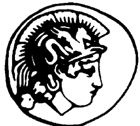

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Insect hemocytes and their role in immunity

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Abstract

The innate immune system of insects is divided into humoral and cellular defense responses. Humoral defenses include antimicrobial peptides, the cascades that regulate coagulation and melanization of hemolymph, and the production of reactive intermediates of oxygen and nitrogen. Cellular defenses refer to hemocyte-mediated responses like phagocytosis and encapsulation. In this review, we discuss the cellular immune responses of insects with emphasis on studies in Lepidoptera and Diptera. Insect hemocytes originate from mesodermally derived stem cells that differentiate into specific lineages identified by morphology, function, and molecular markers. In Lepidoptera, most cellular defense responses involve granular cells and plasmatocytes, whereas in *Drosophila* they involve primarily plasmatocytes and lamellocytes. Insect hemocytes recognize a variety of foreign targets as well as alterations to self. Both humoral and cell surface receptors are involved in these recognition events. Once a target is recognized as foreign, hemocyte-mediated defense responses are regulated by signaling factors and effector molecules that control cell adhesion and cytotoxicity. Several lines of evidence indicate that humoral and cellular defense responses are well-coordinated with one another. Cross-talk between the immune and nervous system may also play a role in regulating inflammation-like responses in insects during infection. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Hemocytes; Encapsulation; Nodulation; Phagocytosis; Clotting; Immunity; Antimicrobial peptides; Cytokines; Lepidoptera; Diptera; *Drosophila*; Insects

1. Introduction

Multicellular animals defend themselves against infectious organisms by two systems known as innate and acquired immunity. The innate immune system relies on germline encoded factors for recognition and killing of foreign invaders, whereas the acquired immune system produces receptors by somatic gene rearrangement that recognize specific antigens and that allow organisms to develop an immunological memory (Fearon, 1997). Insects lack an acquired immune system but have a well-developed innate response. Initial defenses include the physical barriers of the integument or gut, clotting responses by hemolymph, and the production of various cytotoxic molecules at the site of wounding. Foreign entities that pass these barriers and enter the hemocoel must contend with additional cytotoxic molecules as well as an array of different hemocytes.

The insect immune system is further subdivided into humoral and cellular defense responses. Humoral defenses include the production of antimicrobial peptides (Meister et al., 2000; Lowenberger, 2001), reactive intermediates of oxygen or nitrogen (Bogdan et al., 2000; Vass and Nappi, 2001), and the complex enzymatic cascades that regulate coagulation or melanization of hemolymph (Muta and Iwanaga, 1996; Gillespie et al., 1997). In contrast, cellular defense refers to hemocyte-mediated immune responses like phagocytosis, nodulation and encapsulation (Strand and Pech, 1995; Schmidt et al., 2001). While great progress has been made over the last several years in identifying antimicrobial peptides and the signaling pathways that regulate their synthesis, much less is known about control of cellular defense responses. This is due in large part to the small size of many insects, which makes collection of hemocytes and identification of hemocyte-produced effector molecules difficult. It is also often difficult to conduct manipulative experiments on hemocyte-mediated defense responses in

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vivo or to isolate defined populations of hemocytes for use in experiments conducted in vitro.

Dividing the insect immune system into cellular and humoral responses is somewhat arbitrary, as many humoral factors affect hemocyte function and hemocytes are an important source of many humoral molecules. There also is considerable overlap between humoral and cellular defenses in processes like the recognition of foreign intruders. Hemocytes recognize foreignness either by direct interaction of surface receptors on hemocytes with molecules on the invading organism, or indirectly by recognition of humoral receptors that bind to and opsonize the surface of the invader. Inter- and intracellular signaling events must then coordinate effector responses like phagocytosis or encapsulation. The purpose of this review is to summarize our understanding of cellular immune responses. Emphasis is placed on studies with insects in the order Lepidoptera and dipterans like *Drosophila melanogaster*, as the majority of studies on insect hemocytes have been conducted in these taxa.

2. Hemocyte types and hematopoiesis

Insects produce several types of hemocytes that are traditionally identified using morphological, histochemical and functional characteristics (Gupta, 1985; Brehelin and Zachary, 1986). More recently, antibody and genetic markers have been characterized in selected species that more reliably distinguish different hemocyte types from one another (Chain et al., 1992; Mullet et al., 1993; Willott et al., 1994; Strand and Johnson, 1996; Gardiner and Strand, 1999; Lebestky et al., 2000). The most common types of hemocytes reported in the literature are prohemocytes, granular cells (= granulocytes), plasmatocytes, spherule cells (= spherulocytes), and oenocytoids (Fig. 1). These hemocyte types have been described from species in diverse orders including Lepidoptera, Diptera, Orthoptera, Blattaria, Coleoptera, Hymenoptera, Hemiptera, and Collembola (Ahmad, 1988; Azambuja et al., 1991; Ahmad, 1992; Luckhart et al., 1992; Fenoglio et al., 1993; Sonawane and More, 1993; Butt and Shields, 1996; Joshi and Lambdin, 1996; Hernandez et al., 1999; de Silva et al., 2000a).

In larval stage Lepidoptera, granular cells and plasmatocytes are the only hemocyte types capable of adhering to foreign surfaces, and together usually comprise more than 50% of the hemocytes in circulation (Lackie, 1988; Ratcliffe, 1993; Strand and Pech, 1995). In the noctuid moth *Pseudoplusia includens*, two distinct subpopulations of plasmatocytes are distinguished by antibody markers while no antigenic differences are detected among circulating granular cells (Gardiner and Strand, 1999). The other hemocytes described from Lepidoptera are non-adhesive spherule cells, oenocytoids and prohemocytes. Spherule cells have been suggested to transport cuticular components (Sass et al., 1994), while oenocytoids contain cytoplasmic phenoloxidase precursors that likely play a role in melanization of hemolymph (Ashida and Dohke, 1980; Iwama and Ashida, 1986; Jiang et al., 1997). Prohemocytes are hypothesized to be stem cells that differentiate into one or more of the aforementioned hemocyte types. The hemocytes described from *Drosophila* are named somewhat differently from those of most other insects, including lower dipterans such as culicids and simuliids (Lanot et al., 2001). During embryogenesis, the only hemocytes present are called macrophages or plasmatocytes. In larvae, plasmatocytes remain the most abundant hemocyte type in circulation but two other cell types, called lamellocytes and crystal cells, are also present. Like plasmatocytes, lamellocytes are able to attach to foreign surfaces while crystal cells are non-adhesive hemocytes that contain phenoloxidase precursors and appear morphologically similar to what are called oenocytoids in other insects.

