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REVIEW

Modulation of host immunity by haematophagous arthropods*

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The medical and veterinary public-health importance of haematophagous arthropods is immense and continuing to increase because of the emergence of new vector-borne infectious agents and the resurgence of well known ones. Control of blood-feeding arthropods and the pathogens they transmit is compounded by drug, insecticide and acaricide resistance. Novel control strategies are needed. Immunological control is one very promising approach to these problems. In order to develop anti-arthropod vaccines that block pathogen transmission and establishment, the immunological interactions occurring at the interface of the blood-feeding arthropod and host must be characterized. An important component of these interactions is arthropod modulation of the host's innate and acquired, specific immune defences. This review discusses current knowledge regarding the ability of haematophagous arthropods to alter their hosts' immune defences, the impact of those changes on pathogen transmission, the molecular bases for the immunomodulation, and strategies for identification of the molecules in arthropod saliva that are responsible for the immunomodulation.

Blood-feeding arthropods are of vast medical and veterinary importance because of their transmission of infectious agents and also the direct tissue damage caused during their bloodmeals. Hundreds of millions of cases of vector-borne disease occur annually (Gubler, 2000). Arthropod-transmitted diseases were responsible for more morbidity and mortality, between the 17th and early 20th centuries, than all other causes (Gubler, 1998). The past 40 years have witnessed the emergence of previously unknown vector-borne diseases and the resurgence of old foes once thought to be under control (Gubler, 1998; Gratz, 1999; Parola and Raoult, 2001). Since the identification of *Borrelia burgdorferi* in 1982 as the causative agent of Lyme borreliosis, 15, previously unrecognized, bacterial pathogens transmitted by ixodid ticks have been described (Parola and Raoult, 2001). Some arboviruses, including dengue, have expanded their geographical range and caused epidemics (Gubler, 2000). Arthropod-borne emerging infectious diseases of wildlife are potential threats to the health of domestic animals and humans (Daszak *et al.*, 2000).

* The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the U.S. Navy Department or the U.S. Naval Service at large.

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This emergence and resurgence of arthropod-transmitted diseases has been attributed to a variety of elements: population movement; urbanization; deforestation; climate change; inadequate management of sewage; lack of funding for control programmes; a shortage of trained specialists; increased global travel; genetic changes in pathogens; and increased densities of the vectors (Gubler, 1998; Gratz, 1999). Additional factors of great importance are drug resistance in the infectious agents (Molyneux, 1998), widespread insecticide resistance in the insect vectors (Brogdon and McAllister, 1998; Hemingway and Ranson, 2000), and acaricide resistance in ticks (Nolan, 1990; Mitchell, 1996).

As mosquitoes are the most important arthropod vectors of infectious agents to humans, and ticks the second most (Balashov, 1972), it is not surprising that the host–vector–pathogen interactions of these arthropods have been the most intensively studied. However, there are many aspects of these relationships that have not been characterized. Studies need to focus on additional species of blood-feeding arthropods, particularly insects. In light of the current problems in vector control and disease suppression, a great need exists to develop innovative strategies for the control of blood-feeding arthropods and the diseases they transmit. Increasingly, attention has focused on immunology-based control. This review will concentrate on our current understanding of immunomodulation of the hosts' immune defences by blood-feeding insects and ixodid ticks.

CHALLENGES TO SUCCESSFUL BLOOD-FEEDING

In order to obtain a bloodmeal successfully, blood-feeding arthropods must cope with a variety of challenges posed by the host: haemostasis; pain and itch responses; and immune defences (Ribeiro, 1996). For both rapidly engorging and slowly feeding arthropods, the solutions to these problems are found in the pharmacological properties of their saliva.

Host haemostatic defences include coagulation pathways, platelet aggregation and vasoconstriction. Blood-feeding insects and ticks have developed countermeasures to each of these responses in the form of salivary molecules that inhibit an array of components of the coagulation pathways, block platelet aggregation, or act as vasodilators (Ribeiro, 1995; Champagne and Valenzuela, 1996; Stark and James, 1996). Bradykinin is an important mediator of the itch (Alexander, 1986) and pain (Clark, 1979) which could stimulate host grooming and removal of a blood-feeding arthropod. Saliva of at least one tick, *Ixodes scapularis*, contains a kininase that inhibits bradykinin (Ribeiro and Mather, 1998). As histamine can cause the sensation of itch (Alexander, 1986), it is perhaps not surprising that the salivary glands of the blood-feeding bug *Rhodnius prolixus* (Ribeiro and Walker, 1994) and of ticks (Chinery and Ayitey-Smith, 1977; Paesen *et al*., 1999) produce histamine-binding proteins.

Generally, ectoparasitic arthropods induce some level of host immune responsiveness to re-infestation (Wikel, 1999*a*). Ticks can stimulate and elicit host immune responses ranging from no apparent reactivity (Schoeler *et al*., 1999) to those that reduce tick feeding, impair reproductive success, block moulting, and result in death of the tick (Wikel, 1996). The nature of the host response to infestation depends upon both the species of tick and the host (Ribeiro, 1989). Host immunoregulatory and effector elements stimulated by blood-feeding arthropods include antigen-presenting cells, B- and T-lymphocytes, cytokines, antibodies, complement, chemokines, and an array of other biologically active molecules (Sandeman, 1996; Wikel, 1996). In turn, it is not surprising that blood-feeding insects and ticks have developed countermeasures to the innate and specific, acquired immune responses of the host (Wikel, 1982*a*, 1996, 1999*a*, *b*; Wikel and Bergman, 1997; Gillespie *et al*., 2000). A significant advantage is gained by reducing the host's immune response against the molecules in the arthropod's saliva that are essential for

obtaining a bloodmeal. For both rapidly feeding insects and ticks, which feed for days, the reduction of host immunity to the components of their saliva enhances the likelihood that a host will be a suitable source of future bloodmeals.

