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Molecular characterization and related aspects of the innate immune response in ticks

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1. ABSTRACT

Compared to insects, little is known about innate immunity in ticks. This chapter addresses the molecular processes that recognize non-self and the cellular and molecular processes mobilized to phagocytose, engulf, inhibit or kill invaders. We discuss the receptors that recognize pathogen associated molecular patterns (PAMPs) and the putative up-regulation of regulatory cascades that lead, ultimately, to cellular or molecular responses. We describe the molecular events that activate the cellular processes and the array of humoral factors that are mobilized against invading organisms, including antimicrobial peptides, proteases and protease inhibitors, lectins, coagulation factors and others. Special attention is directed to the antimicrobial activity of the midgut, the initial site of contact for microbes ingested with the blood. Blood feeding and digestion alone up-regulates an impressive array of proteins, e.g. oxidative stress reducing proteins, lectins, protease inhibitors, proteases, hydrolases, protein/lipid binding agents. Finally, we compare the innate immune responses of ticks with insects and other invertebrates and note deficiencies in our knowledge tick innate immunity.

2. INTRODUCTION

The evolution of animals from the simplest single-celled organisms to complex metazoans, including humans, would not have been possible without the development of effective defenses against microbial invasion. This ability to defend against infection by pathogenic organisms is termed immunity. An efficient immune system which enables the animal to recognize and eliminate, or at least control invading pathogens, obviously has high adaptive value. The most ancient and widespread form of this defense is the innate immune system which recognizes and responds to conserved features of invading organisms so as to inhibit or destroy them. This system is present in some form or other in all eukaryotes, while acquired immunity (also known as adaptive immunity) is a specialized type found only in vertebrates. The innate immune system uses the panoply of genes that encode intracellular signaling pathways leading from the cell surface to the activation of either the Toll NF-Kappa-B or immune deficiency (Imd) pathways; these are believed to be the central signaling pathways responsible for activation

of the genes that transcribe the effector molecules of the system (1). The well defined host defense system of *Drosophila melanogaster* has recently been reviewed (2). In contrast, this current review emphasizes the less well studied response in ticks.

Ticks have a well developed innate immune system. Nevertheless, despite their ability to resist infection by many microorganisms, they remain important vectors of numerous disease-causing agents. Indeed, ticks surpass the mosquitoes and other blood feeding insects in the variety of different pathogens that they transmit. Ticks have been reported to transmit a wide variety of infectious microbes including bacterial, viral, fungal, even helminth and protozoan pathogens (3).

Microbial and other foreign organisms may enter the tick's body by accidental puncture of the integument or by ingestion during blood feeding. When challenged by such agents, ticks, like insects and other invertebrates, utilize the innate immune system to control or prevent infection. The nature of the innate immune response involves two major components: cellular defenses, namely phagocytosis and encapsulation (or nodule formation) and humoral responses, involving the secretion of transient antimicrobial polypeptides, expressed by the hemocytes, fat body, midgut and in some instances by other internal body organs and tissues. Other peptides, not exclusively antimicrobial, such as lysozyme, lectins and protease inhibitors, are also up-regulated and secreted in response to pathogen challenge (4, 5). Injury (even sterile injury) as well as invading pathogens can induce a proteolytic cascade that also contributes to immune defense, although it must be regulated to limit damage to the body tissues (4). The midgut of ticks and other arthropods presents a special case where feeding up-regulates expression of proteases, cysteine and serine protease inhibitors (cystatins and serpins), lectins, Glutathione S-transferases (GSTs), antimicrobial peptides as well as peroxiredoxins and other oxidative stress reducing proteins which may inhibit or destroy ingested microbes (5, 6).

Progress in our understanding of insect immunity has accelerated rapidly in recent years, facilitated to no small degree by the sequencing of the genomes of several important insect species, namely the malaria mosquito, *Anopheles gambiae* and the fruit fly, *D. melanogaster*. Several excellent reviews have been published on this subject (2, 7). In addition, expressed sequence tag (EST) libraries of specific insect organs have also been published, with the data available in GenBank. This provides opportunities for data mining and characterization of full length molecules from these insect sources as well as the identification of tick homologues. New and suspected candidate genes involved in insect innate immunity have been rapidly identified using high throughput methodologies such as microarrays by which their roles in different tissues, physiological conditions or the insect life cycle have been assessed (8, 9). Proteomics is also being employed to an increasing degree to determine whether functional involvement of insect immune genes actually results in the expression of relevant immune peptides and proteins. Increases in mRNA levels do not necessarily correlate with detectable levels of the cognate proteins. In many cases, the activation of the encoded proteins also depends upon post-translational modifications, e.g. cleavage of a precursor sequence to liberate the mature, active peptide (6).

In contrast to these well studied insects, much less is known about the innate immune system of ticks. Early studies emphasized the role of hemocytes and cellular immunity in the tick's defense against infection. This has changed in recent years since numerous studies have identified the sequences of a large number of tick immune peptides and proteins.

This chapter reviews the molecular basis of the innate immune system in ticks. In the sections that follow, cellular and humoral immune mechanisms will be addressed, including the little that is known about their regulation. Unlike the knowledge available for insects, in particular the host defense system of *D. melanogaster*, we do not have a complete picture of the response or its regulation in ticks.

One aspect that is not discussed in this review is the remarkable ability that pathogenic microbes use to evade or disable the tick's armamentarium of immune responses and mechanisms for transmission to vertebrate hosts. This is an area in which additional knowledge is needed before we can directly relate a microorganism's fight for survival to the tick innate immune response.

3. MOLECULAR BASIS FOR RECOGNITION OF INVASIVE AGENTS

The ability of eukaryotic animals to prevent microbial invasion of body tissues is an essential element in their evolution from the simplest to the most complex species. To do this, the immune system must be able to distinguish self from non-self molecules. Non-self molecules or objects such as components characteristic of bacterial cell surfaces (e.g. peptidoglycans or lipoteichoic acid) stimulate up-regulation of antimicrobial peptides such as defensins, cercropins, attacins and lysozyme that disrupt the cell wall structure, leading to cell death. Other non-self sequences, such as 2-keto-3-deoxyoctonate characteristic of the lipopolysaccharides (LPS) of gram-negative bacteria or the beta-1-3-glucans and beta-1-3 mannans on the cell walls of fungi can result in up-regulation of soluble lectins. Lectins then bind to the target organisms which leads to their aggregation and subsequent walling off by accumulating hemocytes. The cell wall components and foreign molecular structures that induce these responses are known, collectively, as pathogen-associated molecular patterns (PAMPs) because of their ability to trigger various innate immune responses (10). PAMPs are regarded as opsonins because of their ability to trigger immune responses such as phagocytosis, nodule formation, melanization and encapsulation. The molecules are recognized by a highly conserved set of germline encoded pattern recognition receptors (PPRs), which activate the Toll-like and/or Imd pathways of the host cells especially

phagocytic hemocytes and the digestive cells lining the lumen of the midgut (11). Among the most important receptors are the peptidoglycan recognition proteins (PGRPs) which contain a specific domain homologous to bacterial type 2 amidases. Exposure to bacteria activates the Toll signal transduction pathway leading to secretion of antimicrobial peptides, phagocytosis, or other immune responses (12). LPS binds to an unidentified receptor, stimulating the Imd pathway. In insects and horseshoe crabs, up-regulation of PGRPs also activates the prophenoloxidase (proPO) cascade which stimulates localized wound healing, melanization and microbial phagocytosis (13). Recognition of LPS also activates the focal adhesion kinase (FAK)/Src complex in the hemocytes and phagocytosis (14). Once activated, the Toll and Imd pathways possess proteolytic cascades that result in transcription of I-Kappa-B/NF-Kappa-B driven immune response genes (15, 16).