In mammals, all blood cells derive from hematopoietic stem cells that differentiate into different lineages under control of transcription factors like GATA 1–3 and AML1 (Lebestky et al., 2000). Hemocytes first develop in insects during embryogenesis from head or dorsal mesoderm (Hoffmann et al., 1979; Ratcliffe et al., 1985; Tepass et al., 1994). Insects also continue to produce hemocytes during larval or nymphal stages via division of stem cells in mesodermally derived hematopoietic organs and/or by continued division of hemocytes already in circulation (Jones, 1970; Akai and Sato, 1971; Feir, 1979; Ratcliffe et al., 1985). Numerous scenarios have been proposed for the lineage relationships among different hemocyte types. In Lepidoptera, Beaulaton (1979) proposed a linear maturation process in *Bombyx mori* whereby prohemocytes in hematopoietic organs differentiate into plasmatocytes that are released into circulation. Plasmatocytes in circulation then differentiate into granular cells, spherule cells and oenocytoids. The short-term culture experiments of Yamashita and Iwabuchi (2001), however, suggest that *B. mori* prohemocytes are able to differentiate into plasmatocytes, granular cells, or spherule cells. Studies with the noctuid moths *Euxoa declarata* and *Spodoptera frugiperda* suggest a dual origin for hemocytes during larval development. Hematopoietic organs contain almost exclusively putative stem cells and plasmatocytes, while granular cells, spherule cells and oenocytoids are only observed in circulation (Arnold and Hinks, 1976; Hinks and Arnold, 1977; Gardiner and Strand, 2000). Using bromodeoxyuridine (BrdU) incorporation and hemocyte-specific antibody markers, Gardiner and Strand (2000) determined that prohemocytes/plasmatocytes in hematopoietic organs and all cell types in circulation, with the exception of oenocytoids, actively divide. These results also

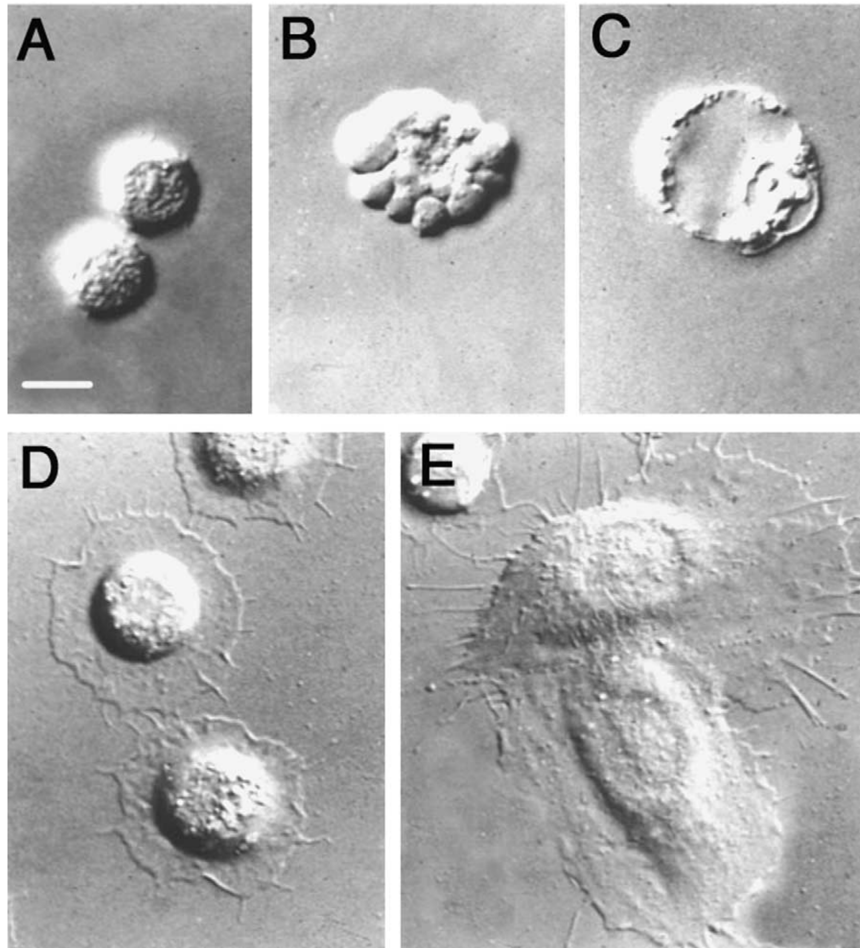

Fig. 1. Nomarski images of the five types of hemocytes found in lepidopterans like *Pseudoplusia includens*. (A) Prohemocytes, (B) spherule cell, (C) oenocytoid, (D) granular cells, and (E) plasmatocytes. Scale bar = 50 μm.

suggested that: (1) stem cells (= prohemocytes) in hematopoietic organs differentiate primarily into plasmatocytes, whereas other hemocyte types differentiate after release into circulation, and (2) maintenance of hemocyte populations in circulation depends heavily on continued division of each cell type after differentiation.

In *Drosophila*, the GATA homolog Serpent (Srp) is expressed in hematopoietic stem cells (Rehorn et al., 1996). Two additional transcription factors, glial cell missing (Gcm) and the AML1-like protein Lozenge (Lz), function downstream of Srp and are required for plasmatocyte and crystal cell development, respectively (Lebestky et al., 2000). Misexpression of Gcm in crystal cells appears to cause transformation of these cells into plasmatocytes based on changes in morphology and expression of the plasmatocyte-specific receptor Croquemort. However, expression of Lz in plasmatocytes does not convert them into crystal cells. A small population of Lz-expressing precursor cells also appear to develop into plasmatocytes instead of crystal cells. Upon maturation, these plasmatocytes no longer express Lz but do express Gcm. Whether these plasmatocytes represent a functionally distinct plasmatocyte sub-population is unknown. Other genes implicated in proliferation and differentiation of hemocytes include the Janus kinase (JAK)/signal transducer and activator transcription (STAT) (Dearolf, 1999; Myrick and Dearolf, 2000) and Toll signaling pathways (Govind, 1999). Identification of homologs for Srp, Lz, and Gcm have not yet been reported from other insects, but expression patterns of these factors in combination with other markers could greatly help in clarifying the relationships among hemocyte types in different insect taxa.

3. Hemocyte types involved in cellular immune responses

As noted in the Introduction, the main defense responses involving hemocytes are phagocytosis, nodulation and encapsulation. Hemocytes also respond to external wounding by participating in clot formation. Phagocytosis refers to the engulfment of entities by an individual cell. Hemocytes phagocytose both biotic tar-

gets like bacteria, yeast, and apoptotic bodies as well as small abiotic targets like synthetic beads or particles of India ink (Yokoo et al., 1995; Hernandez et al., 1999; de Silva et al., 2000b). The particular hemocytes reported to be phagocytic varies among insect taxa, and in some cases discrepancies even exist in the literature among studies on the same species (Anggraeni and Ratcliffe, 1991; Tojo et al., 2000). These differences are likely due in part to difficulties with identifying hemocytes in some insects. In Lepidoptera, granular cells and plasmatocytes are the only hemocyte types reported to be phagocytic, while in *Drosophila* plasmatocytes are the main phagocytic hemocyte (Elrod-Erickson et al., 2000).