ARTHROPOD-BORNE INFECTIOUS AGENTS

An increasing body of evidence supports the view that arthropod modulation of the hosts' immune defences enhances pathogen transmission and establishment. Salivary-gland lysates of the sandflies *Lutzomyia longipalpis* and *Phlebotomus papatasii* dramatically enhanced infection of mice with *Leishmania major* (Titus and Ribeiro, 1988; Belkaid *et al.*, 1998). Infection with Cache Valley virus was enhanced when virus was injected into sites of feeding by *Aedes triseriatus*, *Ae. aegypti* or *Culex pipiens* (Edwards *et al.*, 1998). Saliva of the mosquito *Ae. triseriatus* potentiated transmission of vesicular stomatitis virus (Limesand *et al.*, 2000). Immunomodulation of the hosts' immune defences by tick saliva appears to be central to the successful transmission of Thogoto virus (Jones *et al.*, 1989), *Theileria parva* (Shaw *et al.*, 1993), tick-borne encephalitis virus (Labuda *et al.*, 1993), and vesicular stomatitis virus (Hajnicka *et al.*, 2000).

In addition to arthropod modulation of host immunity, infectious agents themselves have evolved strategies to evade their vertebrate hosts' innate and specific, acquired immune defences (Marrack and Kappler, 1994; Kotwal, 1996; Brodsky, 1999). Examples of such immune-evasion strategies include antigenic variation, molecular mimicry, inhabiting 'immune privileged' sites, immune deviation, inhibition of complement components, enzymes that cleave antibodies, coating with host molecules, and production of molecules that act in a manner similar to host immunoregulatory factors. Arthropod modulation of host immunity could provide the appropriate environment for pathogen transmission and establishment, which would be combined with, or followed by, immune evasion mediated by the infectious agent.

HOST IMMUNE MODULATION BY BLOOD-FEEDING INSECTS

The observations that rapidly feeding insects, such as mosquitoes and biting flies, suppress and/or deviate the immune defences of their hosts seems unexpected in light of the few minutes such arthropods need to acquire a bloodmeal. However, it is important to consider the potential number of bloodmeals that might be obtained from an individual host over a period of months or years. From a population perspective, it would be undesirable for a host to develop antibodies to proteins in insect saliva that are needed for successful blood-feeding.

Onchocerciasis, caused by a nematode which is transmitted by blackflies, occurs in the New World from southern Mexico to the Equator as well as in tropical Africa and a small region of Arabia (Crosskey, 1995). Blackfly bites on BALB/c mice induce antibodies which recognize fewer salivary-gland molecules than antibodies induced by immunization with a salivary-gland extract (Cross *et al.*, 1993*b*). This difference in antibody responses reflects both qualitative and quantitative differences between saliva introduced during feeding and an extract prepared from whole salivary glands (which would also contain molecules associated with gland physiology and cyto-architecture). However, blackflies are capable of modulating the immune defences of their hosts. Cross *et al.* (1993*a*) observed that mice inoculated with a salivary-gland extract (SGE) of the blackfly *Simulium vittatum* have reduced expression of major histocompatibility complex (MHC) class-II antigens on their splenocytes (compared with that of similar cells from mice not exposed to SGE). These changes could reduce antigen-presentation capabilities. No effect was observed on class-II MHC expression by cells from regional lymph nodes or skin.

Addition of *Si. vittatum* SGE to cultures of splenocytes, derived from mice inoculated

intraperitoneally with blackfly SGE, inhibited the in-vitro proliferation induced by a T-lymphocyte mitogen [concanavalin A (Con A)] or a B-lymphocyte mitogen (*Salmonella typhosa* lipopolysaccharide) (Cross *et al*., 1993*a*). In-vivo inoculation of blackfly SGE did not, by itself, alter the in-vitro proliferative responses of lymphocytes to mitogens (Cross *et al*., 1993*a*). Paradoxically, inoculation of sheep erythrocytes in combination with blackfly SGE enhanced the erythrocyte-specific antibody response (Cross *et al*., 1993*a*).

The influence of *Si. vittatum* SGE on cytokine production by splenocytes was assessed by repeatedly inoculating mice with either SGE or saline prior to administration of ovalbumin to generate antigen-specific lymphoblasts (Cross *et al*., 1994*a*). Splenocytes from the ovalbumin-primed mice were then cultured *in vitro* in the presence of ovalbumin, to induce cytokine secretion. Cells from SGE-treated mice produced lower levels of interleukin-5 (IL-5) and IL-10 than the cells from saline-injected controls, although secretion of interferon-γ (IFN-γ), IL-2 and IL-4 was not affected.

