°C for one minute and 72°C for 2 minutes. One final extension at 72°C for eight minutes was performed. The DNA from the reaction was electrophoresed in a 1% agarose gel (Sigma). Primers used for WNV RT-PCR are shown in table 4.1.

Plaque Reduction Neutralization Test (PRNT).

Blood was drawn from mice by cardiac puncture and placed in a sterile 1.7 ml microcentrifuge tube and allowed to coagulate at room temperature. Tubes were centrifuged at 3500 rpm for 10 minutes. Serum aliquots were diluted 1:2 with MEM and frozen at -70°C. Diluted serum was analyzed for WNV antibodies by 80% plaque reduction neutralization test (PRNT) on Vero cells (71). Vero cells were allowed to grow to confluency in 6-well plates in MEM with Earle's salts, L-glutamine, and 100 units/ml penicillin, and 100 μg/ml streptomycin and 10% FBS. The virus used in the test was WNV strain NY99. Second overlay containing neutral red was applied two days after the initial nutrient overlay application. Samples were read the day after the second overlay. Titers were expressed as the reciprocal of the serum dilutions reducing the number of plaques by ≥80% ($PRNT_{80}$).

Viremia titration by plaque assays.

Mice were euthanized at days 4 and 8 post infection. Additional mice were sacrificed at 24, 48 and 72 hours post infection to analyze early viremia. Virus titers in

blood were determined by plaque titration in Vero cells at 37°C as described previously in chapter two.

Q-RT-PCR assay.

In order to measure the cytokine related mRNA production during WNV infection by mosquito bite, Q-RT-PCR assay was used, applying the protocol previously described (chapter 2) using specific primers for each mouse cytokine and β actin. Primer sequences were selected from previous reports (80, 189) and are presented in table 4.1. Total RNA was extracted from spleen at days 4 and 8 as described above. First strand cDNA was amplified from 100ng of extracted RNA, using the ThermoScript RT-PCR system (Invitrogen) and Q-PCR was carried out using the Brilliant SYBR Green QPCR Master Mix (Stratagene) in a Bio-Rad iQ5 system. Three cytokines from the Th1 response (IL-2, IFN gamma and TNF alpha) and two from the Th2 response (IL-4 and IL-10) were measured. Data were compared with the iQ5 system software, provided by the manufacturer. The conditions used for Q-PCR amplification were: 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute followed by incubation at 4°C, if reaction was left overnight.

Statistical analysis.

Q-RT-PCR results were analyzed by non-paired Student's *t-test* for a single mean comparison. Plaque reduction neutralization test results were analyzed assuming a Poisson distribution using a logarithm transformation. Results were presented by group and time with a 95% confidence interval. An exact test of proportions was used to compare morbidity and mortality between groups of animals.

Cytokine	Primer Sequence	Reference
IL-2	(+) 5'CCT GAG CAG GAT GGA GAA TTA CA3'	(80)
	(-) 5'TCC AGA ACA TGC CGC AGA G 3'	
IFNγ	(+) 5'TCA AGT GGC ATA GAT GTG GAA GAA 3'	(80)
	(-) 5' TGG CTC TGC AGG ATT TTC ATG 3'	
TNFα	(+) 5'CAT CTT CTC AAA ATT CGA GTG ACA A 3'	(80)
	(-) 5' TGG GAG TAG ACA AGG TAC AAC CC 3'	
IL-4	(+) 5' ACA GGA GAA GGG ACG CCA T 3'	(80)
	(-) 5'GAA GCC CTA CAG ACG AGC TCA 3'	
IL-10	(+) 5' GGT TGC CAA GCC TTA TCG GA 3'	(80)
	(-) 5' ACC TGC TCC ACT GCC TTG CT 3'	
β-Actin	(+) 5' AGA GGG AAA TCG TGC GTG AC 3'	(189)
	(-) 5' CAA TAG TGA TGA CCT GGC CGT 3'	
WNV	(+) 5' TTG TGT TGG CTC TCT TGG CGT TCT T 3'	(136)
	(-) 5' CAG CCG ACA GCA CTG GAC ATT CAT A 3'	

Table 4.1. Primer sequences used for RT-PCR and Q-RT-PCR analyses (+, forward primer, - reverse primer). Interleukin 2 (IL-2), Interferon gamma (IFNγ), Tumor necrosis factor alpha (TNFα), Interleukin 4 (IL-4), Interleukin 10 (IL-10), Beta actin (β-Actin) and West Nile virus (WNV).

RESULTS

Immunoblot.

Salivary gland homogenate (SGH) proteins from *Cx. tarsalis* were separated by PAGE, transferred to a nitrocellulose membrane, and incubated with serum of vaccinated or unvaccinated control mice in order to confirm immunity after vaccination. Because of the small amount of blood obtained from the caudal vein, serum samples were pooled. Reactivity to SGH antigens of immunized mice and controls were compared. Generation of strong bands showed that immunized mice produce antibodies against mosquito salivary gland proteins, whereas the unvaccinated group had no detectable antibodies by immunoblot assay. The immunoblot results are shown in figure 4.1. Several bands were observed in the Western blot. Prominent reactivity was seen at 17 kDa, 36 kDa 47 kDa, 49 kDa and 68 kDa. Since the proteins were neither heat nor β-mercaptoethanol treated, bands seen at the top of the gel (>100 kDa) could be protein aggregates although no protein analysis was carried out to confirm protein identity.

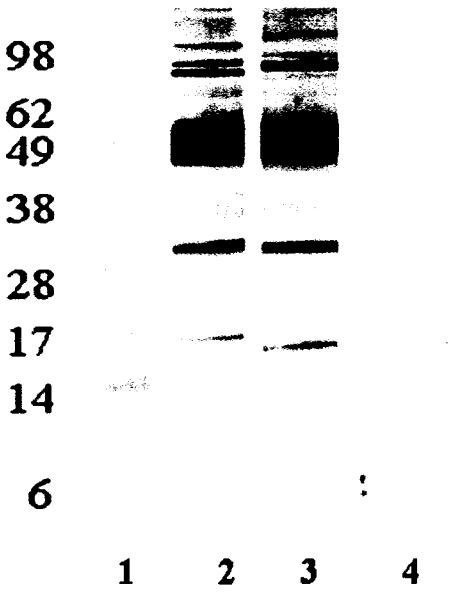

Figure 4.1. Immunoblot analysis using *Cx. tarsalis* salivary gland homogenates to confirm generation of immune response to mosquito salivary proteins in the vaccinated mice used in the experiments 1) Molecular marker, 2) Serum pool from 4 immunized mice, 3) Serum pool from 4 immunized mice, 4) Serum pool from 8 non-immunized mice.

Confirmation of WNV infection in mosquitoes.

Mosquitoes used for this experiment were *Cx. tarsalis* from Bakersfield, California. Mosquitoes either received an infectious blood meal containing $7.3\log_{10}$ pfu/ml or were IT inoculated with 1µl of the same virus stock ($4.3\ \log_{10}$ PFU/mosquito) WNV and were incubated for seven days before feeding on mice. After mosquitoes fed on mice, WNV infection was confirmed by RT-PCR analysis and whole body virus quantitation by plaque assay. The cDNA generated with specific WNV primers by RT-PCR was electrophoresed in a 1% agarose gel stained with ethidium bromide and visualized under UV light. Two or three mosquitoes fed on each mouse. Some mosquitoes were not infected, (figure 4.2), but all the mice were bitten by at least 2

infected mosquitoes. The mosquito titer determined by plaque assay averaged $5.3\log_{10}$ pfu/ml for those fed on both vaccinated and unvaccinated mice.

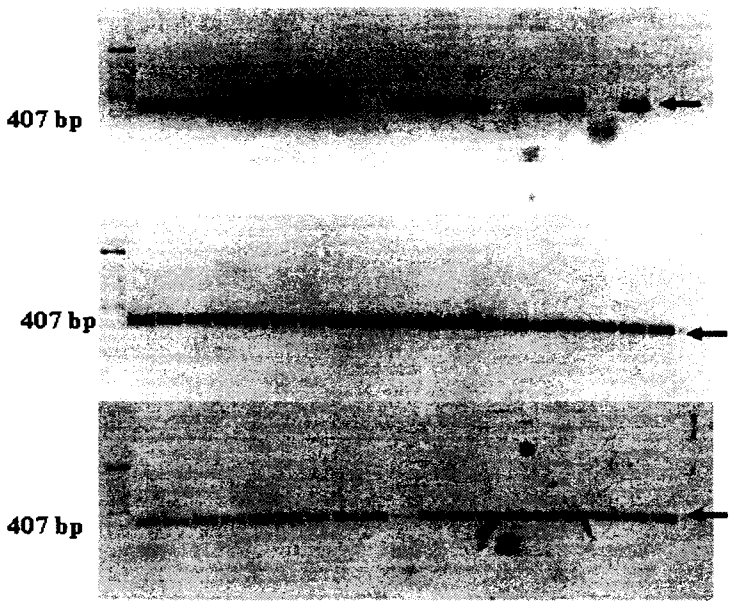

Figure 4.2. Detection of WNV RNA in mosquitoes. Total RNA was extracted from mosquitoes that were infected *per os* with WNV NY99 strain and infection was confirmed by RT-PCR seven days after mosquitoes were offered and infectious blood meal or were IT infected. Each lane represents the amplicon product from a single WNV infected mosquito that fed on mice.

WNV infection of mice.

