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Tick-host-pathogen systems immunobiology: an interactive trio

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1. ABSTRACT

Significant new insights are being made into tick modulation of host immune defenses and the implications of those host defense changes on tick-borne pathogen transmission and establishment of infection. Understanding tick saliva complexity increased with construction and analyses of salivary gland cDNA libraries. High throughput next generation sequencing and advances in proteomics are revealing greater complexity of saliva, nature of gene families and differential gene expression patterns not previously attainable. Combined use of genome arrays and histopathology are defining cutaneous gene expression during the course of infestation with pathogen-free ticks and during infestations with ticks experimentally infected with a tick-borne pathogen. Large data sets are being generated that are of value to researchers. A major challenge remains in linking saliva molecules with specific functions. Systems biology technologies provide the tools for analyses of complex tick-host-pathogen interactions that are the underpinnings for development of novel control strategies for ticks and tick-borne diseases of medical and veterinary public health importance.

2. INTRODUCTION TO TICKS, HOST INTERACTIONSANDTICK-BORNEDISEASES

Ticks are of vast and increasing medical and veterinary importance due to the array of infectious agents they transmit; direct damage caused to the skin of companion, domestic and wildlife animal species; and, their resulting economic impact (1,2,3,4,5). With regard to taxonomy, the order Ixodida contains 907 valid species in the families Argasidae, 186 valid species; Ixodidae, comprised of 721 species; and, the monotypic Nuttalliellidae (6). Questions about tick taxonomic relationships are an active area of study being addressed using a variety of molecular tools (7).

Argasid and ixodid ticks differ significantly in their life cycles, host interactions and other aspects of their biology (8,9,10,11). Argasid ticks develop through two to eight instars with each requiring a blood meal. Ixodid tick larvae, nymphs and adults must blood feed to progress to the next developmental stage. Life cycle adaptations of ixodid ticks result in blood feeding patterns involving one, two or three hosts, dependent upon the tick species. Ticks are telmophages that use their cutting mouthparts to lacerate dermal blood vessels and created feeding “pools” of blood (12). Argasid chelicerae are more robust for cutting compared to those of ixodids that have a well-developed hypostome to facilitate long term host attachment (13). Many ixodid tick species also produce attachment cement to help hold them to the host over the course of feeding that may last more than a week (14). Attachment cement deposit patterns differ among ixodid genera relative to mouthpart structure (15).

Frequency and durations of blood feeding differ between argasids and ixodids and thus host exposure to tick saliva varies as well. Argasid ticks blood feed for up to approximately two hours (13, 16) with a weight increase of as much as twelve fold. A fully engorged female ixodid tick obtaining a blood meal for possibly longer than a week may increase in weight from 100 to 200 fold (14, 17).

The wound created by insertion of tick mouthparts disrupts the epidermis and enters the dermis, stimulating host defenses of pain/itch, hemostasis, inflammation and initiates immune defenses (18,19,20,21,22). The host is exposed to tick saliva during disruption of the skin and throughout the days of blood feeding from the pool-like lesion created (14,23,24). Tick saliva is deposited directly into the feeding site; percutaneously across the epidermis; and, held in attachment cement (25). Tick-borne infectious agents can also be trapped in attachment cement (26). Since the number of ticks infesting an individual animal at one time can be high (27,28), the amount of exposure to the "foreign" proteins in tick saliva can be a significant antigenic challenge.

Acquired, immune response based, resistance to tick feeding develops naturally for some tick-host associations, including laboratory animal species, companion animals, horses and cattle (19,29,30,31,32,33). Acquired resistance to tick feeding is expressed as reduced engorgement, disrupted molting, reduced ova production and death of the feeding tick. However, many ticks successfully parasitize a wide array of hosts without development of significant resistance to infestation, suggesting an overall low immunogenicity for saliva proteins. Acquired resistance to the bites of pathogen-free ticks induces resistance to transmission of a tick-borne pathogen by subsequent feeding of infected ticks of the same species (34). Understanding the complexity of host responses to tick infestation and tick countermeasures to those defenses is increasing at a rapid rate due to the use of powerful cellular, molecular, genomic, functional genomic and proteomic tools (22,35,36,37,38).