4. EPITHELIAL IMMUNITY

Many eukaryotic cells express proteins or peptides on the cell surfaces that prevent microbes from invading the cell. In the blood sucking fly, *Stomoxys calcitrans,* a 42 amino acid-peptide is expressed on midgut epithelial cells that is able to kill the sleeping sickness trypanosome, *Trypanosoma brucei.* The hemolymph extracts also exhibited a broad spectrum of activity against a variety of other microorganisms (17). Lipid binding proteins occur on the midgut epithelial cells of several tick species. The best known is the glycoprotein Bm86, an important target for anti-tick vaccine production. Bm86 and similar proteins in other ixodid ticks (e.g. H186 in *Haemaphysalis longicornis*) could function in pathogen-gut interactions (18). Lectins also occur on the surfaces as well as within the cells of several organs, e.g. the epithelial cells lining the midgut in insects and ticks. An example is seen in the sandfly, *Lutzomyia longipalpis,* where lectins were found in cytoplasmic secretory granules and microvilli along the length of the midgut (19). Lectins have been reported from several species of ticks and play an important role in tick innate immunity. Some are secreted while others occur in the epithelial cells, particularly in the midgut (20). Lectins are discussed further in sections 6.4 and 9.3.

Hemocytes are also important in wound healing. In *I. ricinus,* hemocytes reacted in response to a 25 kDa antigenic protein that is involved in cuticle formation, indicating a capability for sealing off surface injuries (21). In insects, hemolymph coagulation localizes immune effectors in the vicinity of a breach of the cuticle and also restricts the spread of invasive particles throughout the hemocoel (22).

5. HEMOCYTES: MOLECULAR BASIS OF CELLULAR IMMUNE RESPONSE

In ticks, like in insects and other invertebrates, the body tissues are bathed in a fluid known as the hemolymph. Hemolymph consists of watery, protein-rich plasma and hemocytes, both of which play an important role in immune defense.

5.1. Hemocyte cell classification

Four major cell types are generally recognized in tick hemolymph, namely prohemocytes, non-granular plasmatocytes, granulocytes and spherulocytes (23). Granulocytes are further subdivided into type I and type II granulocytes. Some earlier authors also reported the presence of oenocytoids, a cell type that also occurs in insect hemolymph (10), but their occurrence in ticks remains controversial (24). Prohemocytes are small cells (6 - 7 um long) with little cytoplasm around their nuclei, and no granules. These are the stem cells from which all other hemocytes differentiate and, as might be expected, represent a very small proportion of the total population. Plasmatocytes are slightly larger (8 – 12 um long) elongated, often fusiform cells and readily recognized by their long filamentous cytoplasmic extensions (filopodia). Granulocytes are larger cells (15 – 20 um long) and characterized by the presence of numerous intracellular granules; filopodia may also occur. Type I granulocytes have a mix of electron dense and electron lucent granules with a matrix of fibrillar material, all of varying sizes. Type II granulocytes are similar but contain only small electron dense granules. Spherulocytes are small to medium size (8 – 14 um long) oval or suboval cells with large, fibril-filled granules.

5.2. Hemocyte responses to infection

Hemocytes play an important role in the tick's defense against injury as well as microbial infection. Hemolymph coagulates at the site of injury, walling it off and preventing microbes spreading into the body tissues. When exposed to bacteria, viruses, protozoa or other microbes, the hemocytes respond to control the infection. Hemocyte populations increase greatly over a period of several days or until the invading microbes have been eliminated. In the hard tick, *Dermacentor variabilis*, hemocyte populations were reported to increase from a mean of 1,006 ± 441 cells/ul to 6,077 ± 1,596 cells/ul within 48 hours when challenged by injection of the spore-forming bacteria *Bacillus subtilis* (25). This represents approximately a 6.4 fold increase within this brief time period. When challenged with the spirochete *Borrelia burgdorferi* a more rapid response occurred, with a 3.1 fold increase in numbers 1 hour after challenge, but cell numbers declined to normal levels within 24 hours as the spirochetes were lysed and cleared from the system (26). A similar pattern of hemocyte population increase was found in bacteria-challenged black-legged ticks, *Ixodes scapularis*, although to a much lesser degree, with only a 2.6-fold increase in the hemocyte population at one hour (27). Most of the increase in hemocyte abundance may be due to increases in the population of plasmatocytes and granulocytes as seen in *O. moubata* (28). In *O. moubata* nymphs undergoing ecdysis, granulocytes constituted the majority (80%) of the increase in hemocytes (29). However, despite extensive study of the different cell types, the tissue site and regulation of hemocyte blastogenesis remains to be discovered. Similarly, rates of multiplication for the different cell types in response to a specific pathogen have not been fully clarified.

In *O. moubata* plasmatocytes and type I granulocytes destroy small invading microbes such as

bacterial cells and viral particles by phagocytosis, either by engulfing and ingesting them or by forming aggregates around them (28). When bacteria such as the Lyme disease spirochete *B. burgdorferi* are injected into *D. variabilis*, a non-competent vector, they are lysed by antimicrobial peptides (Section 6) and the fragments or even intact spirochetes ingested by phagocytes (30). In *B. burgdorferi* infected black-legged nymphal ticks, only 5.4 percent of the spirochetes in the midgut are found in the hemolymph. Most of these spirochetes are then phagocytosed such that only a very small percentage (0.7 percent) of the thousands of spirochetes in the midgut ever reach the salivary glands (31). Currently it is not understood how ticks deal with typical gram-positive bacterial pathogens such as *Staphylococcus aureus*. It would seem most likely these organisms would be killed primarily by lysis and phagocytosis. In contrast, control of gram-negative bacteria such as *Escherischia coli* appears to be by nodulation, a form of non-melanotic encapsulation (32). These cellular responses are described below.

5.3. Phagocytosis

Phagocytosis is a complex, multi-step process similar to receptor-mediated endocytsosis. Typically, it begins with recognition (Section 3) and binding of the foreign object to the plasmatocytes or granulocyte cell surface receptor, followed subsequently by signal transduction and extrusion of pseudopods that surround and engulf the bound particle (33).

As noted previously (Section 3), in insects the most important signal transduction pathways are the FAK/Src and mitogen activated protein kinase (MAPK) pathways which activate the proPO cascade (33). In addition, factors external to the hemocytes have been reported to enhance the process. Among these is ecdysone, which was shown recently to enhance phagocytic clearing of yeast cells (*Saccharomyces cerevisiae*) in kissing bugs (34). Another important signaling molecule is calreticulin which is up-regulated in response to particle binding and pattern recognition (35). This protein is found in a number of tick species and has a variety of biological functions in addition to a role in innate immunity (36). The trapped particle is internalized by endocytosis into a vesicle that may in turn fuse with lysosomes, forming a phagolysosome where digestive enzymes, especially acid phosphatases and lysozyme complete the killing process. Once inside the phagolysosome, a cascade of intracellular enzymes is activated, leading to the rapid death and digestion of the trapped microbe or particle. Recent evidence also implicates the use of reactive oxygen species (ROS) in the hemocytes of *Rhipicephalus (*previously *Boophilus) microplus,* and this is thought to involve an oxidative burst modulated by protein kinase C, in a manner similar to that found in mammalian leucocytes (37).