Plaque assay revealed that the viremia in unvaccinated mice lasted for at least 72 hours post infection; the viral titer on average was $3.52\log_{10}$ pfu/ml from 2 mice analyzed per time point (figure 4.3 A). Infectious virus was not detected by plaque assay in blood of the vaccinated group as shown in figure 4.3.A RT-PCR was used to confirm viral RNA. Viral RNA was first detected in blood of 1 of 2 unvaccinated mice (50%) but not in vaccinated mice sampled by RT-PCR at 24 hours post challenge. Viral RNA was detected in the serum from the vaccinated group at 48 hours post challenge. RNA was first detected in 2 (100%) sampled vaccinated mice

by RT-PCR at 48 hours post challenge and in 1 of 2 mice (50%) at 72 hours post infection (figure 4.3 B).

A

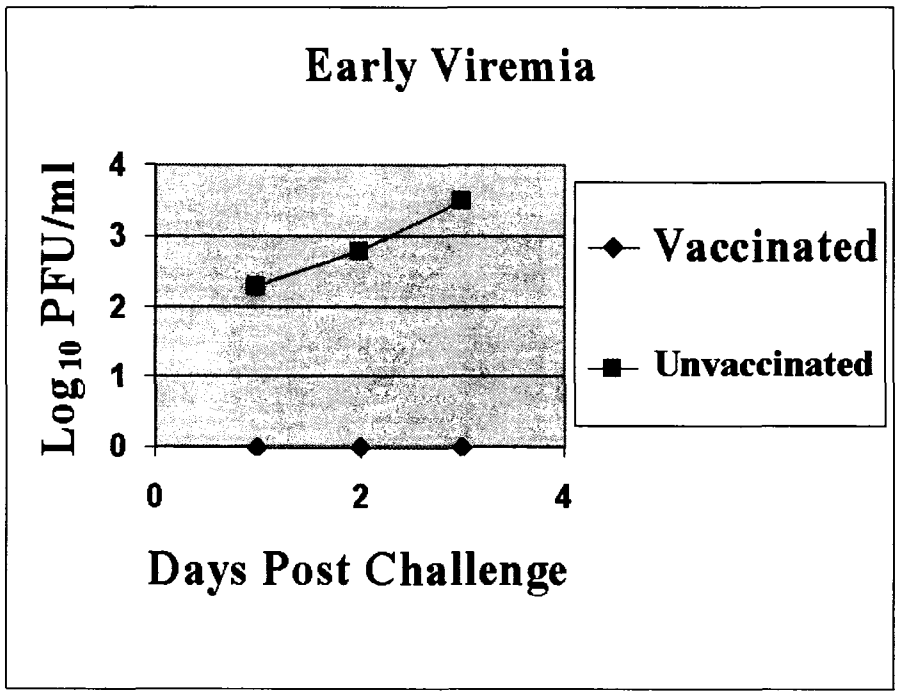

Figure 4.3.A). Detection of WNV viremia in unvaccinated and vaccinated mice at 24, 48 and 72 hours post challenge. The viral titer was lower in the vaccinated group when compared with the control by plaque assay. No statistical analysis was performed due to sample size (Two mice per group were analyzed).

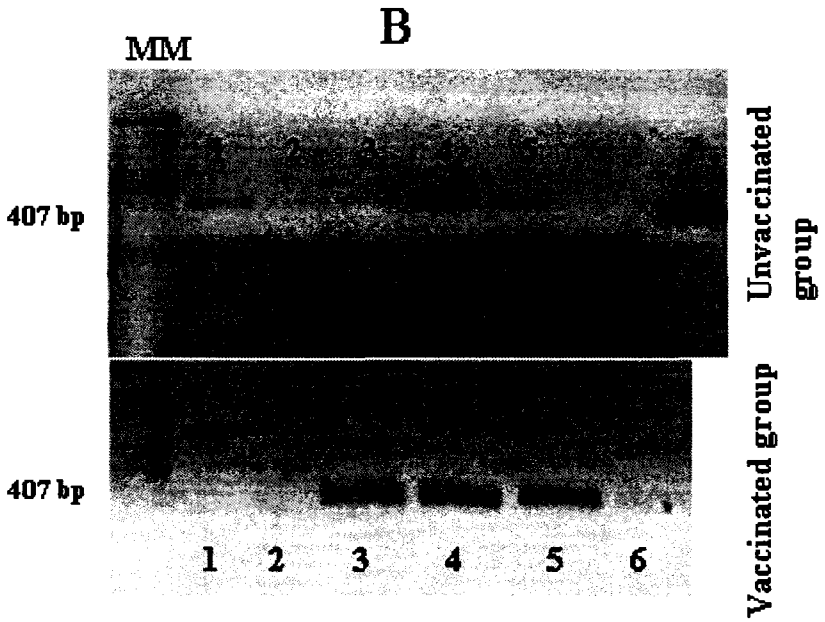

Figure 4.3.B). Detection of viral RNA in unvaccinated and vaccinated mice. Viral RNA was first detected in blood at 24 hours post infection in the unvaccinated and detected in vaccinated group only after 48 hours post infection by RT-PCR. MM, molecular marker; lanes 1 and 2; day one post challenge; lanes 3 and 4, day 2 post challenge; lanes 5 and 6, day 3 post challenge, lane 7 Positive control (shown in control group only).

After mice were fed upon by infected mosquitoes, brain, blood and spleen were collected at days 4 and 8 post challenge to analyze viral dissemination and viral titer. At day 4 post challenge viral RNA was detected by RT-PCR in the brains of 3 out of 8 (37.5%) unvaccinated mice but only one (12.5%) mouse presented splenic viral RNA. In contrast, vaccinated mice did not show evidence of viral RNA in brain, but 37.5% (3 of 8) had viral RNA on their spleens (figure 4.4.A). WNV dissemination to spleen and brains was confirmed by plaque assay (figure 4.4.B). Difference in viral titer was statistically significant between groups for brain ($P<0.0001$), being higher in the unvaccinated group at day four post infection. In contrast, viral titer was significantly higher ($P<0.005$) in the spleens of the vaccinated group at day 4.

A

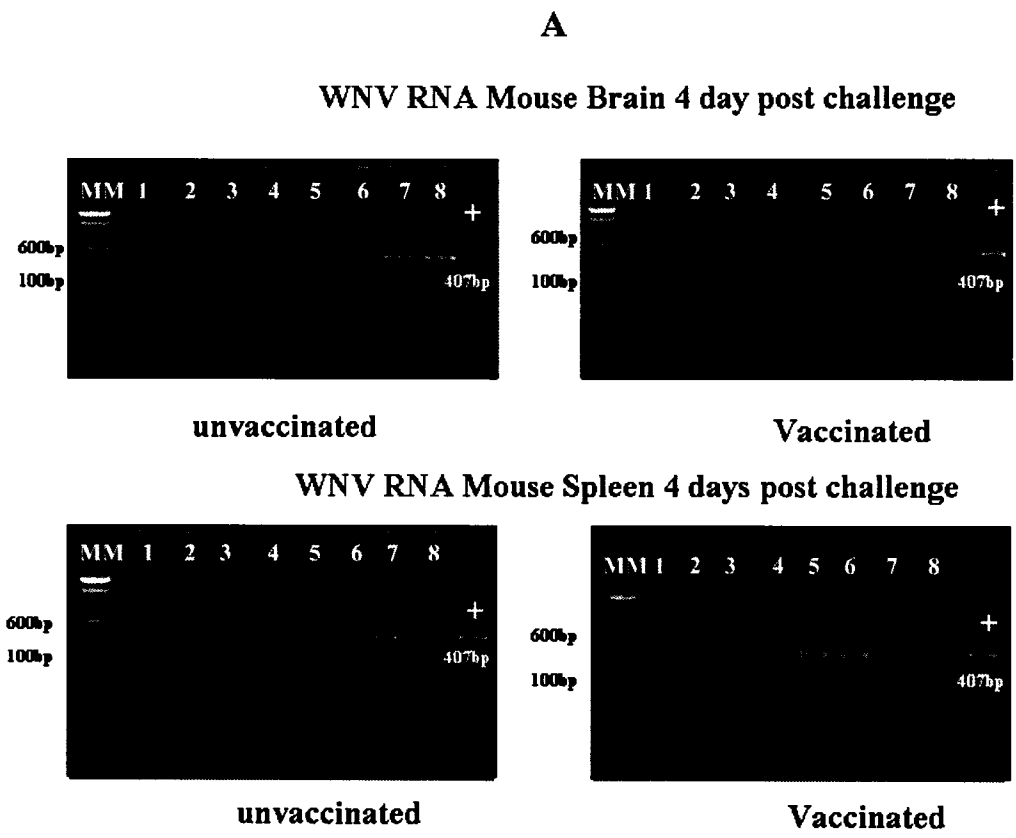

Figure 4.4.A) Detection of viral RNA by RT-PCR in mouse tissues at day 4 post challenge. + represents the positive control of RNA from cell culture infected with WNV. Each lane (1 to 8) represents tissue of an individual mouse.

