Exploring tick saliva: from biochemistry to 'sialomes' and functional genomics

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SUMMARY

Tick saliva, a fluid once believed to be only relevant for lubrication of mouthparts and water balance, is now well known to be a cocktail of potent anti-haemostatic, anti-inflammatory and immunomodulatory molecules that helps these arthropods obtain a blood meal from their vertebrate hosts. The repertoire of pharmacologically active components in this cocktail is impressive as well as the number of targets they specifically affect. These salivary components change the physiology of the host at the bite site and, consequently, some pathogens transmitted by ticks take advantage of this change and become more infective. Tick salivary proteins have therefore become an attractive target to control tick-borne diseases. Recent advances in molecular biology, protein chemistry and computational biology are accelerating the isolation, sequencing and analysis of a large number of transcripts and proteins from the saliva of different ticks. Many of these newly isolated genes code for proteins with homologies to known proteins allowing identification or prediction of their function. However, most of these genes code for proteins with unknown functions therefore opening the road to functional genomic approaches to identify their biological activities and roles in blood feeding and hence, vaccine development to control tick-borne diseases.

Key words: Functional genomics, high-throughput approaches, molecular biology, pharmacology, tick saliva.

INTRODUCTION

The saliva of blood-feeders is a cocktail of potent pharmacologically active components able to disarm the host haemostatic system (Ribeiro, 1987a, 1995) and alter the inflammatory and host immune responses (Wikel, 1999; Gillespie, Mbow & Titus, 2000). The molecules present in tick saliva range from lipids to large proteins and represent a plethora of biological activities which alter the physiology at the feeding site, consequently affecting pathogen transmission (Ribeiro, 1995; Valenzuela, 2002).

Adaptation of ticks to their natural hosts has resulted in the ability of ticks to modulate the host immune and haemostatic response with their saliva. However, with non-natural hosts, tick feeding often results in immune and allergic responses, presumably to the injected salivary proteins, resulting in tick rejection (Ribeiro, 1989). Interestingly, immune responses to tick feeding confer in some cases, protection to pathogen transmission (see the chapter by Nuttall & Labuda in this supplement).

Because of the importance of tick saliva in haemostasis, inflammation, immunity and pathogen transmission, the isolation of the molecules in saliva responsible for these effects is a logical step to pursue. However, the amount of proteins and nucleic acids present in the saliva or salivary gland of ticks is very limited making the isolation of these components sometimes a Herculean task. Recently, strategies based on classical and modern molecular biology techniques (high-throughput approaches) have enabled the isolation and sequencing of a large repertoire of cDNAs from the salivary glands of different tick species. The isolation of these molecules is contributing to our understanding of the role of saliva in blood feeding and pathogen transmission, and it may help in the identification of potential vaccine candidates to control tick-borne diseases.

This review focuses on the diversity of biological activities reported in tick saliva and the emergent trend toward high-throughput approaches to isolate genes from tick salivary glands. The host immune responses to tick salivary proteins or to tick bites have been studied extensively in the past (see chapter by Brossard & Wikel in this supplement). Renewed interest in this topic, together with the large number of isolated salivary genes isolated by high-throughput approaches, may provide better clues of how to design vaccines based on tick salivary proteins to control vector-borne diseases.

HOW TICKS EVADE THE HOST HAEMOSTATIC SYSTEM

Ticks attempting to obtain a blood meal face the vertebrate host's haemostatic system whose role is to prevent blood loss after tissue injury. The three branches of the haemostatic system, vasoconstriction (reduction of the blood flow), platelet aggregation (formation of the platelet plug) and the blood

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Table 1. Anti-haemostatic components identified in tick saliva. Some of these biological activities have been isolated and characterized at the molecular level[a]

Tick species	Vasodilatory factor	Anticlotting factor	Antiplatelet factor
Amblyoma americanum	Prostaglandin E_2, $F_{2\alpha}$[a] (Aljamali et al. 2002; Ribeiro et al. 1992)	Factor Xa inhibitor (Zhu et al. 1997a); Americanin (antithrombin) (Zhu et al. 1997b)	Americanin (antithrombin) (Zhu et al. 1997b)
Boophilus microplus	Prostaglandin E_2[a] (Dickinson et al. 1976)	Antithrombin (Horn et al. 2000)	Antithrombin (Horn et al. 2000)
Dermacentor andersoni		Inhibitor of FV and FVII (Gordon & Allen, 1991)	GPIIa-IIIb antagonist[a] (Wang et al. 1996)
Dermacentor variabilis			
Haemaphysalis longicornis		Madanin 1 and 2 (Iwanaga et al. 2003)	Madanin 1 and 2 (Iwanaga et al. 2003)
Hyalomma truncatum		FXa inhibitor (Joubert et al. 1995)	
Ixodes scapularis	$PG1_2$ (prostacyclin)[a] (Ribeiro et al. 1988)	Tissue factor pathway inhibitor[a] (Francischetti et al. 2002); Factor Xa Inhibitor[a] (Narasimhan et al. 2002)	Apyrase (Ribeiro et al. 1985)
Ornithodorus moubata		Tick anticoagulant peptide (TAP)[a] (Hawkins & Hellmann, 1966); Ornithodorin (anti-thrombin)[a] (Van de Locht et al. 1996)	Apyrase (Ribeiro et al. 1991); Disagregin[a] (Karczewski et al. 1994); Moubatin[a] (Keller et al. 1993); Tick adhesion inhibitor (TAI) (Karczewski et al. 1995)
Ornithodorus savigni		Savignin (anti-thrombin)[a] (Nienaber et al. 1999); Factor Xa inhibitor[a] (Joubert et al. 1998); BSAP1, BSAP2 (Ehebauer et al. 2002)	Savignygrin (disintegrin)[a] (Mans et al. 2002); Apyrase (Mans et al. 2000); Savignin (anti-thrombin)[a] (Nienaber et al. 1999)
Rhipicephalus appendiculatus		Factor Xa inhibitor (Limo et al. 1991)	

