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Impact of Insect Salivary Proteins in Blood Feeding, Host Immunity, Disease, and in the Development of Biomarkers for Vector Exposure

Iliano V. Coutinho-Abreu, **Anderson B. Guimaraes-Costa**, and **Jesus G. Valenzuela***

Vector Molecular Biology Section, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, 20878

Abstract

Functional genomic approaches based on expression of recombinant proteins linked to biochemical and disease model approaches resulted in the discovery of novel biological activities and the role some of these proteins play in disease transmission. Importantly, the expression of salivary proteins was recently shown to be affected by environmental factors and by the presence of the pathogen in the salivary gland. A practical application resulting from insect saliva research is the use of insect antigenic salivary protein as biomarkers of vector exposure in humans and animal reservoirs, an approach that is yielding interesting results in the field.

Introduction

The components and biological effects of the saliva of blood feeding arthropods is a growing area of research that is being cross-fertilized by various disciplines including biochemistry, immunology and molecular biology. Importantly, studies of saliva that focused only on a couple of insect species have expanded to other disease vectors in the last few years. Furthermore, the effect of insect saliva in pathogen transmission and establishment has been expanded to other pathogens.

This review highlights recent work in saliva from vectors of disease with emphasis in the discovery of novel biological activities from salivary proteins, the impact of insect saliva in infection, and the effect of environmental factors and pathogens in the expression of these salivary molecules. This review will also highlight an important contribution and practical application of insect salivary proteins: the use of antigenic proteins as novel biomarkers for vector exposure.

*Correspondence to jvalenzuela@niaid.nih.gov.

Insect saliva in blood feeding: Old problems, smart solutions

To have a successful blood meal, hematophagous insects have developed several strategies to overcome the host hemostasis mechanisms. Vasodilators, inhibitors of the blood coagulation cascade, and inhibitors of platelet aggregation have been identified from the saliva of various vectors of disease [1]. Although we have achieved great knowledge on the composition of saliva (transcripts and proteins), the biological activity of many of the most abundant molecules has remained elusive. Functional genomics approaches based on the expression of recombinant proteins in heterologous systems and in gene silencing have propelled the discovery of novel activities from some of the highly abundant salivary proteins with previous “unknown function”.

Aegyptin, a novel salivary collagen-binding protein from Aedes aegypti

It was recently shown that a 30 kDa recombinant protein, named Aegyptin, specifically binds to collagen, impeding the interaction of collagen with the platelet receptor glycoprotein VI, Integrin α2β1, and von Willebrand factor. This ultimately leads to the inhibition of collagen-induced platelet aggregation and adhesion [2]. Chagas et al [3] used a gene-silencing approach to assess the relevance of Aegyptin in blood feeding. Saliva fromT transgenic mosquitoes lacking Aegyptin failed to inhibit collagen-induced platelet aggregation, exhibited increased probing time, and also ingested less amount of blood when feeding on mice as compared to control group.

The function and structure of the mosquito salivary D7 protein

D7 salivary proteins are found in Nematoceran Diptera and consist of a multigene family distantly related to the odorant binding proteins. The short molecular forms (D7r) have been characterized in the mosquito *Anopheles gambiae* [4]. The D7 proteins were shown to bind biogenic amines, which are important mediators of inflammation and vascular tone. Among the long D7 proteins, the different domains have evolved to bind different ligands. Whereas the C-termini domains of some long D7 proteins, such as the *Aedes aegypti* AeD7, bind to biogenic amines, the N-terminal domains bind to cysteinyl leukotriene, another mediator of allergy and vascular permeability [4]. Interestingly, the N-terminal domain of AnSt-D7L1, a long D7 salivary protein of the mosquito *An. stephensi*, also binds to thromboxane A_2 [5]. The N-termini, on the other hand, lost its ability to bind to biogenic amines [5].

A salivary lipocalin from Rhodnius prolixus is a biogenic aminebinding protein

A salivary protein from *Rhodnius prolixus* was shown to bind biogenic amines and the structure of this protein was recently solved and shown to be a lipocalin [6], a different structure compared to the D& family of proteins, the biogenic amine binding proteins found in mosquitos [4] suggesting a case of convergent evolution. The amine-binding protein (ABP) from *R. prolixus* has some sequence homology to the salivary nitrophorins but ABO does not bind heme. ABP binds serotonin and norepinephrine with high affinity and inhibits biogenic amine-mediated platelet activation.