B

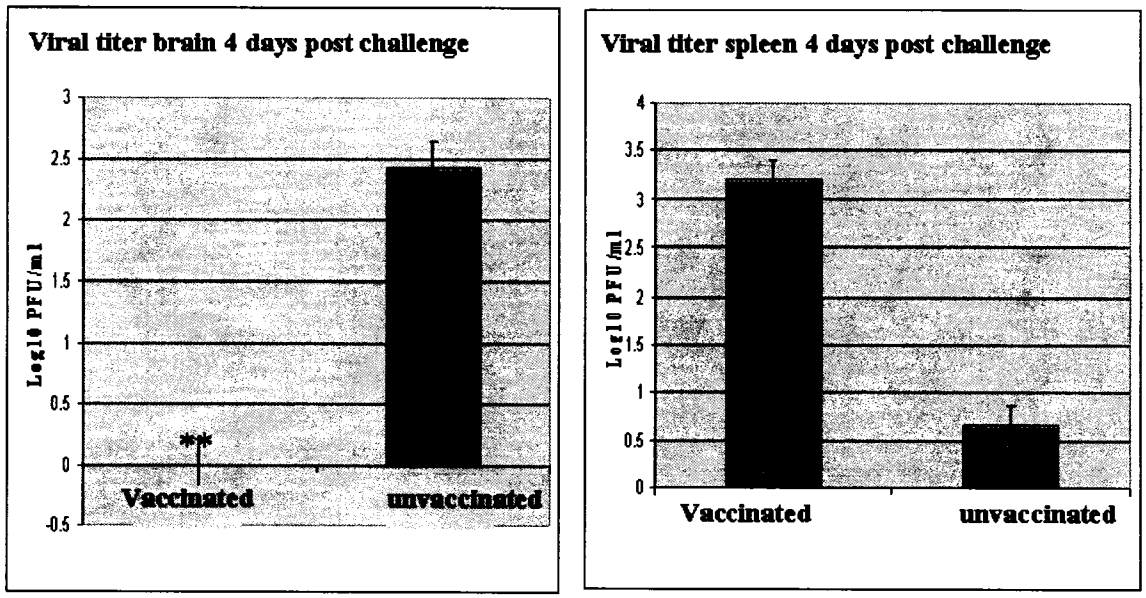

Figure 4.4.B WNV dissemination in tissues of vaccinated and unvaccinated mice 4 days post challenge by infected mosquito bite. MSP immunization of mice alters WNV pathogenesis. Graphics represent mean viral titer by plaque assay at day 4 post challenge. Viral titers in brain (red bars) and spleen (purple bars). Eight mice were analyzed per time point per group; ** No virus detectable.

At day eight post challenge brains of all mice in both groups were positive for WNV RNA by RT-PCR (figure 4.5 A). By plaque assay we found infectious virus in five mice from the unvaccinated group and six of the vaccinated group. An exact test of proportions revealed that viral titer in the brain was statistically higher ($P<0.0098$) in the unvaccinated group than in the vaccinated at day eight post challenge (figure 4.5.B). Neither virus nor viral RNA was found in spleens of unvaccinated or vaccinated mice at day eight post challenge. When vaccinated and unvaccinated groups were compared independently of time post challenge the virus titer was higher ($P<0.0025$) in tissues of the unvaccinated compared to vaccinated mice.

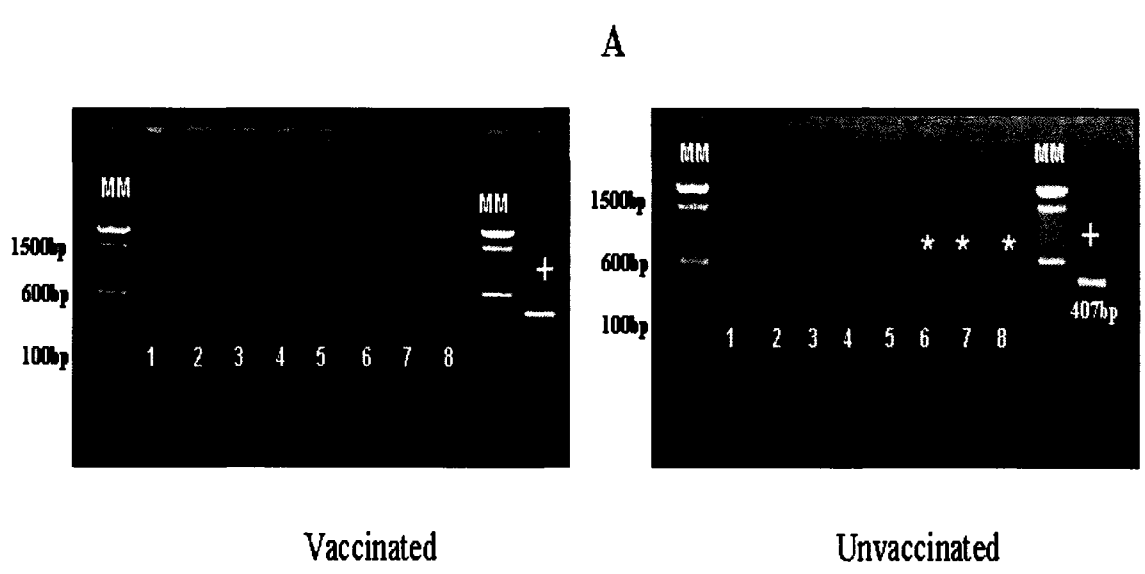

Figure 4.5.A) Detection of West Nile viral RNA in mouse brain. Total RNA was extracted from mouse brains and RT-PCR was performed. Amplicons of WNV were detected in a 1% agarose gel from mouse brains at day 8 post infection. Each lane represents a single mouse. * Mice dead by day 8, + Positive controls.

B

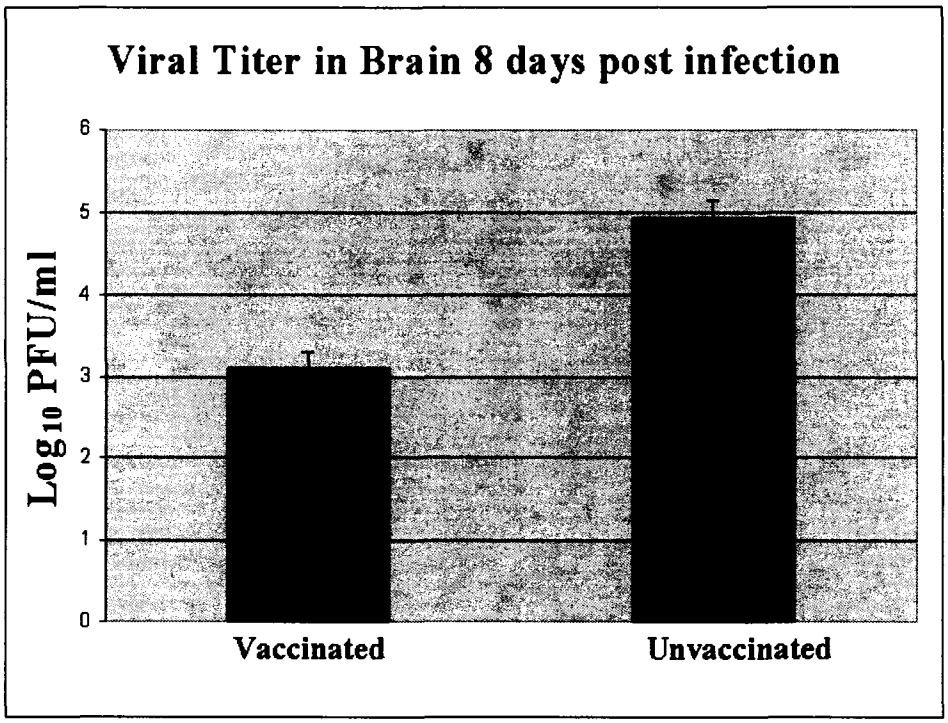

Figure 4.5.B) Mean viral titer in mouse brains 8 days post challenge. Mouse brains were harvested and homogenized, Vero cells were used for plaque assay to compare viral titers in vaccinated and unvaccinated groups. Eight mice were analyzed per group. No infectious virus was detected in spleens of either group at day 8 (Data not shown).

Antibody response.

Antibodies to WNV in serum were measured and the $PRNT_{80}$ titers are shown in the table 4.2. Plaque reduction neutralization test results are summarized and shown graphically (figure 4.6). Analysis indicated a significant difference in antibody titer between groups ($P<0.0008$); and times ($P<0.0001$), with higher anti-WNV antibody production in the vaccinated group than the unvaccinated group.

Mouse Number	Days Post Infection	$PRNT_{80\%}$ titer/Health Status			
		Vaccinated		Unvaccinated	
1	4	8	Healthy	8	Healthy
2	4	16	Healthy	4	Healthy
3	4	16	Healthy	<4	Healthy
4	4	<4	Healthy	<4	Healthy
5	4	8	Healthy	8	Healthy
6	4	<4	Healthy	<4	Healthy
7	4	8	Healthy	8	Healthy
8	4	4	Healthy	4	Healthy
1	8	32	Sick*	16	Dead
2	8	>128	Healthy	32	Sick*
3	8	32	Sick*	8	Sick
4	8	64	Sick	16	Dead
5	8	64	Sick	128	Healthy
6	8	16	Sick*	<4	Dead
7	8	4	Sick*	<4	Sick*
8	8	32	Healthy	32	Sick*

Table 4.2. $PRNT_{80}$ titers in vaccinated and unvaccinated mice at days 4 and 8 post challenge. Health status is also included. *Fur ruffling and hunchback posture.