Ticks are efficient vectors of the widest array of human and veterinary infectious agents transmitted by blood feeding arthropods (1,39,40,41). Multiple factors, including climate change, influence the geographic ranges of both tick species and tick-borne infectious diseases (42). Dynamic nature of human tick-borne infections is reported in recent reviews of coinfections with *Ixodes* transmitted pathogens (43); emerging Powassan virus infections (44); identification of infections caused by *Borrelia miyamotoi* (45); and, emergence of Heartland virus, a novel phlebovirus, in the United States (46,47). Crimean Congo hemorrhagic fever is a zoonotic tick-borne infection of increasing importance in domestic and wild animals (48) with a central role for these species in increasing dissemination of infections (49). Emerging and resurging tick-borne infections are significant global threats to animal health requiring novel control strategies (50). Approaching human and veterinary tick-borne diseases from an integrated "one health" perspective is a logical route (51).

3. HOST DEFENSES AND TICK COUNTERMEASURES

Tick saliva is a highly complex mixture that consists of bioactive proteins, lipids and nucleosides (37,38,52,53). Tick saliva modulates, deviates or inhibits multiple cellular and molecular elements of host defenses of pain and itch, hemostasis, inflammation, innate and adaptive immunity, and wound healing (22,35,36). In addition, saliva of some argasid and ixodid species contain paralysis toxins (54). By creating a unique immunoprivileged environment at the bite site, tick saliva enhances transmission of tick-borne viruses and bacteria (20,22,35,55,56,57). Understanding of the complexity of tick saliva protein families, redundant activities and differential gene expression during blood feeding increased during the past five decades as more sensitive analysis methods evolved (21,37,38,52,58,59,60).

Tick saliva mediated countermeasures of host defenses include pain and itch responses; hemostasis by platelet aggregation, vasoconstriction and coagulation; inflammation, innate immunity and adaptive immune defenses; and, wound healing. Itch responses are simulated by diverse mediators acting on appropriate sensory receptors (61). Histamine is an example of a recognized itch mediator, and anti-histamines are effective itch inhibitors (61,62). Multiple ixodid species produce saliva histamine binding proteins (63) that could reduce bite site itch responses as well as inhibit the direct negative impact of histamine on tick feeding (64). *Dermacentor reticulatus* histamine high affinity binding protein is a lipocalin that also possesses a low affinity serotonin binding site (65). Serotonin is also an itch mediator (66). Tick saliva kininases are potential itch inhibitors as well (67,68). Identification of tick saliva molecules that inhibit host itch and pain responses will likely increase in diversity and redundancy within a species as a result of additional proteome characterization and functional analyses.

Hemostasis is a significant threat to successful tick feeding that requires continuous availability of blood in the feeding lesion over a period of several days to more than a week (17). Blood feeding arthropods evolved diverse countermeasures to the hemostasis events of platelet aggregation, vasoconstriction and coagulation (69,70,71). Platelet aggregation inhibitors are present in saliva of multiple tick species (69,71,72,73). Tick saliva contains limited vasodilator activity that is likely limited to prostaglandin E2 and adenosine (52). Multiple tick species evolved saliva inhibitors of coagulation pathway molecules (70) with activated factor ten (Xa) being a common target of inhibition (71,74,75). Together, these numerous saliva factors ensure that ticks can maintain blood flow into the pool-like feeding lesion over the days required for full engorgement.

Deviating, modulating or suppressing host inflammation, innate and adaptive immune responses are important challenges for ticks during the course of a single infestation as well as over the life span of the host for whom multiple infestations will be a distinct possibility, especially for domestic animals and wildlife. Ticks evolved an impressive array of saliva molecules that target nearly all aspects of vertebrate immune defenses and the scope of understanding of tick countermeasures to host immune defenses continues to increase as reported in recent reviews of the subject (22,35,36,76). As discussed in subsequent sections of this manuscript, the scope of discovery of how ticks affect host immune defenses; increased with the application of new technologies including the recent use of genome arrays to characterize tick feeding induced changes in cutaneous immunity (77,78). Likewise, next generation sequencing and proteomics are broadening understanding of number of different molecules, scope of activities, regulation, redundancies and putative roles for tick saliva molecules (37,38,79,80,81). Generation of these large data sets are the foundation for a more global perspective of tick-host interactions that contribute to development of studies linking specific immunomodulatory events with specific saliva molecules.