Triplatin, a salivary protein from Triatoma infestans, is a novel platelet aggregation inhibitor and a vasoconstriction inhibitor

Triplatin was shown to be an inhibitor of collagen-induced platelet aggregation and proposed to antagonize the collagen receptor glycoprotein VI (GPVI). Recently, triplatin was shown to inhibit platelet aggregation induced with low dose of collagen but it did not bind the collagen receptor GPVI. Triaplatin was also shown to bind Thromboxane A_2 and prostanglandin $F2_{alpha}$ and PGJ_2 and to inhibit vasoconstriction [7].

The salivary Antigen 5 from Triatoma infestans and Dipetalogaster maxima functions as an antioxidant by scavenging O^{-}_{2}

The presence of antigen 5 family of proteins has been reported in the saliva of many blood feeding insects. However, the function of this protein remained elusive for many years. Recently, the biological function of the antigen 5 salivary protein from *Triatoma infestans* and *Dipetalogaster maxima* was elucidated and shown to be a superoxide dismutase that binds Cu^{2+} and scavenges O^{-}_{2}. The salivary antigen 5 inhibited platelet aggregation induced by collagen and blocked neutrophil oxidative burst [8]. This family of proteins represents a novel family of antioxidants present in the saliva of blood-feeding insects.

The yellow salivary proteins from sand flies bind biogenic amines

One of the most abundant salivary proteins from sand flies with “unknown function” is the yellow related family of proteins. It was recently shown that the yellow related proteins LJM17, LJM11, and LJM17 bind biogenic amines, including serotonin, catecholamines, and histamine, counteracting this way the hemostatic system [9]. The kissing bug *Rhodnius prolixus* has also a novel biogenic amine binding protein; however, the sequence and structure of this protein does not resemble the protein in sand flies and belongs to the lipocalin protein family, representing a case of convergent evolution [6].

Lufaxin, the anticoagulant from sand flies

A salivary protein of 38 kDa of unknown function was demonstrated to be the anticoagulant in sand flies. The salivary protein named Lufaxin is a potent and specific inhibitor of Factor Xa [10] and a potent inhibitor of inflammation. Lufaxin has no homologies to any other proteins in accessible databases and so far it has only been identified in the salivary glands of sand flies [10].

Sand fly salivary protein SP15 is a novel inhibitor of contact pathway

The recombinant protein SP15 from the sand fly *Phlebotomus duboscqi* was shown to bind negatively charged surfaces. These anionic surfaces serve to stabilize complexes of the blood coagulation cascade. By binding to these negatively charge surfaces, PdSP15 inhibits the activation of factors FXII and FXI, therefore preventing the process of coagulation and bradykinin production [11].

Insect saliva in pathogen transmission

Effect of mosquito saliva on virus infection

The powerful effects of saliva in the host hemostatic system and inflammatory system may have consequences in virus transmission as demonstrated in other diseases. The saliva from *Ae. aegypti* was shown to enhance Dengue virus infection in *ex vivo* human keratinocytes [12]. Importantly, this effect correlated with the down-regulation of the expression of several antimicrobial peptides, including β-defensin, LL-37, Elafin, and S100A7 and the down-regulation of the anti-virus cytokines, IFN-a, IFN-B, and IFN-y [12]. Recently, a salivary protein with a molecular weight of 34 kDa only present in *Aedes* and *Culex* mosquitoes was shown to enhance Dengue virus replication and suppress the innate immune response of the host [13]. Conway and colleagues have identified a mosquito salivary protease CLIPA3 that enhances DENV infectivity and dissemination both *in vitro* and *in vivo* models of infection [14]. CLIPA3 is proposed to cleave extracellular matrix proteins, allowing DENV particles to ultimately interact with local permissive cells [14].

A salivary endonuclease helps Leishmania to escape from the innate immune system

A sand fly salivary endonuclease named Lundep (LJL138) was shown to have a direct impact on *Leismania* infection. Neutrophils are the first cells recruited to the site of a sand fly bite, where they play a pivotal role during infection. Neutrophils can eliminate microorganisms through the release of extracellular traps (NETs) that are structures composed by DNA, histones, cytoplasmic, and granular proteins. Lundep has a potent endonuclease activity and degrades the NETs formed at the site of the bite allowing the parasites to escape NET-mediated killing [15], facilitating therefore *Leishmania* survival [15].