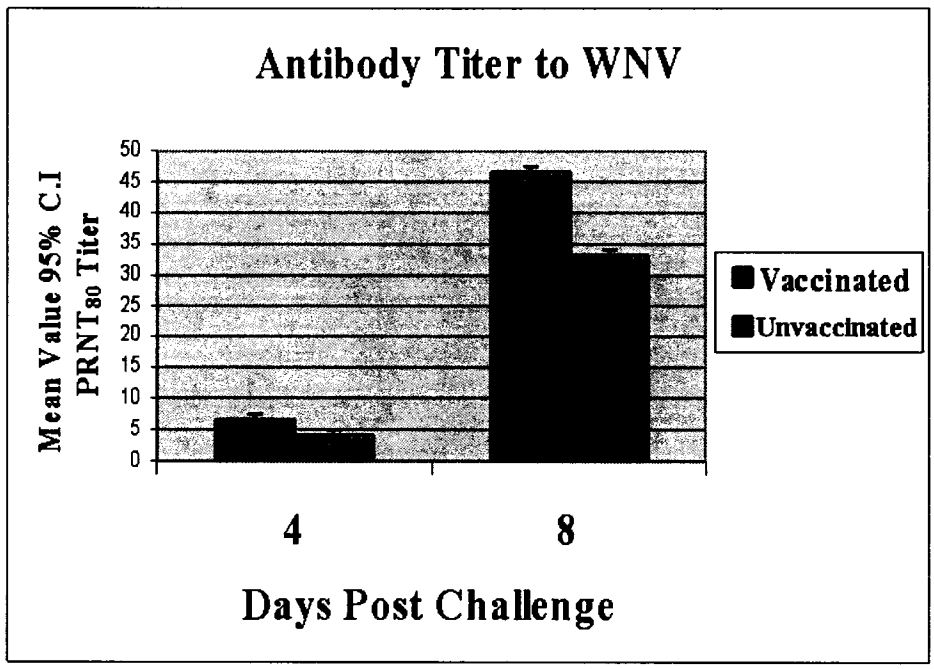

Figure 4.6. Neutralizing antibody titers in WNV infected mice. Analysis compares groups (8 mice per group) at 4 and 8 days post challenge. Analysis indicates a significant difference between groups (P<0.0008); and times (P<0.0001).

Health status.

Health status was monitored by the clinical signs shown during the course of infection. Based on clinical signs, no significant difference was found when comparing morbidity in vaccinated and unvaccinated groups (P= 0.728). Independent of treatment a high degree of morbidity was observed at 8 days post challenge when compared to 4 days post challenge (P<0.0001). Mortality rate was observed to be higher 37.5% (3/8) in the unvaccinated group. In contrast no fatalities occurred in the vaccinated group by day eight post challenge. Analysis by an exact test of proportions revealed that mortality was significantly higher (P<0.003) in the unvaccinated group.

Flow cytometry analysis.

Flow cytometry analysis of cytokines production from gated $CD3^+/CD8^+$ and $CD8^-$ (gated as CD4) T-cells revealed very small differences between cellular immune response of vaccinated and unvaccinated mice. Production of Th1 cytokines in $CD8^-$ (gated as $CD4^+$) cells was decreased in the vaccinated group as compared to the control. There was an increase in IL-4 production in the vaccinated group; in contrast IL-10 was decreased in expression in the vaccinated group as can be seen in figure 4.7. At day 4 post challenge expression of Th1 cytokines (IL-2, IFNγ), except TNFα were slightly increased in $CD8^+$ cells of the vaccinated group compared to the unvaccinated, whereas Th2 cytokines (IL-4 and IL-10) were decreased in the vaccinated mice (figure 4.8). $CD8^+$ cells at day 4 post challenge were present in higher numbers in the vaccinated group than in the unvaccinated group.

For $CD4^+$ cell population by day 8 all the Th1 and Th2 cytokines were expressed at higher levels in the unvaccinated group, whereas expression levels were lower in the vaccinated group as shown in figure 4.9. At 8 days post challenge, the Th2 cytokine IL-10 was expressed at higher levels in $CD8^+$ cells in the unvaccinated group compared to the vaccinated; IL-4 was, nevertheless, produced more abundantly in the vaccinated group (figure 4.10). Flow cytometry analysis showed no statistical differences in cytokine production; therefore Q-RT-PCR was used to determine Th1/Th2 cytokine mRNA production in splenocytes.

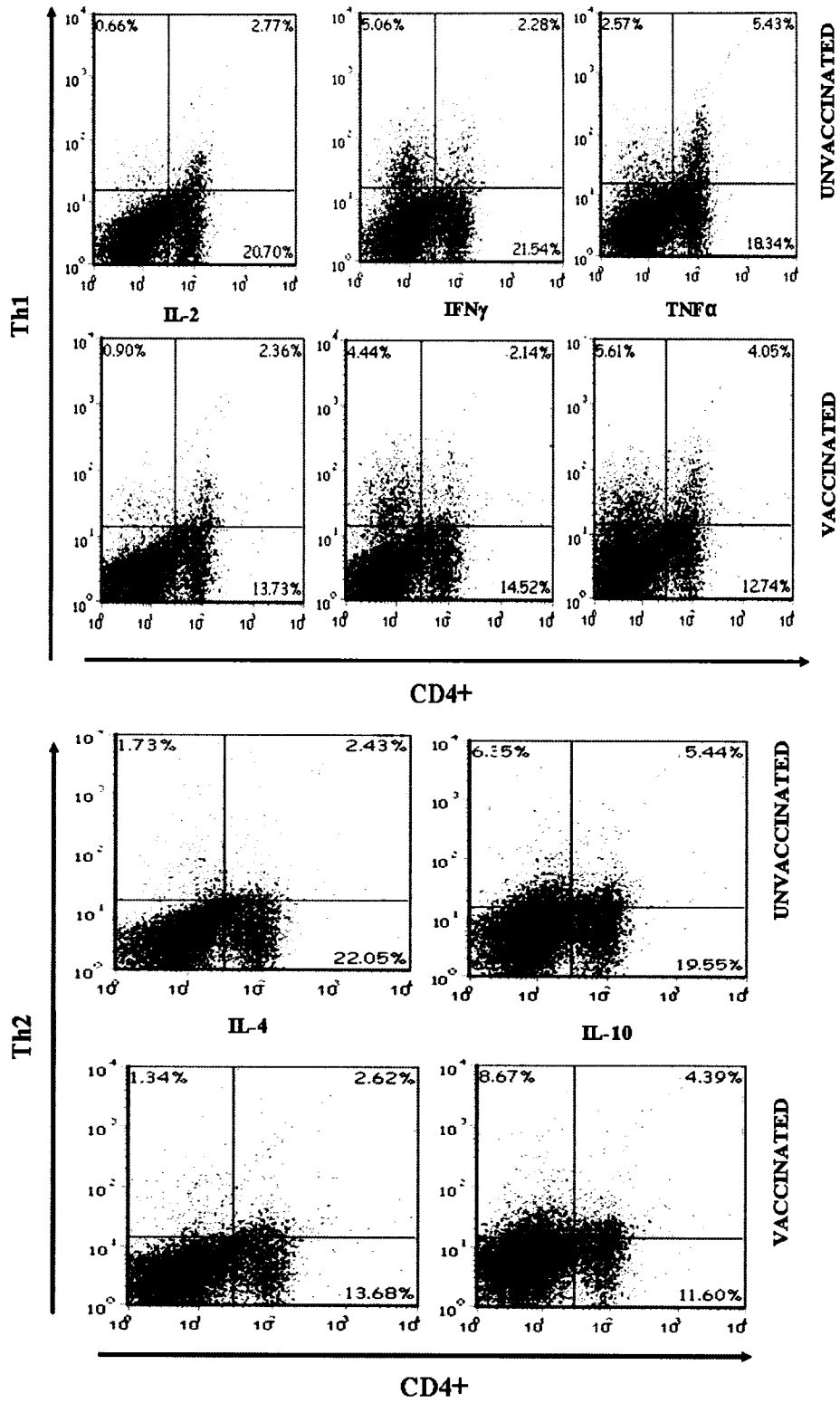

Figure 4.7. Flow cytometry analysis of mice splenocytes labeled $CD4^+$ cells producing Th1/Th2 cytokines 4 days post infected mosquito bite. Splenocytes were collected and labeled for Th1 or Th2 cytokines and analyzed by flow cytometer at 25000 events per cytokine. (n=4).

