Innate immunity of insects

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Insects are particularly resistant to microorganisms. Their host-defense system relies on several innate reactions: upon injury, the immediate onset of two proteolytic cascades leading to localized blood clotting and to melanization, the latter process involving production of cytotoxic molecules (namely reactive oxygen intermediates); the phagocytosis of bacteria and the encapsulation of larger parasites by blood cells; the induced synthesis by the fat body of a battery of potent antimicrobial peptides/polypeptides which are secreted into the hemolymph where they act synergistically to kill the invading microorganisms. The insect host defense system shares many of the basic characteristics of the mammalian acute phase response, especially at the level of the coordinate control of gene expression, where similar cis-regulatory and inducible transactivators appear to play key functions. The powerful techniques developed to study the genetics of Drosophila provide a unique opportunity to dissect the development and differentiation of this primordial immune system and may contribute to our understanding of the innate immune response in higher organisms.

Introduction

Insects have been remarkably successful in evolution. Current estimates are that they account for 90% of all extant animal species; with the exception of the seas, insects colonize all ecological niches. Consequently, they are confronted by an extremely large variety of potentially harmful microorganisms. At the turn of this century, researchers had already fully appreciated that insects are able to build up an efficient defense against microorganisms and by 1930 it was understood that this defense involved both a cellular and a humoral facet [1]. So far the cellular facet has been analyzed essentially in morphological terms, and we have little information about the molecular mechanisms that mediate the recognition of invading microorganisms and their subsequent phagocytosis or encapsulation. In contrast, we have gained a better insight into the humoral facet. We have learned that a septic injury induces the insect fat body (a functional homologue of the mammalian liver) and some blood cells (evocative of the myeloid cell lineage) to rapidly and transiently synthesize a battery of potent antibacterial and antifungal peptides that are released into the blood and synergistically act to destroy the invading microorganisms (see reviews in [2,3,4,5]). This response lacks both specificity and memory and is an innate non-adaptive defense reaction that exhibits striking similarities to the vertebrate acute-phase response [3,4]. This review will first focus on recent developments in the study of the humoral response, namely on the structure of the immune-induced antibacterial peptides and on the control of the coordinate expression of their genes. In all insects, an immediate result of septic injury is the induction of two proteolytic cascades that lead to clotting and melanization and it is believed that these reactions play a major role in defense. In addition, proteins situated upstream in these cascades can bind microbial determinants and thus serve as recognition molecules. Next, the two cascades and the problem of recognition of infectious non-self will be analyzed in a section devoted to the induction of the immune response. Finally, potentially interesting fields of research on the cellular reactions will be presented and discussed.

Inducible antibacterial and antifungal peptides

Cecropins and defensins

More than 50 antibacterial molecules, which are all cationic peptides or polypeptides, have now been isolated from the blood of immune-challenged insects [5]. Two well defined antibacterial families have been studied in detail: the cecropins, which act on Gram-positive and Gram-negative bacteria [2], and the insect defensins, which act on Gram-positive bacteria [6]. Cecropins are 4 kDa peptides present in Lepidoptera (an order of insects that includes moths and butterflies) and Diptera (an order of insects containing the two-winged flies). They have a hydrophobic amidated

Abbreviations

LPS—lipopolysaccharide; Dif—dorsal-related immunity factor.
carboxyl terminus and a hydrophilic basic amino-terminal region connected by a flexible hinge; both the amino- and carboxyl-terminal regions adopt a mainly helical formation [2,7,8]. Cecropins are membrane-active antibiotics and experiments conducted with artificial membranes have indicated that they have channel-forming properties and can permeabilize the lipid bilayer [9].

Insect defensins (sapecins) are widely distributed among insects [5-6]. They are 4 kDa peptides consisting of three distinct domains: a flexible amino-terminal loop, a central amphipathic α-helix and a carboxy-terminal antiparallel β-sheet. The α-helix is stabilized via two disulfide bridges linked to one of the strands of the β-sheet, and the amino-terminal loop is linked via one disulfide bridge to the other β-strand [10,11]. This structural organization is evocative of the channel blocker toxin charybdotoxin from scorpion venom, which also has a well defined central α-helix linked to a carboxy-terminal β-sheet by two disulfide bridges [12], but differs markedly from mammalian defensins which are all β-sheet in structure [13*]. Insect defensins form voltage-dependent channels, leading to the rapid leakage of K+ and other ions [14*] (see also [15] for the activity of the sapecin B isoform).