Salivary glands of female mosquitoes (the males do not blood-feed) contain molecules which modulate components of host innate and specific, acquired immune defences (Wikel, 1999*a*). An SGE of female *Ae. aegypti* inhibited release of a pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α), from rat mast cells (Bissonette *et al*., 1993). Mast cells participate in immune- and non-immune-mediated, acute and persistent, inflammatory responses (Van Loveren *et al*., 1997). They are situated perivascularly in the dermis and their granules contain a variety of biologically active molecules, including TNF-α, acid hydrolases, histamine, prostaglandins, leucotrienes, and chemotactic factors for eosinophils and neutrophils (Van Loveren *et al*., 1997). Mast cells have the unique ability to release pre-formed TNF-α (Van Loveren *et al*., 1997). The inhibitory activity of the *Ae. aegypti* SGE, which was lost upon boiling, was attributed to a molecule with a molecular mass of at least 10 kDa (Bissonette *et al*., 1993).

Another SGE of female *Ae. aegypti* suppressed secretion of IL-2 and IFN-γ (but not that of IL-4 and IL-5) when added to cultures of Con-A-stimulated splenocytes derived from naive BALB/c mice (Cross *et al*., 1994*b*). Interestingly, this SGE did not affect cytokine secretion by antigen-activated splenocytes derived from ovalbumin-primed mice (Cross *et al*., 1994*b*). Lymphocytes pre-treated with the *Ae. aegypti* SGE had markedly suppressed proliferative responses upon stimulation with optimal concentrations of exogenous IL-2 (Cross *et al*., 1994*b*); the effect on IL-4 stimulation was less intense. As more of the relationships between blood-feeding arthropods and their hosts are studied, suppression of Th1 cytokines is emerging as a common theme (Wikel, 1999*a*, *b*).

Systemic cytokine responses of C3H/HeJ mice were determined 4–10 days after 10–25 *Ae. aegypti* or *Cx. pipiens* had fed to repletion on each mouse (Zeidner *et al*., 1999). Production of IFN-γ was significantly down-regulated 7–10 days after feeding by *Cx. pipiens* and 7 days after *Ae. aegypti* feeding. The Th2 cytokines IL-4 and IL-10 were significantly up-regulated 4–7 days after feeding by either mosquito species. In a manner similar to *Ae. aegypti* feeding, injection of sialokinin I, a neuropeptide occurring in *Ae. aegypti* salivary glands, into C3H/HeJ mice resulted in down-regulation of Th1 cytokines and significant up-regulation of Th2 cytokines 4 days after inoculation. Modulation of T-lymphocyte function can alter the host's responses to mosquito salivary proteins and possibly facilitate pathogen transmission and establishment. More effort needs to be directed toward determining the immunomodulatory properties of mosquito saliva from diverse vector species.

Sandflies are the most extensively studied blood-feeding insect in regard to modulation of host immune defences (Wikel, 1999*a*; Gillespie *et al*., 2000). Investigations originated with the observation that salivary gland lysates of *Lu. longipalpis* enhance infection with *Le. major* (Titus and Ribeiro, 1988). This property of the lysates was subsequently attributed to maxadilan, a 7-kDa protein, which has both

vasoactive and immunomodulatory properties (Gillespie *et al*., 2000). In fact, maxadilan is the major vasodilator and factor enhancing infection in sandfly saliva. Both sandfly saliva and maxadilan inhibit macrophage function, and T-lymphocytes are impacted as a result of the changes induced in macrophages (Theodos and Titus, 1993).

Maxadilan acts upon macrophages by inhibiting the production of nitric oxide (Gillespie *et al*., 2000) and secretion of TNF-α (Bozza *et al*., 1998; Soares *et al*., 1998) and by augmenting production of prostaglandin E_2 (PGE_2), IL-6 and IL-10 (Bozza *et al*., 1998; Soares *et al*., 1998). The protein's effects upon T-lymphocytes include the inhibition of activation and delayed-type hypersensitivity reactions in mouse foot-pads (Qureshi *et al*., 1996). An effect on T-lymphocyte proliferation was determined by adding maxadilan to splenocytes stimulated with either Con A or anti-T-lymphocyte receptor immobilized to the culture plate. The observed modulation of macrophage and T-lymphocyte function could have arisen to prevent development in the host of immune responses to the salivary-gland proteins, introduced into the bite site, that are essential for successful blood-feeding. In addition, the immunological environment created is favourable for the transmission and establishment of *Leishmania*.

Sibling species within the *Lu. longipalpis* complex differ significantly in their amounts of maxadilan mRNA (Yin *et al*., 2000). Likewise, amino-acid content differed by as much as 23% for maxadilan from different sibling species. Significantly, all the variants possessed similar vasodilatory activity. This finding led to the conclusion that differences in vasodilatory activity of *Lu. longipalpis* saliva reflected differences in the amount of maxadilan present in the saliva of sibling species. Differences in maxadilan in these sibling species correlate with differences in the clinical manifestations of *Le. chagasi* transmitted by these sandflies (Yin *et al*., 2000).