Much less is known about the surface receptors on the hemocytes of ticks. Tick hemocytes recognize and phagocytose both gram positive bacteria and spirochetes, e.g*., B. burgdorferi.* Most likely, this is accomplished by the presence of PGRPs on the surface of the cells. In contrast to insects, recognition of LPS on the surface of gram-negative bacteria leads to microbial control by nodulation rather than phagocytosis, as described below (Section 5.4). Small (1 – 2 um) inanimate objects such as fluorescent-coated beads are also recognized and ingested by phagocytes patrolling the tissues, even though they are not coated with PAMPs (28). In studies done in our laboratory, fluorescent-labeled beads administered to *D. variabilis* females by capillary oral feeding were observed to pass from the midgut into the hemolymph where they were phagocytosed by granulocytes (Sonenshine *et al* unpublished). Little else is known about the molecular factors that initiate and control phagocytosis by tick hemocytes.

5.4. Nodulation

In insects, nodule formation is a major, yet poorly understood process mediated by eicosanoids, prostaglandins and lipoxygenase-derived products (38). Recently, a novel protein, known as Nodular, was found to be up-regulated following bacterial challenge and shown to be involved in nodulation in a silkmoth (*Antheraea mylitta*) (39). This protein was shown to bind microorganisms and their ligands. In ticks, hemocytes recognize components of the surfaces of bacteria (such as LPS) and respond by nodulation. In this process, if it is similar to that described in the silkworm, the first steps would involve hemocyte aggregation resulting in entrapment of the bacteria (39). Opsonizing molecules such as lectins may also cause the bacteria to aggregate. A lectin with high hemagglutinating activity, Dorin-M, was found in the hemolymph plasma of the soft tick *O. moubata* (40). Dorin-M was isolated from hemocytes of *O. moubata,* while a similar molecule Ixoderin A was found in the hemocytes and midgut of *I. ricinus* (20). Lectin recognition leads to recruitment of hemocytes that form a thick mass around the bacterial aggregate, thereby walling it off and eventually digesting it. This process, termed nodulation, is similar to melanotic encapsulation observed in insects, but without melanin (32).

5.5. Encapsulation

Pathogens (parasitoids and nematodes) that are too large to be destroyed by phagocytosis or nodulation may be eliminated by encapsulation. Encapsulation in insects is broadly similar to nodulation but with noteworthy differences, in particular the presence of melanin. The melanization and production of toxic free radicals, such as quinines or semiquinones, and asphyxiation result in destruction of the parasite (41). In the typical encapsulation response, hemocytes accumulate around the parasite, foreign object, or clump of microbes and become organized in concentric layers. In insects, the process also includes melanin. Initially, granulocytes degranulate and deposit a matrix-like material around the particles. Subsequently, plasmatocytes attach to the matrix, undergo apoptosis, become highly flattened and form a thick layer encapsulating the particle. An example of this process was described by Eggenberger *et al.* who injected plastic particles into the hemocoel of *D. variabilis*, and observed a sequence of encapsulation events as described previously in insects but without melanization (42). Similar results were reported with bacterial aggregates without the hemocytes

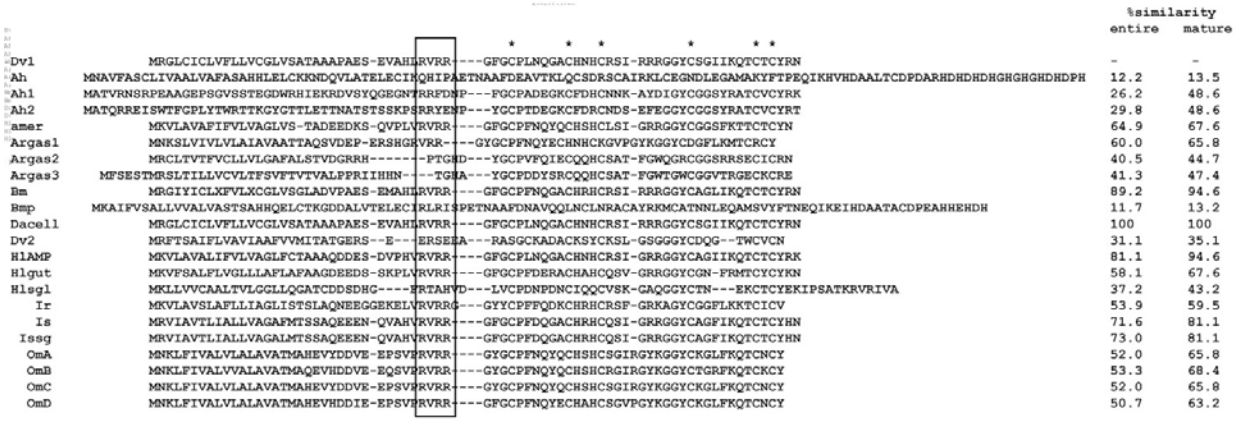

Figure 1. Amino acid sequence alignment (cds-region) of defensins or defensin-like peptides reported from ixodid and argasid ticks deposited in GenBank. Percent similarity to *D. variabilis varisin* of entire peptide and mature peptide region (after cleavage site) is shown. The * indicates position of the highly conserved cysteine residues while the boxed region indicates the RVRR cleavage site. GenBank accession numbers are given for each sequence (scientific names in brackets): Dv1 = (*Dermacentor variabilis,* varisin) AY181027; Ah = (*Amblyomma hebraeum,* hebraein) AY437139; Ah1 = (*A. hebraeum*) AY437137; Ah 2 = (*A. hebraeum*) AY437138; Amer = (*Amblyomma americanum,* americin) DQ864986; Argas1 = (*Argas monolakensis*) DQ886900; Argas2 = (*Argas monolakensis*) DQ886769; Argas3 = (*Argas monolakensis*) DQ886902; Bm = (*Rhipicephalus (Boophilus) microplus)* AY233213; Bmp = *(Rhipicephalus microplus,* microplusin*)* AY233212 ; (Dacell = (*Dermacentor andersoni*) EF060192; Dv2 = (*D. variabilis*) AY159879; HlAMP (*Haemaphysalis longicornis*) = AB105544; Hlgut = (*Haemaphysalis longicornis* gut) EF432731; Hlsgl = (*Haemaphysalis longicornis* sal.gland) EF432732; Ir = (Ixodes ricinus) EF067917; Is = (*I. scapularis,* scapularisin) AY660970; Issg = (*I. scapularis* salivary glands) AY775825; OmA = (*Ornithodoros moubata*) BAB41028; OmB = (*O. moubata*) BAB41027; OmC = (*O. moubata*) BAC22074; OmD = (*O. moubata*) BAC22073.

forming the circular layers seen in encapusulation processes (32).

6. HUMORAL FACTORS: MOLECULAR BASIS OF HUMORAL IMMUNE RESPONSE

In invertebrates, invasion of the animal's body tissues by foreign organisms induces or up-regulate expression of molecules that act directly on the invader. Those that are secreted into the hemolymph plasma are termed humoral factors. Included are a variety of antimicrobial peptides, lectins, lysozymes, coagulation factors, proteases and protease inhibitors.

6.1. Antimicrobial peptides

Antimicrobial peptides (AMPs) in the hemolymph include a variety of relatively small (ca 4 - 20 kDa) molecules that bind to the cell wall or cell membrane of the invading microbe and disrupt its structure and/or membrane potential, eventually killing the target cell. Antimicrobial peptides are generally fast acting agents and effective at micro- or even nanomolar concentrations.