Proline-rich and glycine-rich peptides

To date, the other characterized inducible antibacterial peptides are either proline-rich peptides or glycine-rich polypeptides. The proline-rich peptides are small in size (15–34 residues) and have high proline and arginine contents. Among the new members of this family that have recently been discovered, drosocin and pyrthocoracin are remarkable in that they carry an O-glycosylated substitution that is necessary for their full biological activity [16*,17]. The proline-rich peptides are primarily active against Gram-negative but are also active against Gram-positive bacteria. Their mode of action has only been investigated using apidaecin, which was found not to be membrane-active. D-enantiomers of apidaecin are devoid of biological activity, suggesting that the effect of this peptide involves stereospecific recognition of a chiral cellular target [18], in contrast to cecropins [19] and insect defensins [14*].

The glycine-rich family of inducible antibacterial molecules includes several 9–30 kDa polypeptides, all of which have a higher than average percentage of glycine residues (10–22%). They are predominantly active on Gram-negative bacteria. Although two members of this family (attacins [20] and sarcotoxins II [21]) were described several years ago, several more members have been reported recently: the 74-residue coleopteracin from the Coleopteran Zophobas [22] (18% glycine residues), the 133-residue hemipteracin (found in Hemiptera) [17] (15% glycine residues) and the 94-residue hymenoptaecin (found in Hymenoptera) [23] (20% glycine residues). The lethal effects of hymenoptaecin against Escherichia coli were shown to be secondary to sequential permeabilization of the outer and inner membranes [23]. Diptericins are peculiar in that they contain a short proline-rich domain at their amino-terminal and a large (~60 residues) glycine-rich domain at their carboxyl terminus. Both domains carry an O-glycosylated substitution that is necessary for full biological activity (P Bulet, et al., unpublished data).

Other peptides/polypeptides

Other peptides or polypeptides participating in the insect defense system include the ubiquitous lysozyme and the peptides produced in the genital tracts of the male Drosophila (andropin, a 34-residue peptide [24]) and female Ceratitis capitata (ceratotoxins, 40-residue, lysine-rich peptides [25]). In Drosophila, several lysozyme isoforms are found, mainly in the digestive tract, and their expression is repressed upon infection [26]. In contrast, immune challenge of Manduca leads to an increase in lysozyme gene transcription, predominantly in the fat body [27]. All the antibacterial peptides or polypeptides mentioned so far are inactive at their physiological concentrations (0.1 to 10 μM) against eukaryotic cells, including fungi, although Drosophila also produces a strongly antifungal peptide devoid of antibacterial activity in response to immune challenge. This peptide, drosomycin, has 44 residues and contains eight cysteine residues engaged in four intramolecular disulfide bridges [28*]. As with most of the inducible antibacterial peptides, drosomycin is synthesized in the fat body and secreted into the hemolymph. Drosomycin shares significant homology with a family of 5 kDa cysteine-rich plant antifungal peptides that were recently isolated from seeds of Brassicaceae [29]. This emphasizes that plants and insects can rely on similar molecules in their innate defense. A constitutively expressed 27 kDa histidine-rich protein that exhibits antifungal activity under certain test conditions was isolated from Sanaphaga hemolymph [30].

Immune gene expression

Several genes encoding inducible antibacterial peptides have been cloned recently in Drosophila (reviewed in [31]), Hyalophora cecropia and Sanaphaga peregrina (reviewed in [4]). As a rule, these genes are silent in the absence of immune challenge. Injury and injection of bacteria or fungi, or their cell wall determinants, can induce the expression of these genes, usually within half an hour. The intensity of transcription peaks from 12 to 48 h, after which time transcription decreases. The major site of antibacterial-peptide gene expression is the fat body, but some blood cells (including a tumorous blood cell line [32]) also express these genes. Additionally, the larval integument of bacteria-challenged Lepidoptera has been shown to express the cecropin genes [33].
To date, although a few of the characterized genes contain small introns, many are intronless. The corresponding messengers mostly encode conventional precursor polypeptides, with a signal peptide, a short or moderately long (2-35 residues) prosequence, and the sequence of the antibacterial peptide. A remarkable exception is the precursor for the proline-rich apidaecin which contains multiple copies of different isoforms of bioactive apidaecins.