coagulation cascade (formation of the blood clot) pose a real threat to ticks when obtaining blood from the host. These three branches are well interconnected making haemostasis a redundant system, thereby increasing the challenge for blood feeders. The redundancy of the system is exemplified during platelet activation; these cells are essential in forming the platelet plug, but additionally, when activated, they release two potent vasoconstrictors, serotonin and thromboxane A_2, resulting in a decrease of blood flow. Additionally, activated platelets expose a negative charge on their membrane comprising exposed phosphatidyl serine phospholipids. These phospholipids are used for the formation of protein complexes such as the 'tenase complex' composed of factor VIII (FVIII), factor IXa (FIXa) and factor X (FX). The tenase complex is required for the formation of factor Xa (FXa) and the activation of the blood coagulation cascade. Another example of redundancy is in the blood coagulation branch. In addition to being a crucial enzyme in this cascade, thrombin also cleaves the thrombin receptor on platelets causing platelets to activate and aggregate. Therefore, the haemostatic system poses interesting problems for blood feeders, specificity and redundancy. How did ticks solve these problems? Ticks have been in the business of blood feeding for a long time. At least 120 million years of evolution and adaptation to their host's haemostatic system have created a repertoire of potent bioactive salivary molecules with vasodilatory, anti-platelet and anti-coagulant activities. In many cases molecules display more than one anti-haemostatic activity to combat the specificity and redundancy of the haemostatic system. In the next section examples of the different molecules which have been characterized from the saliva of different ticks and examples of their potent biological activities will be described. A summary of these molecules is presented in Table 1.

TICK VASODILATORY ACTIVITIES

The way ticks are able to disarm the vasoconstriction branch of the haemostatic system is by the presence of salivary vasodilators. Vasodilators are molecules which increase blood flow by antagonizing vasoconstrictors produced by the haemostatic system following tissue injury. Vasodilators act directly or indirectly on smooth-muscle cells, thereby activating enzymes such as adenylate cyclase or guanylate cyclase. This leads to the formation of intracellular cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP), respectively, which results in muscle relaxation, therefore resulting in an increased blood flow. Interestingly, all known tick salivary vasodilators reported to date are lipid derivatives. No salivary protein reported in ticks so far appears to function as a vasodilator (other blood feeders have peptides or enzymes for this function).

As examples of tick salivary vasodilators we have from the hard tick *Ixodes scapularis* a salivary arachidonic acid lipid derivative prostacyclin (Ribeiro, Makoul & Robinson, 1988), as well as prostaglandin E_2 (PGE$_2$) (Ribeiro *et al*. 1985). This molecule is a short-acting vasodilator and also an inhibitor of platelet aggregation (counteracts the redundancy of the system) that exerts its effect by increasing cAMP in smooth muscle cells resulting is vasorelaxation. *I. scapularis* is not the only tick containing this vasodilator; the lone star tick *Amblyomma americanum* saliva also contains the vasodilator PGE$_2$ and additionally PGF$_{2\alpha}$ (Ribeiro *et al*. 1992; Aljamali *et al*. 2002). The presence of PGE$_2$ has also been reported in *Boophilus microplus* (Dickinson *et al*. 1976), *Haemaphysalis longicornis* and *I. holocyclus* (Inokuma, Kemp & Willadsen, 1994). The synthesis and role of salivary gland prostaglandins are discussed in the chapter by Bowman & Sauer in this Supplement.

TICK ANTI-PLATELET FACTORS

Platelet aggregation is the first line of defence to avoid blood loss during tissue injury. Platelets are activated by diverse agonists including thrombin, collagen and ADP, after which they aggregate to form the platelet plug, promote clotting and release vasoconstrictor substances. The ability of ticks to counteract platelet aggregation resides in the presence of specific molecules that block platelet interaction or enzymes that destroy platelet agonists.

Integrin αIIbβ3 (glycoprotein IIb-IIIa, GP IIb-IIIa) is an inactive receptor on resting platelets. When activated, this receptor regulates aggregation and adhesion of platelets (Ferguson & Zaqqa, 1999). This glycoprotein receptor binds fibrinogen resulting in platelet–fibrinogen–platelet interaction or platelet aggregation by fibrinogen crosslinking. ADP secreted by activated platelets provokes integrin and Ca^{2+}-dependent platelet aggregation. Thrombin, ADP and adrenalin increase the receptor affinity to their ligands (plasma protein, fibrinogen and von Willebrand factor) which are responsible for binding to platelets during aggregation.