Nodulation refers to multiple hemocytes binding to aggregations of bacteria while encapsulation refers to the binding of hemocytes to larger targets like parasitoids, nematodes and chromatography beads. Nodule and capsule formation look nearly identical at the ultrastructural level (Ratcliffe and Gagen, 1976, 1977) which suggests they are essentially the same process albeit against different targets. Unlike phagocytosis, nodulation and encapsulation result in the formation of an overlapping sheath of hemocytes around a target. Again, the two types of hemocytes most often observed in capsules are granular cells and plasmatocytes in Lepidoptera, and lamellocytes in *Drosophila* (Schmidt et al., 2001; Vass and Nappi, 2001). Nodules and capsules melanize in some insect species but not others (Strand and Pech, 1995). For those species in which melanization occurs, oenocytoids or in *Drosophila* crystal cells may also play a role in capsule formation.

The distribution of granular cells and plasmatocytes in capsules formed by some lepidopterans appears random (Wiegand et al., 2000) while in others it is highly organized, with granular cells being the first cells to bind to the target and plasmatocytes attaching thereafter (Sato et al., 1976; Schmit and Ratcliffe, 1977, 1978; Lackie et al., 1985; Pech and Strand, 1996). For example, encapsulation of Dowex 1X-2 chromatography beads by hemocytes from the moth *P. includens* begins when individual granular cells attach to the beads. Thereafter, plasmatocytes attach to the target and one another, forming a multicellular sheath. Capsule formation then ends when a monolayer of granular cells attach and apoptose on the capsule periphery (Pech and Strand, 1996, 2000) (Fig. 2). Neither granular cells nor plasmatocytes form capsules alone, but plasmatocytes readily encapsulate this target following attachment of granular cells. While Dowex 1X-2 beads are encapsulated more rapidly in vivo, capsule architecture is virtually identical to capsules formed in vitro (Pech and Strand, 1996; Loret and Strand, 1998). Lavine and Strand (2001a) elaborated on these studies by finding that many other abiotic targets are also only encapsulated by plasmatocytes following attachment of granular cells. However, some targets can be encapsulated by plasmatocytes in the absence of

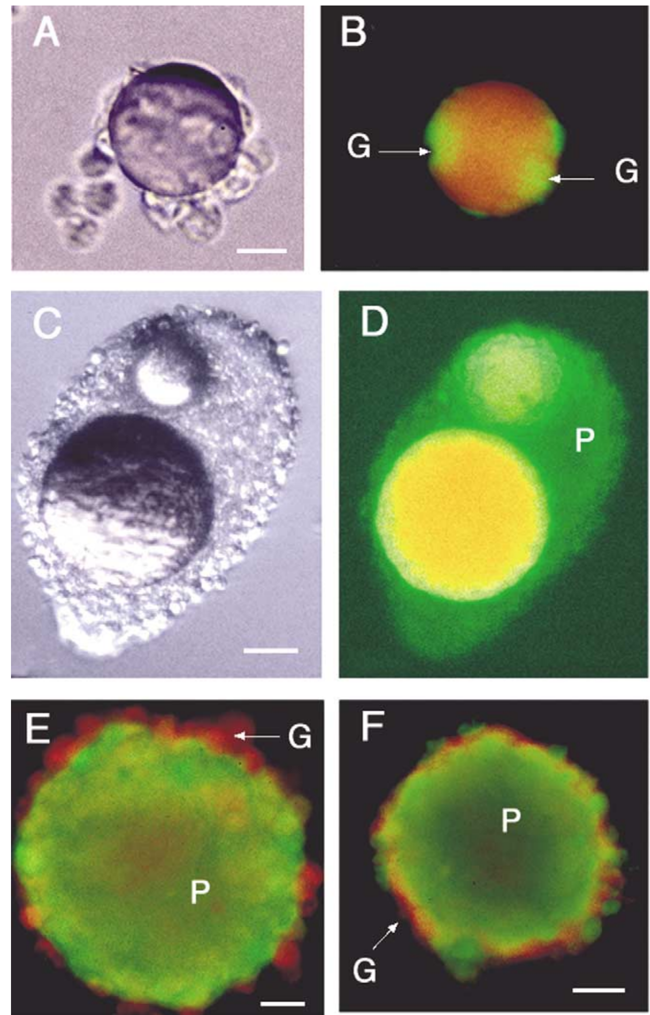

Fig. 2. Encapsulation of a foreign target (Dowex 1X-2 chromatography bead) by hemocytes from *Pseudoplusia includens*. Fluorescent images of hemocytes labeled with the granular-cell-specific antibody marker 48F2F5 and plasmatocyte-specific marker 52F3A5 or 49B11C6. (A) Light microscopy image 30 min after presentation of the target to hemocytes. Some hemocytes have already bound to the target's surface. Scale bar = 20 μm. (B) Fluorescent image of the same target after double-labeling for granular cells (green) and plasmatocytes (red). Only granular cells (G) are attached to the target. (C) Light microscopy image 12 h after presentation of the target to hemocytes. Overlapping layers of hemocytes have now attached to the target. Scale bar = 120 μm. (D) Fluorescent image of the same target after double-labeling granular cells (red) and plasmatocytes (green). The overlapping layers of hemocytes attached to the target are almost exclusively plasmatocytes (P). (E) Fluorescent image of a 16 h old capsule. The core of the capsule is comprised primarily of plasmatocytes (P) (green) while rounded granular cells (G) (red) are attached to the capsule periphery. (F) Fluorescent image of a 30 h old capsule. Granular cells (G) around the capsule periphery have apoptosed, leaving a flocculent outer layer labeled by monoclonal antibody (mAb) 48F2D5 (G, arrow). Scale bar for A–D is 30 μm. Figure adapted from Pech and Strand (1996, 2000).

granular cells if they are preincubated in plasma. These results indicate that both humoral and cellular receptors are involved in recognition of encapsulation targets. After granular cells or humoral opsonins bind to the target, they induce plasmatocytes to change from non-adhesive to strongly adhesive cells that form the majority of the capsule. Encapsulation is then terminated by the attachment of granular cells to the capsule periphery.