The upstream regions of the genes encoding inducible antibacterial peptides contain a number of motifs showing sequence homology to cis-regulatory elements recently characterized within the promoters of acute-phase response genes in mammals (reviewed in [3,4], see also [35]). Some of the more prominent motifs are κB-related elements [36], sequences related to the interleukin-6 response elements (NF-IL6 binding motifs) and interferon-γ responsive half-sites [35]. The relevance of the κB-related sites to the induced expression of the insect genes was ascertained by several methods (DNase I protection and electrophoretic mobility-shift assays) but particularly, in the case of Drosophila, by the establishment of transgenic fly-lines carrying constructs in which reporter genes were fused to wild-type or variously mutated promoter sequences from inducible antibacterial genes [37**,38**]. Reporter genes carrying a minimal promoter downstream of multimerized κB-related elements can be induced by bacterial challenge, not only in transgenic flies but also when the reporter genes are transfected into tumorous blood cells [37**,38**,39]. In the case of the diptericin gene, it must be noted that the κB-related sequences confer only a moderate level of expression (less than 5% of the normal response). Additional proximal regulatory elements, most probably the sequences homologous to mammalian interleukin-6 response elements and interferon-γ responsive half-sites, are necessary to upregulate expression [39]. These data are in agreement with several recent reports on the control of acute phase response genes in mammals that point to interactions of different regulatory elements [35,39].

The involvement of κB-related cis-regulatory motifs in the control of immune gene expression in insects has raised considerable interest and prompted the question of whether the transactivating proteins binding to these motifs in immune-challenged insects were also members of the Rel/NF-κB family, which mediates many of the innate immune responses in mammals. A first hint came from studies on immune-challenged Hyalophora cecropia that resulted in the partial characterization of an immune-responsive factor (Cecropia immune-responsiveness factor [CIF]) that was found to recognize Cecropia κB-related motifs and to share certain similarities with mammalian NF-κB. CIF appears to be a homodimer of a 65 kDa protein [40]. Recent studies have also identified a 59 kDa κB-related protein in the flesh fly, Sarcophaga peregrina [41]. Subsequently, it was shown in Drosophila that both dorsal, an established member of the Rel/NF-κB family, and Dif (for dorsal-related immunity factor), a novel member of this family, can bind to κB-related sites in the promoters of immune genes [42*,43*]. In transfection experiments of immunoresponsive tumors, the dorsal protein can sequence-specifically transactivate reporter constructs via κB-related promoter sequences [42*]. Furthermore immune challenge induces or upregulates the expression of the dorsal gene in fat-body cells of Drosophila, and leads to a rapid nuclear translocation of both dorsal and Dif proteins, which are predominantly cytoplasmic in the absence of challenge [42*,43*]. This result is consistent with the activation mechanism observed for the other members of the Rel/NF-κB family, which are sequestered in the cytoplasm by binding to proteins (I-κB or cactus) that inhibit their nuclear translocation (reviewed in [44]). This inhibition is released by various extracellular stimuli: in the case of mammalian lymphocytes binding of interleukin-1 to its transmembrane receptor leads to dissociation of I-κB from NF-κB [45], and in the case of dorsoventral patterning in the early Drosophila embryo, binding of the extracellular protein spatzle to the transmembrane receptor Toll initiates an intracellular regulatory cascade which leads to the dissociation of the I-κB homologue, cactus, from the dorsal protein (reviewed in [46]). Actually, recent data suggest that the nuclear localization of the dorsal protein during the immune response of Drosophila is controlled by a Toll signalling pathway similar to that directing the nuclear import of dorsal during dorsoventral patterning in the embryo [47]. The respective roles of dorsal and Dif in the immune response, the mechanisms of their activation, and their interactions within the transcription complex during the coordinate control of immune gene expression, are among the most challenging questions in this field.