One strategy employed by ticks to counteract platelet aggregation is to bind to the GP IIb-IIIa receptor, therefore inhibiting platelet–fibrinogen–platelet interaction, even if platelets are activated. This anti-platelet strategy is used by the soft ticks *Ornithodoros moubata* and *O. savygni*. These ticks contain a small protein (*ca.* 7 kDa) named disagregin and savignygrin, respectively which bind to GPIIb-IIIa in platelets (Karczewski, Endris & Conolly, 1994; Mans, Louw & Neitz, 2002*a*). Disagregin uses a motif that is different from known GPIIb-IIIa antagonists to bind to the receptor, while savignyrin uses the classical Arg-Gly-Asp (RGD) motif to bind to GPIIb-IIIa. The hard tick *Dermacentor variablilis* saliva contains a protein named variabilin (4·9 kDa)

that contains an RGD motif and blocks this receptor. However, this peptide has little sequence homology to other GPIIb-IIIa antagonists (Wang *et al*. 1996). It is interesting to note that when different tick species use the same strategy to counteract a biological activity they may still use different proteins.

In addition to having disagregin in the saliva, *O. moubata* also produces a salivary anti-platelet factor named moubatin (17 kDa). Structurally, moubatin belongs to the family of lipocalins, which are beta-barrel structures that, in general, bind small hydrophobic molecules (Keller *et al*. 1993; Mans, Louw & Neitz, 2003). Salivary proteins with lipocalin structure have been described in the kissing bug *Rhodnius prolixus* (Francischetti, Andersen & Ribeiro, 2002*a*) and in the tick *Rhipicephalus appendiculatus* (Paesen *et al*. 1999). Moubatin inhibits platelet aggregation induced by collagen though the mechanism of action is not clear. However, because of the similarity in structure between moubatin and the platelet inhibitor RPAI-1 from *R. prolixus* (both being lipocalins), it may be possible that moubatin binds ADP with high affinity, thereby preventing platelet aggregation caused by ADP released from collagen-activated platelets.

The strategy used by most blood feeders to block platelet aggregation is to destroy or hydrolyze the platelet agonist ADP. This is achieved by the presence of a salivary enzyme named apyrase (EC 3.6.1.5). This enzyme hydrolyses the phosphodiester bonds of nucleoside tri- and di-phosphates but not monophosphates. Apyrase activity has been reported in the saliva of many ticks including *I. scapularis*, *O. moubata* (Ribeiro *et al*. 1985; Ribeiro, Endris & Endris, 1991) and *O. savygni* (Mans *et al*. 2000).

Two classes of apyrases have been isolated and characterized at the molecular level in haematophagic arthropods, the 5′-nucleotidase family of apyrases, present in mosquitoes such as *Anopheles gambiae* (Arca *et al*. 1999) and *Aedes aegypti* (Champagne *et al*. 1995), while the *Cimex* family of apyrases is present in bed bugs (Valenzuela *et al*. 1998) and sand flies (Valenzuela *et al*. 2001*a*). In ticks, apyrases have not been characterized at the molecular level. However, we have reported a cDNA encoding for a secretory 5′-nucleotidase in the salivary glands of *I. scapularis* (Valenzuela *et al*. 2002). Further experimental evidence is needed to demonstrate this cDNA codes for the apyrase previously characterized in the saliva of this tick.

Thrombin, the protease activated at the end of the blood coagulation cascade, is a potent agonist of platelet activation. Therefore, inhibition of thrombin activity is of central importance to blood feeders. Salivary anti-thrombin from soft ticks, including *O. moubata* and *O. savigny*, have been characterized as anticoagulants as well as inhibitors of platelet aggregation induced by thrombin (Nienaber, Gaspar & Neitz, 1999).

TICK ANTICOAGULANT ACTIVITIES

The blood-coagulation cascade involves the sequential activation of pro-enzymes ultimately resulting in thrombin activation, which in turn cleaves fibrinogen into fibrin. Polymerization of fibrin results in blood clot formation. Inhibitors of the blood-coagulation cascade are the most characterized entities from the saliva of ticks.

The most intensively studied anticoagulant in soft ticks is the tick anticoagulant peptide (TAP) isolated from the saliva of the tick *O. moubata* (Waxman *et al.* 1990). It is a specific inhibitor of FXa with a molecular mass of 6977 daltons. FXa is involved in the activation of thrombin, hence the importance of blocking the activity of this protease for haematophagous arthropods. TAP binds FXa with a dissociation constant of 180 pM. The structure of this protein has been determined by means of NMR (Antuch *et al.* 1994), as well as X-ray crystallography (St Charles *et al.* 2000). *O. savignyi* saliva also contains a FXa inhibitor with 46% identity to TAP (Joubert *et al.* 1998). *R. appendiculatus* salivary anticoagulant activity also inhibits the activation of FXa from human plasma but it does not inhibit the activity of FXa toward a chromogenic substrate (Limo *et al.* 1991). This inhibitor is probably directed to the exosite of FXa. FXa inhibitors were reported from the saliva of the lone star tick, *Amblyomma americanum* (Zhu *et al.* 1997*b*) and from the saliva of *Hyalomma truncatum* (Joubert *et al.* 1995).