Effects of environmental factors and pathogens in the expression of Insect salivary proteins

At the time when pathogens are transmitted, the salivary glands secrete a specific cocktail of molecules in which composition and amounts are defined by the vector's age, environmental seasonality, and the presence of the transmitted pathogen. In tropical areas, vectors are adapted to face the seasonal differences in sugar and blood source availability between rainy and dry seasons [16,17]. As drought reduces sugar content produced by plants, insect vectors need to bear special adaptations in order to obtain a sugar meal (reviewed in [16]). In the sand fly *P. papatasi*, up-regulation of five transcripts encoding for secreted salivary gland proteins are only observed in sand flies caught during the dry season [18]. This plasticity in salivary gland gene expression seems also to be associated with the transmission of *Leishmania*, which is more prevalent in the dry season [16,19].

The age of the insects apparently modulates the differential expression of salivary gland genes [20,21]. The salivary glands of older *An. gambiae* mosquitoes (21 days old) secrete six additional salivary proteins than their younger counterparts (8 days old) [20]. In the sand fly *P. papatasi*, on the other hand, only the salivary protein SP44 is consistently down-regulated in older flies [21]. Due to the extrinsic incubation period faced by pathogens

during development in their insect vectors, pathogen transmission is mostly accomplished by older vectors that may display a unique saliva composition.

Invasion of the vector salivary glands by viruses and parasites is an intrinsic step for most vector-borne pathogens in their journey to a new host. The invasion of vector's salivary gland by pathogens causes disturbances in the cells homeostasis, leading to not only activation of the tissue remodeling machinery [22,23], but also modulations in the expression of salivary proteins [20,22–26]. The later can have a significant impact in the pathogen transmission.

Modulation in the expression of genes encoding saliva components due to pathogen invasion of vectors' salivary glands seems to be specific to the vector-parasite pair. Whereas *Plasmodium falciparum* invasion of the mosquito *An. gambiae* salivary glands leads to up-regulation in the expression of the gSG6 salivary protein [26], *P. berghei* invasion in the same vector leads to reduction in the expression of gSG6 and other three secreted proteins (apyrase, D7-related 1, and AEDA), and up-regulation of the protein gVAG [20]. Although infection with DENV-2 virus does not seem to induce differential expression in salivary gland genes encoding secreted proteins in the mosquitoes *Ae. aegypti* and *Ae. albopictus* [25,27,28], analysis of the actual saliva reveals reduced amounts of adenosine deaminase, aegyptin, and serpin [24]. Infection of *Ae. aegypti* salivary glands by chikingunya virus, on the other hand, leads to up-regulation in the expression of apyrase and D7 proteins [22]. Colonization of the tsetse fly *Glossina morsitans morsitans* salivary gland by *Trypanosoma brucei* also causes significant down-regulation of 27 genes encoding secreted proteins, including antigen-5, tsal1–2, tsgf1–2, 5'-nucleotidase, and sgp3 [23].

Alteration of the salivary components in the mosquito and tsetse saliva may explain the interrupted/delayed feeding pattern of pathogen-infected vectors and also the increase in the probing frequency, which also facilitates transmission to multiple hosts [23,24]. The roles of the up-regulated salivary components for pathogen transmission, on the other hand, have yet to be unveiled. Insect vector salivary gland gene expression is fine-tuned to face the challenges of obtaining nutrients in a changing environment. In addition, aging-dependent changes in salivary gland gene expression as well as modulation by pathogens seem to fulfill the pathogens needs to get established in the host skin and escape the vertebrate immune effectors.

The use of insect salivary proteins as biomarkers for vector exposure

In addition to the biological activities identified in vector saliva, a useful property of some of these vector proteins is becoming an important tool for the study of vector borne disease in endemic areas. The conventional approach to study vector surveillance or the risk of exposure to vector bites has been by entomological methods that include identification of breeding sites and insect trapping. These methods for large-scale measurements are cost prohibitive Furthermore, these methods can measure density of insects, but they cannot directly evaluate the human exposure to insect or vector bites. A complementary approach has been undertaken by various research groups to overcome this limitation [29–41]. This method consists in using antigenic vector salivary proteins as biomarkers of human exposure

to insect bites. The premise is simple: it is known that some of the insect salivary proteins are antigenic in humans; therefore, humans exposed to insect bites will mount an antibody response to some of the injected salivary proteins and the antibody levels to specific proteins can be used to assess the level of vector exposure. The use of salivary proteins as biomarkers of vector exposure has been used in various vectors of disease including ticks, kissing bugs, sand flies, mosquitoes, and tsetse flies [29–42]. The use of recombinant proteins or synthetic peptides has advanced considerably this approach in the last few years.