In insects, a remarkable array of antimicrobial peptides have been described, with additional ones being added to this list every year. The fat body is considered to be the primary site for biosynthesis of these defensive compounds, especially defensins (43). The hemocytes and the midgut also serve as important sites of AMP expression and secretion (10). Insect AMPs include defensins and cercropins that attack Gram-positive bacteria or both Gram-positive and Gram-negative bacteria, attacins and sarcotoxins that are bacteriostatic against many species of gram-negative bacteria, and a variety of proline- or glycine-rich AMPs that are produced by a few insect species or specific families (10).

To date, far less is known about the AMPs of ticks compared to insects. At present, the only known humoral AMPs described from ticks are defensins, lysozymes, lectins, proteases and protease inhibitors.

6.1.1. Defensins

The best known AMPs in ticks are the defensins, a family of relatively small (3 - 6 kDa) cysteine-rich cationic peptides. The first evidence of tick defensins was found in the hard tick, *D. variabilis* and the soft tick, O. *moubata* (44, 45). In ticks, like in insects, most defensins are 3- to 6-kDa, arginine-rich, cationic beta-sheet peptides that contain 6 – 8 cysteine residues that are folded in a precise manner by 3 or 4 disulfide bridges. The disulfide bonds stabilize the molecule and maintain the tertiary structure known generically as the "defensin fold" (46). Comparison of the defensin sequences with defensins from insects and other invertebrates indicates that the mature peptides are highly conserved, while the leader regions show much more variation (Figure 1). Their spatially separated hydrophobic and charged regions enable them to insert into the bacterial membrane and cause voltage dependent channels or pores to form, gradually disrupting the membrane and killing the cell (47). At least 20 different defensins have been identified in ticks from 11 different ixodid and argasid species (Table 1). Most are small cationic molecules similar to the insect group of defensins. They range from 67 – 92 amino acids in length, including the prepro-region which is cleaved when the

Table 1. Summary of tick defensins and their characteristics

Tick species	Name	Length (aminoacids)		Primary Tissue Source (s)	GenBank Accession No.	Refernce
		Prepro defensin	Mature Peptide			
A. hebraeum	Defensin Ah1	84	61	Hemolymph	AY437137	(58)
A. hebraeum	Defensin Ah2	84	61	Hemolymph	AY437138	(58)
A. hebraeum	Hebraein	123	102	Hemolymph	AY437139	(59)
A. americanum	Americin	72	37	Hemolymph	ABI74752	(57)
A. monolakensis	Defensin	77	49	Salivary glands	DQ886769	(129)
A. monolakensis	Defensin	72	37	Salivary Glands	DQ886900	(129)
A. monolakensis	Defensin	68	50	Salivary Glands	DQ886902	(129)
R. (Boophilus) microplus	Preprodefensin	97	38	Hemocytes	AY233213	(130)
R. microplus	Microplusin	110	90	Hemolymph	AY233212	(130)
D. andersoni	Defensin	74	38	Cultured cells	EF060192	(65)
D. variabilis	Varisin	74	38	Hemolymph	AY181027	(131)
D. variabilis	Defensin 2	67	33	Midgut	AY159879	(52)
H. longicornis	Preprodefensin	73	37	Midgut	EF432731	(93)
H. longicornis	Antimicrobial peptide	74	38	-----	AB105544	(132)
H. longicornis	Preprodefensin	81		Salivary glands	EF432732	(93)
I. ricinus	Preprodefensin	76	37		AY335442	(110)
I. scapularis	Scapularisin	74	38	Hemocytes	AY660970	(55)
I. scapularis	Defensin	74	38	Salivary glands	AY775825	Alarcon-Chaidez *et al*. unpublishe
O. moubata	Defensin A	73	37	Midgut	BAB41028	(45)
O. moubata	Defensin B	73	37	Midgut	BAB41027	(45)
O. moubata	Defensin C	73	37	Midgut	BAC22074	(50)
O. moubata	Defensin D	73	37	Fat body	BAC22073	(50)

mature peptide is secreted. Most have a signal peptide with the sequence "RVRR" between the prepro- and mature regions of the peptide (Figure 1). Mature peptides range from 37 – 61 amino acids in length. Several species have multiple isoforms, with differential expression seen in different tissues. Analysis of an amino acid alignment of different defensins (full length protein sequences) shows that most align very closely, especially in the mature region. The exceptions are the three defensins from the hard tick *Amblyomma hebraeum*, one from the salivary glands of the hard tick *Haemaphysalis longicornis*, and microplusin from *R. microplus* (Figure 1).

In *D. variabilis,* defensin appears to be up-regulated in response to challenge with *B. burgdorferi* or *Bacillus subtilis,* leading to lysis of these microbes. Defensin alone is effective after prolonged incubation, however, when combined with chicken lysozyme, 65 percent of cultured *B. burgdorferi* are killed within 1 hour (44). This indicates a possible synergism between these two molecules.

In the soft tick, *O. moubata,* 4 different isoforms, A – D, have been identified. Injection of bacteria or bacterial wall components (peptdioglycan or lipotechoic acid) directly into the hemocoel of these argasid ticks up-regulates defensin expression in the granulocytes as determined by semiquantitative RT-PCR (48). Using an ELISA assay defensin-like materials appear to increase approximately 2-fold after bacterial challenge. All 4 defensin isoforms are expressed, but defensin A, B and C are more strongly expressed in the midgut than in the fat body, indicating that contact with the outer membrane of the midgut was stimulatory. Defensin D is more strongly expressed in the fat body (49). Defensin C and D isoforms are strongly up-regulated in the midgut after blood feeding and are believed to play an important role in midgut immunity (50) (see below section 9.2). This supports the idea that defensins are differentially regulated in various tissues in response to various stimuli. Whether different receptors and signaling pathways are involved needs to be investigated.

In *D. variabilis,* the major defensin of hemolymph (known as varisin) is produced primarily in the hemocytes, but transcript for varisin is also found in the fat body and midgut upon microbial challenge or blood feeding (51-53). In addition, transcript is prevalent in all life stages, including the embryonated egg. In this tick, as in mussels, it appears that injection of bacteria such as *B. burgdorferi* results in rapid release of stored defensin peptide from the hemocytes, contributing to early clearing of spirochetes from circulating hemolymph (54). *B. burgdorferi* could not be cultured from hemolymph recovered from the injected ticks, even as early as one hour after injection. Preliminary evidence indicated that injection also stimulated up-regulation of defensin transcript and eventual production of new defensin peptide to replenish the depleted stores (51). A second defensin isoform, defensin 2, was described recently from the midgut (Section 9.2) (52).

In the blacklegged tick, *I. scapularis,* a defensin, scapularisin, was identified with 78.9 percent similarity to the mature defensin from *D. variabilis.* The same isoform of the gene was found in the hemolymph, midgut and fat body. However, no evidence of defensin peptide could be demonstrated in ticks injected with *B. burgdorferi (*55).

Intact spirochetes remained viable (as demonstrated by culture) for up to 24 hours post-injection. In addition, spirochetes incubated in the presence of *I. scapularis* hemolymph remained viable and active (30). Thus, although defensin transcript is present in this species, the peptide does not appear to contribute to immune defense. The reason for this unusual finding is unclear. It is not known whether defensin peptide is translated or, if translated, perhaps it is not cleaved and secreted as the mature, active peptide (55). In the mosquito it has been suggested that defensin may have an alternative role other than bacterial killing (56). This is in part because even though transcript levels increase, peptide levels do not follow. The authors also suggest the occurrence of multiple levels of regulation from induction to defensin peptide production. Such regulation may also explain the results observed in some ticks where no mature defensin peptide is seen.