Another strategy employed by ticks to inhibit the blood coagulation cascade is to block thrombin activity. Thrombin is the last enzyme in the blood coagulation cascade and is a strong agonist for platelet aggregation. Thrombin inhibitors have been described in the saliva of the ticks *O. savigny*, *O. moubata*, *A. americanum*, *B. microplus* (Horn, Dos Santos & Termignoni, 2000) and *Haemaphysalis longicornis* (Iwanaga *et al.* 2003). Americanin, the salivary anti-thrombin from *A. americanum* is a specific, reversible and slow tight-binding inhibitor of thrombin (Zhu *et al.* 1997*a*). The salivary antithrombin from *O. savigny* is a 12·4 kDa protein named savignin (Nienaber *et al.* 1999). It is a slow, tight-binding inhibitor of thrombin and interacts with the active site as well as with the binding exosite of this protease (Mans, Louw & Neitz, 2002*b*). Savignin is 63% identical to ornithodorin, the salivary anti-thrombin from *O. moubata*. The mechanism of action of these anti-thrombins differs from the classical mechanism found in the Kunitz family of protease inhibitors. Soft tick anti-thrombins insert their N-terminal residues into the thrombin active site inhibiting the activity of this protease, whereas traditional Kunitz-type inhibitors use a central, reactive loop.

Recently, two other anticoagulants have been identified from the salivary glands of the tick *O. savignyi*. These two anticoagulants termed BSAP1 and BSAP2 have molecular masses of 9·3 and 9·2 kDa, respectively, and are inhibitors of the extrinsic pathway of the blood coagulation cascade (Ehebauer *et al.* 2002), no sequence information is available for these proteins.

A novel salivary anticoagulant was recently isolated from the saliva of *I. scapularis*. This anticlotting factor named ixolaris, is an inhibitor of the tissue factor (TF) pathway (Francischetti *et al.* 2002*b*). Tissue factor is the initiator of the blood coagulation cascade. In response to tissue injury, membrane-bound tissue factor is exposed on the endothelium and on mononuclear cells. It binds FVIIa to form the complex TF/FVIIa, which converts FX to FXa. The active protease, FXa subsequently activates prothrombin to thrombin, and finally thrombin converts fibrinogen into fibrin to form the blood clot. Ixolaris is a small protein of 140 amino acids containing 10 cysteines and 2 Kunitz-type domains (Francischetti *et al.* 2002*b*). It is a fast and tight-binding inhibitor acting in the picomolar range. It specifically inhibits the activation of FX by TF/FVIIa. This salivary protein interacts with FX and FXa but not with inactive FVII. The second Kunitz domain of ixolaris is proposed to bind to the exosite of FXa and the first domain to FVIIa but only when FVIIa is in complex with tissue factor/FX and FXa, thus functioning as scaffolds for the inhibition of ixolaris to the activation of FX by FVIIa/tissue factor.

I. scapularis saliva also contains other anticoagulant besides ixolaris. Recently, Narasimhan *et al.* (2002) reported the purification of a salivary component affecting the blood coagulation cascade. This resulted in the isolation of a cDNA coding for a protein homologous to Salp14, a previously characterized antigenic protein with a molecular weight of 13·97 kDa present in the saliva of this tick. Recombinant Salp14 was shown to inhibit FXa but not other proteases. It will be interesting to determine if the two salivary anti-coagulants are secreted simultaneously during feeding or if there is a temporal expression of these proteins.

Mans, Louw & Neitz (2002*c*) have studied the origins of the blood coagulation cascade inhibitors from the saliva of the genus *Ornithodoros*. Their detailed analysis, based on predicted structure, showed that the FXa inhibitors and anti-thrombin genes share a common ancestor and they predicted that the anti-thrombin genes preceded the FXa inhibitor genes and that platelet aggregation inhibitors share a common ancestor with the FXa and thrombin inhibitors. Soft tick serine proteases inhibitors differ significantly from other canonical protease inhibitors. Furthermore, comparison of soft tick anticoagulants with hard tick anticoagulants suggested that hard tick salivary anticoagulant evolved independently.

ADDITIONAL TICK SALIVARY ANTI-HAEMOSTATIC ACTIVITIES

Other biological activities which may be related to haemostasis have been described in the saliva of ticks. Phospholipase A_2 activity was detected in the saliva of *A. americanum* (Bowman *et al.* 1997). This salivary activity hydrolyses arachidonyl phospatidylcholine, is activated by submicromolar calcium and has a predicted molecular mass of 55 kDa. It has been suggested that this phospholipase may be involved in producing PGE_2 from host substrates, and that it may also be responsible for the haemolytic activity reported in *A. americanum* saliva (Zhu *et al.* 1997*c*).

Recently both a gelatinase and fibrinolytic activities were identified in the saliva of *I. scapularis* (Francischetti, Mather & Ribeiro, 2003). These proteolytic activities are metal-dependent and target gelatin, fibrin, fibrinogen and fibronectin, but not collagen or laminin. These activities may confer additional anticoagulant activity by preventing the formation of the fibrin clot or dissolving the already formed blood clot.