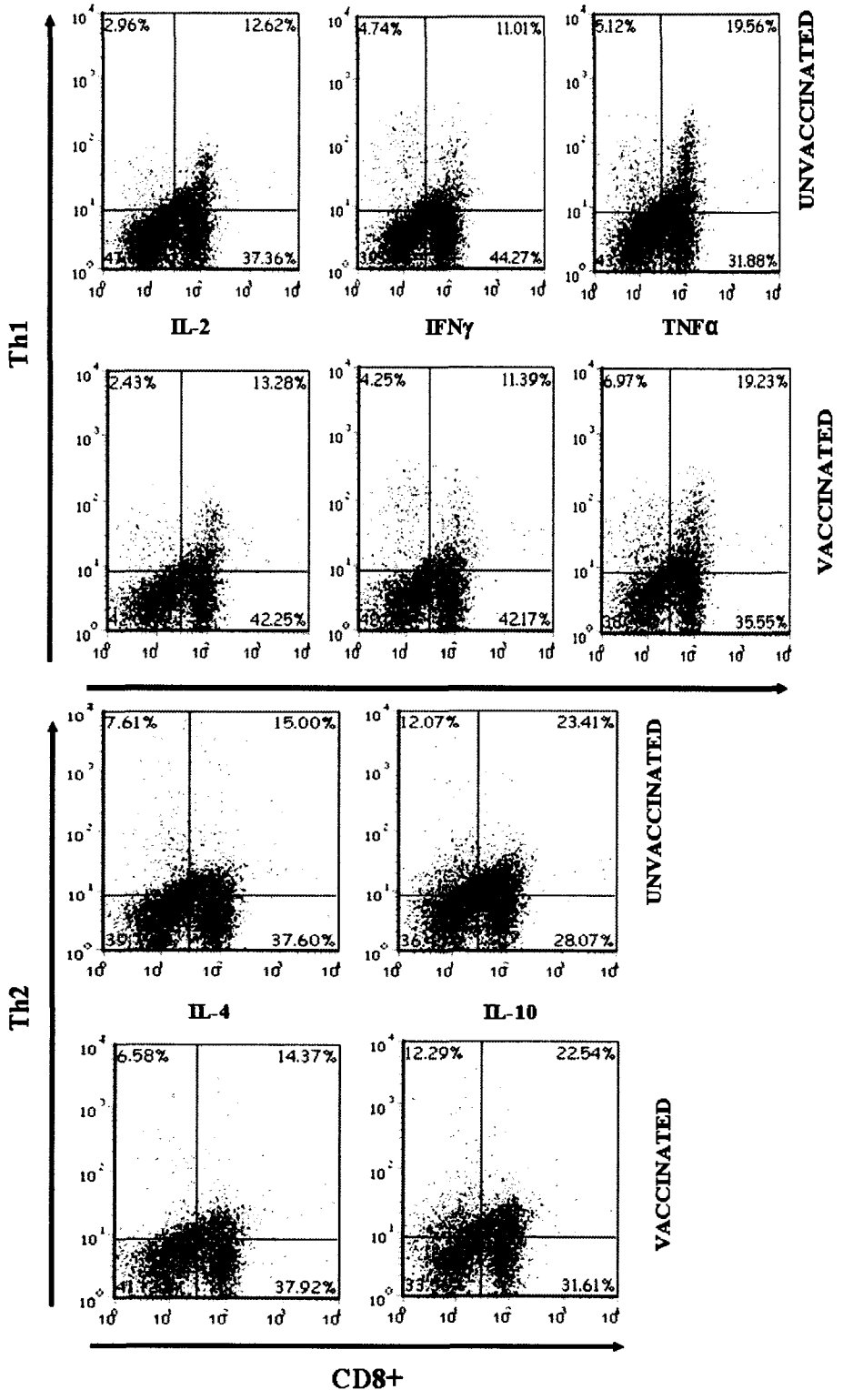

Figure 4.8. Flow cytometry analysis of mice splenocytes labeled $CD8^+$ cells producing Th1/Th2 cytokines 4 days post infected mosquito bite. Splenocytes were collected and labeled for Th1 or Th2 cytokines and analyzed by flow cytometer at 25000 events per cytokine. (n=4).

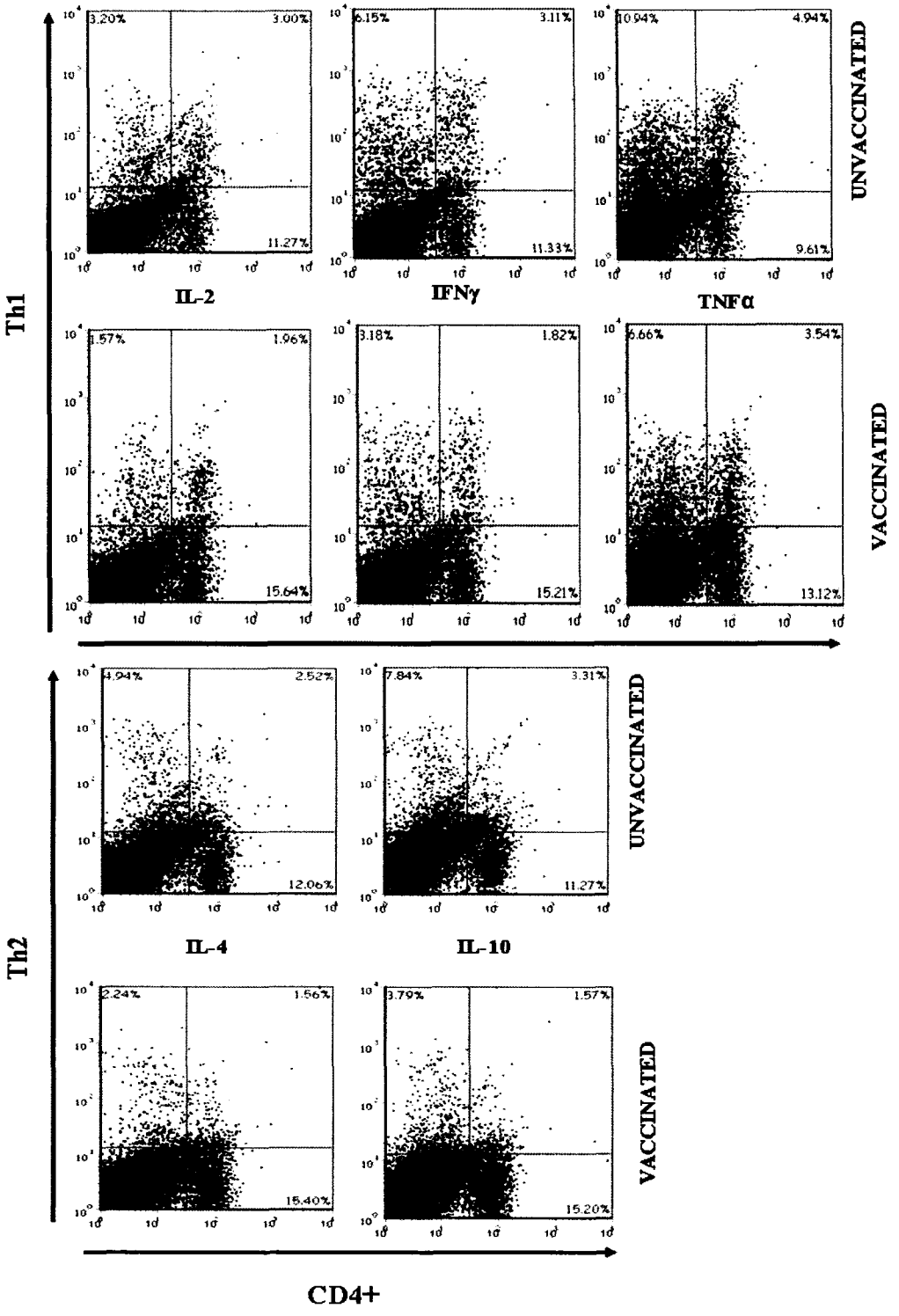

Figure 4.9. Flow cytometry analysis of mice splenocytes labeled $CD4^+$ cells producing Th1/Th2 cytokines 8 days post infected mosquito bite. Splenocytes were collected and labeled for Th1 or Th2 cytokines and analyzed by flow cytometer at 25000 events per cytokine. (n=4).

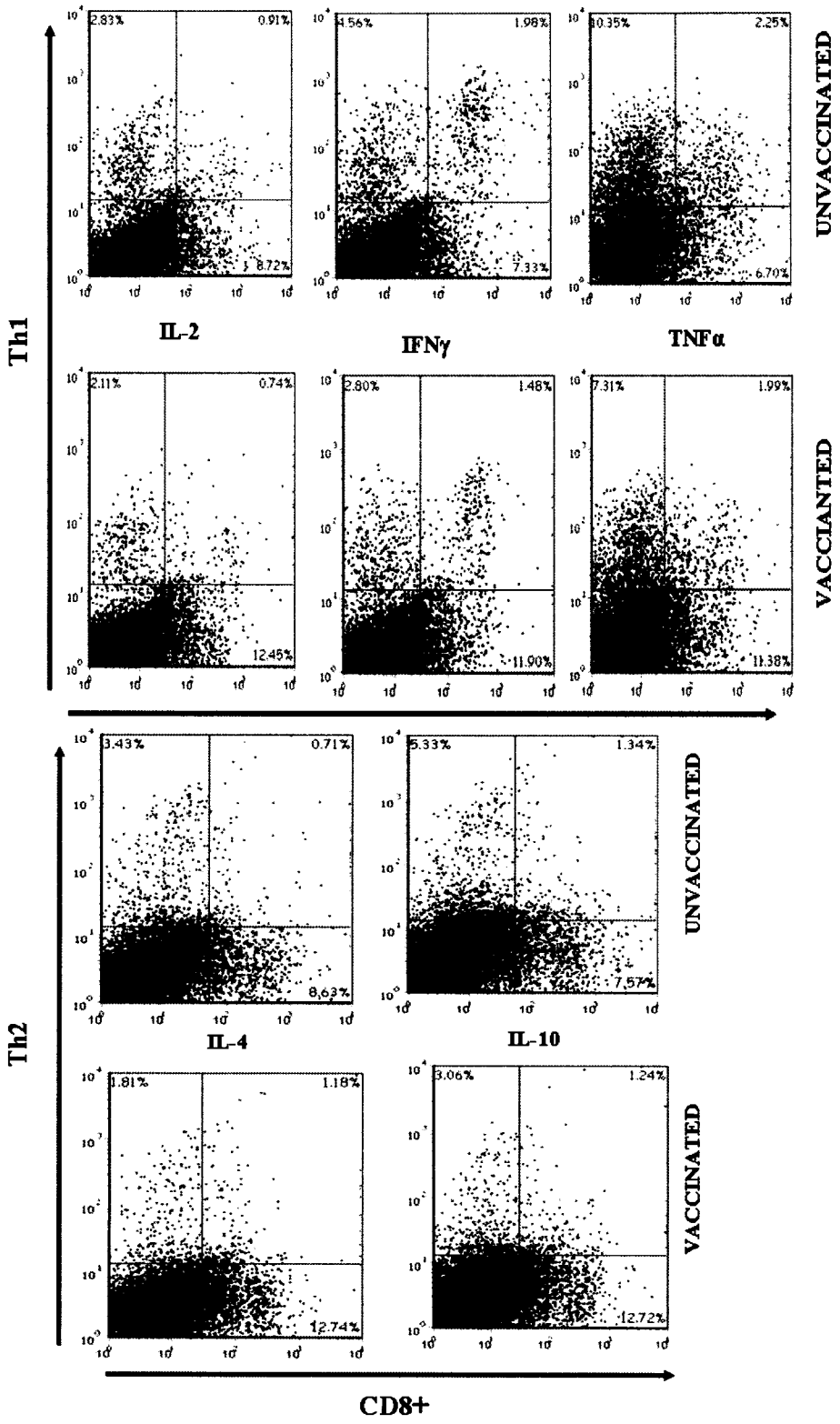

Figure 4.10. Flow cytometry analysis of mice splenocytes labeled $CD8^+$ cells producing Th1/Th2 cytokines 8 days post infected mosquito bite. Splenocytes were collected and labeled for Th1 or Th2 cytokines and analyzed by flow cytometer at 25000 events per cytokine. (n=4).