Tick saliva modulates activities of cellular and molecular mediators of inflammatory and innate immune responses. NK cell target cell binding and killing are suppressed by saliva of several tick species (82,83) that could result in impaired antivirus defenses. Likewise, tick suppression of type I interferon production can contribute to a bite site favorable for establishment of tick-borne virus infection. Innate immune defenses are further reduced by tick saliva modulation of the alternative pathway of complement activation by inhibiting C3b deposition (84,85) and dissociation of the active C3 convertase (86,87,88).

Keratinocyte derived proinflammatory cytokines are suppressed by tick saliva that inhibits Toll-like receptor agonist stimulation (89). This finding is significant due to the observation that during tick feeding saliva is introduced percutaneously around the bite site; directly into the feeding lesion; and, it is trapped in attachment cement that is in contact with the epidermis (25). Tick mediated reduction of proinflammatory cytokines impairs the role of these molecules in activation of endothelium, chemoattraction and activation of neutrophils, monocytes/macrophages, dendritic cells as well as other cell types; orchestrates cytokine and chemokine production by other cells; and, creates an environment that contributes to determining the nature of the subsequent adaptive immune response (90,91). For the interested reader, an excellent textbook for explanation of immune phenomena is Abbas *et al.* (92).

Neutrophils are the first cells attracted to a site of inflammation for destruction of microbes, and they also contribute to tissue repair (93,94,95). Tick saliva down regulates neutrophil phagocytosis (96); inhibits their chemotaxis and activation at tick feeding sites (97); and, reduces the oxidative burst essential for killing phagocytosed microbes (98).

Movement of neutrophils and other leukocytes from intravascular to extravascular sites of inflammation and immune responses depends upon coordinated interactions among proinflammatory cytokine and chemokine activation of endothelium that expresses intercellular adhesion molecules, selectins as well as activation of leukocyte surface integrins, to facilitate adhesion to endothelium and subsequent diapedesis into the extravascular compartment (99). Tick saliva reduces neutrophil β2 integrin expression (100,101) and the lymphocyte integrins, lymphocyte function associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4) (102). Salivary gland extracts of two tick species differentially suppress skin endothelial cell expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM-1), E-selectin, and P-selectin (103). Implications of these leukocyte molecule changes induced by tick saliva factors are an impaired inflammatory response that results in a reduced threat to the feeding tick and development of an environment that is immunologically more favorable for introduction and establishment of tick-borne infectious agents.

Skin dendritic cells are a heterogeneous population of antigen presenting cells that are most effective at presenting processed peptide in the context of major histocompatibility antigen to naïve T lymphocytes in draining lymph nodes and spleen (104). Dendritic cell interactions with T lymphocytes involve multiple sequential receptor-ligand interactions of costimulatory molecules, cytokines and intracellular signaling pathways, resulting in helper and cytotoxic T lymphocyte effectors and memory responses. Tick infestation can reduce dendritic cell numbers at feeding sites (105). Tick saliva impairs dendritic cell differentiation and expression of costimulatory molecules (106); diminishes their chemotactic responses; reduces stimulation of T lymphocytes (107); and, down regulates proinflammatory cytokine expression while increasing anti-inflammatory cytokine production (108). Among the best characterized molecular relationships between tick and host is the action of tick saliva on dendritic cell innate receptors, signal transduction pathways and inflammasome function (109,110,111).

Dendritic cell cytokine production helps orchestrate the nature of the T lymphocyte response that develops upon antigen exposure. Tick infestation is frequently associated with development of helper

T lymphocyte responses with polarization to a Th2 cytokine profile that is accompanied by down regulation of Th1 cytokines (22,36). In addition to suppression of the proinflammatory cytokines TNF, IL-1, IL6 and chemokines, expression of the Th1 polarizing cytokine IL-12 is reduced (108,112,113). *Ixodes ricinus* saliva Th2 polarization suppression of Th1 cytokines is accompanied by an absence of Th17 activation (114). Tick modulation of dendritic cells reduces innate and adaptive immune responses that could be disruptive to successful blood feeding as well as provide an environment that favors establishment of some types of tick-borne infectious agents.