In the lone star tick, *A. americanum,* the transcript for a single isoform of a defensin, americin*,* was found in hemocytes, midgut, fat body and salivary glands. In contrast to the defensins from the bont tick, *A. hebraeum,* which had only 44.7 percent and 42.1 percent amino acid similarity, the americin mature peptide has 73.7 percent and 71.1 percent amino acid similarity with the mature peptides from *I. scapularis* and *D. variabilis,* respectively. Similarity with 11 other tick defensins ranged from 42 to 71 percent (57).

Most tick defensins are small cationic peptides. However, unusual defensins have also been reported. Two novel non-cationic defensins were identified in a cDNA library of the synganglion of the hard tick *A. hebraeum (*58). The two defensins each have 92 amino acid residues in the preprotein with a predicted 41 amino acid mature peptide. Despite some novel features, they show similarity to defensins from other ticks, in particular the alignment of the cysteine residues (Figure 1). *A. hebraeum* defensin peptide 2 was isolated from fed female hemolymph and shown to be active against gram-negative as well as gram-positive bacteria (58). Another even more remarkable antimicrobial peptide is hebraein*,* a novel 11 kDa molecule purified from the hemolymph of the same hard tick, *A. hebraeum.* Hebraein was found to have multiple histidine residues as well as the 6 cysteine residues characteristic of most arthropod defensins. Characterization of the gene and peptide indicated that it is translated as a 123 amino acid pre-protein which is cleaved to a mature peptide of 102 amino acids. It is anionic and has an all alpha-helical structure instead of the typical beta-sheet structure found in most arthropod defensins. Comparison of its sequence with known proteins indicated little similarity with other defensins. However, it did show similarity to microplusin, an antimicrobial protein produced by *R. microplus*, with both proteins having the same cysteine motif (59). Like the other anionic antimicrobial peptides from this species, hebraein was active against both gram-negative and gram-positive bacteria. Protein profiling of tick hemolymph suggested that hebraein is up-regulated by blood feeding (59).

Another novel defensin is longicin, an antimicrobial peptide from the tick, *H. longicornis* (60). This AMP is similar to the small cationic defensins from other arthropods, including most ticks, with a characteristic beta-sheet at the C-terminus, as well as the typical 6 cysteine residues. However, the most unusual feature of longicin is its ability to inhibit eukaryotic pathogens, especially *Babesia* species and malaria-like parasites of cattle and other mammals. This was the first report of a tick defensin active against eukaryotic parasites (60). It is discussed further in section 9.2.

In the defensin genes of the soft tick, *O. moubata,* introns appear frequently. All four *O. moubata* defensin genes have four exons and three introns, similar to the gene pattern seen in some other invertebrates such as mussels, but not in insects. Most of the ixodid defensins described to date (*D. variabilis, I. scapularis* or *A. americanum*) lacked introns (55, 57). However, a defensin with two introns separating the three exons was reported in the European tick, *Ixodes ricinus* (61). This defensin also has two distinct isoforms, with isoform 1 being four times more abundant than isoform 2. The mature isoforms are 97 percent identical to one another, with only three amino acid substitutions. Whether there is a relationship between the presence of introns and multiple isoforms is currently unknown, but it is interesting that introns and multiple isoforms seem to go together.

6.1.2. Lysozymes

Lysozymes are small proteins (approximately 14 kDa) that can serve as digestive enzymes and antimicrobials against a variety of different microorganisms. Lysozymes act against bacteria by hydrolyzing the bonds between the N-acetyl-muramic acid and N-acetyl-D-glucosamine residues (alternating sugar residues) that make up the peptidoglycan backbone. This results in disruption of the integrity of the cell wall. Clear evidence for the role of lysozyme in tick innate immunity was demonstrated in the hard tick, *D. variabilis* which expresses a 121 amino acid long (mature protein) C-type lysozyme (62). Transcript levels were elevated in hemolymph following injection of *E. coli*, reaching a 17-fold increase within 72 hours post-challenge. Comparison of tissue levels showed that *D. variabilis* lysozyme was most abundant in hemolymph but present in very low levels in the midgut or other organs. Blood feeding did not result in increased lysozyme expression in this hard tick, consistent with the findings of lysozyme transcript but not the active protein in the midgut of this species (53, 62). In the hemolymph, lysozyme may act synergistically with defensin in disrupting the bacterial cell wall, thereby greatly accelerating the killing action and perhaps broadening the range of bacteria inhibited by the activity of the antimicrobial peptides (44). In the hemocytes, lysozyme occurs in the cisternae of the endoplasmic reticulum and in the primary lysosomes (63). Lysozyme antimicrobial activity has been reported from the hemocytes of *Ixodes ricinus*, from cell lines derived from *I. scapularis* and *D. andersoni* and from *Ixodes persulcatus (*21, 64, 65). Most reports showed that lysozyme acted on the cell wall of gram positive bacteria. A noteworthy finding was that lysozyme expression was not up-regulated

in *I. scapularis* or *D. andersoni* cell cultures in response to challenge with *Rickettsia peacockii*, but expression was up-regulated after stimulation of the cells with *E. coli* and *M. luteus (*65). This may indicate that tick endosymbionts could avoid recognition by the tick's innate immune system .

In contrast to hard ticks, the pattern of lysozyme expression appears to be different in soft ticks. *O. moubata* expresses a 124 amino acid C-type lysozyme similar to the lysozymes found in hard ticks (66). Comparison of its phylogenetic relationships suggests that the *O. moubata* lysozyme is a digestive-adaptive C-type lysozyme. In studies done with this tick, blood feeding was found to stimulate increased expression in the midgut but not in the hemolymph. However, the authors found no evidence that transcription of the lysozyme gene is up-regulated in response to bacterial infection, perhaps because it was already strongly expressed in the midgut (66).

6.2. Proteases

In mosquitoes, serine proteases are reported to be up-regulated in response to invasion of the hemolymph by malarial parasites, contributing to the normal innate immune response (67). This is believed to be an important factor in the refractoriness of these mosquitoes for a number of malaria parasites. In tick hemolymph, an immune-responsive factor D-like serine protease was expressed in hemocytes in response to *E. coli* challenge (68). This protease shows high sequence similarity (54 percent identity) to a serine protease from hemocytes of the horseshoe crab, *Tachypleus tridentatus (*69). Proteins with similar Clip domains are found in a variety of invertebrates suggesting that such molecules are widespread (68). The precise role of the antimicrobial proteases in tick innate immunity needs further investigation. The role of proteases in the midgut is described in section 9.4

6.3. Protease inhibitors

Proteases are important virulence factors used by infectious pathogens, both prokaryotes and eukaryotes, during various stages of the infection process. Protease inhibitors, although not being directly antimicrobial, are produced by many invertebrate and vertebrate species that selectively target pathogen proteases in various ways (70). Inactivating these important virulence factors for the pathogen may prevent survival of the organism in the vector.

In ticks, a serine proteinase inhibitor (serpin) and an α-macroglobulin type protease inhibitor have been reported. The serpin reported from *D. variabilis* was found primarily in the hemocytes and has the characteristic "clip domain" found in pro-clotting enzymes of vertebrates. However, the active site serine was replaced by glycine (68). This protein also shares similarity with a phenoloxidase activating cofactor, perhaps adding evidence for the existence of a phenoloxidase (PO) pathway in ticks. The presence of PO in ticks is a subject of debate. PO was reported to be present in the hemolymph plasma during ecdysis in 4th instar nymphs of the argasid tick *O. moubata*, but no evidence of enzyme was detected in hemolymph or tissues of three different species of ixodid ticks (71, 72).