The literature offers little insight into the hemocytes involved in clot formation, although we would speculate that granular cells and plasmatocytes are likely key participants in Lepidoptera. As with melanizing capsules, oenocytoids or crystal cells may also play an important role in clotting given that external wounds usually melanize. However, epidermal tissue and plasma are other sources of phenoloxidases that could play an equally important role in melanization. In summary, all of the hemocyte-mediated defense responses discussed above involve the following sequence of events. The foreign target or wound site first has to be recognized as foreign and then one or more hemocytes must be activated to perform a particular effector response. The biochemical and molecular factors involved in regulating these events are discussed below.

4. Recognition of foreignness

In the absence of an acquired response, the biggest challenge to the innate immune system of insects is how to recognize pathogens and other entities from self. This has been addressed in vertebrates by the evolution of pattern-recognition receptors (PRRs) that bind conserved pathogen-associated molecular pattern (PAMP) molecules produced by microorganisms like bacteria and fungi (Fearon, 1997). Most identified PAMPs are cell-wall components like lipopolysaccharide (LPS), peptidoglycan, and β-1,3-glucans that are recognized by either humoral or cellular PRRs. Humoral PRRs like mannose-binding protein bind to the PAMP, which results in opsonization of the infectious organism. The bound PRR is then recognized by a cell-surface protein like the C1q receptor expressed by macrophages. In contrast, cellular PRRs are expressed on the surface of immune cells and bind PAMPs directly (Aderem and Underhill, 1999).

4.1. Humoral PRRs involved in recognition of microorganisms

Several factors including lectins, hemolin, LPS-binding protein, gram-negative bacteria recognition protein, peptidoglycan recognition protein, and the thioester-containing protein αTEP1 have been identified from insects as candidate PRRs (Bulet et al., 1999; Schmidt et al., 2001). While the receptors on hemocytes or other tissues that recognize these PRRs are unknown, studies do indicate that some of these factors enhance phagocytosis or nodulation responses by hemocytes after binding to microorganisms. For example, the mosquito *Anopheles gambiae* produces αTEP1 which is related to vertebrate complement factors and α_2-macroglobulins (Levashina et al., 2001). αTEP1 binds to gram-negative bacteria and suppression of this factor by RNA interference reduces phagocytosis by a hemocyte-like *A. gambiae* cell line. LPS-binding protein from *B. mori* (BmLBP) binds to rough strains of *Escherichia coli*, which in turn are nodulated more quickly than smooth strains of *E. coli* that are not recognized by BmLBP (Koizumi et al., 1999). A polyclonal antibody generated against BmLBP also appears to block the opsonizing activity of *B. mori* plasma since it reduces nodulation of rough strain *E. coli* in in vitro assays. The immunoglobulin (Ig)-superfamily protein, hemolin, binds LPS but whether this facilitates any kind of effector responses by hemocytes is unclear (Zhao and Kanost, 1995; Faye and Kanost, 1998). A number of lectins have been purified from insects and suggested to function as humoral PRRs (Pendland et al., 1988; Yu and Kanost, 2000). However, in most instances, the ligands for these lectins are unknown.

4.2. Cellular PRRs involved in recognition of microorganisms

Less is known about the cellular PRRs on hemocytes involved in recognition of microorganisms. Mammalian immune cells express several Toll-like receptors that are considered cellular PRRs, because they directly recognize LPS and other microbial products. These receptors in turn activate signal transduction by way of NF-κB that results in production of pro-inflammatory cytokines (Akira, 2001). The *Drosophila* genome contains *Toll*, seven additional *Toll*-related genes (*Toll-3-8*, *18-wheeler*) and the gene *immune deficiency* (*imd*) (Imler and Hoffmann, 2000). *Toll*-like genes have also been identified in other insects including mosquitoes and the Tsetse fly, *Glossina papalis papalis* (Luo and Zheng, 2000). In *Drosophila*, genetic studies indicate that Toll-, 18w- and imd-mediated signaling pathways activate different suites of antimicrobial genes. However, Toll does not appear to be a legitimate PRR, because a protealytically cleaved form of the *spatzle* (*spz*) gene product is its only known extracellular ligand (Levashina et al., 1999). One potential humoral PRR involved in initiating the proteolytic cascade that results in cleavage of Spatzle and activation of Toll is a peptidoglycan recognition protein that may recognize gram-positive bacteria (Michel et al., 2001). Analysis of the *Drosophila* genome database further reveals the presence of at least 12 genes with sequence homology to known peptidoglycan recognition proteins (Werner et al., 2000). Several of these genes have transmembrane domains that suggest they are cell-surface receptors, but to our knowledge no functional

experiments have been conducted on any of these factors. The humoral PRR hemolin lacks a transmembrane region but membrane extracts from hemocytes suggest this factor may exist in a membrane-associated form in Lepidoptera (Bettencourt et al., 1997).

Another potential class of cellular PRRs involved in recognition of microbes is the integrins. This large family of (α,β)-heterodimeric transmembrane proteins are produced by metazoans in a variety of tissues and cells. Ligand specificity is determined by the specific α- and β-chain and can vary greatly among family members. In mammals, most integrins bind extracellular matrix (ECM) proteins like laminin, collagen IV, and fibronectin that contain the recognition sequence Arg–Gly–Asp (RGD) (Gonzalez-Amaro and Sanchez-Madrid, 1999). However, some integrins, like CD11b/(CD18), bind bacterial cell wall components like LPS. The *Drosophila* genome contains five α- and two β-subunits, and three complete integrins have been functionally characterized (Hynes and Zhao, 2000). Each of these integrins is essential for developmental processes but none have been implicated in recognition of microorganisms. However, studies with the Mediterranean fruit fly, *Ceratitis capitata*, indicate that soluble RGD decreases phagocytosis of *E. coli* by hemocytes, as does a monoclonal antibody directed against a murine integrin β-subunit (Foukas et al., 1998).

4.3. Recognition of other foreign targets and altered self

It is not surprising that insects have evolved PRRs that recognize bacteria and fungi, because these microorganisms have surface proteins that are biochemically distinct from the hosts they infect. However, insect hemocytes also recognize many other targets that are less molecularly distinct from themselves. These range from other biological entities like protozoans, nematodes, insect parasitoids and xenobiotic transplants of tissues from other insect species (Lackie, 1988; Strand and Pech, 1995; Gillespie et al., 1997) to self or conspecific tissues that have a damaged basement membrane (BM) (Rizki and Rizki, 1980a, 1980b; Karacali et al., 2000). Hemocytes also recognize abiotic targets like nylon, latex, and chromatography beads that are made of materials insects are unlikely to ever have encountered during evolutionary time. These observations emphatically underscore that the insect immune system recognizes a much broader suite of molecules than just the PAMPs on microorganisms. The question is, how insects are able to do this in the absence of an acquired immune system? Few answers are currently available, but we think it highly unlikely that insects express receptors individually tailored to recognize the eclectic assemblage of targets hemocytes are able to phagocytose or encapsulate. Instead we would hypothesize that insects more likely rely on receptors with promiscuous binding properties that interact with a variety of pattern molecules normally not encountered in the hemocoel.