Macrophages are widely distributed throughout tissues where they can act as antigen presenting cells for memory T lymphocytes (115). The three macrophage subpopulations are classically activated M1, regulatory M2 and wound healing macrophages (116). The M1 macrophages produce proinflammatory cytokines that also contribute to Th1 and Th17 polarization of helper T lymphocyte responses (117). Regulatory M2 macrophages are associated with suppression of inflammation by producing IL-10 and transforming growth factor-β (117,118). Tick saliva inhibits macrophage elaboration of the proinflammatory cytokines TNF and IL-1 (119,120) as well as chemokines, including IL-8 (121,122). Tick saliva inhibition of proinflammatory cytokines can occur concurrently with enhanced IL-10 production, resulting in reduced inflammatory response at the bite site (120). Reduced macrophage influx and activation also results from a tick saliva mimic of macrophage migration inhibition factor (123).

The overall modulatory properties of tick saliva on host endothelial cells, neutrophils, dendritic cells and macrophages create an environment where the inflammatory and innate immune response cells and molecular mediators are reduced in their ability to respond to injury and infectious agents. In addition, subsequent helper T lymphocyte responses are deviated to a Th2 profile associated with a less protective response (22,35,36,76). The challenging tasks remaining are identification of the molecules specifically responsible for modulation of these multiple host defenses.

Tick saliva induced effects on T lymphocytes include suppression of proliferation, cytokine elaboration and Th2 polarization (22,35,36,76). Both male and female tick salivary glands contain T lymphocyte specific inhibitors of proliferation (124). *Ixodes scapularis* saliva binds IL-2 that serves as an autocrine and paracrine driver of proliferation of antigen activated T lymphocytes (125). Likewise, production of the helper T lymphocyte effector cytokine INF-γ is inhibited by *Ixodes scapularis* feeding (126). One of the most thoroughly characterized tick saliva proteins is Salp15 that acts upon the T lymphocyte costimulatory molecule CD4 to block proliferation by inhibiting post-antigen engagement calcium influx, signal transduction and IL-2 production (127,128,129). *Ixodes scapularis* sialostatin L inhibits cytotoxic CD8+ T lymphocyte proliferation (130). Suppression of helper CD4+ T lymphocyte derived IL-2 inhibits the ability of cytotoxic T lymphocytes to respond.

Tick induced host Th2 helper T lymphocyte polarization is reported for numerous tick species (22,35,36,76). An *Ixodes scapularis* saliva sphingomyelinase-like molecule is capable of inducing the prototypic CD4+ T lymphocyte Th2 cytokine IL-4 indicative of Th2 polarization (131). Additional Th2 polarizing factors are induced by *Ixodes ricinus* saliva (132).

Tick saliva also directly impacts B lymphocyte function by impairing proliferation and indirectly by reducing T lymphocyte helper signals essential for antibody class switching. Suppression of B lymphocyte proliferation is linked to tick saliva proteins (133,134). Concurrent with Th2 polarization is down regulation of Th1 helper cell responses with suppression of IFN-γ that can potentially reduce essential T lymphocyte help for an antibody class switch from IgM to IgG. Tick infestation suppresses both IgM and IgG antibody responses (135). A Th2 polarization favors production of other immunoglobulin isotypes, specifically IgE. Holstein cattle susceptible to *Rhipicephalus* (*Boophilus*) *microplus* infestation develop increased levels of IgE when compared to a tick resistant breed (136). Tick bite induced anaphylaxis is a recognized medical problem, resulting from bites of selected tick species (137). However, local cutaneous hypersensitivity can be valuable in alerting a host to the presence of a tick and thus stimulating grooming behavior to remove the tick.

4. IDENTIFYING BIOLOGICAL ACTIVITIES OF SALIVA

Pace of research determining the actions of tick saliva increased significantly during the past few decades. Early studies assessed the ability of experimental tick infestations to induce resistance to subsequent tick exposures (138). Subsequently, histological studies provided insight into host defense mechanisms at tick feeding sites on naïve and repeatedly infested laboratory animals, cattle and humans (139,140,141,142,143,144). Results of histological studies informed experiments examining specific immune response elements and their relationship to tick saliva exposure. Identification of specific activities in saliva began by using tick salivary gland extracts prepared at distinct time points during feeding to assess their influence on host immune defenses. These whole salivary gland extract studies were followed by biochemical fractionation of salivary

gland extracts or saliva to isolate and identify specific biological activities (18,20,21,22,23,145,146). These experiments yielded some of the first descriptions of specific activities of salivary gland derived molecules linked to modulation of host defenses. At that point in time, our understanding greatly underestimated the complexity of tick saliva.