The saliva of *P. papatasi*, the Old-World vector of *Le. major*, contains potent inhibitors of protein phosphatase 1 and protein phosphatase 2A of mouse macrophages (Waitumbi and Warburg, 1998). Likewise, *P. papatasi* saliva down-regulated the expression of the gene for inducible nitric oxide synthase and the production of nitric oxide by macrophages. It has been suggested that these changes are the basis by which *P. papatasi* saliva enhances *Leishmania* infection (Waitumbi and Warburg, 1998). A salivary-gland lysate of *P. papatasi* up-regulated IL-4 mRNA in mice (Mbow *et al*., 1998). In the same study, the effects of the lysate on immune responses to *Le. major* were monitored by determining the levels of cytokine mRNA in the lymph nodes draining cutaneous lesions in CBA/CaH-T6J mice. The enhancement of disease by the *P. papatasi* lysate correlated with a reduction in IL-12, IFN-γ, and inducible nitric oxide synthase, while IL-4 was elevated (Mbow *et al*., 1998). Recent analyses of *Lutzomyia* and *Phlebotomus* SGE by electrophoretic methods revealed highly complex populations of proteins that differed both between and within species from different geographical regions (Volf *et al*., 2000). The salivary glands of *P. argentipes* contain adenosine/AMP, which has immunomodulatory properties in addition to anti-platelet and vasodilatory activity (Ribeiro and Modi, 2001). It seems highly likely that additional immunomodulatory molecules will be found in the saliva of these important vectors. Different strategies of host immunomodulation appear to have evolved for Old-World and New-World sandflies.

The blood-feeding bug *Rho. prolixus* is an important vector of *Trypanosoma cruzi*, the causative agent of Chagas disease. The salivary glands of *Rho. prolixus* contain a nitrosyl-heme protein, nitrophorin (Ribeiro and Walker, 1994). Nitrophorin releases nitric oxide, which acts as a vasodilator and inhibitor of platelet aggregation. Histamine displaces the nitric oxide from nitrophorin. The antihistamine property of the *Rho. prolixus* nitrophorin was confirmed by its ability to inhibit histamine-induced contractions of guinea-pig ileum (Ribeiro and Walker, 1994). Histamine is an important mediator released from mast cells at sites of tissue injury (Van Loveren *et al*., 1997). Spontaneous and mitogen-induced pro-

liferation was assessed *in vitro* for splenic lymphocytes of CBA mice each of which had been bitten by three adult *Rho. prolixus* (Kalvachova *et al*., 1999). Both types of responses were suppressed by the blood-feeding. The most dramatic effects were at 4 days post-feeding, when responses to the polyclonal activators Con A, phytohaemagglutinin (PHA) and *Escherichia coli* lipopolysaccharide (LPS) were all suppressed. The most intense suppression was 85% in cultures stimulated with PHA. Although *Tr. cruzi* is transmitted in the faeces of the bug, vector-induced host immunomodulation could facilitate infection of the host, since the trypanosomes are deposited in the vicinity of the feeding site.

Clearly, blood-feeding insects modulate or deviate the hosts' innate and specific, acquired immune defences. However, only a limited number of these important phenomena and vectors has been studied. Efforts should focus on the identification of the molecules in saliva that are responsible for these events.

HOST IMMUNE MODULATION BY TICKS

Ixodid ticks remain attached to their hosts and acquire a bloodmeal over a period ranging from days to weeks (Balashov, 1972; Sonenshine, 1991). Salivary-gland-derived proteins introduced into the host by such ticks have an array of pharmacological activities (Champagne and Valenzuela, 1996; Ribeiro, 1995; Wikel, 1996, 1999*b*). Furthermore, tick saliva changes in protein composition over the course of feeding (McSwain *et al*., 1982). The extended period of exposure to tick saliva provides ample opportunity for the host to develop innate and specific, acquired immune responses to those molecules. In fact, both natural and laboratory hosts can develop immunologically-based resistance to tick feeding (Willadsen, 1980; Wikel, 1982*a*, 1996; Brossard and Wikel, 1997). Acquired resistance to tick infestation is expressed as reduced engorgement, decreased numbers and viability of ova, impaired moulting, and death of feeding ticks (Wikel, 1996, 1999). The immunological basis of acquired resistance to ticks has already been the topic of several recent reviews (Allen, 1989; Brossard and Wikel, 1997; Wikel, 1996, 1999; Wikel and Bergman, 1997; Willadsen and Jongejan, 1999). Immune regulatory and effector mechanisms linked to acquired resistance include antigen-presenting Langerhans cells, cytokines, circulating and homocytotropic antibodies, T-lymphocytes, complement, granulocytes, and a variety of bio-active molecules.

In light of the long duration of contact with the host and the potential for immune-mediated rejection, it is not surprising that ticks have developed strategies to modulate or deviate their hosts' immune defences. Increasingly, tick-induced modification of the hosts' immune defences is recognized as an important factor not only in successful feeding but also in pathogen transmission (Ribeiro, 1995; Zeidner *et al*., 1996, 1997; Wikel, 1996, 1999; Wikel and Bergman, 1997; Gillespie *et al*., 2000). An extension of these observations is gaining increased attention as a novel approach to the development of vaccines to block transmission of tick-borne infectious agents (Ribeiro, 1995; Wikel, 1996, 1999; Wikel and Bergman, 1997).

By using cobra venom factor *in vivo* to deplete the complement component C3, Wikel and Allen (1977) demonstrated that complement played a role in acquired resistance to *Dermacentor andersoni*. However, since C3 is a key component of both pathways, the relative importance of the classical and alternative pathways of complement activation could not be ascertained by this approach. Subsequently, the alternative pathway of complement activation was established to be involved in the expression of acquired resistance (Wikel, 1979). Saliva of *Ixodes dammini* (= *I. scapularis*) inhibited activation of the alternative pathway in human, rat, mouse, guinea pig and hamster sera, by inhibiting C3b deposition on activating surfaces and the release of the anaphylatoxin C3a (Ribeiro, 1987). The inhibitory activity was reported to elute as a single gel-filtration peak at 49 kDa (Ribeiro, 1987). Addressing a similar question,

salivary gland lysates of *I. ricinus*, *I. hexagonus* and *I. uriae* inhibited activity of the alternative pathway of complement, but the inhibitory ability varied with the species of mammal used as a source of the complement (Lawrie *et al.*, 1999). Saliva of *I. scapularis* inhibited the action of guinea-pig anaphylatoxin (Ribeiro and Spielman, 1986). An *I. scapularis* salivary-gland protein that inhibits the alternative pathway of complement was recently purified, cloned and expressed (Valenzuela *et al.*, 2000). This 18.5-kDa protein was not similar to any protein detailed in several sequence-analysis databases.