Consider first the recognition of altered self tissues. While still poorly defined, the BM of insects and vertebrates contains many components including proteoglycans, insoluble fibrous proteins, like Type IV collagen, and soluble multiadhesive matrix proteins. BM lines the hemocoel and all internal organs of insects, and hemocytes normally do not attach or attach only weakly to this substrate. However, several lines of evidence indicate that hemocytes are sensitive to changes in this matrix. First, hemocytes are known to be involved in remodeling of the BM which is broken down and rebuilt during metamorphosis (Wigglesworth, 1973; Ball et al., 1987; Nardi and Miklasz, 1989; Nardi et al., 2001). Granular cells appear to be the main hemocyte involved in secretion of BM components in Lepidoptera (Nardi and Miklasz, 1989; Nardi et al., 2001), and, in the dipteran *Sarcophaga peregrina*, a 200 kDa surface protein has been implicated in binding of granular cells to basal lamina during metamorphic reprogramming (Kurata et al., 1991, 1992). Second, a number of studies report that hemocytes aggregate at external wound sites or encapsulate tissue transplants from conspecifics if they have a damaged BM (Rizki and Rizki, 1980b; Lackie, 1988). The *Drosophila* melanotic tumor mutants *tu-W* and $tu(l)Sz^{ts}$ similarly exhibit an autoimmune response whereby hemocytes encapsulate their own fat body. Studies with these mutants suggest that encapsulation is due to recognition of an altered BM (Rizki and Rizki, 1974, 1980a).

Taken together, these trends argue that detection of altered self by hemocytes involves changes to the BM. The lack of response by hemocytes to undamaged BM could be due to either the absence of surface receptors on hemocytes that recognize BM components, or to the presence of molecules on BM that inhibit hemocyte binding. In vertebrates, inhibitors of cell adhesion such as thrombospondin, tenascin, SPARC, and various mucopolysaccharides localize to BM (Greenwood et al., 1998; Iozzo, 1998). Hemocytes from *P. includens* and *Manduca sexta* likewise adhere poorly or not at all to commercially available BM preparations like Matrigel or highly purified polysaccharides like reagent-grade agarose (Pech et al., 1995; Lovallo and Cox-Foster, 1999; Strand and Clark, 1999). In contrast, hemocytes may bind to sites where the BM is altered, because of structural modifications to glycoconjugants or because novel ligands are presented. An example of the former occurs in the wax moth, *Galleria mellonella*, which normally does not encapsulate self tissues but rapidly encapsulates self tissues pretreated with neuraminidase that removes sialic acid residues (Karacali et al., 2000). Removal of sialic acid could either unmask antigens recognized as foreign or removal of these residues alters

the glycoprotein in a way that allows soluble or cell-surface receptors to bind.

A possible example of hemocytes recognizing altered self by novel molecules is phagocytosis of apoptotic bodies. In *Drosophila*, the surface receptor Croquemort is specifically expressed on plasmatocytes, and Croquemort null mutants are unable to phagocytose apoptotic cells (Franc et al., 1996, 1999). Although the ligand on apoptotic cells recognized by Croquemort is unknown, apoptotic and necrotic cells present novel surface molecules like phosphatidyl serine (PS) that are absent on healthy cells. Croquemort also shows some sequence similarity to CD36, which is a type of scavenger receptor (SR) found on mammalian macrophages. SRs are defined primarily by their promiscuous binding of characteristic, though not invariant, polyanionic ligands like modified lipoproteins, anionic polysaccharides, and anionic phospholipids. Some SRs are also able to bind prokaryotic PAMPs like LPS and lipoteichoic acid, nucleic acids, and even abiotic surfaces like glass (Platt and Gordon, 1998). In vertebrates, such ligand promiscuity implicates SRs in recognition of molecules associated with both altered self and foreign invaders. However, Croquemort does not appear to be a typical SR, because it does not bind the standard ligands recognized by other family members (Franc et al., 1996). Plasmatocytes from Croquemort null mutants also retain the ability to phagocytose bacteria (Franc et al., 1999), suggesting that this receptor may function primarily in recognition of apoptotic cells. A novel scavenger receptor (dSRC-I) has been identified from *Drosophila* that does bind polyanionic ligands (Pearson et al., 1995). However, dSRC-I does not as yet have a known function in immunity.

The molecular mechanisms by which hemocytes recognize altered self may also be involved in recognition of foreign targets, like xenotransplants and parasitoids, that are also of insect origin. Differences in BM and other surface components are likely to increase with phylogenetic distance between species. Consistent with this assumption, hemocytes tend to encapsulate xenobiotic transplants from phylogenetically more distant species of insects more readily than tissues from closely related species (Lackie, 1988). The similarity of the surface components of parasitoids to the insect host's own BM may help determine the ability of the host to mount a hemocytic defense response. For example, hemocytes from obligate hosts usually do not bind to the eggs or larvae of parasitoids. However, mechanical or enzymatic disruption of surface factors or membranes from the parasitoid egg or larva almost always results in rapid encapsulation (Schmidt et al., 2001). Unfortunately, the specific surface features and receptors involved in these interactions are unknown.

Unlike the complexities of BM and other biological surfaces, chromatography beads are made of well-defined materials that could provide insight about the types of surfaces hemocytes recognize (Vinson, 1974; Lackie, 1983; Wiesner, 1992; Zahedi et al., 1992; Lavine and Strand, 2001a). Yet, comparisons among species identify few unifying themes as to the types of beads hemocytes most avidly encapsulate. For example, Lavine and Strand (2001a) used 19 different bead types as encapsulation targets in *P. includens*. Encapsulation assays revealed that some beads are recognized by granular cells and then encapsulated by plasmatocytes, others are encapsulated directly by plasmatocytes if opsonized by humoral molecules in plasma, and some beads are never encapsulated. Beads with certain functional groups (sulfonic, diethylaminoethyl, and quaternary amine) are encapsulated more readily than other functional groups tested, and positively charged beads are better encapsulated than negatively or neutrally charged beads. In contrast, targets with carbohydrate moieties on their surface (neutral dextran, neutral agarose, *N*-acetyl-glucosamine, *N*-acetyl-galactosamine) are the most poorly encapsulated targets. These results indicate that: (1) both humoral and cellular receptors are involved in recognition of abiotic targets just as they are with microorganisms, and (2) the importance of humoral and cellular receptors differs as a function of bead matrix, charge and functional group. However, when these beads were tested in other noctuid moths, some responses were similar to *P. includens* while others differed greatly. For example, CM-Sephadex is rarely encapsulated by *P. includens* but is always encapsulated by *Spodoptera frugiperda*, and DEAE-Sepharose is strongly encapsulated by *P. includens* but is rarely encapsulated by *S. frugiperda*. Thus, while we would expect that insects in the same family use the same types of molecules as recognition receptors, affinities for specific ligands can still greatly differ.

