CMLS, Cell. Mol. Life Sci. 59 (2002) 363–372
1420-682X/02/020363-10 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

The coagulation of insect hemolymph

U. Theopold[a,*] D. Li[b], M. Fabbri[b], C. Scherfer[a] and O. Schmidt[b]

[a]Department of Molecular Biology and Functional Genomics, Stockholm University, 106 91 Stockholm (Sweden), Fax +46 8 152350, e-mail: uli@molbio.su.se
[b]Department of Applied and Molecular Ecology, Adelaide University, Glen Osmond SA 5064 (Australia), e-mail: otto.schmidt@adelaide.edu.au

Received 24 July 2001; received after revision 13 September 2001; accepted 28 September 2001

Abstract. In contrast to both vertebrates and non-insect arthropods, little is known about the coagulation of hemolymph (hemostasis) in insects. We discuss the integration of the hemostatic response with other branches of the insect immune system. We also describe the present stage in the characterization of both soluble and cellular factors that contribute to hemostasis in insects. The factors of the well-characterized clotting cascades of vertebrates, primitive chelicerates and crustaceans are used to assess the implications of sequencing the whole *Drosophila* genome for searching candidate genes involved in hemostasis. Some striking similarities between blood clotting in vertebrates and the reaction of insect cells involved in hemolymph coagulation have implications for a phylogenetic comparison of hemostasis between divergent animal classes.

Key words. Insect immunity; blood clotting; phosphatidylserine; phenoloxidase; coagulogen; transglutaminase; *Drosophila melanogaster.*

The role of coagulation in insect immunity

Much of the evolutionary success of insects and other arthropods has been attributed to their efficient immune system. Like vertebrates, insects rely on both soluble factors and immune-competent cells to defend themselves against foreign intruders [1–6]. In contrast to vertebrates, there is little evidence for adaptive changes of the immune response during the individual's life [2]. Nevertheless, there is strong induction of gene expression for a number of immune components following the first encounter of the foreign antigen, including components of the signal transduction machinery and effector molecules [7]. Induction by microbial elicitors has been used successfully as a hallmark to identify immune molecules and differential expression screens as a method of isolating the corresponding complementary DNAs (cDNAs) and genes [8–12].

* Corresponding author.

In addition to de novo induction of immune components, there are a number of reactions that take place immediately after an insect has been wounded (the so-called wound response) and are often the basis for subsequent immune responses involving microbe-specific reactions [13–18]. A large fraction of these initial responses serve to seal a wound in order to avoid loss of body fluid and at the same time restrict the movement of microbes [17, 19]. The activities that contribute to stopping loss of body fluids are collectively called the hemostatic system. The formation of a clot, which seals the wound, is achieved in arthropods as in vertebrates through the activity of clotting systems [19, 20]. Many arthropods contain enzyme cascades, which result in the formation of a clot by protein cross-linking. Since arthropods have an open circulatory system, they use clot formation much more extensively than vertebrates without the potential danger of thromboses. One of the cascades leading to cross-linking is the clotting (or coagulation) system, which ultimately

leads to deposition of specialized clottable proteins, a number of which have been isolated ([19, 21], and below). The second is the PPO activating cascade, which leads to the (mostly enzymatic) activation of phenoloxidase (PO) [22]. PO has a number of activities, including immune functions as well as developmental functions, for example the hardening of the insect cuticle [23]; a function of PO during the wound response has been suggested as well [24, 25]. Most of the recent investigations on the PPO-activating cascade have focused on its immune function, in particular its activation by microbial elicitors. Here, we discuss both cascades in a number of invertebrate species and speculate on the implications for insects, where less is known about the physiology of coagulation, but genomic *(Drosophila melanogaster* [26]) and biochemical data are available.