Basophils were found to be the predominant type of infiltrating cell at the sites of tick attachment on laboratory animals (Allen, 1973) and cattle (Allen *et al.*, 1977) that had previously been infested with ixodid ticks. Mediators released by basophils, particularly histamine, were thought to play an important role in tick rejection by hosts expressing acquired resistance. Histamine levels are elevated in the skin of cattle resistant to *Boophilus microplus* infestation (Willadsen *et al.*, 1979). Significant increases in tissue histamine were observed at the sites of tick attachment on guinea pigs resistant to *D. andersoni*, and concomitant administration of type-1 and type-2 anti-histamines abrogated the expression of resistance (Wikel, 1982*b*). Furthermore, histamine was shown to disrupt the feeding of *D. andersoni in vitro* (Paine *et al.*, 1983).

In light of the potential for histamine to disrupt tick feeding and the proximity of basophils and mast cells in the skin to tick feeding sites, the presence of histamine-blocking molecules in tick salivary glands is not unexpected. Histamine blockers have been found in the salivary glands of *Rhipicephalus sanguineus* (Chinery and Ayitey-Smith, 1977). Soluble receptors have also been detected in the salivary glands of *Rhi. appendiculatus*: two in the glands of the females and one in those of the males (Paesen *et al.*, 1999). The histamine-binding proteins from the females are more closely related to each other than to the male-derived protein. The male-derived protein is also present in nymphs and larvae. These soluble, histamine-binding proteins are highly specific and differ from mammalian histamine receptors. Tick histamine-binding proteins are lipocalins with two binding pockets that trap hydrophilic molecules (Paesen *et al.*, 2000). Their predicted role is to reduce inflammation during blood-feeding (Paesen *et al.*, 1999).

Natural-killer (NK) cells are lymphocytes that are distinct from B and T cells, and they are an important component of innate immune responses to infection (Trinchieri, 1989; Biron *et al.*, 1999). An SGE prepared from 6-day-fed female *D. reticulatus* reduced the effector function of normal human NK cells by 14% to 69% (Kubes *et al.*, 1994). The supernatant solution produced by centrifuging the homogenized salivary glands of 5-day-fed female *I. ricinus* (at $10\,000 \times g$) suppressed the ability of NK cells from uninfested mice to kill YAC-1 cell targets (Kopecky and Kuthejlova, 1998). Non-stimulated NK cells and those stimulated *in vitro* with polyinosinic–polycytidylic acid (polyIC) were suppressed by 31% and 26%, respectively. SGE inhibited the LPS induction of NK-cell activity but not the Con-A-mediated induction. In addition, *I. ricinus* SGE reduced polyIC-induced interferon, as measured by an assay that detects production of IFN-α, -β and -γ (Kopecky and Kuthejlova, 1998). An SGE from female *D. reticulatus* also inhibited the antiviral effects of IFN-α and IFN-β produced by mouse fibroblasts (Hajnicka *et al.*, 2000).

In vitro, saliva collected from 5-day-fed female *I. scapularis* inhibited the following functions of normal peritoneal neutrophils from rats: anaphylatoxin-induced aggregation; chemical-induced secretion of granule β-glucuronidase; zymosan-induced secretion of superoxide; and phagocytosis of *Bor. burgdorferi* (Ribeiro *et al.*, 1990). The movement of leucocytes into tissues from the vascular compartment involves interactions between activated endothelial cells and adhesion molecules on the leucocyte surface (Springer, 1995). Splenic lymphocytes of mice infested with the pathogen-free nymphs of *D. andersoni* had reduced expression of some of these adhesion molecules: leucocyte function-associated antigen-1 (LFA-1) and very late activation-4

(VLA-4) integrins (Macaluso and Wikel, 2001). In-vitro exposure of normal lymphocytes to *D. andersoni* SGE or saliva resulted in suppressed expression of both VLA-4 and LFA-1. Although the hosts' innate defences are modulated by molecules derived from tick salivary glands, the contribution of these changes to successful feeding and pathogen transmission remains to be fully defined.

Macrophages are tissue-dwelling phagocytic cells that play important roles in both innate and adaptive immune responses, particularly those directed towards infectious agents. SGE prepared from female *D. andersoni* reduced the LPS-stimulated production of the pro-inflammatory cytokines IL-1β and TNF-α by splenic macrophages from tick-naive mice (Ramachandra and Wikel, 1992). The same SGE suppressed the production of both IL-1β and TNF-α by normal, bovine, peripheral-blood-derived macrophages (Ramachandra and Wikel, 1995). Saliva collected from partially fed, female *I. scapularis* significantly suppressed the production of nitric oxide by peritoneal macrophages collected from uninfested mice (Urioste *et al.*, 1994). This suppressive activity was linked to a protein in the saliva and not to PGE_2. Saliva of *Rhi. sanguineus* inhibited the killing of *Tr. cruzi* by IFN-γ-activated macrophages (Ferreira and Silva, 1998); the impaired ability to kill the intracellular pathogens correlated with a 69% reduction in the production of nitric oxide. An SGE of 5-day-fed *I. ricinus* females inhibited the killing of *Borrelia afzelii* by mouse peritoneal macrophages by 43% (Kuthejlova *et al.*, 2001). The production of nitric oxide by macrophages was reduced by 26%–57% when the cells were exposed to high and low doses of spirochaetes, respectively. In addition, SGE reduced concentrations of superoxide anions in the macrophages by as much as 27%.