One factor potentially involved in recognition of chromatography beads is a glutamine-rich protein purified from the beetle *Tenebrio molitor* (Cho et al., 1999). This protein exists as two isoforms, with molecular masses of 56 kDa and 48 kDa. Neutralization experiments using antibodies against each isoform suggest that both are required for opsonization of targets. Studies with *P. includens* indicate that, in the absence of plasma, addition of the tripeptide RGD to culture medium dose-dependently inhibits binding of *P. includens* plasmatocytes (strongly) and granular cells (weakly) to polystyrene. This indicates that, similar to some microorganisms (see above), recognition of some abiotic targets also involves an RGD-dependent mechanism (Pech and Strand, 1996). Recent studies further indicate that *P. includens* granular cells and plasmatocytes express three alpha and one beta integrin subunit (Lavine and Strand, 2001b). Functional studies are still in progress but expression of these subunits by hemocytes strongly suggests that integrins play a role in recognition of both microbial and abiotic targets.

5. Hemocytic-mediated effector responses

5.1. Phagocytosis

Phagocytosis is a widely conserved cellular response that occurs in many Protozoa and all metazoan phyla. It has been most extensively studied in mammalian leukocytes like macrophages (Aderem and Underhill, 1999). An extensive review of the mechanisms controlling formation and maturation of the phagosome in macrophages is beyond the scope of this article. However, the process of phagocytosis begins when a target binds to its cognate receptor. This activates signaling cascades that regulate formation of a phagosome and target ingestion via an actin polymerization-dependent mechanism. The phagosome matures into a phagolysosome when effector molecules are introduced by vesicle fusion events. These effector molecules then kill and/or degrade the target. Phagocytosis has not been extensively studied in insect hemocytes. However, antibodies to some mammalian signaling molecules that regulate phagocytosis cross-react with proteins in hemocyte lysates from the fruit fly, *C. capitata*. This has led to the suggestion that the signal transduction pathways regulating phagocytosis are conserved among insects and mammals (Foukas et al., 1998; Metheniti et al., 2001).

Mammalian phagocytes are well known to generate reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) which are released into the phagosome or extracellularly, and which are toxic to a variety of microorganisms (Nathan and Hibbs, 1991; Robinson and Badwey, 1994). ROI or RNI have also been detected in the hemolymph and/or hemocytes of many insects. For example, increased superoxide levels occur in the hemolymph and hemocytes of the West Indian leaf cockroach *Blaberus discoidalis* after exposure to heat-killed *E. coli*, LPS, and laminarin (Whitten and Ratcliffe, 1999). *E. coli* growth is also enhanced when ROI scavengers or inhibitors of the mammalian respiratory burst are added to *B. discoidalis* hemolymph, suggesting that ROI is an important killing mechanism (Whitten and Ratcliffe, 1999). Although not directly related to phagocytosis, infection of the mosquito *Anopheles stephensi* by *Plasmodium berghei* and *Plasmodium falciparum* increases nitrite/nitrate levels which are end products of the RNI generating conversion of L-arginine to L-citrulline by nitric oxide synthase in hemolymph (Luckhart et al., 1998). *Plasmodium* infection also induces increased expression of an *A. stephensi* nitric oxide synthase gene (Luckhart et al., 1998; Luckhart and Rosenberg, 1999). Addition of L-arginine to *A. stephensi*'s diet decreased the mean number of *Plasmodium* ookinetes in infected mosquitoes, while addition of an L-arginine analog to the diet resulted in increased numbers of ookinetes relative to controls (Luckhart et al., 1998).

Some of the effects of ROI and RNI may be due to their roles in immune-related signal transduction pathways, rather than to any direct cytotoxic effects on parasites or pathogens. In mammals, ROI such as H_2O_2, and RNI such as NO, function as second messengers in signal transduction pathways that include activation of NF-κB (Kaul and Forman, 1996). These types of activities have been poorly studied in insects, although exogenous H_2O_2 is reported to increase expression of the antimicrobial peptide attacin in the giant silkmoth, *Hyalophora cecropia* (Sun and Faye, 1995). Superoxide anion, H_2O_2 and NO have also been detected during cellular immune responses in *Drosophila* (Nappi et al., 2000), but whether these factors are related to NF-κB activation is unknown.

Although the fat body is usually considered the most important source of antimicrobial peptides, hemocytes also produce these molecules in some insects (Lowenberger, 2001). To our knowledge, antimicrobial peptides from insect hemocytes have not been implicated in killing of phagocytosed bacteria. However, studies with a mollusc indicate that hemocytes release the antibacterial peptide mytilin into both the medium and phagolysosomes when phagocytosing bacteria (Mitta, 2000).

5.2. Encapsulation and nodulation

Once a target is recognized, formation of a capsule or nodule requires that circulating hemocytes change from non-adhesive to adhesive cells that are able to bind to the target and one another. Changes in the adhesive state of mammalian immunocytes are regulated by signaling molecules (cytokines), cell adhesion molecules, and their cognate receptors. For example, specific chemokines activate monocyte adhesion by inducing the rapid export of adhesion molecules from cytoplasmic granules to the cell surface (Baggiolini et al., 1997). In lepidopterans like *P. includens*, binding of granular cells or humoral PRRs to a foreign surface similarly induces a rapid change in the adhesive state of plasmatocytes which, as previously discussed, are the main capsule-forming cells.

The most potent plasmatocyte activator identified to date is a 23-amino-acid cytokine called plasmatocyte spreading peptide (PSP) (Clark et al., 1997) (Fig. 3). Both purified and synthetic PSP induce plasmatocytes to aggregate or adhere to foreign surfaces within seconds at concentrations $\geq$100 pM. PSP is expressed as a propeptide of 142 residues with the PSP sequence located at the C-terminus (Clark et al., 1998). This biologically inactive precursor is then cleaved by an unknown protease to release the mature peptide. The structure of PSP consists of a disordered N-terminus (residues 1–6), and a well-structured core (residues 7–22) stabilized by a disulfide bond, hydrophobic interactions, and a short β-hairpin turn (Volkman et al., 1999). PSP homologs have been identified from a number of other lepidopterans,