Biomarkers for mosquito exposure

The salivary recombinant gSG6 protein was the first molecule to be used as marker for mosquito bite exposure, and it was tested in field conditions in Burkina Faso [38] and in Tanzania [43]. Further work with this molecule resulted in the development of gSG6-P1, a small synthetic peptide that is part of the gSG6 molecule. This peptide was successfully used as marker of *An. gambiae* exposure [34] in Senegal [44] and Kenya [45]. Recently another salivary protein was shown to be a marker of *An. gambiae* exposure. The cE5 salivary protein was shown to be recognized by individuals exposed to *An. gambiae* bites [46].

It was previously shown that salivary gland homogenates of *Ae. aegypti* can work successfully as marker of mosquito exposure in Dengue endemic areas [32]. Recently, the N-terminus of the 34kDa salivary protein from *Ae. aegypti* was synthesized and recognized by sera of children living in *Aedes* prevalent area. For *Ae. albopictus* a good number of candidates have been identified as markers for exposure. The salivary proteins, D7, adenosine deaminase, serpin, and apyrase were identified by a 2D immunoproteomic approach as promising biomarkers of *Aedes* exposure [32].

Biomarkers for sand fly exposure

The recombinant salivary LJM17 and LJM11 proteins were shown to be recognized by individuals living in areas where the sand fly *Lu. longipalpis* is prevalent [40]. Individuals living in *Lu. intermedia* prevalent areas did not recognize these proteins, suggesting LJM17 and LJM11 are specific markers for *Lu. longipalpis* sand flies. These two proteins were also recognized by sera of dogs naturally exposed to *Lu. longipalpis* bites [40] and by sera of sentinel chickens [39].

A different salivary protein was identified as a biomarker for *Phlebotomus* sand fly exposure [42]. Sera of individuals living in *P. papatasi* prevalent areas recognized the salivary protein PpSP32 [36]. This protein was not recognized in sera from individuals living in areas where *P. perniciosus* is prevalent, suggesting PpSP32 protein is specific for *P. papatasi* exposure. PpSP32 was used successfully in Tunisia as a marker of *P. papatasi* exposure and recently in Saudi Arabia [37], suggesting that PpSP32 will work as a biomarker in different geographical regions where *P. papatasi* is prevalent. Recent work with *P. pernicious* salivary proteins identified a number of recombinant proteins as potential markers of vector exposure, specifically for dogs, a key reservoir of the disease [33]. The yellow related protein and the apyrase were recognized by sera of dogs exposed to bites of this sand fly species and are the best candidates as markers for *P. perniciosus* exposure [33].

Concluding remarks

The genomics and proteomics era in insect salivary gland research has brought new insights into the proteins present in vectors of disease and other insects; unfortunately for many of these sequences, particularly the abundant salivary proteins, their biological activities could not be predicted. Simple functional genomic approaches based of expression of recombinant proteins linked to clever biochemical, immunological, and disease model approaches have resulted in the discovery of their biological activities and their role in disease transmission. This will likely continue with many other salivary proteins which activities remain to be elucidated. The effect of environmental factors and the presence of the pathogen in salivary protein expression is an area that is beginning to be explored and will definitely yield further information for the early events on disease transmission. Live imaging of salivary components in the vertebrate skin can potentially unveil additional functions performed by salivary proteins, such as immune cell recruitment, interaction with immune effectors, and pathogen chemotaxis [47,48]. Live imaging can as well shed light on the potential roles of vector saliva in blood intake and digestion [49]. The use of vector salivary proteins as biomarker for vector exposure is one of the most useful and practical products resulting so far from the research on insect saliva. The impact of these biomarkers in epidemiological studies is now well documented for malaria research and will likely impact studies for many other vector-borne diseases.

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Highlights

- Novel biological activities from vector salivary proteins of “unknown” function
- Salivary proteins affects innate cells allowing the parasite to get established
- Parasites and viruses affect the expression of vector salivary proteins
- Salivary proteins are becoming attractive biomarkers for vector exposure