5. SALIVARY GLAND EXTRACTS TO ACTIVITIES OF INDIVIDUAL MOLECULES

Biochemical fractionation of tick salivary glands or saliva used to isolate and characterize specific reactive molecule(s) generally requires large numbers of starting material, often thousands of pairs of salivary glands, or considerable volumes of collected saliva; use of fractionation and isolation procedures that ensured retention of activity; and, Edman degradation to obtain N-terminal amino acid sequence of isolated active saliva proteins (86,147,148). Although these approaches seem limiting today compared to currently available technology to identify tick saliva molecules, their use at the time represented significant steps forward in understanding tick-host interactions.

Several examples are provided of biochemical fractionation procedures to identify saliva activities. Wang and Nuttall (147) identified and isolated IgG-binding proteins in salivary gland extracts of *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *Ixodes hexagonus*. In this study, salivary glands were dissected from equal numbers of six day fed male and female ticks followed by affinity column chromatography to isolate IgG-binding saliva protein. Eluted proteins were precipitated and analyzed by SDS-polyacrylamide gel electrophoresis. A Sepharose column served as a negative control. Multiple IgG-binding proteins were identified as to molecular weights in unfed and six day fed salivary gland extracts of *Rhipicephalus appendiculatus* females and males and unfed salivary glands of female and male *Amblyomma variegatum* and *Ixodes hexagonus*.

Alternative complement pathway inhibitor activity is present in *Ixodes scapularis* saliva (84). A combination of biochemical isolation, N-terminal amino acid sequencing, cloning and expression were used to identify the inhibitor that acts by uncoupling factor Bb from the alternative pathway C3 convertase (86). This study represents the combination of identification of a saliva molecule by isolation procedures with random screening of a salivary gland cDNA library, cloning and expression of an active protein for functional analyses.

Female *Ixodes scapularis* engorged for four to five days prior to collection of saliva induced by pilocarpine. This study is an example of the challenging requirements for large amounts of starting material (86). When saliva was subjected to molecular sieving followed by reverse-phase HPLC, the active fraction was considered to be at too low a concentration to provide a reliable N-terminal amino acid sequence. Since both saliva and salivary gland homogenates have alternative pathway inhibitory activity, approximately 500 pairs of salivary glands were homogenized and subjected to reverse-phase chromatography. Active fractions were concentrated and subjected to Edman degradation N-terminal amino acid sequencing. Obtaining a PCR product based upon N-terminal sequence primers failed. A full-length clone of the complement inhibitor was identified by random screening of an *Ixodes scapularis* cDNA library followed by expression of the cDNA in COS cells. Recombinant expressed and native protein both have similar characteristics and alternative pathway inhibitory activities.

A *Dermacentor andersoni* suppressor of lymphocyte proliferation is an example of the progression from observation to identification, isolation and molecular characterization of a bioactive saliva molecule. Lymphocytes isolated from *Dermacentor andersoni* larvae infested guinea pigs have reduced *in vitro* proliferative responses to a T lymphocyte mitogen compared to similar cells from uninfested controls (149). *Dermacentor andersoni* salivary gland extract suppressed normal lymphocyte responsiveness to the T lymphocyte mitogen concanavalin A, demonstrating the saliva link (119). Salivary glands obtained from approximately 1,000 four day fed female *Dermacentor andersoni* were homogenized and subjected to low speed centrifugation to remove larger cellular debris (150). Pellet resulting from low speed centrifugation was treated with octylthioglucoside, a low molecular weight nonionic detergent, followed by a second low speed centrifugation with the resulting supernatant and sodium dodecyl sulfate treated pellet subjected to centrifugation at 105,000 x g. The supernatant obtained from the original low speed centrifugation was subsequently centrifuged at 105,000 x g. Further fractionation was achieved by a series of differential centrifugations, use of defined molecular weight concentrators and fractionation by preparative SDS-PAGE (150). Preparative SDS-PAGE fractionation resolved lymphocyte *in vitro* proliferation suppression activity to a 36 kDa protein whose ability to suppress lymphocyte concanavalin A responsiveness was reduced by immunoprecipitation by a specific antibody to the 36 kDa protein (148). In addition, the N-terminal 27 amino acid sequence was determined (148). A cDNA encoding the 36kDa *Dermacentor andersoni* immunosuppressant protein was isolated by 3' rapid amplification of cDNA ends using a set of nested forward degenerate oligonucleotide primers corresponding to the N-terminal amino acid sequence (124). Immunoblot analyses detected a peak expression of the immunosuppressant at the six day

of engorgement and present in both female and male salivary glands.