Q-RT-PCR.

Production of mRNA of Th1/Th2-related cytokines in the spleens of unvaccinated and vaccinated mice was measured by Q-RT-PCR. Amaounts measured for of all Th1 mRNA cytokines were higher in the vaccinated than in the unvaccinated mice at day 4 post challenge (figure 4.11). For Th2 cytokines, IL-4 mRNA level did not differ statistically between vaccinated and control groups at day 4 post challenge; however IL-10 mRNA was statistically higher in the vaccinated mice (figure 4.11). By day 8 post challenge, mRNA for IL-2 (Th1 cytokine) and IL-10 (Th2 cytokine) were higher in the control group. Interestingly, production of IFN gamma mRNA remained higher in the vaccinated group than in the unvaccinated group. Expression of TNF alpha mRNA increased by day 8 in the unvaccinated group, although there was no statistical difference compared to the vaccinated. IL-4 mRNA had increased by day 8 post infection to a statistically significantly higher level in the vaccinated group. The results are summarized in figures 4.11 and 4.12.

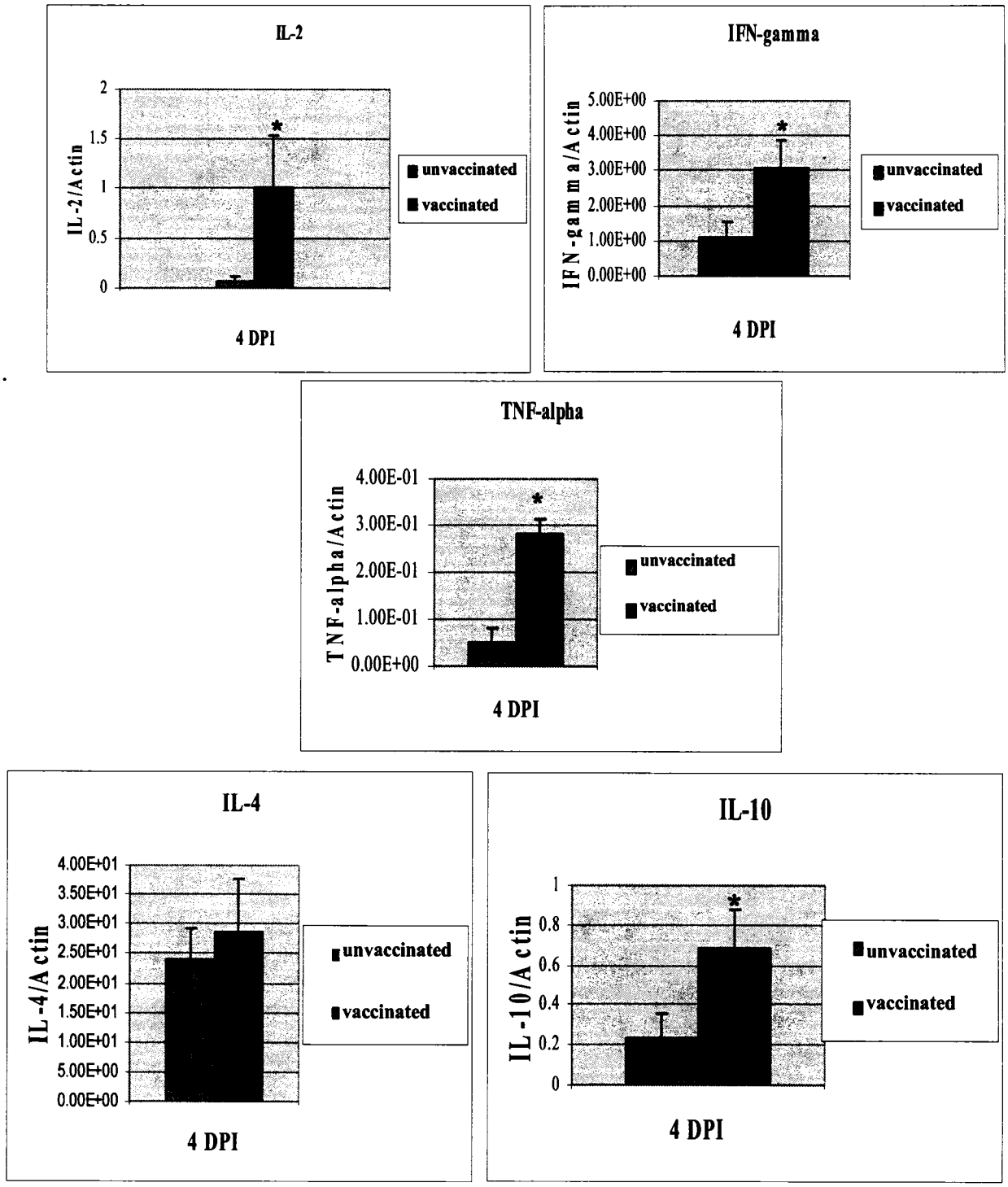

Figure 4.11. Cytokine mRNA production determined at 4 days post challenge in splenocytes of both unvaccinated and vaccinated mice by Q-RT-PCR. The y-axis depicts the ratio of amplified cytokine cDNA to housekeeping gene (β actin) of each sample (ratio using standard error of the mean). Data shown are representative of two similar experiments (n=8) * ($P<0.05$) compared to control.

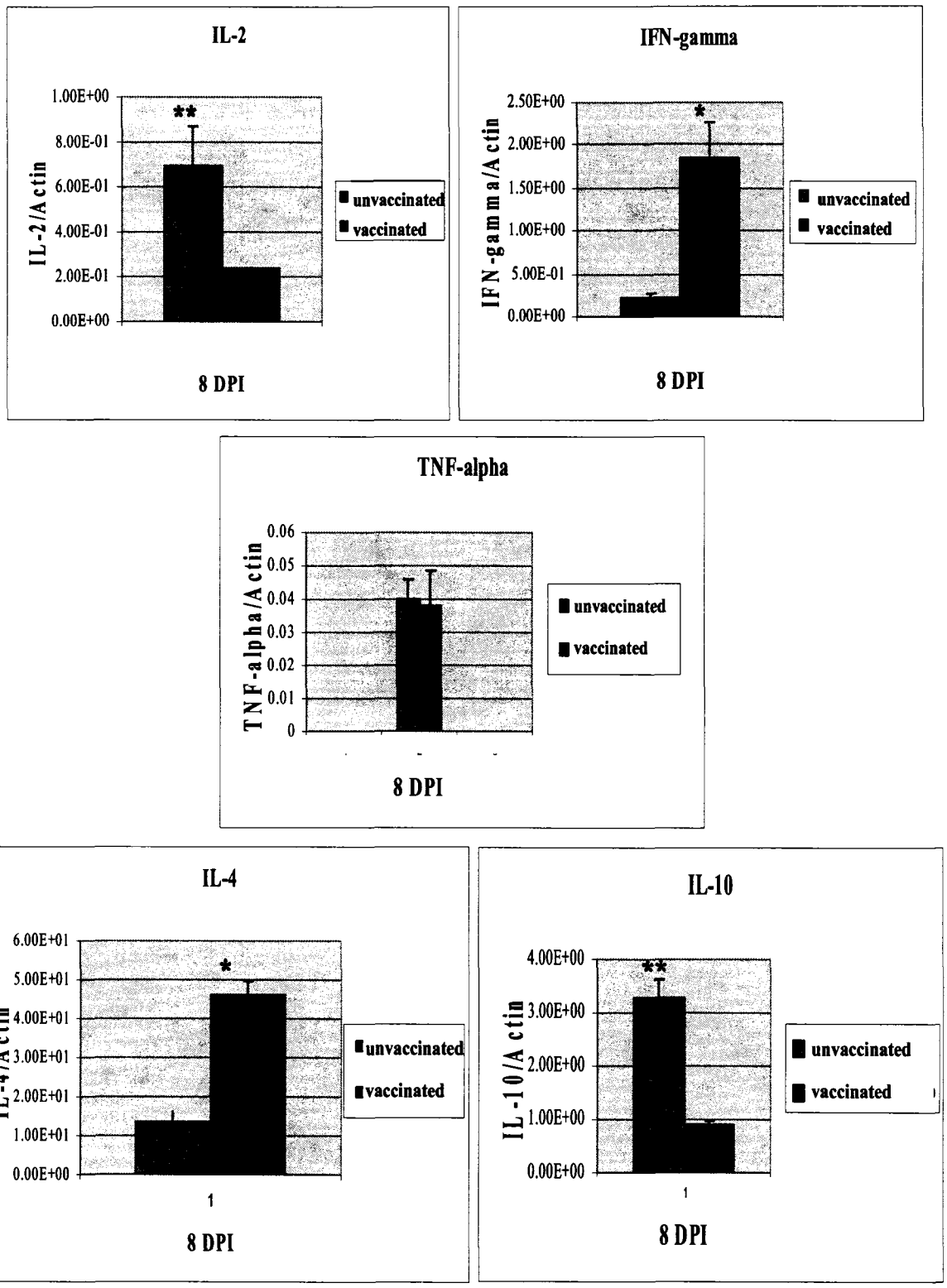

Figure 4.12. Cytokine mRNA production determined at 8 days post challenge in splenocytes of both unvaccinated and vaccinated mice by Q-RT-PCR. The y-axis depicts the ratio of amplified cytokine cDNA to housekeeping gene (actin) of each sample (ratio using standard error of the mean). Data shown are representative of two similar experiments (n=8) *($P<0.05$) compared to control or, ** compared to vaccinated.