ANTI-INFLAMMATORY PROPERTIES OF TICK SALIVA

Ticks stay on the host for a long period of time compared to other blood feeding arthropods (Binnington & Kemp, 1980). Ticks damage the host with their mouthparts and inject salivary components into the skin. The natural consequence of these actions would be an inflammatory response of the host which may impair blood feeding and lead to rejection of the tick (Ribeiro, 1989). Inflammation is the response to localized injury involving neutrophils, macropahges, mast cells, basophils, eosinophils and lymphocytes as well as chemokines, plasma enzymes, lipid inflammatory mediators and cytokines. The main function of neutrophils is to engulf and kill invading microorganisms by secreting inflammatory mediators and oxygen radicals. *I. scapularis* saliva has been reported to inhibit key pro-inflammatory activities of neutrophils such as aggregation following activation by anaphylotoxins, the release of enzymes, production of oxygen radicals and the phagocytosis of bacteria (Ribeiro, Weis & Telford, 1990).

Cell recruitment during the inflammatory response is triggered by chemokines, which are mainly derived from macrophages. The chemokine interleukin-8 (IL-8) is a potent chemo-attractant for neutrophils. IL-8 triggers a G-protein-mediated activating signal that results in neutrophil adhesion and subsequent trans-endothelial migration to the injury site. Anti-IL-8 activity was recently reported from saliva of various ticks including *Dermacentor reticulatus*, *A. veriegatum*, *R. appendiculatus*, *Haemaphysalis inermis* and *I. ricinus* (Hajnicka *et al.* 2001).

Anti-histaminic activity

Histamine is a very potent inflammatory mediator and a vasoactive factor that binds to H1 and H2 receptors, causing oedema and erythema by dilating and increasing the permeability of small blood vessels. Histamine is released from mast cells and basophils, often, but not always, via an IgE-dependent mechanism and is also released by platelets of many mammals. Additionally, histamine is a regulator of T cell response, binding to the lymphocyte H1 receptor results in a positive Th1 response while binding to H2 receptor results in inhibition of Th1 and Th2 responses (Jutel *et al.* 2001). *R. appendiculatus* has a set of novel salivary histamine-binding proteins (Paesen *et al.* 1999). The structure of this high-affinity histamine-binding protein is a lipocalin. Most lipocalins are beta-barrel structures with only one binding site for hydrophobic molecules. Interestingly, *R. appendiculatus* histamine-binding proteins have two binding sites, one displaying higher affinity for histamine than the other. Tick histamine-binding proteins were shown to out-compete histamine receptors for the ligand and decrease the effects of histamine. A protein homologous to *R. appendiculatus* histamine-binding protein was identified in the salivary glands of the tick *I. scapularis* (Valenzuela *et al.* 2002) and *A. americanum* (Aljamali *et al.* 2003) suggesting these ticks also have these anti-histaminic activities.

Anti-serotonin activity

Serotonin, another mediator of the inflammatory response, is secreted by tissue mast cells (in rodents) and has similar activities to histamine. A serotonin-binding protein was isolated from the salivary glands of *D. reticulatus* (Sangamnatdej *et al.* 2002). This protein is similar in structure to the *R. appendiculatus* histamine-binding protein. It has a predicted molecular weight of 22 kDa and contains two binding sites, one of which binds histamine, while the other is slightly larger and is able to accommodate and bind serotonin.

Anti-complement activity

Another important part of the inflammatory response is the alternative pathway of the complement cascade, which is important for the evasion of pathogens (Joiner, 1988). From this cascade the inflammatory mediators C5a and C3a are produced (Hugli & Muller-Eberhard, 1978). These two anaphylotoxins are chemotactic for neutrophils and can cause histamine release from mast cells and basophils. The final step of the complement cascade is the formation of the membrane attack complex which causes lysis of invading organisms. Modulation or inhibition of the complement cascade may be advantageous for blood

feeders in avoiding inflammatory reactions and in preventing lysis of the tick gut. The tick *I. scapularis* has a salivary protein that specifically inhibits the alternative pathway of complement (Ribeiro, 1987*b*). Isac (*I. scapularis* anticomplement) is a novel 18 kDa protein that inhibits the formation of C3 convertase, thereby acting as a regulator of the complement cascade (Valenzuela *et al.* 2000). Isac inhibits the interaction of factor Bb to C3b, a mechanism resembling those used by regulators of the complement cascade. However, the sequence of isac is not homologous to any known complement cascade regulators.

Kininase activity

Bradykinin is an important mediator of the inflammatory response. When coagulation FXII is activated following tissue injury, it activates prekallikrein to kallikrein. Kallikrein activates high molecular weight kininogen to release bradykinin. This nonapeptide is a mediator of pain and causes oedema formation by increasing capillary permeability. *I. scapularis* saliva contains an activity that proteolytically cleaves bradykinin (Ribeiro *et al.* 1985) and is reportedly a metallodipetidylcarboxypeptidase (Ribeiro & Mather, 1998).

TICK SALIVA AND PATHOGEN TRANSMISSION

Many pathogens are transmitted from the salivary glands of the vector to the skin of the host via salivary fluid. Because saliva of blood feeders changes the physiology of the host at the feeding site by injecting an array of bioactive molecules, some pathogens co-injected with saliva may be more infective. One of the first demonstrations of this phenomenon was reported in the sand fly–*Leishmania* interaction. *Leishmania major* parasites in the presence of sand fly salivary gland extract were more infective than parasites injected alone (Titus & Ribeiro, 1988). Other insect salivas were reported to have similar effects. The bite of the mosquito *Aedes triseriatus* potentiates vesicular stomatitis (New Jersey) virus infection in mice, while *Ae. triseriatus*, *Ae. aegypti* (Limesand *et al.* 2000), and *Culex pipiens* feeding enhances infection of Cache Valley virus in mice (Edwards, Higgs & Beaty, 1998).