**Induction of the immune response**

Injury in insects and the other Arthropods induces two major proteolytic cascades, which result in hemolymph coagulation and melanization. In insects, our knowledge of blood clotting and wound sealing is still mostly morphological, but a significant breakthrough in our understanding of the biochemistry of these mechanisms has come from studies on a member of another Arthropod class, the horseshoe crab Limulus (reviewed in [48**]). We can anticipate that essentially similar results will be reported for insects in the near future. The hemolymph of this species is very sensitive to bacterial lipopolysaccharide (LPS) which causes a rapid coagulation response. In response to LPS, the single hemocyte type of Limulus undergoes aggregation and release of granule contents into the hemolymph, where they form an insoluble gel. Four components have been characterized in this coagulation response that comprise a cascade of three serine protease zymogens (factor C, factor B, pro-clotting enzyme) and one clottable protein, coagulogen. Factor C, which is sensitive to LPS, contains five complement (C1r
and C1s)-related domains, an epidermal growth factor (EGF)-like domain, a C-type lectin domain and a putative amino-terminal LPS-binding domain. This structure is similar to that of the selectin family of cell adhesion molecules. A β-(1,3)-glucan mediated clotting pathway has also been characterized in the hemocytes of Limulus [48°] which in addition contain a cysteine-rich 8 kDa protein that is readily cross-linked intermolecularly by a transglutaminase that shows significant sequence similarities to the mammalian transglutaminase factors [48°].

Melanization is the result of the other proteolytic cascade that is induced upon injury. The key enzyme in this cascade is phenoloxidase, which is present as a precursor form, prophenoloxidase, in the hemocytes or in the plasma (reviewed in [49]; see also [50] for crustaceans). Activation of the phenoloxidase system occurs as a result of a Ca2+-requiring cascade involving serine proteases. It can be elicited by microbial components, namely β-(1,3)-glucan and peptidoglycan, that bind to proteins upstream in the cascade. Phenoloxidase has also been recently isolated in a cockroach [51]. The reason for the great interest in melanogenesis is that dopamine metabolism is an important source of cytotoxic molecules (semi-quinones and reactive oxygen species) that destroy intrahemocoelic microorganisms sequestered within melanotic capsules. Melanin itself probably also acts as a scavenger for free radicals, thus preventing the cytotoxic molecules from harming surrounding tissues (reviewed in [52]).

The coagulation and melanization cascades undoubtedly play a major role in the innate response of insects to infection. As cell wall components of microorganisms are among the elicitors of these cascades, they are part of the system by which infectious non-self molecules are recognized. Other recognition systems obviously exist and current efforts are devoted to the characterization of binding proteins that mediate phagocytosis by blood cells and induce the synthesis of immune peptides in fat-body cells. Several types of proteins that recognize β-(1,3)-glucan or LPS have been described (see for example [53]). In crayfish (Crustacea), a hemocyte receptor was partially characterized that binds a circulating glucan-binding protein after it has reacted with β-(1,3)-glucan [54]. Hemolin, a 48 kDa member of the immunoglobulin family which was isolated from the hemolymph of Lepidoptera, recognizes and binds to bacteria and possibly opsonizes them for phagocytosis [55,56]. Its role in the immune defense system is underlined by the observation that the expression of its gene is upregulated by immune challenge.

*Drosophila* macrophages in embryos and Schneider cells exhibit scavenger receptor mediated endocytosis with basic characteristics similar to those of mammalian scavenger receptors [57]. In mammals, these receptors exhibit an unusually broad ligand specificity, including polyanions, modified proteins, certain purino nucleotides, certain polysaccharides and phospholipids, and LPS. Also in *Drosophila*, fat-body cells and blood cells were recently shown to express a gene coding for a transmembrane receptor belonging to the CD36/rat lymphosomal integral membrane protein II (LIMP II) superfamly of mammals (N Franc, JL Dimarcq, F Golzené, JA Hoffman, M Lagueux, unpublished data). In mammals, CD36 in addition to its role as a cell adhesion molecule during clotting is also involved in the recognition of non-self or abnormal self molecules.

Although our information on recognition of infectious non-self molecules remains fragmentary, it is now generally assumed that the receptors recognize highly conserved microbial structures that are the products of complex biosynthetic pathways shared among broad classes of microorganisms (pattern recognition receptors [58]). The characterization of these receptors and the mechanisms by which they induce the immune responses is another challenging problem in this field of research.