An SGE of partially fed *Rhi. appendiculatus* females inhibited both transcription and secretion of IL-1α, TNF-α, and IL-10 *in vitro* by the mouse-macrophage cell line, JA-4, stimulated with LPS (Gwakisa *et al.*, 2001). When compared with control JA-4 cells stimulated with LPS, the presence of SGE reduced the levels of secreted cytokines by 67.8% (IL-1α) to 82.0% (IL-10). Suppression of mRNA transcription was suppressed by 36.9% for IL-1α and 31.5% for IL-10 (values less than those observed for the secreted cytokines). In addition, the production of nitric oxide by LPS-stimulated JA-4 cells was significantly reduced by exposure to SGE (Gwakisa *et al.*, 2001).

A homologue of one pro-inflammatory cytokine, macrophage migration inhibitory factor (MMIF), has been detected in both the midguts and salivary glands of 3-day-fed female *Amblyomma americanum* (Jaworski *et al.*, 2001). Although antibodies to the tick MMIF did not cross react with the human or mouse homologues, both the tick and human MMIF were essentially similar in their ability to inhibit macrophage migration. The presence of the MMIF in tick saliva remains to be confirmed. The obvious question is why would tick salivary glands contain a molecule with pro-inflammatory activity? Possible roles for the tick MMIF include increasing inflammation at the bite site (and so enhancing blood flow to the site) and inhibiting potentially deleterious macrophages from migrating toward the mouthparts of the tick (Jaworski *et al.*, 2001). Mammalian MMIF is a pleotropic protein, expressed by a wide range of tissues, that plays a number of roles in the immune response, including glucocorticoid-induced modulation of immune responses (Nishihira, 2000). In addition to uncertainty as to its classification as a cytokine, hormone or enzyme, MMIF has been linked to tumour growth and angiogenesis as well as wound repair (Nishihira, 2000). Characterization of the role of this interesting molecule in tick feeding and pathogen transmission is eagerly awaited.

Working with guinea pigs infested with adult *D. andersoni*, Wikel (1982*c*) was the first to report that tick infestation can induce suppression of the proliferative responses of the host's T-lymphocyte to mitogens. In-vitro proliferation stimulated by Con A or PHA was reduced during both the initial and a subsequent infestation. Interestingly, the magnitude of suppression was greater during the

first infestation. The lymphocytes of the infested animals were not suppressed in their responsiveness to the B-lymphocyte mitogen LPS. The peripheral-blood lymphocytes of purebred *Bos taurus* were reduced in their in-vitro responsiveness to PHA after a third and fourth infestation with *D. andersoni* (10 female and five male ticks/cow; Wikel and Osburn, 1982). SGE were prepared from unfed and feeding female *D. andersoni* and tested for their ability to suppress the proliferation, *in vitro*, of normal mouse splenocytes stimulated with Con A (Ramachandra and Wikel, 1992). Although proliferation was reduced by only 5.5% by the SGE of the unfed females, the extracts prepared on the first day of engorgement suppressed responses by 84.9%, indicating a rapid increase in the appearance of immunosuppressive moieties in the salivary glands. Interestingly, the in-vitro proliferation of B-lymphocytes in the presence of LPS was significantly enhanced. These SGE were not cytotoxic. Similar changes were observed for peripheral-blood lymphocytes of purebred *Bos indicus* or *Bos taurus* cultured in the presence of the same *D. andersoni* SGE (Ramachandra and Wikel, 1995). Although SGE suppressed the Con-A-driven responsiveness of T-cells of both *Bos* species to a similar extent, the baseline responses of *Bos indicus* were appreciably higher than those of *Bos taurus*.

Significant progress has been made in characterizing one of the proteins in the saliva of *D. andersoni* that inhibits the Con-A-driven proliferation of T-lymphocytes *in vitro*. Preparative SDS–PAGE was used to identify a soluble suppressive protein in an SGE of 4-day-fed females (Bergman *et al.*, 1995). This reactivity was subsequently linked to a 36-kDa protein, the N-terminal amino-acid sequence of which was determined (Bergman *et al.*, 1998). Bergman *et al.* (2000) then used nested, forward, degenerate, oligonucleotide primers, corresponding to the N-terminal sequence of the 36-kDa protein, to isolate a cDNA encoding the immunosuppressant protein—by 3′ rapid amplification of the cDNA ends (RACE). The sequence of this novel protein was determined and found to contain five potential glycosylation sites, one myristylation site, and potential phosphorylation sites.