This phenomenon has also been reported in ticks and the concept of saliva-enhanced transmission of tick-borne diseases is discussed in the chapter by Nuttall & Labuda in this Supplement. The saliva of the tick *R. appendiculatus* enhanced Thogoto virus transmission (Jones, Hodgson & Nuttall, 1989) and, in combination with interleukin-2, increased *Theileria parva* infection in lymphocytes (Shaw, Tilney & McKeever, 1993). In another example, the saliva of the tick *D. reticulatus* promoted vesicular stomatitis virus growth *in vitro* (Hajnicka *et al.* 1998), while the

saliva of *D. reticulatus*, *I. ricinus* and *R. appendiculatus* enhanced tick-borne encephalitis virus transmission (Labuda *et al.* 1993). *I. ricinus* saliva increased bacteraemia (*Borrelia afzelii*) in C3H mice (Pechova *et al.* 2002) and exacerbated the proliferation of the bacterium *Franciscella tularensis* in mice (Krocova *et al.* 2003).

Results from these studies prompted the search for the salivary protein(s) responsible for this effect with the objective to produce salivary vaccines able to neutralize this enhancing effect. So far such a vaccine does not exist; the search for this salivary component(s) has not been easy. The small amounts of protein and transcripts present in the salivary glands of ticks, together with the complexity of tick salivary proteins made this task more difficult. In the next section, recent approaches taken to overcome these problems, particularly to describe the different type of transcripts and proteins we can find in ticks are discussed.

BEYOND SINGLE PROTEIN–SINGLE GENE: TICK SALIVARY GENE TRANSCRIPT-WIDE ANALYSIS (EXPLORING TICK SIALOMES)

Classical biochemical and molecular biology protocols have allowed the identification, isolation and characterization of a number of tick salivary proteins. However, this number is very limited mainly due to the small amount of material present in the saliva. The conventional strategy is to identify a biological activity, isolate the corresponding protein, obtain some sequence information from the protein and use this information to design a DNA probe or primer, which can be used to screen salivary gland cDNA or a cDNA library. The clone encoding the protein responsible for the observed activity can then be isolated. To confirm or validate the correct gene, biologically-active recombinant protein is produced and its activity tested. This approach recently took a dramatic shift, moving from one protein – one gene to the use of high-throughput approaches allowing the isolation of large sets of tick salivary transcripts or genes. Following are some of the strategies and novel molecules characterized by these approaches.

Tick salivary gland cDNA library screening

One of the first approaches to isolate tick salivary gland genes or transcripts was reported by Das *et al.* (2001). The strategy was based on the construction of an expression cDNA library from the salivary glands of the deer tick *I. scapularis*. This cDNA library was screened with the serum of rabbits infested repeatedly with this tick. Rabbits exposed to tick bites reject ticks and make antibodies to salivary proteins. The screening resulted in the isolation of 47 clones, out of these, 14 different salivary tick cDNA were identified. These salivary proteins represent

promising vaccines candidates to control pathogen transmission. With this strategy a salivary protein named Salp15, was shown to block CD4+ T cell activation (Anguita *et al.* 2002).

PCR subtraction

Another strategy used to isolate tick genes was reported by Leboulle *et al.* (2002*b*) and is based on PCR subtraction and the construction of a full-length cDNA library from salivary glands of engorged ticks. Messenger RNA from the salivary glands of unfed *I. ricinus* was subtracted from the mRNA of salivary glands of blood-fed ticks. This resulted in the enrichment of salivary gland mRNA or transcripts expressed during blood feeding. With this strategy 27 different cDNA were identified from 96 randomly selected clones. Most of these clones had no homology to other proteins deposited in Gene-Bank. Only three cDNA had significant homologies to other proteins. These were proteins homologous to the human tissue factor pathway inhibitor, snake metalloproteases, and a human thrombin inhibitor (this last clone also had significant similarities to a monocyte/neutrophil elastase inhibitor). Nine clones were selected from the full-length cDNA library and 5 of these had significant homology to proteins deposited in GeneBank, these were an *Anopheles gambiae* uncharacterized mRNA, an *Ae. aegypti* chitinase, rat leukocyte common antigen-related protein, an aminotransferase and a human interferon-related protein. With this approach, the first salivary immunosuppressive molecule from the salivary glands of *I. ricinus* (iris) was identified. This cDNA had similarities to monocyte/neutrophil elastase inhibitor and was later demonstrated to have immunosuppressive activity (Leboulle *et al.* 2002*a*).