### Cellular reactions

The cellular reactions of insects in response to invading microorganisms include a diversity of processes such as phagocytosis, cell aggregation, nodule formation and large-scale encapsulation. Although the two latter processes are known to usually involve localized melanisation, no significant progress has been made in recent years towards the understanding of the molecular mechanisms underlying these cellular reactions. This situation may change now that *Drosophila* blood cell lines are available that can be induced to mount an immune response [32,57,59]. Another area which might add momentum to studies on the molecular aspects of the blood cell response are investigations on melanotic tumors in *Drosophila*. This species contains predominantly one blood cell type in its circulation, the plasmocyte, which is phagocytotic and can differentiate into lamellocytes which encapsulate foreign materials such as parasites (reviewed in [60]). An additional blood cell type, the crystal cell, provides the phenoloxidase required for melanin formation in this capsule. In normal development, during the larval-pupal transition, the plasmocytes differentiate into lamellocytes which are involved in sequestering larval tissues during metamorphosis. Several mutant *Drosophila* strains have been described that exhibit a melanotic tumor phenotype. In these mutants, lamellocyte differentiation is induced by metabolic products from aberrant tissues; the lamellocytes encapsulate these tissues in a manner similar to the encapsulation of foreign objects. This process, referred to as melanotic tumor formation, is often considered to be an autoimmune response, as melanotic tumors are non-invasive [61]. In this context, it is highly relevant to note that dominant gain-of-function alleles of *Toll* and loss-of-function alleles of *cactus*, two genes involved in the control of the nuclear localization of the Rel/NF-κB protein dorsal [47] also exhibit a melanotic tumor phenotype. In keeping with this, in three other melanotic tumor mutants, the
dorsal protein was shown to be nuclear in the absence of an external immune challenge [47]. Furthermore, in transgenic fly lines, overexpression of the p50 subunit of mammalian NF-κB in the form of a lacZ fusion protein, induces the formation of melanotic tumors, while also upregulating the expression of antibacterial peptide genes (S Govind, B Lemaitre, LH Huang, JA Hoffmann, R Steward, unpublished data). These data point to a role of Rel proteins in the cellular response. The melanotic tumor mutants will provide a potentially powerful tool for unravelling the mechanisms of this response, as illustrated by the recent molecular characterization of several melanotic tumor suppressor genes that share significant homologies with mammalian genes involved in the immune response (e.g. hopscotch [62,63]) or in cell proliferation (gene encoding ribosomal protein S6 [64,65]).

Concluding remarks

The innate defense of insects starts with the initiation of two major proteolytic cascades, which then lead to localized blood clotting and melanization at the site of injury and around invading microorganisms. It is then followed by the phagocytosis of microorganisms by specialized cells, or by formation of capsules around larger-sized parasites by certain blood cells. Concomitantly, the synthesis of a battery of potent, generally small-sized and cationic antibacterial and antifungal peptides is induced, predominantly in the fat body. These peptides are released into the blood and synergistically kill the invading microorganisms. Recognition of infectious non-self molecules is mediated by proteins (either in circulation or on cell membranes) that bind preferentially to bacterial or fungal cell wall components such as LPS, peptidoglycan, β-(1,3)-glucan, but probably also to polyanions and cell debris liberated at the wound site. These recognition proteins, now referred to as pattern recognition receptors, await structural and functional characterization. Our current understanding is that there is no clonal distribution of these receptors and that the insect host defense system lacks specificity and memory. The control of gene expression during the immune response shares essential characteristics with that of the mammalian acute phase response, and we surmise that several of the regulatory pathways are similar in between insects and mammals. The genetics of Drosophila provide a unique opportunity to dissect genetically the development and the differentiation of a primordial immune system, and researchers in the field may anticipate that the study of insect immunity will contribute significantly to our understanding of the innate immune response in general.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest


Insect defensins permeabilize the cell membrane of a Gram-positive bacterium by forming discrete channels, resulting in leakage of intracellular potassium, decreased ATP concentration and inhibition of respiration.
Although their structures differ, mammalian and insect defensins have similar mechanisms of action.


This paper reports the interesting observation that the precursor polypeptide of apidaecin contains multiple copies of apidaecin and isomers.


An excellent review of the important contribution of the laboratory to the study of clotting in an arthropod.


57. Abrams JM, Lux A, Steller H, Krieger M: Macrophages in Drosophila embryos and L2 cells exhibit scavenger recep-