Coagulation in non-insect species

Most of the research on arthropod clotting systems has been performed in non-insect species, including crustaceans and two ancient chelicerates, the horseshoe crabs *Limulus polyphemus* and *Tachypleus tridentatus*. Since there are a number of excellent reviews on clotting in horseshoe crabs [20, 27], we summarize only those aspects that might have implications for the insect clotting system, in particular the domain structure of the proteins involved. The LPS-activated clotting system in the horseshoe crab comprises three proteases (factor C, factor B and proclotting enzyme) which activate each other sequentially in a proteolytic cascade, leading finally to the precipitation of the clottable protein coagulogen into insoluble coagulin. In addition to a serine protease domain, the clotting factors contain a number of domains, including epidermal growth factor (EGF)-like domains; sushi domains, which are also found in mammalian complement factors; C-type lectin domains and clip (or disulfide-knotted) domains. The latter domain shows similarities to antibacterial peptides (big defensin) and may be released during the activation process of the cascade. It was speculated that this and other peptides released during the activation of the cascade have antibacterial activity, adding to the efficiacy of the clotting cascade, which leads to both immobilization and killing of bacteria. Antibacterial activity was recently shown for the clip domain of the PPO-activating enzyme from crayfish [28]. Gelation in horseshoe crabs does not seem to involve crosslinking through the activity of a transglutaminase as observed in other arthropods (see below), although transglutaminase activity has been found in horseshoe crab hemocytes (summarized in [20]). A second activation pathway, which is initiated by the fungal elicitor β-glucan, involves the heterodimeric factor G, which contains some additional domains worth mentioning. The α subunit is composed of several regions with similarities to bacterial enzymes involved in carbohydrate degradation (glucanases and xylanases). Since some of the functionally essential amino acids of the active sites are missing in these domains, they are likely to act as lectins, which retain substrate specificity of the enzymes but lack their activity. A mechanism to keep the coagulation reaction localized may involve a tight regulation by serpins (serine protease inhibitors). An important feature of the horseshoe crab clotting system is the recognition of microbial elicitors, which bind to factor C (in the case of lipopolysaccharide, LPS) or factor G (in the case of β-glucan). The sensitivity of horseshoe crab hemolymph towards LPS is in fact the basis for the so-called *Limulus* test, which is used in the clinical field. To our knowledge, there are no reports showing activation of the system in the absence of microbial components, even though the effective closure of epidermal lesions is an essential requirement in aquatic organisms.

In crustaceans, clotting has been shown to depend on the activity of the calcium-dependent enzyme transglutaminase, which has cross-linking activity. Transglutaminase is released by hemocytes or muscle cells upon wounding and cross-links clottable hemolymph proteins. Clotting proteins have been isolated from a number of crustacean species, including a crayfish [21, 29], shrimp [30] and sand crayfish [31]. They are often large (~200 kDa) lipoproteins with similarity to vitellogenins [21].

As in the horseshoe crab, the blood clotting system in vertebrates is dependent on the activity of a proteolytic cascade (including the protease thrombin) leading ultimately to the precipitation of fibrin. It also involves a thrombin-activated transglutaminase (blood coagulation factor XIIIa), which stabilizes fibrin aggregates through cross-linking. Although both horseshoe crabs and vertebrates employ proteolytic cascades to achieve hemostasis, there is little similarity in the primary structure of the proteins involved in both reactions. Although sequence similarity might be rare, protein domains are shared between members of protein cascades from different animal groups [32]. The best-studied example is the von Willebrand factor (vWF), which plays a central role in blood clotting. Domains with similarity to vWF are present in at least one insect protein that is involved in hemolymph clotting [33]. One protein that does show high similarity between arthropods and vertebrates is in fact transglutaminase, which is involved in clotting reactions in both crayfish and vertebrates (see above).

The PPO-activating cascade

The PPO activating system is a proteolytic cascade that is activated by a number of microbial elicitors. A concept of immune recognition that has emerged in recent years is

that microbial elicitors (pathogen-associated molecular patterns, PAMPs) are recognized by specialized binding proteins (the so-called pattern recognition proteins, PRPs), leading to the activation of a range of immune functions [2, 34]. Such immune functions include phagocytosis, the PPO-activating cascade and the production of antibacterial peptides. The PPO-activating cascade consists of a number of proteins, including several proteases, which ultimately activate PO. The activated PO is involved in several reactions, including melanin formation, production of reactive oxygen species [35] and to some extent sclerotization as part of the formation of the cuticle, capsule formation and wound healing. The concept of pattern recognition has gained strong support through the identification of a number of proteins that recognize microbial elicitors and subsequently activate the PPO-activating cascade [36–39]. Together with some older findings, the PPO-activating cascade is usually discussed as an integral part of innate immunity, which is activated in the presence of PAMPs and not as part of the hemostatic system. While this picture is certainly true, there are some additional aspects of the PPO-activating cascade that might link PO activity to other functions, including hemolymph clotting. A link between both systems has been suggested by others as well [40]. Some of these aspects include:

- the presence of multiple PO genes in several species, which may differ in their enzymatic activity and substrate specificity. For example, *Drosophila* contains three and *Anopheles* at least six different PPO genes (see also below the specific aspects of the *Drosophila* genes);
- the possible activity of regulatory proteins within the PPO-activating cascade, including dopachrome-converting enzyme and quinone isomerase [41]. Shifts in the relative concentration of key metabolites of the PPO-activating cascade are expected to influence PO activity, leading in some cases to an increase of sclerotization at the expense of the production of antibacterial quinoid intermediates of melanin biosynthesis [35];
- early findings that PPO can also be activated by nonmicrobial elicitors like denaturing agents or denatured proteins, which may indicate a role for PO in housekeeping functions to be activated in the absence of microbial elicitors. Alternative nonmicrobial elicitors include certain phospholipids, which are released from damaged cells [41], and denatured lipophorin [42];
- an unsolved issue is the mechanism by which PO is transported into hemolymph and to the cuticle [24] in the absence of any signal peptide in the primary sequences, which code for PPOs [43–46]. There have been recent reports of a mechanism which allows transport of proteins across membranes independent of the presence of a signal peptide in the primary sequence [47, 48]. One of these proteins (the engrailed homeoprotein) associates with specialized membrane structures, so-called microdomains, before getting access to a compartment which is involved in secretion [49]. It remains to be determined whether this mechanism also applies to PO, or other proteins, which are apparently secreted in a nonclassical pathway [50, 51].

Although the PPO-activating cascade has recently been discussed foremost as an independent part of humoral immunity, it is likely that PO cooperates with other branches of the immune system, including the clotting system. By binding to the clot, it might enhance the cross-linking activity and/or add antibacterial activity to other immune mechanisms that are localized there [52] and aid in wound healing [53].

The coagulation cascade in insects

In contrast to other arthropods, little is known about the clotting system in insects. Similar to the situation in vertebrates and non-insect arthropods, hemolymph coagulation in most insects involves cellular and humoral components which are inactive on their own [19]. Full clotting activity has been shown to depend on both cellular and humoral procoagulant activity in a number of species. Much of the early work has been on the morphological characterization of the cellular reactions that are found in different insect species [19], leading to a classification of four different types of coagulation patterns [54]. At present, the molecular characterization of the proteins involved in hemolymph clotting is lagging behind the detailed morphological characterization of the cell types involved.

Humoral procoagulant activity

When the sequencing of the *Drosophila* genome reached completion, there was hope that *Drosophila* homologues could be identified for many of the known immune molecules from other systems, including components of the clotting cascade. But as mentioned above, only a few arthropod proteins involved in hemolymph clotting can be considered true orthologues of vertebrate clotting factors. Even comparison among different classes of arthropods shows that different proteins are utilized for establishing a functional clot. Therefore, it is not surprising that sequence searches for most members of the hemostatic system of crustaceans, chelicerates or humans fail to detect any orthologues in the *Drosophila* genome. One of the genes that can be positively identified by performing sequence comparisons is, just like in crustaceans, the

enzyme transglutaminase (or factor XIIIa in vertebrates), for which there is high similarity to a protein in the *Drosophila* genome (CG7356). No obvious similarities between *Drosophila* proteins and crayfish clotting protein or the horseshoe crab coagulogen can be found. Although vitellogenin also exists in insects, its function in the clotting system may be different from crustaceans, since a vitellogenin-like protein has recently been implied in the melanization reaction [55]. Nevertheless, in a number of insect species one coagulogen has been identified as a lipid-carrying protein called lipophorin [56–59]. Lipophorin is a multifunctional protein in insects that consists of three subunits, all of which have been also implied in immunity. In addition, there has been a report on a 22-kDa protein from *Manduca sexta* (hemofibrin), which forms a fibrous clot that can be visualized using electron microscopy [60].