Another family of protease inhibitors includes the alpha$_2$-macroglobulins, which act by entrapping the proteases secreted by invading microorganisms within a "molecular cage" (bait region) formed when these molecules recognize substrate. Proteases trapped in the bait region undergo proteolytic cleavage, which in turn activates thioester rupture (rupture of 4 thioester bonds). Both bait region cleavage and thioester rupture is essential in stabilising alpha$_2$-macroglobulin complexes. This is followed by conformational changes that complete the entrapment- and inactivation processes. The alpha$_2$-macroglobulins and their entrapped proteases are then transported to circulating hemocytes, where they are internalized and degraded in secondary lysosomes of these phagocytic cells (73). An alpha$_2$-macroglobulin, an abundant acidic glycoprotein, that inhibits trypsin and other endopeptidases was sequenced from the hemolymph plasma of the soft tick, *O. moubata* (74). Molecular characterization studies showed that the molecule consists of two subunits generated from a precursor polypeptide by post-translational processing (75). Evidence for an alpha$_2$-macroglobulin was also found following sequencing of a cDNA library of the salivary glands of the hard tick *I. scapularis (*76). The role of these alpha$_2$-macroglobulins in tick innate immunity or pathogen entry needs to be addressed since alpha$_2$-macroglobulins are broad spectrum protease inhibitors capable of reacting with all four catalytic classes of proteases. Midgut protease inhibitors are specifically discussed in section 9.5.

6.4. Lectins

Lectins are proteins or glycoproteins that contain binding sites for specific mono- or oligosaccharides (77). They are capable of binding to these carbohydrate moieties commonly found on the cell walls/membranes of bacteria, yeast and protozoan pathogens. Originally defined by their role as hemagluttinating agents, lectins are now recognized as important mediators of the innate immune response in invertebrates as well as vertebrate animals where they can also activate the complement pathway. These proteins also bind to microbial pathogens which enables hemocytes to recognize and engulf them in a process known as opsonization. This involves carbohydrate recognition by ficolins and mannose binding lectins (78, 79). In addition, lectins can also bind to one another. In the horseshoe crab, *Tachypleus tridentatus,* the TPL-1 and TPL-2 lectins form clusters of interlocking molecules, binding to one another as well as to microbes (78). The clustering effect enables them to immobilize invading organisms, a process that has also been shown to occur in insects, ascidians, crustaceans and ticks (10, 79, 20, 80). Lectin binding causes the entrapped, immobilized organisms to aggregate, whereupon they are surrounded by degranulating hemocytes that destroy them by encapsulation or nodulation. Lectins are also involved in recognition, opsonization, phagocytosis and destruction of infecting microbes by tick cells (63).

Much less is known about the role of lectins in ticks. Lectins have been identified in the hemolymph plasma of the soft tick *O. moubata*, from the hemocytes of the hard tick, *I. ricinus*, from the saliva of the cattle tick *R. microplus* and *O. moubata* and from various body

tissues from the soft tick *Argas polonicus* and from *Ornithodoros tartakovskyi*, *O. tholozani* (*papillipes*), and *Rhipicephalus appendiculatus* (20, 40, 77, 81, 82, 83). Lectins may be secreted into the hemolymph or may be localized on the cell surfaces of the different tissues. Lectins have even been found in the folds of Gene's organ, the organ that secretes the waxy material that coats the eggs of ticks (81). In the soft tick, *O. moubata*, the hemolymph lectin Dorin M is a 640 kDa glycoprotein synthesized in the hemocytes and secreted into the hemolymph plasma, that may play a role as a pattern recognition molecule (80). Study of the molecular structure showed that it has a fibrinogen-like domain that recognizes carbohydrate sequences, especially sialic acid and N-acetyl-D-glucosamine, indicating that it is very similar to the tachylectins of the horseshoe crab *T. tridentatus*. It is also similar to the fibrinogens found in the blood of many vertebrates and is related to the ficolin family of proteins (80). Current evidence suggests that there is only one isoform of Dorin-M in *O. moubata* and that it is mainly expressed in the hemocytes and salivary glands, with some expression in the malphigian tubules, but no expression in either the midgut or ovary (80). Another potential lectin in *O. moubata*, OMFREP, has been identified in hemocytes (20). OMFREP shows a similar tissue distribution to Dorin M (20). Recently, a different lectin, Omgalec, with a broad tissue- and life stage distribution was discovered in this same species (84). In the hard tick, *I. ricinus,* two lectins, Ixoderin A and B, have been identified that show different tissue expression. These lectins, like OMFREP and Dorin M all show a fibrinogen-domain similar to the ficolins of mammals. Ixoderin A is expressed in the hemocytes, salivary glands and midgut while Ixoderin B is expressed only in the salivary glands (20), and shows differences that may reflect a different function to Ixoderin A. The reason for the selective expression of these different isoforms in the different tissues is unknown, although the high degree of tissue specificity raises the possibility that they have distinctly different roles. Like limulin, the sialic acid-specific lectin of the horseshoe crab, *Limulus polyphemus,* tick lectins may recognize a wide range of Gram-negative bacteria due to its specificity for sialic acid, N-acetyl-D-glucosamine and other cell wall carbohydrate moieties (77). In addition to their role in controlling invasive microorganisms, another intriguing hypothesis suggested by several investigators is that the vector lectins may facilitate pathogen transmission (summarized by 77, 80).

6.5. Host serum proteins

Several host serum proteins with potential antimicrobial activity, e.g., immunoglobulins such as IgG, cross the midgut into the hemolymph where they can inhibit or kill microorganisms, especially if host complement is also present. Calreticulin has been shown to bind host complement component C1q and potentially prevents classical pathway activation (85). Since calreticulin is found in a large number of tick species determining whether it has any function in innate immunity, such as protection of tick tissues from host complement activation, needs to be established (86). Host immunoglobulins (IgGs) ingested with the blood meal migrate into the hemolymph where they are sequestered by immunoglobulin-binding proteins (IGBPs). IGBPs are believed to protect the tick from the potentially harmful effects of host IgGs in the skin lesion during blood feeding. IGBPs also transport host IgGs from the ingested blood back into the host via the saliva, thereby modulating the ability of the host to reject the feeding tick (87).

6.6. Transferrin

In mosquitoes, transferrin synthesis and secretion are increased on exposure of mosquito cells to bacteria and in response to filarial worm infestations, suggesting that this protein participates in the insect's immune response (88, 89). In the filarial vector, *Culex quinquefasciatus,* transferrin is known to function in immune defense, along with other defense molecules such as defensin, gambicin and cercropin (89). Transferrin from the host blood has also been reported to cross into the hemolymph of ticks, however, it may not inactivate bacteria or other microbial invaders (63). Only a single study on the potential antimicrobial effect of transferrin in ticks has been done, using a similar protein called lactoferrin. This study with *Rickettsia* spp. showed no sensitivity of the microbes to lactoferrin, but confirmed sensitivity towards other AMPs (90).