These low throughput approaches (38) link a specific tick salivary gland molecule with a defined function effecting host physiology. The same challenges exist for assessing the data derived from high throughput transcriptomic and proteomics strategies, that of linking a specific protein or family of proteins to modulation of a host defense. These efforts require specific selection and use of appropriate bioassays.

6. SALIVARY GLAND TRANSCRIPTOMES TO NEXT GENERATION SEQUENCING AND PROTEOMICS

Development of salivary gland cDNA libraries prepared at different time points during feeding provided major advances for identification of proteins expressed in tick salivary glands. Biologically active tick saliva molecules were identified, cloned, expressed and characterized starting from cDNA library Sanger sequencing, screening, subsequent amplification of the desired open reading frame, cloning, expression, purification and analysis of function (65,124,151,152). Addition of proteomic analyses with random Sanger sequencing of cDNA library clones advanced salivary gland genes discovery and functional analyses revealing in a higher level of salivary gland complexity than previously realized (59,60). Magnitude of complexity of salivary gland gene families, functional diversity, redundancy and differential gene expression are further revealed by next generation RNA or DNA sequencing technologies and high throughput proteomics (79,80, 81). These technological advances are described in an excellent review by Chmelař *et al.* (38). Addition of *Ixodes scapularis* genome sequencing provides another valuable data set for analyses of tick interactions with their hosts (153).

Early transcriptomic and proteomic studies provided the foundation to build upon for subsequent next generation sequencing and use of new tools for identification of tick salivary gland molecules (38). Salivary gland multigene families were discovered in early transcriptome analyses of cDNA libraries (59,60). Next generation sequencing findings expands the richness of the members of salivary gland gene families, resulting in an increased appreciation of the biological functions of these proteins (53,79,154). A 24 hour *Ixodes scapularis* fed female phage display cDNA library was screened with antibodies followed by next generation sequencing and bioinformatics analyses to identify immunogenic saliva proteins (155). From the same research group, over 800 putative immunoproteins were identified in *Amblyomma americanum* salivary gland using a similar immunological screening of a phage display cDNA library combined with Illumina next generation sequencing (156).

Differential expression of genes during tick salivary gland infection with tick-borne pathogens provides valuable insights into saliva molecules that could contribute to successful pathogen transmission and establishment in the host. Examples of infected salivary gland transcriptome analyses are *Rhipicephalus appendiculatus* infected with *Theileria parva* (157); *Ixodes scapularis* infected with *Borrelia burgdorferi* (59); *Rhipicephalus* (*Boophilus*) *microplus* with *Anaplasma marginale* (158); and, *Ixodes ricinus* infected with *Bartonella henselae* (159).

Accumulation of extensive molecular data sets underpins systems biology approaches to achieve genome-wide analyses of specific response networks, such as inflammation, in a variety of complex human diseases (160), veterinary infectious diseases (161) and host-parasite interactions (162,163). Current systems biology approaches depend upon recent major technological advances. High throughput DNA sequencing was significantly advanced by development of strand specific RNA sequencing methodologies (164,165). Proteomics serves as an increasingly powerful tool for global identification and quantification of proteins during different physiological states (166). Transcripts identified can be successfully used to identify proteins in a strategy known as proteomics informed by transcriptomics (167). Next generation deep transcriptome sequencing methodologies combined with liquid chromatography and mass spectrometry proteomic analyses are powerful methodologies successfully applied to the study of salivary gland derived molecules in tick-host-pathogen interactions (37,38,154). The strength of these approaches can define in greater detail, than previously possible, specific gene families and their differential expression during infestation; the relationships between salivary gland proteins and specific tick transmitted infectious agents; and, transcriptomics and proteomics of tick tissues of interest. The ultimate goal still remains to definitively link a specific salivary gland derived molecule with a specific role within the host.