DISCUSSION

Salivary effects in the enhancement of infection by pathogens transmitted by arthropod vectors has been reported elsewhere (68, 149, 264, 294), but little is known about the effect of host immune response against mosquito saliva on viral pathogenesis. Arthropod saliva contains proteins capable of regulating inflammation and modulating cytokine effects (79). In our study we immunized NIH-Swiss mice with salivary gland homogenate of *Cx. tarsalis* to determine the effect of prior immunity to mosquito salivary proteins on WNV pathogenesis when the virus is delivered by *Cx. tarsalis* bite. Previous exposure to sand fly saliva protects mice against cutaneous leishmaniasis (117, 270). I found that immunization of mice with salivary gland homogenates modulates WNV infection. Mice infected by mosquito bite that had not been immunized with mosquito salivary gland homogenates had higher viremia titers at 24, 48 and 72 hours post infection compared to those mice that were immunized with mosquito salivary gland homogenates. Although not statistically significant due to simple size, early viremia observation is supported by the data that, as early as 4 days post feeding, 37.5% of the mice in the unvaccinated group had detectable viral RNA on their brains, whereas virus and viral RNA were detected in the spleens but not the brains of vaccinated mice at day 4 after infected mosquito challenge. This observation suggests that early viremia and viral dissemination to the brain are likely to occur when an immune response to mosquito salivary proteins is not present. There is a viremia delay in mice immune to salivary proteins.

After virus is inoculated into the skin of the vertebrate host it is likely to be carried by Langerhans cells from the skin to the lymph nodes, where primary

replication takes place. Then viruses disseminate to spleen, and later to the brain (240). The potentiation of WNV encephalitis by mosquito feeding has been demonstrated previously (244); however, that experiment was in animals that were immunologically naïve to mosquito salivary proteins. Mosquito feeding can modulate the Th1/Th2 cytokine response, increasing production of the Th2 related cytokines and decreasing the Th1 cytokines (245, 309). It is also known that the Th1 cytokine response is important in protecting mice during early WNV infection (252, 289). These data imply that cytokine modulation by MSP has an effect on viral pathogenesis.

Cellular immune response to viral infection in mice is related to the CD8+ T cells; three phases related to this response have been described; first is a period of activation and expansion, second is the killing of infected cells, and third is the establishment or maintenance of memory (115). Primary or memory CD8+ cells have proved to efficiently kill target cells that display WNV antigens (249) and CD4+ cells have been observed to respond more vigorously in the periphery (127). In spite of the attempt to confirm these observations in the present study, data obtained from flow cytometry analysis were not conclusive. Results yielded by flow cytometry analysis to determine cytokine expression showed very small differences between vaccinated and unvaccinated mice, with no statistical difference between groups, possibly as a result of low number of mice analyzed or due to inappropriate choice of cell markers.

In order to further investigate the changes in Th1/Th2-related cytokines, quantitative RT-PCR was used to measure cytokine mRNA (80). Splenocyte analysis revealed significant differences in the levels of all analyzed cytokines with the exception of IL-4, which did not differ statistically between groups early in the

infection (figure 4.11). These findings could be explained by the fact that unvaccinated mice have a Th2 response. Indeed, all their cytokine production from the Th1 subset was decreased compared to the vaccinated group early in the infection (figure 4.11). The increase of IL-4 mRNA in the vaccinated group can be explained by the fact that mice were immunized with salivary gland homogenates and a subsequent exposure to the antigen would trigger an antibody response in the vaccinated group (figures 4.11 and 4.12). IL-4 is secreted by Th2 cells and is strongly cytokine priming, co-stimulating and activating B cells (81). IL-4 also induces antibody class switch to IgG1 in mice, and stimulates the up-regulation of Class II MHC expansion in resting B-cells (81).

Antibody titer to WNV was demonstrated to be higher in the vaccinated than in the unvaccinated group at both days 4 and 8 post challenge (figure 4.6). Production of antibodies is important during WNV infection (63). In mice genetically deficient in B cells, viral dissemination to the brain could be demonstrated as early as 4 days post infection and one of the main reasons was the lack of antibody response (63).

Another Th2 cytokine subset analyzed was IL-10. IL-10 mRNA expression increased early during the infection in the vaccinated group, but we did not observe a decrease in the production of Th1 cytokines although IL-10 is known to interfere with the ability of monocytes and macrophages to produce Th1 cytokines (110). At day 8 post infection, IL-10 production was increased significantly in the unvaccinated group compared to the vaccinated. This observation demonstrates to some extent that a Th2 response occurred in the unvaccinated group. Probably the increase of IL-10 mRNA production in the vaccinated mice 4 days after challenge was merely transient and although mRNA was expressed, poor translation might have occurred due to tight

cytokine expression control or due to high expression of Th1 cytokines (81). Thus there was not a significant effect of IL-10 on response of the Th2 cytokines. This hypothesis is supported by the observation that the vaccinated mice had significant increases in Th1 cytokines early post challenge. IL-2, IFN gamma and TNF alpha were significantly increased early in the infection as demonstrated by Q-RT-PCR assay. IL-2 is secreted by Th1 cells. IL-2 induces proliferation of T_H cells and T_c cells, and supports long-term growth of antigen specific T-cell clones as well as enhancing their activity (81). By day 8 post infection, the activity of IL-2 had significantly declined in the vaccinated group and increased in the unvaccinated group, which suggested that the immune response in the control group was affected by mosquito feeding at early stages post challenge. This effect can be seen as down regulation of Th1 cell activity, thereby affecting viral pathogenesis late in the course of infection. IL-2 is also known for priming antigen specific T-cell clones, but its cytokine mRNA is very short-lived because of an instability sequence in the 3' untranslated region which allows tight cytokine regulation (110). IFN gamma mRNA was also analyzed. It was found that independently of the time observed, IFN gamma was increased in the vaccinated group of mice compared with the unvaccinated. IFN gamma is known to be important during WNV infection. It has been observed that lack of IFN gamma production or signaling results in increased vulnerability to WNV infection, thus lack of IFN gamma production has been related to early central nervous system infection and greater viral replication in lymphoid tissues (252). The effect of mosquito saliva or mosquito salivary gland homogenates in down regulation of IFN gamma has been described (245, 309). It was clear in these experiments that generation of antibodies against salivary proteins of the mosquito *Cx.*

tarsalis blocked the factor that may be involved in regulation of IFN gamma production during mosquito feeding. There was a significant decrease in the production of IFN gamma at day 4 and 8 post challenge in the unvaccinated group (figures 4.11 and 4.12). IFN gamma production has been attributed to the subset of cells known as γδ T-cells. γδ T-cells are responsible for facilitating adaptive immunity against WNV infection in mice. Mice deficient in γδ T-cells are more susceptible to WNV infection (290, 291). In mice capable of mounting a normal immune response, γδ T-cells expand rapidly in the spleen and peritoneal cavity early during WNV infection. This subset of cells produces IFN gamma and enhances perforin expression by splenic T cells. This observation strongly suggests that IFN gamma production early during infection limits viral load and might help to prevent viral dissemination to the brain. TNF mRNA alpha was also analyzed; interestingly TNF alpha was up-regulated significantly in the vaccinated mice compared to the unvaccinated. The up-regulation was observed in the vaccinated group at day 4 post challenge. At day 8 the unvaccinated group showed a significant increase of TNF alpha compared to day 4. This late increase in TNF alpha supports the observation that vaccination against mosquito salivary proteins improves immune response early in the course of infection, but it is also sustained at late time points. TNF alpha has been previously reported as an important cytokine to control dengue virus, which is also a flavivirus (184). After virus deposition in the skin WNV is encountered by antigen presenting cells such as Langerhans cells. TNF alpha production was increased when myeloid dendritic cells had been previously exposed to mosquito saliva (1). This last finding supports the data presented here. The immunization of mice with salivary gland homogenates is important in increasing

production of TNF alpha early in the infection. Previous reports suggested that TNF alpha could be in part responsible for early WNV neuroinvasion due to permeability of blood-brain barrier (292). However this was not corroborated by our data; our finding agrees with other reports in which early production of TNF alpha is a protective factor against WNV neuroinvasion (252). Interestingly, mRNA for two of the cytokines analyzed here, IFN gamma and TNF alpha, were shown to be increased early during WNV infection. This increase correlated with lower viral load and protected against early viral infection of the CNS. The synergistic activity of IFN gamma and TNF alpha seems to confer antiviral properties (170).

Based on these studies, vaccination with mosquito salivary gland homogenates has an effect on viral replication. It delays viremia and produces a more robust immune response during early infection; nevertheless late infection and viral dissemination to the brain cannot be prevented solely by immunization with MSP. Ex vivo studies will be necessary to better compare the role of antigen presenting cells during infection in previously immunized animals as well as using more accurate markers of immune response such as anti CD4/CD8 antibodies instead of using anti CD3 as marker to improve cytokine analysis. Clearly immunization and immune response analysis must be better optimized in future experiments. In addition, viral pathogenesis should be analyzed by histopathologic examination of various tissues. Nonetheless the data presented are one step towards increasing basic information for target vector immunogens for vaccines. Combining not only antigens from the pathogen but also the vector salivary antigens in vaccines may provide improved immunity for vector-borne disease.