A surprisingly high number of protease genes are present in the *Drosophila* genome, including a number of proteases with clip domains, which are present in protease members of the PPO-activating cascade in other insects [61] and crustaceans [62]. Some of these proteases might be involved in digestion and others in proteolytic cascades during developmental processes, but some may be involved in immune functions. A number of observations show that members of proteolytic cascades can be involved in both development and immunity [2, 63]. This suggests that it might be necessary to reconsider some of the proteases that have been identified due to their developmental phenotype as multifunctional proteases with both developmental and immune functions. Included in this class of proteins may be gene products which show high similarity to certain proteases but lack some of the amino acids known to be necessary for catalytic activity. One such protein is the *masquerade* gene product, which has been identified as a developmentally important protein in *Drosophila* [64] and shows similarity to proteins with immune function in crayfish [62, 65] and insects [66].

There are a number of proteins from other insects, which have been implied in induction of coagulation reactions, including M13 [67] and scolexin [68] from *M. sexta* as well as hemocytin, a multidomain protein from *Bombyx mori*, which has significant similarity to vWF [69].

Although true homologues of proteins involved in clotting in other species are somewhat difficult to identify in the *Drosophila* genome [32, 70], there are a number of annotated genes which might turn out to play an important role in hemolymph clotting or related processes. Here we provide a list of some examples, which is far from being complete but points towards some potentially interesting genes and their products. There are a number of predicted gene products which contain domains known to exist in clotting factors from other species. These domains include the immunoglobulin fold, the EGF-like domain, domains with similarity to vWF, sushi domains, clip domains and C-type lectin domains [26, 71, 72]. In most cases the functional importance of these domains has still to be confirmed. One also has to keep in mind that the annotation of many genes is preliminary and might change in years to come. Nevertheless, there are some predicted genes with unique combinations of domains that seem worthy of further functional analysis:

- a protein with similarity to *Bombyx* hemocytin exists in the *Drosophila* genome (CG7002) and will most likely turn out to be involved in immune reactions;
- three genes coding for POs, one of which seems to be expressed in SL2 cells, as there are at least two ESTs (expressed sequence tags) which have been derived from these cells. SL2 cells are usually used as a negative control to assay PO activity, opening the possibility that traditional PO assays may leave some PO-like activity undetected;
- a number of *Drosophila* homologues of a gene for dopachrome conversion enzyme from *Aedes aegypti*, which can be found in GenBank [AF288384, unpublished]. One of these homologues is the *yellow* gene, mutants of which show defects in melanization. Homologues from other species include a gene for a protein from royal jelly (RJP57-1, [73]);
- members of the pentraxin family in *Drosophila*. Pentraxins include C-reactive protein (CRP) and a CRP-related protein from horseshoe crab, which are involved in the activation of the complement system, and the clotting system respectively;
- in addition to PO activity there is a laccase-like activity which can be identified in the insect cuticle [74]. Since to our knowledge no laccases have been cloned from insects, we used plant laccase genes to search the *Drosophila* genome and identified four genes with significant similarity (CG7871, CG3759, CG19408, CG5959). Interestingly, one of these (CG3759) displays additional similarity to the type C domain of vWF. It remains to be investigated in which tissues these genes for laccase-like proteins are expressed.

Cellular aspects of the coagulation cascade

In contrast to the humoral part of the clotting system, which shows little similarity between vertebrates and insects, there are a number of similarities on a cellular basis between the reaction of hemocytes in insects and platelets in vertebrates. This includes the mechanisms that contribute to clot formation in insects. They comprise the release of granular components [13] as well as the formation of membranous vesicles, so-called microparticles, which are released from blood cells [54].