7. BITE SITE GENE EXPRESSION

Saliva composition and temporal changes in molecules expressed that occur during tick feeding interact with an array of cells, receptors and chemical mediators involved in maintaining cutaneous homeostasis as well as responding to injury and infection of the skin (90,91,168). Genome arrays and histopathology reveal very complex tick saliva induced modulation of host defenses and other changes at tick feeding sites on laboratory animals (77) and humans (78). Results demonstrate that tick saliva modulates,

deviates, suppresses or enhances numerous immune defense pathways to create an environment favorable for blood feeding and for pathogen transmission and establishment of host infection. Real time PCR analysis conducted for selected genes of lymph nodes draining sites of injection of *Ixodes scapularis* salivary gland extract and Powassan virus revealed temporal variability post-infection for expression of chemokines, proinflammatory cytokines and anti-inflammatory cytokines (56). Tick transmission models of tick-borne infectious agents provide the next step in determining host skin gene expression patterns at the tick-host interface. Host responses to feeding by *Ixodes scapularis* nymphs, experimentally infected with Powassan virus, were analyzed by pathway specific PCR arrays using bite site extracted RNA isolated at different times after initiation of blood feeding (171). Results were significant upregulation and down regulation of immune response genes at three and six hours after tick attachment.

Host cutaneous gene expression profiling was performed using T helper lymphocyte, wound healing, and signal transduction PCR arrays, during initial and second infestations of mice with pathogen-free *Ixodes scapularis* nymphs (77). Significant differences in gene expression occurred during primary and secondary infestations with 35 genes differing during the first exposure and 138 genes differing during the secondary exposure when compared with uninfested controls. Examples of upregulated genes during a primary infestation include: cytokines, chemokine signaling, NOD-like receptor signaling, inflammatory response, chemotaxis and immune response. Secondary infestation was characterized by upregulation of numerous genes associated with immune cell activation and signaling pathways that would indicated development of acquired immunity as a consequence of a primary infestation that is expanding significantly with initiation of a secondary infestation. These gene expression findings can be correlated with histological comparison of *Ixodes scapularis* nymph bite sites during primary and secondary infestations of mice (144). A very limited inflammatory response was observed surrounding tick mouthparts during a primary infestation; however, similar sites during a secondary infestation were characterized by an intense cellular influx that suggested a strong response to introduced saliva.

Similar microarray analyses combined with histopathology were performed during primary and secondary infestations with pathogen-free *Dermacentor andersoni* nymphs (170). Histologically, a small feeding lesion was developed at 12 hours of an initial infestation accompanied by mild inflammation. The inflammatory cell influx did not increase by 48 hours of the primary infestation, a finding consistent with that reported for *Ixodes scapularis* infestation (144).

Cutaneous gene expression and histological responses were assessed at one, three, six and 12 hours of attachment of pathogen-free *Ixodes scapularis* larvae (169). These time points were selected, since it is reported that *Ixodes scapularis* nymphs transmit Powassan virus within 15 minutes of host attachment (172). Number of cutaneous genes either up regulated or down regulated at one and three hours post-attachment were few compared to the six or twelve hour tissues (169). Up regulated genes at six hours of tick attachment included negative regulators of myloid cell differentiation, which correlates with a subsequent neutrophil influx; B and T lymphocytes and Toll-like receptor signaling; and, anti-microbial response that would reflect innate immunity. At 12 hours of tick feeding, genes associated with inflammation and chemotaxis were up regulated. In addition to an influx of neutrophils, mast cell degranulation was observed at three hours that indicates initiation of an inflammatory response.

Human skin reactions to *Ixodes ricinus* infestation were examined by analyses of mRNA for cytokines, chemokines, leukocyte markers and histologically, using a comparison timeline of less than or more than 24 hours of host attachment (78). Bite sites of less than 24 hours displayed increased transcripts for proinflammatory cytokines as well as neutrophil and macrophage chemoattractants. Histologically, macrophages and dendritic cells were predominant at that time. At greater than 24 hours of tick feeding, the trend was for innate immune response elements to decline and lymphocytes to increase; however, these changes were not found to be statistically significant.