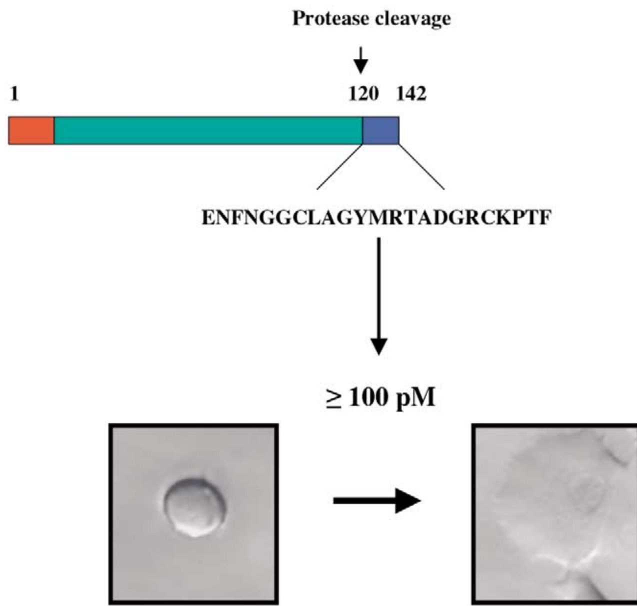

Fig. 3. Processing and activity of the cytokine plasmatocyte spreading peptide (PSP). The 142-amino-acid PSP precursor protein consists of a strongly hydrophobic signal sequence domain at its N-terminus (red), a central domain (green), and a C-terminal domain that encodes PSP (blue). Two basic residues (Lys^{117} and Arg^{119}) N-terminal from Glu^{120} serve as potential recognition sties for endoproteolytic cleavage of the precursor protein to yield the mature peptide. Dose–response studies indicate that PSP dose-dependently induces the attachment and spreading of plasmatocytes to foreign surfaces and one another at concentrations ≥100 pM. At concentrations of ≥10 nM, PSP induces plasmatocytes to aggregate or adhere in less than 30 s.

and in some cases these homologs have also been shown to be plasmatocyte activators (Wang and Jiang, 1999; Strand et al., 2000). Based on the consensus sequence of their N-termini (Glu–Asn–Phe–X–X–Gly), these molecules are referred to as the ENF-peptide family (Strand et al., 2000). Comparison with other proteins reveals that, despite sequence identity at only four positions, the core region of PSP adopts a very similar structure to the C-terminal subdomain of human epidermal growth factor (hEGF) and the fifth domain of the anticoagulant protein thrombomodulin (hTM5). In contrast, the N-terminus of PSP is similar only to other members of the ENF-peptide family (Volkman et al., 1999). Mutational analysis indicates that specific residues in both the disordered and ordered domains of the molecule are required for biological activity (Clark et al., 2001a, 2001b). Several other partially purified factors have been reported to induce changes in hemocyte adhesion (Chain and Anderson, 1982; Davies et al., 1988). Injection of certain inhibitors of eicosanoid biosynthesis into *M. sexta* reduces nodulation of bacteria, leading to the suggestion that cyclo- and lipoxygenase products may also regulate hemocyte adhesion responses (Miller et al., 1994). However, whether any of these factors are signaling molecules or possible homologs of the ENF-peptide family is unknown.

The next question of interest is how these extracellular signaling molecules induce a change in the adhesive state of plasmatocytes. Studies with PSP suggest that binding to its cognate receptor causes plasmatocytes to export cytoplasmically stored adhesion molecules to their surface (Strand and Clark, 1999). In vertebrates, four main families of receptors mediate cell adhesion events: cadherins, Ig-superfamily members like N-CAMs, selectins and integrins. The identification of multiple integrin subunits and the fact that soluble RGD inhibits plasmatocyte adhesion circumstantially suggest that integrins are involved in plasmatocyte binding during encapsulation (Pech et al., 1995; Lavine and Strand, 2001b). Integrins have also been implicated in regulating adhesion of crustacean and molluscan hemocytes (Johansson, 1999). Neutralization experiments with the antibody M13 further suggests that an unidentified protein on the surface of plasmatocytes from *M. sexta* is also required for aggregation and binding to foreign surfaces (Wiegand et al., 2000).

Examination of capsules from a number of species indicates that termination of an encapsulation response occurs when a BM-like layer appears on the capsule's periphery (Grimstone et al., 1967; Pech and Strand, 1996; Liu et al., 1998). In *P. includens*, this BM-like layer is produced by a monolayer of granular cells that attaches to the periphery of the capsule at the end of an encapsulation response (Pech and Strand, 1996, 2000). The signal(s) from plasmatocytes that recruits granular cells to the periphery of the capsule is unknown, but the role of granular cells in terminating capsule formation is notable as granular cells are also the main hemocyte type involved in secretion of the BM components that line the hemocoel (see Section 4.3). In effect, the periphery of the capsule likely assumes the characteristics of intact BM, which creates a self surface to which plasmatocytes no longer bind. In *H. cecropia* and *M. sexta*, the Ig-related protein hemolin inhibits hemocyte aggregation (Ladendorff and Kanost, 1991; Bettencourt et al., 1997) but whether this activity has any role in capsule termination is unknown.

Once a capsule has formed, the encapsulated organism almost always dies. Several factors including asphyxiation, production of toxic quinones or hydroquinones via the prophenoloxidase (PPO) cascade, ROI and RNI, and antibacterial peptides have been suggested to function as killing agents (Salt, 1970; Gillespie et al., 1997; Nappi et al., 2000). However, as with phagocytic responses by insect hemocytes, there is little direct experimental evidence to support a role for any of these factors in killing encapsulated targets. For example, resistant strains of *Drosophila* that encapsulate the parasitoid *Leptopilina boulardi* produce increased levels of superoxide anion, H_2O_2 and nitrite compared with susceptible strains (Nappi et al., 1995; Nappi and Vass, 1998; Nappi et al., 2000). Yet, superoxide anion and H_2O_2 do not appear to

be toxic to *L. boulardi*, and it is equally unclear whether elevated levels of these factors actually exist in the capsule itself. Although some insects produce capsules that never melanize, capsules formed by *Drosophila* and many other species often do. This has led to the suggestion that cytotoxic quinoid intermediates produced during melanization reactions may serve as killing agents (Nappi et al., 2000). Again, though, no studies we are aware of support this experimentally.