Most prominent among the biochemical similarities is the exposure of negatively charged phospholipids (visualized

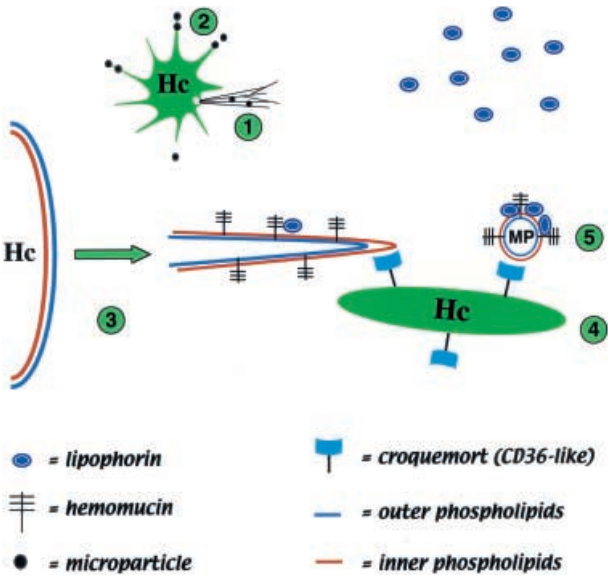

Figure 1. Scheme of hemolymph coagulation in insects. Coagulation of insect hemolymph comprises the activation of both hemocyte and hemolymph coagulogen activity. Hemocytes (Hc) show two reactions, namely degranulation (1), and the formation of microparticles (2). Microparticle (MP) formation is accompanied by a reversal in membrane polarity, leading to the exposure of (mostly negatively charged) lipids from the inner leaflet of the membrane lipid bilayer on the cell surface (3). These lipids can be recognized by receptors like the *Drosophila* croquemort protein or other CD36-like proteins on hemocytes, which are not involved in clot formation during subsequent wound healing (4). The hemocyte coagulogen attracts hemolymph coagulogen, comprising lipophorin (5) and possibly other factors. See text for further details.

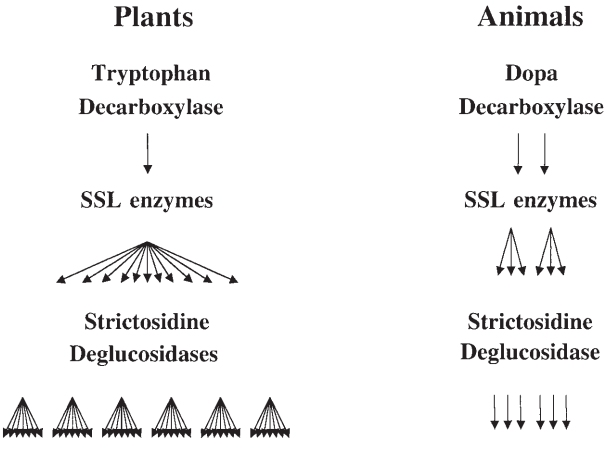

Figure 2. Biosynthetic pathway involving strictosidine synthase-like (SSL) enzymes in plants and animals. The pathway has diversified at least at two levels in plants, leading to a multitude of isoforms of SSL enzymes and strictosidine deglucosidases. The resulting branched pathway might be involved in the production of different alkaloids. In contrast, there are only few members of the two enzyme families in animals pointing towards a more restricted function.

by annexin V labeling), during clotting. These phospholipids are usually confined to the inner leaflet of the cell membrane but become exposed both during vertebrate blood clotting [75] and hemolymph coagulation in insects [76]. The rearrangement of phospholipids is achieved through the activity of scramblases [77, 78]. There are two annotated genes with similarity to vertebrate scramblases in the *Drosophila* genome (CG6590 and CG1893). It is believed that the negatively charged phospholipids play a role in the elimination of cells during wound healing. Scavenger receptors with affinity for these lipids have been identified in both vertebrates [79–81] and insects [4]. In *Drosophila*, the *croquemort* gene product belongs to the scavenger receptor family [82]. This protein has mostly been discussed as an important scavenger receptor for apoptotic cells, which share exposure of aminophospholipids with cells that are involved in clotting [83]. A possible role for croquemort in wound healing remains to be studied.

Blood clotting in vertebrates similarly involves the production of membraneous vesicles which are released from platelets and also show strong annexin V staining [84, 85]. The formation of microparticles is dependent on calcium [84]. In insects, similar structures have been described [54, 86]. More recently, the formation of these 'insect microparticles' could also be shown to depend on calcium, and positive labeling of insect microparticles with annexin V was observed as well [76].