7. COAGULATION FACTORS

Hemolymph clotting is the first response to wounding in insects and many other invertebrates, limiting fluid loss and initiating the healing process. Hemolymph coagulation also serves as an important immune defense by immobilizing bacteria, protozoan parasites or other microbes. The mesh of clot fibers subsequently hardens and darkens, undergoing the melanization process mediated by the phenoloxidase pathway. In horseshoe crabs, Tachypleus tridentatus and other species, injury (or microbial invasion) stimulates release of proteins known as proxins from the hemocytes as well as expression of transglutaminase (TGase). The latter promotes cross-linking of proxins with the plasma protein coagulin, resulting in the formation of stable coagulin fibrils. A second hemocyte-derived protein, stabilin, also interacts with proxin and contributes to the formation of the fibril mesh. In addition, stabilin binds to gram positive and gram negative bacteria, immobilizing them within the clot where they are more susceptible to destruction by AMPs (91). In insects, several different molecules, especially hemolectin and fondue, were identified as contributing to initiating the clotting process. Nevertheless, the process of hemolymph clotting in insects and most other arthropods is poorly understood (92). A similar clotting process occurs in ticks when injured by cuts or wounds. Hemolymph clots at the site of injury, limiting further loss and preventing microbial invasion. However, the molecular basis of the clotting mechanism is unknown.

8. SALIVARY GLAND ANTIMICROBIAL ACTIVITY

Some of the same classes of immune-related proteins and peptides described previously have been

reported in the saliva, or at least in the salivary glands of different tick species. Among the most important are 1) lectins, including the ixoderins A and B (section 6.4), which are also found in the hemocytes and midgut in *I. ricinus* and OmFREP from the salivary glands and hemocytes of *O. moubata.* Both lectins contain fibrinogen-related domains with high sequence similarity to mammalian ficolins (20); 2) peptidoglycan recognition proteins; 3) ML-domain proteins that recognize lipids important in innate immunity; 4) antimicrobial peptides and proteins, including lysozyme, defensins, microplusin and histidine-rich defensin-like proteins. Defensin transcript has been reported from the salivary glands of several tick species (57, 61, 93); and finally, 5) the histidine-rich proteins, microplusin and hebraein.

9. MIDGUT ANTIMICROBIAL ACTIVITY

The midgut is the first site of contact between ingested blood and microbes, and the tick's internal tissues. In contrast to the haematophagous insects, relatively little is known about protein expression in the tick's midgut or how it responds to microbial challenge. However, rather than being a benign environment, the tick midgut expresses an impressive arsenal of antimicrobial agents, substantially greater than previously recognized. The following discussion reviews the midgut's multiple defenses that severely limit the opportunities for infection by invading microorganisms.

9.1. Peritrophic membrane

During feeding in ixodid ticks, a peritrophic membrane (PM) is formed from extracellular secretions, resulting in a chitin-enriched covering that protects the delicate epithelial cells. The PM divides the midgut lumen into two regions, the endoperitrophic space, encompassing the great bulk of the lumen, and the narrow ectoperitrophic space adjacent to the epithelial cells of the lumen (94). It also serves as a barrier against microbial penetration of the midgut epithelium. In mosquitoes, for example, ookinetes of the malaria parasite *Plasmodium gallinaceum* secrete chitinases that lyse portions of the membrane, allowing them access to the underlying midgut epithelium (95). In *I. ricinus,* the PM begins to form as early as 18 hours after the commencement of feeding and remains intact for many days after repletion. The membrane ranges in thickness from as little as 0.30 - 0.48 um in larvae to as much 0.04 - 0.93 um in replete females. In these ticks, the fully formed membrane initially forms about 0.2 - 0.8 um from the epithelial cells but gradually contracts and withdraws away from the epithelium after repletion (96). In studies on 5 different species of *Ixodes,* the PM was found to be replenished throughout the feeding process; each new generation of midgut cells synthesized its own matrix which was deposited on the apical surface of each new cell (97). Some microbial pathogens, e.g., *Babesia microti,* are capable of penetrating the peritrophic matrix by means of a specialized structure, the arrowhead organelle (94). However, it is not known whether prokaryotes can traverse the PM in ticks. Most probably they cannot, and those that are ingested late in the feeding cycle are likely to be excluded from direct contact with the midgut epithelium. Some prokaryotic pathogens express outer surface proteins that bind to receptors found on the epithelial cells lining the midgut. One example is *B. burgdorferi*, in which the outer surface protein OspA binds to the *I. scapularis* receptor TROSPA, enabling the bound spirochetes to conceal themselves among the microvilli (98). Another is *Anaplasma marginale,* wherein an outer surface protein, MSP1a, binds to unknown receptors in the midgut lining of *D. variabilis* or *D. andersoni,* enabling some of the ingested bacteria to invade and colonize the epithelial cells (99). Presumably, microbes that lack these features remain trapped in the midgut lumen and are less likely to survive and spread into the tick's body tissues.

9.2. Antimicrobial peptides.

In haematophagous insects, microbes ingested with the blood meal encounter an array of antimicrobial agents, including lectins, lysozyme, defensins, cercropins and many others (10, 17, 100-103).

In contrast to insects, the tick's molecular response to microbes ingested during blood feeding is not as robust. The midgut of the soft tick, *O. moubata,* secretes defensin peptides which effectively control gram positive bacteria (50). Whether lysozyme contributes to this defensive response is uncertain; it was found to be strongly up-regulated by blood feeding in this species, but not by bacterial challenge (66). The midgut lysozyme found in *O. moubata* has a pI of 9.7; on the other hand it has a pH optimum of 5-7. This raises questions as to a role in defense in the tick midgut (104). Whether lysozyme expression in *O. moubata* is increased in response to bacterial infection is unknown, but transcription is increased during the bloodmeal (66). In addition to its expression in the gut, lysozyme is also expressed in hemocytes and the salivary glands (66). In contrast to *O. moubata*, *D. variabilis* lysozyme levels did not increase following blood meal digestion, but a role in innate immunity was demonstrated (62). Other studies indicated that gram-negative bacteria such as *E. coli* were not immediately inhibited by the midgut contents of *O. moubata*, even though expression of defensin was slightly up-regulated after *E. coli* ingestion (105). Some *E. coli* survived in the guts of these ticks, although in diminishing numbers, for as long as 20 days and were destroyed gradually only after they were endocytosed within the midgut epithelial cells. None were able to penetrate into the hemocoel (105). Whether these lysozyme and defensin-like antimicrobial peptides also occur in the midguts of ixodid ticks is uncertain. In *D. variabilis*, midgut lysozyme mRNA levels are not significantly affected by blood-feeding *(*52, 62). Moreover, no evidence of either the lysozyme or defensin peptides has been observed in other studies of the midgut of this tick (53). Attempts to re-culture bacteria, *B. burgdorferi, E. coli* and *Bacillus subtilis* from the midgut following their ingestion by capillary oral feeding were unsuccessful, although the spirochetes of *B. burgdorferi* remained intact for up to 3 hours (53). The persistence of intact spirochetes was especially noteworthy, since these bacteria were previously shown to be susceptible to lysis by defensin and lysozyme (44). However, it is possible that the defensin peptide was not detected or was inactivated at the time the samples were collected. Defensin from the blood-sucking

fly *Stomoxys calcitrans* has been shown to bind with a serine protease to form an SDS-stable complex with an apparent molecular weight greater than 26 kDa (106). Whether such complexes form in the midgut of *D. variabilis* is unknown, but this may explain why the peptide has not been detected. Ceraul *et al.* reported increased expression of varisin (defensin 1) and a second defensin, defensin 2, in the midgut following blood feeding (52). Varisin levels increased 35-fold compared to a 5-fold increase for defensin 2. When challenged with *Rickettsia montanensis*, varisin expression was increased approximately 2.6 and 1.7 fold after 24 and 48 hours post-injection, respectively, while a 1.9 fold increase in defensin 2 was only observed at 24 hours post-injection. These findings indicate that varisin may play an important role in protection of the midgut against microbial challenge during feeding, but the role of defensin 2 is unknown (105).