Proliferative responses, induced *in vitro* by Con A, were assessed in lymphocytes collected at various times from rabbits infested three times with adult *I. ricinus* (twice with either 25 or five pairs of ticks/rabbit and then with 15 pairs/rabbit; Schorderet and Brossard, 1993). Proliferation was reduced during each infestation, the greatest reduction being associated with the third exposure to ticks. A different pattern of modulation of Con-A-driven responses was observed in splenocytes derived from BALB/c mice infested with *I. ricinus* nymphs (Borsky *et al.*, 1994). Reduced proliferation was observed for the spleen cells isolated after the first and third infestations but the splenocytes obtained after the second exposure to nymphs showed higher levels of reactivity to Con A than those obtained from the tick-naive controls. The in-vitro proliferative responses of splenic T- and B-lymphocyte were assessed for BALB/c mice given four sequential infestations with *I. ricinus* nymphs (Dusbabek *et al.*, 1995). When compared with the corresponding cells from normal controls, the cells from the infested mice reacted similarly or with slightly enhanced reactivity to Con A or PHA. However, their responses to B-cell mitogens were suppressed. In another study involving the infestation of BALB/c mice with *I. ricinus* nymphs, lymph-node cells collected 9 days post-infestation had reduced in-vitro proliferative responses to Con A and increased responses to LPS (Ganapamo *et al.*, 1996*a*).

Ixodes ricinus saliva significantly inhibited the Con-A- or PHA-driven proliferation of the normal spleen cells of mice *in vitro*, in a dose-dependent manner (Urioste *et al.*, 1994). This reduction in responsiveness occurred in parallel with a decrease in the IL-2 secretion by the splenocytes exposed to the saliva. Few differences were detected in regard to the Con-A- or LPS-stimulated, in-vitro responses of splenocytes from C3H/HeN mice that were tick-naive or had been infested one to four times with *I. scapularis* (Schoeler *et al.*, 2000*a*). However, antigen-specific proliferative responses to soluble, salivary-gland proteins of

I. scapularis did develop in the mouse lymphocytes during the course of the infestations. Concurrent with the development of these responses was a decrease in expression of the Th1 cytokines, IL-2 and IFN-γ, and an up-regulation of the Th2 cytokines, IL-4 and IL-10 (Schoeler *et al.*, 1999).

Peripheral-blood lymphocytes of cattle infested with *Boo. microplus* larvae had lower proliferative responses to PHA after the second infestation than similar lymphocytes obtained from uninfested cattle (Inokuma *et al.*, 1993). Infestation of dogs with adult *Rhi. sanguineus* reduced the responses of the host's cells to Con A, PHA and pokeweed mitogen (Inokuma *et al.*, 1998); similar to the pattern reported by Wikel (1982*c*), suppression was greater during the first than the second infestation. A *Rhi. sanguineus* SGE also suppressed the mitogen responses of peripheral-blood lymphocytes from uninfested dogs, the suppressive activity being attributed to salivary proteins (Inokuma *et al.*, 1998). Infestation of dogs and mice with adult *Rhi. sanguineus* reduced Con-A-induced T-cell proliferation by as much as 83% (Ferreira and Silva, 1998).

After finding that ticks modulated lymphocyte proliferation, the next logical area to assess was cytokines, since they orchestrate many aspects of the immune response. In addition to suppressing secretion of IL-1β and TNF-α by normal mouse macrophages, *D. andersoni* SGE, prepared from unfed ticks and during the course of engorgement, inhibited secretion of the Th1 cytokines IL-2 and IFN-γ (Ramachandra and Wikel, 1992). Two infestations of BALB/c mice with *D. andersoni* nymphs resulted in reduced tick feeding and diminished secretion of IL-2, but not of IFN-γ, during the second infestation (Macaluso and Wikel, 2001).

Rhipicephalus appendiculatus SGE inhibited the LPS-induced production of mRNA specific for IL-1α, IL-1β, IL-5, IL-6, IL-7, IL-8, IFN-α, and IFN-γ by normal, human, peripheral-blood leucocytes (Fuchsberger *et al.*, 1995). Infestation by *Rhi. sanguineus* or saliva from this tick modulated cytokine secretion by the macrophages and lymphocytes of C3H/HeJ mice (Ferreira and Silva, 1999). Six days after a primary infestation, the Con-A-stimulated lymph-node cells from the mice were reduced in their ability to produce IL-2 (by 37.2%) and IFN-γ (by 67.0%), while IL-10 secretion was increased 6.5-fold and IL-4 secretion remained unchanged. This Th2 polarization was increased after a fourth infestation, including a significant enhancement of IL-4 levels. Exposure of activated macrophages or splenocytes to *Rhi. sanguineus* saliva reduced IL-12 secretion.

Several studies have addressed the ability of *I. scapularis* or *I. ricinus* to modulate T-lymphocyte cytokines. The general pattern of infestation- or tick-saliva-induced changes consists of a down-regulation of Th1 cytokines with a concurrent increase in Th2 cytokines. Clearly, the point during or after infestation at which cytokines are measured makes a difference in the profile observed. Saliva of partially fed *I. scapularis* females inhibited IL-2 secretion by Con-A-activated T-cells (Urioste *et al.*, 1994). Recently, a soluble, IL-2-binding protein has been described in the saliva of *I. scapularis* (Gillespie *et al.*, 2001). On the final day of each of four repeated infestations of BALB/c or C3H/HeN mice with pathogen-free *I. scapularis* nymphs, Con-A-stimulated splenocyte secretion of the Th1 cytokines, IL-2 and IFN-γ, was reduced whereas that of the Th2 cytokines, IL-4 and IL-10, was enhanced (Schoeler *et al.*, 1999). Cytokine polarization to a Th2 profile was even more intense for mice infested with *I. pacificus*, with the exception of IFN-γ (Schoeler *et al.*, 2000*b*).