It should be mentioned that the exposure of negatively charged phospholipids is a feature that apoptotic cells and cells involved in coagulation share with bacteria [87, 88]. Although the exact mechanism for how antibacterial peptides act on the bacterial membrane remains to be determined in many cases, a common feature seems to be the affinity of these – mostly basic – peptides for the negative charge on the bacterial surface [88]. Thus, it is expected that many antibacterial peptides should also bind to cells which expose a negative charge and act in helping to solubilize cellular lipids and clean up cellular debris. The activity of some antibacterial peptides against tumor cells might be explained in a similar way, since a number of them expose negative charge on their surface [89, 90], a possibility which would impact on the choice and design of potential peptides with antitumor activity [91].

The strictosidine-synthase family

Similar to their vertebrate counterparts, insect microparticles are enriched in certain proteins, one of which is hemomucin, a protein that comprises a typical mucin domain as well as a domain with similarity to strictosidine synthase (SS, [92]), a plant enzyme involved in the production of monoterpenoid indole alkaloids [93, 94]. A number of these alkaloids are used for treatments of neu-

rological disorders [95]. This includes reserpine, which interferes with dopaminergic signal transduction [96]. Hemomucin shares similarity to SS with a number of similar proteins from other insects [76, 97] as well as zebrafish, mouse and humans. Interestingly, the human member of the gene family is strongly expressed in the brain, the very organ many of the plant alkaloids act upon [98].

The function of hemomucin and the other animal members of the SSL family remains to be established, but data so far point towards a role in the coagulation process, at least for the *Drosophila* members [76]. Because the substrates of the plant members of this family belong to the biogenic amines, it is tempting to speculate that the insect members also metabolize such derivatives. A number of biogenic amines are in fact known to play a role in sclerotization reactions [99] and the regulation of hemocyte activity [86, 100]. In addition, dopamine-like substances are induced during bacterial infections of insects [101]. When we searched for possible animal homologues of additional members of the plant pathway involved in the production of indole alkaloids, we identified two possible homologues from *Drosophila* of the next enzyme upstream. They include a dopa decarboxylase, which has been isolated from mutants due to a defect in learning behavior [102, 103]. A dual function in both the immune and nervous system might therefore turn out to be a common feature of members of this biosynthetic pathway.

In the search for new strategies to identify proteins involved in hemolymph coagulation, we have chosen two approaches:

1) Based on previous findings that hemocytes release highly glycosylated proteins during coagulation reactions [17, 54], we have used lectin affinity purification to identify and purify novel factors of the coagulation cascade.
2) By performing an in vitro coagulation reaction and using the cross-linked clot for immunizing rabbits, we obtained an antiserum with specificity for clot components. Since PO activity was not inhibited, we expected to isolate components of both the clotting cascade and the PPO-activating cascade. The antiserum has been used successfully to isolate cDNAs from an expression library [D. Li et al. unpublished].

These approaches allowed us to identify candidates for novel proteins which are involved in coagulation as well as previously known components of both the PPO-activating cascade and the clotting reaction, like lipophorin and PO. cDNAs coding for novel candidate clotting factors include a distant relative of the α-crystallin/small heat-shock protein family [104, 105]. Members of this family are known to have a tendency to aggregate and are substrates for transglutaminases, both features which suit a protein involved in clotting [104]. One of the emerging themes of this work seems to be the dual expression of proteins in both the salivary glands and in hemocytes, which has been previously shown for hemomucin [106] but now also for a lepidopteran silk protein and a product of the *Drosophila* 71E puff [unpublished results]. The latter protein is a relative of the salivary gland glue proteins ([107], M. Fabbri et al. unpublished). One may speculate that a tendency for proteins to precipitate and form a coagulum upon contact with air predisposes them for a dual use as part of salivary secretions and of the hemocyte coagulum.

Outlook

In recent years, a concept for the recognition of molecular patterns has emerged from a number of investigations trying to explain how immune responses are activated. As mentioned before, according to this idea, the innate immune system uses evolutionarily conserved molecular patterns that are characteristic for pathogens as distinctive features to activate immune responses [34]. These PAMPs are recognized by specialized receptors, many of which have been isolated [2].