Massive cDNA sequencing and computational biology

We recently reported a strategy based on massive cDNA sequencing, proteomics and computational biology to isolate and identify salivary gland genes from *I. scapularis* (Valenzuela *et al.* 2002). This approach is based on the construction of a high quality full-length cDNA library, massive sequencing of a large set of transcripts and customized analysis to create a databank of tick salivary genes. The starting material was less than 50 salivary gland pairs and the isolated mRNA was reverse transcribed, amplified by PCR and fractionated to decrease the bias of small fragments into the lambda vector. Lamda phages (735) were randomly picked from a plated cDNA library and the PCR reaction was performed with vector specific primers flanking the inserted cDNA. Using this approach, we avoided the tedious procedure of *in vivo* excision which leads to misrepresentation of large or rare clones. These PCR products were sequenced and analyzed using customized programs which allows for a thorough analysis and better organization to access the obtained information. We identified 410 different clusters of unique families. Of these clusters, eighty seven transcripts had the presence of a signal secretory peptide. These transcripts were fully sequenced and grouped according to their abundance in the cDNA library (Valenzuela *et al.* 2002).

Nene *et al.* (2002) reported the isolation of 2109 non-redundant transcripts from the salivary glands of the tick *Amblyomma variegatum*. Their approach was based on a construction of a cDNA library from 400 salivary gland pairs and the sequencing of randomly obtained plasmids. The resulting sequences were analyzed using the TIGR gene index system (Quackenbush *et al.* 2001). Besides house-keeping gene transcripts, a large set of proteinases were identified including cathepsin L-like protease, a probable zinc protease, serine protease inhibitor 4, and serine protease inhibitor 2. Other transcripts coding for glutathione S-transferase, calreticulin and proteins similar to some of the immunodominant antigens from the saliva of *I. scapularis* (Das *et al.* 2001) were also identified. A large set of proteins rich in glycine were identified and separated in 11 distinct families. These proteins are probably part of the cement secreted during attachment of the tick in the host. A cDNA homologous to the immunosuppressant protein from *D. andersoni* (Bergman *et al.* 2000) was also identified. Many other proteins were identified and can be accessed on the www.tigr.org/ tdb/tgi.shtml site.

Functional genomics of tick salivary gene transcripts

As described above, conventional molecular biology and high-throughput approaches has led to the identification of a large set of gene transcripts from the salivary gland of ticks. Only three years ago, just a handful of tick salivary DNA sequences were deposited in GeneBank. Because the number of DNA sequences has increased dramatically at a very fast pace, this poses some questions. What do we do with all the information generated? How can we use it more effectively? How can we find the biological function of these many transcripts? These questions do not only pertain to tick research, but also to genomic research on other organisms (such as humans, mouse, fly, mosquito, parasites).The field of functional genomics tries to deal with these types of questions. Functional genomic approaches intend to identify the biological activity or function of a gene or large set of genes, based on biochemical assays, genetic manipulations, RNA interference and the expression of recombinant proteins in mammalian, insect, yeast and bacterial expression systems. Other approaches include DNA microarray analysis as well as proteomic analysis including mass spectroscopy and Edman degradation analysis. In practice, the

functional genomic approach of choice depends very much on the area of research.

In most genomic approaches (including ticks) about 50% of the transcripts are similar to proteins of known function, including of course house-keeping genes. The other 50% are transcripts of unknown function. Although the information generated by these approaches can be seen as purely descriptive, it raises a large number of hypotheses which can be immediately tested. Probably the best option is to start with the 'low hanging fruits' or the obvious transcripts which are predicted to have a particular activity. For tick gene transcripts, the expression of recombinant proteins in heterologous systems has given the most interesting results. For example, one of the transcripts of *I. scapularis* salivary glands coded for a protein similar to a tissue factor pathway inhibitor. This protein, named ixolaris, was expressed in insect cells and its biological activity detected in supernatants (Francischetti *et al.* 2002*b*). The immunosuppressor from the salivary glands of *D. andersoni* was also expressed using these insect cells (Alarcon-Chaidez, Muller-Doblies & Wikel, 2003). However, when the same protein was expressed in bacteria it lost its biological activity. These results suggest that eukaryotic expression systems are better to express certain proteins when the functional activity is dependent on the proper folding and post-translational modifications. Another example comes from a novel transcript from the salivary glands of *I. ricinus*, named iris (*Ixodes ricinus* immunosuppressor) (Leboulle *et al.* 2002*a*). This transcript codes for a protein similar to the leukocyte elastase inhibitor. When expressed in a mammalian cell line (CHO-K1 cells) the recombinant protein was able to modulate T cell and macrophage responsiveness and inhibited secretion of pro-inflammatory cytokines (Leboulle *et al.* 2002*a*). Antibodies raised against recombinant iris demonstrated the presence of this protein in the saliva of *I. ricinus*. In another example, an antigenic protein named Salp15 from the salivary glands of *I. scapularis* coded for a protein with weak homology to inhibin A, a member of TGFβ super-family, suggesting Salp15 may have some immuno-modulatory activities. Expression of Salp15 in *Drosophila* cells resulted in an active protein capable to block CD4 + T cell activation (Anguita *et al.* 2002).

Currently there are a number of expression vectors with high-throughput cloning capabilities. The problem still remains to find a robust expression system to accommodate the large repertoire of expression constructs. Insect cell and mammalian cell expression systems seem better at producing certain functional proteins. However, the amount of protein produced is still very limited, but recent advances in insect cell expression systems can now produce fairly substantial quantities of recombinant protein. Protein expression in bacteria or cell-free expression systems can produce very large amounts of material but with less probability to obtain a soluble, functional protein. Baculovirus expression systems produce large amounts of material in the active conformation but the cloning process is lengthy. Technological development of recombinant protein expression systems will have a great impact in functional genomics of any organism.