Infestation of C3H/HeJ mice with *I. scapularis* nymphs infected with *Bor. burgdorferi* resulted, by 10–12 days post-infestation, in an up-regulation of IL-4 and IL-10 and a down-regulation of IL-2 and IFN-γ (Zeidner *et al.*, 1997). Similar infestation of BALB/c mice resulted in only transient changes in IL-2, IFN-γ and IL-4 secretion, but IL-10 levels were elevated (Zeidner *et al.*, 1997). Passive reconstitution of IL-2, IFN-γ and TNF-α provided 95% protection of C3H/HeJ mice against tick-transmitted *Bor. burgdorferi* (Zeidner *et al.*, 1996).

Repeated infestations of BALB/c mice with *I. ricinus* nymphs caused increased secretion of IL-4 and decreased production of IFN-γ by lymph-node cells draining the infestation site (Ganapamo *et al.*, 1995). Production of the IL-4 was linked to the ability of the lymph-node cells to proliferate in response to tick SGE. Lymphocytes from similar sites produced significant levels of IL-2 upon Con-A stimulation, and the levels of IL-2 increased from the first to the third exposure to *I. ricinus* nymphs (Ganapamo *et al.*, 1996*b*). Nine days after a first infestation, Con-A-stimulated lymph-node cells produced IL-10, and at nine days after a third exposure the IL-10 levels were increased along with those of IL-5 (Ganapamo *et al.*, 1996*a*). An SGE of *I. ricinus* up-regulated IL-10 and down-regulated IFN-γ production by normal, BALB/c splenocytes (Kopecky *et al.*, 1999). Addition of anti-IL-10 neutralizing antibodies to splenocyte cultures abolished the suppressive effects on IFN-γ production. Administration of anti-IL-4 and anti-IFN-γ antibodies did not effect tick attachment or engorgement weights (Christi *et al.*, 1998). Interleukin-4 produced after infestation with *I. ricinus* nymphs infected with *Bor. burgdorferi* reduced the IgG_{2a} antibody response to the spirochaetes (Christi *et al.*, 2000). Proteins in *I. ricinus* SGE stimulated enhanced IL-4 production (Mejri *et al.*, 2001).

In light of the profound effects of tick infestation and salivary-gland-derived molecules on T-lymphocyte function, the fact that tick feeding suppresses the antibody responses of the host is not unexpected. Infestation of guinea pigs with adult *D. andersoni* reduced the IgM-attributable plaque-forming cell responses of the hosts after immunization with sheep erythrocytes (Wikel, 1985). Antibody responses returned to normal by the fourth day post-infestation. Rabbits immunized, during infestation with *Rhi. appendiculatus*, with bovine serum albumin and Freund's complete adjuvant developed significantly less intense antibody responses than uninfested controls (Fivaz, 1989). Likewise, *Rhi. sanguineus* infestation of dogs reduced immunization-induced antibody responses even 7 weeks after initial immunization (Inokuma *et al.*, 1997).

Tick infestation modulates a variety of elements of the innate and specific, acquired immune responses. Increasingly, these changes are linked not only to successful blood-feeding but also to pathogen transmission. Reversing these tick-induced changes to host defences could be the potential basis for novel interventions to prevent tick-borne diseases. The emphasis must now be placed on the identification of the molecules, in the salivary glands of ticks, that are responsible for these changes.

WHAT NEXT?

What are the logical approaches to the identification and characterization of the molecules responsible for arthropod modulation of host immune defences? A combination of genomic and proteomic strategies will probably provide the best insights. A powerful approach to identifying the spectrum of genes expressed in the salivary glands of a blood-feeding arthropod involves the expressed sequence tag (EST). An EST is a partial nucleic-acid sequence derived from a cDNA generated from the mRNA present in the tissue of interest (Adams *et al.*, 1991). The EST approach is dependent upon the information in public databases used to compare gene sequences, predicted amino-acid sequences and motifs. The EST database, dbEST, is the fastest growing division of GenBank (Pandey and Lewitter, 1999). A potential pitfall is that very few blood-feeding arthropods have been the focus of EST studies; those organisms that have been studied include *Lu. longipalpis* (Ramalho-Ortigao *et al.*, 2001), *An. gambiae* (Dimopoulos *et al.*, 2000), *Boo. microplus* (Crampton *et al.*, 1998), and *Am. americanum* (Hill and Gutierrez, 2000). As a consequence, genes unique to blood-feeding arthropods might not be encountered in database searches. Other strategies are needed to identify the functions of genes that do not have homologies with sequences in the public databases. Combining

genomics with proteomics provides the way to study gene expression at the protein level (Naaby-Hanseb *et al*., 2001). Saliva can be fractionated by two-dimensional gel electrophoresis and tandem mass spectrometry then used to determine the amino-acid sequence of each protein (which can be compared with translated EST sequences, to identify the encoding gene). In addition, traditional biochemical fractionation methods, combined with appropriate assays for activities of interest, provide an essential, parallel strategy for analysis. Determination of the N-terminal sequence of each isolated and active protein can be used to design primers for use in the PCR-based, rapid amplification of the cDNA ends, to facilitate cloning of full-length cDNA (Das *et al*., 2001). The information gained from these studies will increase our understanding of the complex interactions involved in successful blood-feeding and pathogen transmission. Novel control strategies will almost certainly emerge from this new knowledge.

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