While we agree that this concept applies to essential activation processes in the hemostatic response, wounds nevertheless have to be sealed even in the absence of microbial elicitors. Therefore, other signals have to be recruited for activation of clotting cascades [16, 108]. These may include physical signals such as an oxidizing environment, which has been shown to induce immune responses [109], or biochemical clues such as intracellular components, which are released upon wounding [110]. As a framework, concepts of vertebrate immunity, which focus more on the internal regulation of immune responses, seem more fruitful in this context. One such concept is the idea that immune reactions rely on so-called danger signals, which are produced by cells that have undergone damage, for their activation [110–112]. Such signals may include nucleic acids, typical intracellular proteins or immature proteins. Wounding may also lead to changes in hemolymph and fat body [109] proteins, which could potentially be used as activating signals. While these signals might activate mostly the clotting system, there is also evidence that under certain circumstances, the PPO-activating cascade relies on such signals for its activation [113]. PPO has in fact been shown to be activated at a low calcium concentration and in the absence of microbial elicitors [114].

There is also recent evidence for cooperation between the clotting system and the PPO-activating cascade [115]. These studies were performed in chelicerates, where the PO activity resides in a member of the hexamerin family, a situation that is different from insects. This protein is hemocyanin, normally the oxygen transporter in the he-

molymph of chelicerates, which can be activated to perform PO activity. This activation can also be achieved by nonenzymatic means [116]. Likewise, in the horseshoe crab, where the focus has been on activation by microbial elicitors, there is evidence indicating that the clotting system is linked to the activation of hemocyanin through a nonenzymatic activity of clottable protein [115, 116]. Studies on the PPO-activating system and on hemolymph coagulation in arthropods are thus expected to further add to an integrated view of the innate immune system.

A number of recently developed or refined techniques used in functional genomics are also expected to add substantially to our understanding of hemolymph clotting. Genes that have recently been found to be induced during immune reactions using microarrays include a laccase, an annexin and the yellow gene [Bruno Lemaitre, personal communication], findings that are compatible with the ideas proposed here.

Note added in proof. The *Drosophila* hemacytin homologue: Goto A., Kumagai T., Kumagai C., Hirose J., Narita H. et al. (2001) A Drosophila haemocyte-specific protein, hemolectin, similar to human von Willebrand factor. J. Biochem. **359:** 99–108.

Microarrays comparing immune-induced and non-induced tissues: De Gregorio E., Spellman P. T., Rubin G. M. and Lemaitre B. (2001) Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. Proc. Natl. Acad. Sci. USA **98:** 12590–12595.

The dopachrome conversion factors from *Aedes aegyptii*. Johnson J. K., Li J. and Christensen B. M. (2001) Cloning and characterization of a dopachrome conversion enzyme from the yellow fever mosquito, *Aedes aegyptii*. Insect. Biochem. Mol. Biol. **31:** 1125–1135.

Acknowledgments. We thank Kenneth Söderhäll and the reviewers for fruitful comments on the manuscript. The authors' work is supported by grants from the Swedish Research Council and the Australian Research Council. D.L. and M.F. are supported by Postgraduate Scholarships from Adelaide University, C.S. by a fellowship from the German Academic Exchange Service (DAAD).