RNA interference (RNAi) is another functional genomic tool which relies on the ability to silence the expression of a gene by degrading a targeted mRNA (Brooks & Isaac, 2002). RNAi has been recently demonstrated to work in ticks. Expression of a transcript coding for a histamine-binding protein from the salivary glands of *A. americanum* was reduced by injecting ticks with double stranded RNA (dsRNA) from this clone. Furthermore, ticks treated with dsRNA resulted in lower histamine-binding ability and increased oedema around the feeding sites (Aljamali *et al.* 2003). RNAi may help identify the function and significance of tick salivary gene transcripts with no clear predicted function.

Functional genomics and vaccine development

Because tick salivary proteins have become an attractive target for vaccines to control vector-borne diseases, the resulting gene transcripts generated using high-throughput approaches are ready to be evaluated for their potential as vaccines. The questions are how to select the right candidates and how to test them. At least two non-exclusive alternatives exist. The first is to select the candidate(s) based on their anti-haemostatic, anti-inflammatory or immunosuppressor functions, and attempt to neutralize the activity, thereby potentially affecting pathogen transmission. The second is to select a protective molecule irrespective of its function. This strategy is based on the premise that pre-exposure to tick feeding in non-natural hosts results in a strong immune reaction to the injected salivary proteins (Trager, 1939). Resistance to ticks or tick immunity (Brown & Askenase, 1985) is believed to be due to antibodies (Wikel & Allen, 1976; Brossard, 1977), complement (Wikel, 1996), or to a delayed type hypersensitivity response (Brossard, Monneron & Papatheodorou, 1982). Immune responses to ticks are described in detail by Wikel & Brossard in the chapter in this Supplement.

Immune responses to tick feeding interfere with pathogen transmission. Bell, Stewart & Wikel (1979) demonstrated that *D. andersoni* pre-exposed rabbits were partially protected to the challenge to tick-transmitted *Francisella tularensis*. Similarly, mice pre-exposed to uninfected *I. scapularis* were protected to subsequent challenge of *Borrelia burgdorferi*-infected nymphs (Wikel *et al.* 1997). In another study, guinea pigs pre-exposed to uninfected *I. scapularis* were protected to *B. burgdorferi*-infected ticks (Nazario *et al.* 1998). Jones & Nuttall (1990) reported

that animals pre-exposed to uninfected *R. appendiculatus* resulted in decreased virus transmission between ticks. Several mechanisms underlying the observed protection phenomenon have been postulated and are discussed in the chapter by Nuttall & Labuda in this Supplement.

Based on the two vaccination strategies mentioned above, the steps to follow for the first vaccination strategy are quite clear: isolation of the salivary protein of known biological activity (anti-haemostatic, anti-inflammatory or immunomodulator), production of its recombinant protein and vaccination of animals with this molecule to test for inhibition of the activity, and then determination of the consequent degree of protection against the pathogen. For the second vaccination strategy, the candidates could be any of the transcripts isolated by high-throughput approaches. The number of candidates can be reduced to those representing secreted proteins or proteins believed to be secreted in the saliva of the tick. Even so, the problem remains the high number of candidates and how to test them. A possible solution may be using a DNA vaccination strategy protocol. DNA is easier to synthesize than proteins and DNA immunization has proven successful to produce either antibodies or cellular responses to the injected antigen (Whitton *et al.* 1999). For example, we demonstrated that DNA vaccination of a salivary gene (*PpSP15*) from the sand fly *Phlebotomus papatasi* produced a strong immune response in animals and conferred protection against *Leishmania major* infection (Valenzuela *et al.* 2001*b*). This protection was mainly due to a delayed type hypersensitivity response and not to antibodies against this salivary protein suggesting the parasite was killed indirectly by the local immune response generated by the salivary protein. DNA vaccination of tick genes may give us better clues of which of these many molecules are capable of producing a protective immune response. More importantly, we wish to determine if a particular type of immune response is sufficient to block pathogen transmission. Recently, Almazan *et al.* (2003) immunized animals with a cDNA expression library of a cell line of *I. scapularis* (IDE8) and identified a number of cDNA capable to decrease tick infestations. This approach clearly demonstrates the potential use of nucleic acids in tick vaccine research.

Alternatively, candidate molecules can be produced as recombinant proteins in large amount in a prokaryotic system (*E. coli*). This is a quite challenging task due to the large number of transcripts, and with the handicap of producing proteins which may be lacking the proper folding or post-translational modifications.

CONCLUSIONS

The area of tick saliva research has taken a great leap forward in recent years. Molecular biology and high-throughput approaches (PCR subtraction, massive cDNA sequencing, proteomics and computational biology) are increasing our knowledge of the proteins present in the salivary glands of ticks. This new information together with the vast knowledge acquired over the last three decades on the pharmacology of tick saliva and immune responses to tick salivary proteins will probably open new venues to the understanding of tick saliva on blood feeding and pathogen transmission. Ultimately this information may generate better strategies for the use of tick salivary antigens as vaccines to control vector borne diseases.

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