Another source of antimicrobial peptides in the midgut are products resulting from the digestion and breakdown of hemoglobin by the midgut cells. In soft ticks, *O. moubata*, and in hard ticks, *R. microplus* and *D. variabilis,* small fragments (3 –11 kDa) resulting from the digestion of alpha- and beta-chain hemoglobin are antimicrobial, with activity against gram positive bacteria (53, 107, 108).

9.3. Midgut lectins and pathogen recognition proteins.

Several lectins have been reported from ticks, as noted previously (section 6.4). However, the only midgut lectins described to date is ixoderin A, reported from the hard tick *I. ricinus* and omgalectin, a novel lectin from the midgut and other tissues of the soft tick, *O. moubata (*20, 84). A similar lectin with high affinity to these previously recognized lectins was found in a cDNA library constructed from the midgut of *D. variabilis.* It contained a signal peptide, suggesting that it may be a secreted protein *(*5). Lectins bind to sialic acid, hexosamines and other compounds characteristic of the cell walls of bacteria and fungi, resulting in clumping that reduces the ability of these microorganisms to disperse. Lectins also act as pattern recognition proteins, thereby opsonizing invading microbes and enhancing their susceptibility to attack by other antimicrobial agents in the tick midgut.

ML-domain containing proteins are implicated in lipid recognition, in particular as pathogen recognition proteins (109, 110). Among the best known as Bm86 and Bm95 from the midguts of the cattle tick, *R. microplus (*111, 112). In *I. ricinus,* ML domain and von Willebrand factor-containing proteins were induced after *B. burgdorferi* infection but not by blood feeding alone (110). These and similar proteins in other tick species have been targeted as antigens for vaccines useful in controlling tick infestations and the transmission of tick-borne disease agents (113). Their precise role in pathogen recognition and control is not understood.

9.4. Proteases

A large variety of cysteine-, aspartic- and serine proteases have been identified in the midgut of ticks. Most of these are believed to be involved in bloodmeal digestion. Among the most important are the serine proteases, which function as hemolytic agents in the midgut lumen, and cysteine and aspartyl proteases because of their role in hemoglobin digestion, e.g., longespin, an aspartyl protease found in the salivary glands and midgut of *H. longicornis (*114-116). Whether any of these proteases also play a role in immune defense is unknown. However, evidence from insects suggests that metalloproteases may be important in cellular immune defense (117). Evidence for three metalloproteinases has been found in a cDNA library from the midgut of *D. variabilis* (5). These proteinases had very little similarity to proteases of ticks, other acarines, or insects, suggesting these may be novel proteinases.

9.5. Protease inhibitors

Protease inhibitors are important as innate immune proteins by compromising virulence factors expressed by pathogens invading host tissues. A number of protease inhibitors have been reported in ticks. However, most were from the salivary glands or hemolymph. Among the most important are the Kunitz-domain serine proteases found in a number of different tick species from both the Ixodidae and the Argasidae, that function as inhibitors of thrombin, factor X (FX), activated factor X (FXa), tissue factor pathway and kallikrein-kinin systems so as to prevent blood coagulation (118-120). Similar molecules have also been found in the tick midgut (5). However, none are known to prevent microbial infection. Less common are the serine protease inhibitors which disrupt serine proteases secreted by a variety of invasive microbes and play an important role in preventing microbial infections (121). In *R. appendiculatus* four serine protease inhibitors (serpins) were described from salivary glands, midguts and other internal tissues (119). A large number of transcripts for serpins were described from the lone star tick, *A. americanum,* many of which (11 out of 15) were ubiquitously expressed in the midgut as well as in the ovary and salivary glands. At least three were most strongly expressed in the midgut (118). A different type of serpin was reported from the midgut of *H. longicornis (*120).

Another important group of antimicrobial proteins are the cysteine proteases inhibitors (cystatins). Cysteine proteases secreted by infectious bacteria or pathogenic protozoa serve as important virulence factors. Cystatins disrupt the activity of these enzymes, thereby minimizing the ability of the microbes to colonize the tick's digestive tract and other tissues. One such cystatin reported from the midguts and hemocytes of *H. longicornis* was found to be highly effective against *Babesia* spp. When *H. longicornis* were exposed to *Babesia gibsoni* or *B. bovis*, cystatin expression was 1.8 times greater than in uninfected controls. The recombinant protein also inhibited growth of *B. bovis* grown in culture (122). Both secreted and intracellular cystatins have been reported from ticks, including the midgut (123). All of the different types of protease inhibitors remain to be tested for any effect on the innate immunity in ticks.

9.6. Oxidative stress/detoxifying proteins

Haematophagous arthropods have molecules that protect the delicate midgut epithelial cells against the

harmful effects of oxidative stress that results from reactive oxygen species (ROS, e.g. H_2O_2) and reactive nitrogen species (RNOS) produced during blood feeding and digestion. In mosquitoes, as in many other animals, enzymes such as catalase are up-regulated to cope with ROS cellular toxicity (124). Other enzymes, e.g. peroxiredoxins, members of a family of antioxidant peroxidases, also function as antioxidants. Microbial infection is also known to cause oxidative stress, leading to up-regulation of stress reducing proteins (6). However, although ROS and RNOS are cytotoxic to pathogens and parasites, suppression of oxidants in the midgut may be exploited by the microbial invaders to facilitate infection of the host's tissues (125). Some insects have evolved enzymes to enhance the lethal action of ROS against invading microbes while simultaneously protecting their tissues with antioxidants. For example, in the fruit fly (*D. melanogaster),* the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme, known as the dual oxidase (dDuox) enzyme system, controls microbes in the gut by generating oxidative bursts that kill or inhibit microbial growth (126). Glutathione S-transferases (GSTs), members of a diverse family of enzymes found in virtually all living organisms, also play a central role in the detoxification of stress-causing agents, particularly by removing toxic oxygen free radical species and harmful toxicants (127). GSTs are also believed to be important in suppressing microbial infection. Two GST isoforms were reported from the midgut of *D. variabilis,* $DvGST_1$, and $DvGST_2$, both of which were up-regulated during blood feeding; $DvGST_2$ was also found in the tick ovary (128). In *I. ricinus* GST and several other genes were up-regulated in response to *B. burgdorferi* infection (110).

Other oxidative stress reducing compounds have been found in the midguts of ticks, including thioredoxins, glutaredoxins, glutathione peroxidases and at least one species of Cu/Zn superoxide dismutase (5). Although of obvious importance in reducing the oxidative stress involved in hemoglobin digestion, their role in curtailing microbial infections, if any, is unclear.

10. CONCLUSIONS AND PERSPECTIVES

Ticks have a robust innate immune system that presents a complex array of cellular and humoral defenses to prevent microbial infection. In addition to physical barriers, ticks express a variety of antimicrobial proteins, proteases, protease inhibitors, opsonins, anti-oxidants and other lethal molecules when challenged by invading microbes. Organisms recognized as foreign are also destroyed by phagocytic hemocytes or trapped in clusters where they are engulfed by hemocytes and destroyed. Many of these same responses are also activated by blood feeding, providing an early barrier to ingested microbes. However, pathogens that survive and colonize the tick's body organs usually bind to receptors on target cells and, in most cases, are internalized and sheltered from immune destruction. Further study using transcriptomics, proteomics, microarrays and other modern tools may be expected to discover how tick-borne pathogens and other microbes are recognized as well as how the successful pathogens evade or escape the tick's innate immune system. Pathogen recognition and how the tick regulates its response is an area in which considerable research still needs to be carried out. Is the regulation of the response in a tick similar to that of *Drosophila* or other invertebrates? This aspect remains to be examined in depth in the ticks.

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