5.3. The role of hemocytes in wounding and clotting

PAMPs like LPS activate a complicated proteolytic cascade that results in rapid clotting of hemolymph in crustaceans and in chelicerates. Elegant studies in the horseshoe crab, *Limulus polyphemus*, have identified several important humoral- and hemocyte-produced components of this clotting cascade that ultimately result in precipitation of the protein coagulogen into insoluble coagulen (Muta and Iwanaga, 1996; Iwanaga et al., 1998). The hemolymph of some insects also coagulates upon external wounding but little is known about these clotting systems save their dependence on both humoral factors and hemocytes. A lectin and the serine protease scolexin have been implicated in clotting of hemolymph in *M. sexta* (Minnick et al., 1986; Finnerty et al., 1999), while a multidomain protein with some similarity to the vertebrate clotting protein von Willibrand factor has been suggested to play a role in clotting in *B. mori* (Kotani et al., 1995). Hemocytes also contribute to clot formation in insects by aggregating at wound sites (Gregoire, 1974). Damaged epidermal cells release a partially purified protein named hemokinin that induces rapid aggregation of hemocytes in *H. cecropia* (Cherbas, 1973). Whether hemokinin is a signaling molecule or an adhesion protein is unknown.

6. Coordination of the insect immune system

The preceding discussion makes clear that the humoral and cellular arms of the insect immune system both contribute to defense against pathogens. This is well illustrated in *Drosophila*, where disrupting phagocytosis does not reduce survival of wild-type flies injected with *E. coli*, but does reduce survival of *imd* flies that are unable to synthesize antibacterial peptides in response to gram-negative bacteria (Elrod-Erickson et al., 2000). Other *Drosophila* mutants, like *domino*, reduce the number of circulating hemocytes while *bc* inhibits hemolymph phenoloxidase activity. *Imd*, *domino*, *bc*, and *bc/imd* double mutant flies all show similar survival rates to wild-type flies when injected with bacteria. In contrast, *domino/imd* and *domino/bc* double mutants, and *domino/imd/bc* triple mutants all show greatly increased mortality (Braun et al., 1998). While uncharacterized pleiotropic effects could clearly contribute to these outcomes, these results overall suggest that both humoral and cellular immunity are essential for survival.

Few pathways linking humoral and cellular defense responses have been identified, although several lines of evidence suggest such linkages exist. For example, hemocytes do not appear to be an important source of antimicrobial peptides in *Drosophila* as *domino* mutants show wild-type levels of synthesis after injection with bacteria or fungi (Braun et al., 1998). However, *domino* and other hematopoietic mutants like *l(3)hem* show decreased levels of the antimicrobial peptide diptericin when infected per os, which suggests that hemocytes may coordinate antimicrobial peptide responses in the absence of physical injury (Basset et al., 2000). Migration of hemocytes to sites of injury or infection may also contribute to humoral defense responses. As previously noted, bacterial PAMPs stimulate hemocytes from *Limulus* and molluscs to rapidly discharge clotting factors and/or antimicrobial peptides (Iwanaga et al., 1998; Mitta, 2000), while hemocytes from insects likely release PPOs, proteases and other immune-related molecules (Jiang et al., 1997; Wittwer and Wiesner, 1998; Muller et al., 1999). A role for the PPO cascade in pattern recognition and defense is also suggested from studies with certain mosquitoes that form melanotic as opposed to cellular capsules around foreign targets (Richman and Kafatos, 1996; Gorman and Paskewitz, 2001).

A final point of interest is the potential cross-talk that may occur between the immune and nervous system. Studies with vertebrates indicate that several neuropeptides and biogenic amines produced in the central nervous system (CNS) are also produced and have biological effects on immunocytes (Roitt et al., 1993). For example, neural signaling molecules like the opioid peptide Met-enkephalin stimulate lymphocyte proliferation as well as migration and release of pro-inflammatory cytokines by macrophages (Salzet et al., 2000). The biological effects of Met-enkephalin are unlikely indirect as vertebrate immune cells have opioid receptors and produce the appropriate proteases to process opioid precursor proteins, like proenkephalin, to their mature peptides. Interestingly, opioid precursor proteins also encode antibacterial peptides, like enkelytin, that are released along with opioid peptides after processing. This arrangement of signaling and killing factors could result in both an immediate antibacterial response as well as recruitment of immune cells and signaling of danger to the CNS.

Proenkephalins containing opioid peptide sequences and enkelytin have been identified in molluscs and leeches, but not in insects (Stefano et al., 1998). However, a partially analogous situation exists in the case of ENF peptides, like PSP, that are processed from precursor proteins and are expressed in the CNS, fat body, and immune cells (Clark et al., 1998; Hayakawa and Nogu-

chi, 1998). Besides being potent activators of plasmatocytes, ENF peptides also have important effects on larval growth (Hayakawa, 1990; Strand et al., 2000). The titers of ENF-peptide family members like growth-blocking peptide (GBP) fluctuate in concert with the larval molting cycle, which circumstantially suggests a role for this molecule in maintenance of normal homeostatic activity. However, stress factors like parasitism or low rearing temperatures induce a rapid and significant elevation in hemolymph GBP titer that correlates with reductions in larval growth rates and delays in molting (Hayakawa, 1990; Ohnishi et al., 1995). Given that insects often exhibit delays in growth after wounding or infection (Lackie, 1988; Strand and Pech, 1995), it is tempting to speculate that, in addition to maintenance functions, ENF peptides also serve as inflammatory mediators following noxious perturbations. Localized release from hemocytes and/or rapid processing of propeptide already in circulation would clearly promote rapid adhesion of plasmatocytes to sites of injury or infection. However, decreased larval mobility and delays in growth due to a systemic elevation in peptide titer could also facilitate the healing process before the cuticle is next shed.

7. Conclusions

Considerable strides have been made in the last few years to develop new tools for identifying hemocytes and understanding their differentiation. Progress has also been made in developing methods for collecting cells and conducting manipulative experiments on isolated sub-populations. These kinds of studies have enhanced our understanding of the role different hemocytes play in specific defense responses. The concept of insects recognizing microorganisms using PRRs that bind conserved microbe-specific surface factors logically extends ideas originally conceived with the innate immune system of vertebrates in mind. In the absence of an acquired immune response, however, recognition of more closely related foreign targets, like parasitoids, or alterations to self are more difficult to explain, as these targets lack surface molecules distinctly different from self tissues. Understanding how the insect immune system is able to distinguish such entities from self is an important question for the future. Our understanding of the signaling factors that regulate hemocyte-mediated defense responses like encapsulation is also at an early stage. Genetic screens in model species like *Drosophila* offer one approach to identification of these signaling factors, as might expressed sequence tag (EST) or other genomic screens targeted at specific defense responses like encapsulation. However, such molecular approaches will still have to be complemented by bioassay-driven, biochemical approaches since many studies already indicate that proteolytic cascades and other post-translational processing events are critical to production of biologically active molecules. Lastly, ROI and other factors that hemocytes might produce to kill phagocytosed or encapsulated organisms have been repeatedly discussed in reviews, but scant experimental progress has actually been made on the subject in the past 10 years. The availability of hemocyte markers, along with in vitro methods for manipulating encapsulation responses, opens the door for developing appropriate bioassays to identify hemocyte factors involved in killing protozoan and metazoan parasites.

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