Immune response gene transcripts were also examined in peripheral blood cells of cattle breeds resistant and susceptible to *Rhipicephalus* (*Boophilus*) *microplus* infestation (173). Chemokine expression was altered in response to infestation as might be expected from earlier studies. The most intriguing reported observation was the suggestion that gamma/delta T lymphocyte heightened activity correlated with resistant animals, a finding that requires further investigation.

Next logical step is to add a tick-borne pathogen into the examination of changes occurring at the tick-host interface. Pathway-specific PCR arrays were used to examine gene expression at *Ixodes scapularis* feeding sites by pathogen-free or Powassan virus infected ticks at three and six hours post-attachment (171). Inflammatory response genes were up regulated at three hours while down regulation of host genes was more pronounced at six hours of infestation. Powassan virus transmission from the tick to the host can occur within 15 minutes of initiation of feeding (172). Using the same tick-host-pathogen model, neutrophils and mononuclear cells

were recruited earlier to the virus infected bite site, and virus antigens were observed in macrophages and fibroblasts (174).

Transcriptional profiling of tick bite sites for pathogen-free compared with ticks infected with specific tick-borne infectious agents provides a comprehensive picture of how tick feeding temporally influences the local environment. Similar analyses are valuable when extended to lymph nodes draining the tick bite site. The persistent question that must be addressed is how to link observed specific changes in the host environment with specific saliva molecules introduced into the host.

8. SYSTEMS BIOLOGY OF TICK-HOST-PATHOGEN INTERACTIONS

Systems biology is a powerful approach that can be used to analyze the complex interplay between ticks and host responses at the feeding site at selected time points during the course of infestation. A goal would be to correlate concurrent real time salivary gland transcript and protein expression with changes in skin gene expression and then more systemically at lymph nodes draining the bite site. Once these patterns are defined and correlated temporally, then a tick transmitted infectious agent can be added to assess changes in tick saliva and the host response to both tick and microbe. An example of a tool that could be incorporated to obtain a more focused understanding of gene expression at a skin feeding lesion is laser capture microdissection (175) combined with transcriptome analyses of regions of the dissected tissue sections (176). Tick mouthparts create a pool-like lesion that differs both structurally and in cell composition from center to periphery as well as during primary and subsequent infestations (144). Laser capture microdissection could be used to obtain sections across tick feeding lesions to compare gene expression in different regions, resulting in a more refined picture of cellular and molecular interactions within bite site microenvironments.

Large data sets are being obtained for salivary gland transcriptomes and proteomes and for host skin gene expression at bite sites of uninfected and infectious agent transmitting ticks. As stated earlier, linking specific saliva molecules with defined changes in the host remains a major objective. That goal has been achieved for a number of examples of tick modulation of host immune defenses by utilizing various experimental strategies, some of which are described in this manuscript. Next generation sequencing is providing far more detailed data on gene families and temporal expression of individual transcripts.

Cloning and expression of individual gene products to determine biological roles is time consuming and labor intensive, even when strong indications of function are provided by bioinformatics analyses. An alternative screening approach that could be useful was employed to describe a Th2 polarizing saliva molecule in the mosquito, *Aedes aegypti* (177). Selected salivary gland cDNAs were cloned into a DNA vaccine vector. To assess the salivary gland protein of interest, DNA vaccine vectors were injected into normal skin of an appropriate host species and real-time RT-PCR was used to assess cytokine gene expression in the skin. A similar approach might prove useful when screening tick saliva genes hypothesized to be linked with a specific biological function.

9. CONCLUSIONS

Significant advances have been achieved during the past 50 years in understanding the relationships between tick saliva, modulation of host defenses, acquisition of resistance to tick feeding as well as transmission and establishment of tick-borne infectious agents. These advances in large part are linked to the development of increasingly powerful tools for study of the cellular and molecular interactions, mediators and signaling pathways. The current development of large data sets resulting from next generation sequencing, proteomics and transcriptomics at tick bite sites increasingly provide the tools for the application of systems analyses to achieve remarkable insights not previously obtainable into tick-host-pathogen interactions. The resulting knowledge will lead to new strategies for tick and tick-borne pathogen control and potentially new biological therapeutics based upon tick saliva molecules. This is indeed an exciting field of endeavor with great potential for students and investigators to make meaningful contributions to human and veterinary medicine and public health.

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