CHAPTER 5

SUMMARY OF DISSERTATION

Mosquito salivary proteins enhance pathogenesis of some arbviruses. This study was conducted to test the hypothesis that immunity to mosquito salivary proteins affects the pathogenesis of flaviviruses. The role of the different mosquito salivary proteins in the enhancement of flavivirus infection is not yet well understood.

Here the effect of antibodies to mosquito salivary proteins on viral pathogenesis was studied in a retrospective human study involving dengue patients and a prospective study of WNV infection in a mouse model. In addition, specific immunogenic salivary proteins from *Cx tarsalis* and *Ae. aegypti* mosquitoes were identified that may be potentially related to viral infection enhancement.

In order to identify specific salivary proteins that are secreted during mosquito feeding and could be related to viral pathogenesis, a novel technique to obtain and concentrate soluble proteins released in saliva was developed. After concentrated MSP were fractionated by gel electrophoresis, immunogenic proteins were revealed in an immunoblot and individual proteins were also identified by mass spectrometry analysis. In *Cx tarsalis* a 49 kDa protein was identyfied that functions in tryptophan metabolism (figure 2.3). Interestingly in mammals this protein has been associated with regulation of the immune response.

Since there was no information about this protein in *Cx. tarsalis*, we attempted to clone and sequence cDNA. Although the complete sequence was not obtained, we determined the nucleotide sequence of an 800 base pairs 3' terminal segment specific for *Cx tarsalis* tryptophan oxygenase (figure 2.5). Protein function assay as well as immunization of mice with specific MSP will be important to elucidate relationship of this salivary protein with viral pathogenesis.

Another protein identified in *Ae. aegypti* was a 36 kDa protein that was abundantly secreted during blood meal or probing. Q-RT-PCR was used to determine the importance of expression of the 36 kDa protein during mosquito feeding. This study revealed that mRNA of the 36 kDa protein is induced and significantly increased during blood feeding, indicating its importance during the acquisition of a blood meal (figure 2.8). This 36 kDa protein is related to the D7 family of proteins and *in vitro* assay showed that it may function as an IFNγ regulator by binding and sequestering the cytokine (figure 2.10). Interaction of the D7 protein with IFNγ enhances DENV2 replication in cell culture early in infection (figure 2.11).

The role of D7 proteins of mosquito saliva is not completely known; nevertheless the D7 mRNA is highly abundant during feeding. One important function of these proteins is sequestration of bioamines that may be immune mediators that play an important role in anti viral state during early viral infection. Generation of a recombinant D7 protein and functional assays will be necessary to understand how this protein can be potentially used as immunogen component for anti-arthropod/anti-viral vaccines.

Experiments were conducted to characterize the immune response to mosquito salivary proteins during dengue infection (chapter 3). In a retrospective analysis in a human population exposed to DENV2, immunoblot analysis revealed that a higher proportion of patients with secondary DENV2 infections had antibodies directed to a 36 kDa MSP than patients who were not infected. It was observed also by immunoblot that a higher proportion of patients with DHF had antibodies to a 68 kDa mosquito anticoagulant protein than patients who had only DF or were not infected. (figures 3.2 and table 3.1A). Importantly, both groups of DENV2-infected patients independent of

clinical signs (non hemorrhagic and hemorrhagic), had a higher proportion with antibodies to total *Ae. aegypti* salivary proteins than uninfected patients as measured by ELISA (table 3.1B), indicating a higher exposure of the infected population to mosquito bite. Lack of an animal model for DENV infections precludes controlled studies of the role of antibodies to MSP in modulating infection. Monitoring immune response to MSP in human populations during mosquito season would generate important information to identify viral transmission in higher exposed populations that might be at risk of contracting severe forms of the disease. Efforts can then be concentrated in monitoring immune responses to individual proteins that can be used as risk factor markers.

In order to further investigate the effect of immune response to MSP, mice were vaccinated with *Cx. tarsalis* mosquito salivary proteins (chapter 4). Immunoblot analysis revealed that antibody generation against salivary proteins was achieved by immunization (figure 4.1). Viral RNA was detected in the blood of vaccinated mice at 48 hours post challenge but plaque assay did not detect infectious virus in blood of the vaccinated group at 24, 48 or 72 hours post challenge. In the unvaccinated group, plaque assay detected viremia at 24, 48 and 72 hours post challenge (figure 4.3 A and B). At day 4 post challenge viral RNA and infectious virus were detected in brains of unvaccinated mice, but not in brains of the vaccinated group. On the other hand, vaccinated mice had higher viral titers in spleens compared to unvaccinated (figures 4.4.A and 4.4 B). By day 8 post challenge both groups showed viral dissemination in brain (figure 4.5 A) but viral load was significantly lower in the brain of the vaccinated group (figure 4.5 B). Antibody generated to WNV was also measured, vaccinated group had higher antibody response ($PRNT_{80\%}$) to WNV compared to control group (figure 4.6)

RNA extracted from spleen at day 4 and 8 post challenge was analyzed by Q-RT-PCR to quantify Th1/Th2 cytokine mRNA. Immunization with *Cx. tarsalis* MSP improved production of Th1 cytokines 4 days after mosquito bite, whereas unvaccinated group had significant up-regulation of Th2 cytokines (figures 4.11). A switch in cytokine production was observed by day 8 (figure 4.12). Mortality was also greater in the control group by day 8, whereas no fatalities occurred in the vaccinated group, although sick animals were observed in both groups (table 4.2).

Based on these results it was demonstrated that there is a modulation of WNV infection when antibodies to mosquito salivary proteins are present. The importance of immune response to MSP in populations exposed to mosquito bite might to some extent modulate arthropod-borne viral infections. Therefore, groups or individuals in exposed populations could be protected from severe disease by reacting against salivary proteins that may regulate an important antiviral immune response; nevertheless solely a response to mosquito salivary proteins is not completely effective in controlling viral infections. These findings demonstrate the importance in understanding and assessing the response to mosquito bite during viral infections. Immunity to MSP is an important component in the dynamic of arthropod-borne pathogens transmission. Using these basic findings new approaches such as immune response screening in naturally exposed populations, will help in the understanding the role of immunity to vector's saliva and arbovirus pathogenesis. Characterizing immunoglobulin isotypes, natural desensitization, and responses to individual proteins as well as cytokine fluctuation will complete the integration of better data to understand transmission and viral pathogenesis related to vectors of disease and the diseases they cause.

Observations drawn from these data strongly suggest that the proteins of mosquito saliva change pathogenesis of *flaviviruses.* Nevertheless immunity against mosquito salivary proteins alone does not protect from viral dissemination once infection is acquired, therefore generation of vaccine candidates that contain not only viral antigens, but also specific components of mosquito salivary proteins must be considered for further research.

Finally one important topic to be addressed in future research will be the aspects of immunizing populations in which constant exposure to mosquito bites occur. Understanding whether immune response will be achieved and maintained in these populations, or whether desensitization may happen as result of overexposing the immune system to abundant antigens is important. Knowledge about these factors will also be a key in understanding the potential of subunit vaccines containing mosquito salivary proteins in populations were they are required the most.

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APPENDIX

Touch-down PCR protocol.
With annealing temperatures declining from 60°C to 41°C over 20 cycles, followed by 39 cycles with annealing temperatures at 54°C.

Cycle	Temperature (°C)	Time (minutes)	Cycle	Temperature (°C)	Time (minutes)
1	94	4	38	94	1
2	94	1	39	**50**	1
3	**60**	1	40	72	2
4	72	2	41	94	1
5	94	1	42	**49**	1
6	**59**	1	43	72	2
7	72	2	44	94	1
8	94	1	45	**48**	1
9	**58**	1	46	72	2
10	72	2	47	94	1
11	94	1	48	**47**	1
12	**57**	1	49	72	2
13	72	2	50	94	1
14	94	1	51	**46**	1
15	**58**	1	52	72	2
16	72	2	53	94	1
17	94	1	54	**45**	1
18	**57**	1	55	72	2
19	72	2	56	94	1
20	94	1	57	**44**	1
21	**56**	1	58	72	2
22	72	2	59	94	1
23	94	1	60	**43**	1
24	**55**	1	61	72	2
25	72	2	62	94	1
26	94	1	63	**42**	1
27	**54**	1	64	72	2
28	72	2	65	94	1
29	94	1	66	**41**	1
30	**53**	1	67	72	2
31	72	2	68	94	1
32	94	1	69	**54**	1
33	**52**	1	70	72	2
34	72	2	71	**To 68, 39 times**	---------
35	94	1	72	72	2
36	**51**	1	73	4	Hold
37	72	2			

Polymerase Chain Reaction (PCR) "Hot Start" protocol

Reagent	**Volume (µl)**
10 X Buffer $MgCl_2$	2.5
2.5 mM dNTP	2.0
$MgCl_2$	0.75
Forward primer (25 ng/ µl)	1
Reverse primer (25 ng/ µl)	1
cDNA (from reverse transcription)	5
dH_20	12.65
Taq DNA polymerase	0.1
Total	25

Run: 94°C for 5 minutes
30 cycles of
94 °C for 1 minute
50 °C for 1 minute
72 °C for 1 minute
1 cycle
72 °C for 8 minutes

In order to increase $MgCl_2$ use the 10 X Buffer $MgCl_2$ and adjust to 3 mM (it is supplied in a 50mM tube)