1 Gillespie J. P., Kanost M. R. and Trenczek T. (1997) Biological mediators of insect immunity. Annu. Rev. Entomol. **42:** 611–643
2 Hoffmann J. A., Kafatos F. C., Janeway C. A. and Ezekowitz R. A. (1999) Phylogenetic perspectives in innate immunity. Science **284:** 1313–1318
3 Lehrer R. I. and Ganz T. (1999) Antimicrobial peptides in mammalian and insect host defence. Curr. Opin. Immunol. **11:** 23–27
4 Franc N. C. and White K. (2000) Innate recognition systems in insect immunity and development: new approaches in *Drosophila*. Microbes Infect. **2:** 243–250
5 Medzhitov R. and Janeway C. Jr (2000) Fly immunity: great expectations. Genome Biol. **1:** 106
6 Kimbrell D. A. and Beutler B. (2001) The evolution and genetics of innate immunity. Nat. Rev. Genet. **2:** 256–267
7 Medzhitov R. and Janeway C. A. Jr (1999) Innate immune induction of the adaptive immune response. Cold Spring Harb. Symp. Quant. Biol. **64:** 429–435
8 Dushay M. S., Åsling B. and Hultmark D. (1996) Origins of immunity: relish, a compound rel-like gene in the antibacterial defense of *Drosophila*. Proc. Natl. Acad. Sci. USA **93:** 10343–10347
9 Kang D., Liu G., Gunne H. and Steiner H. (1996) PCR differential display of immune gene expression in *Trichoplusia ni*. Insect Biochem. Mol. Biol. **26:** 177–184
10 Lemaitre B., Reichhart J. M. and Hoffmann J. A. (1997) *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc. Natl. Acad. Sci. USA **94:** 14614–14619
11 Hedengren M., Åsling B., Dushay M. S., Andó I., Ekengren S., Wihlborg M. et al. (1999) Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. Mol. Cell. **4:** 827–837
12 Engström Y. (1999) Induction and regulation of antimicrobial peptides in *Drosophila*. Dev. Comp. Immunol. **23:** 345–358
13 Rowley A. F. and Ratcliffe N. A. (1976) The granular cells of *Galleria mellonella* during clotting and phagocytic reactions in vitro. Tiss. Cell **8:** 437–446
14 Ratcliffe N. A. and Rowley A. F. (1979) Role of hemocytes in defense against biological agents. In: Insect Hemocytes: Development, Forms, Functions and Techniques, pp. 331–414, Gupta A. P. (ed.), Cambridge University Press, London
15 Gupta A. P. (1984) Cellular elements in the hemolymph. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 3, pp. 401–451, Kerkut G. A. and Gilbert L. I. (eds), Pergamon Press, Oxford
16 Trenczek T. (1988) Injury and immunity in insects. In: Endocrinological Frontiers in Physiological Insect Ecology, pp. 369 ff., Sehnal B. et al. (eds), Wroclaw Technical University Press, Wroclaw, Poland
17 Gupta A. P. (1991) Insect immunocytes and other hemocytes: roles in cellular and humoral immunity. In: Immunology of Insects and Other Arthropods, pp. 19–118, Gupta A. P. (ed), CRC Press, Boca Raton, Fl
18 Hoffmann J. A. (1995) Innate immunity of insects. Curr. Opin. Immunol. **7:** 4–10
19 Bohn H. (1986) Hemolymph clotting in insects. In: Immunity in Invertebrates, pp. 189–207, Brehélin M. (ed.), Springer, Heidelberg
20 Iwanaga S., Kawabata S. and Muta T. (1998) New types of factors and defense molecules found in horseshoe crab hemolymph – their structures and functions. J. Biochem. **123:** 1–15
21 Hall M., Wang R., van Antwerpen R., Sottrup-Jensen L. and Söderhäll K. (1999) The crayfish plasma clotting protein: a vitellogenin-related protein responsible for clot formation in crustacean blood. Proc. Natl. Acad. Sci. USA **96:** 1965–1970
22 Söderhäll K. and Cerenius L. (1998) Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Opin. Immunol. **10:** 23–28
23 Nappi A. J. and Vass E. (1993) Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. Pigment Cell. Res. **6:** 117–126
24 Asano T. and Ashida M. (2001) Cuticular pro-phenoloxidase of the silkworm, *Bombyx mori*. Purification and demonstration of its transport from hemolymph. J. Biol. Chem. **276:** 11100–11112
25 Chase M. R., Raina K., Bruno J. and Sugumaran M. (2000) Purification, characterization and molecular cloning of prophenoloxidases from *Sarcophaga bullata*. Insect Biochem. Mol. Biol. **30:** 953–967
26 Adams M. D., Celniker S. E., Holt R. A., Evans C.A., Gocayne J. D., Amanatides P. G. et al. (2000) The genome sequence of *Drosophila melanogaster*. Science **287:** 2185–2195
27 Muta T. and Iwanaga S. (1996) The role of hemolymph coagulation in innate immunity. Current Opin. Immunol. **8:** 41–47
28 Wang R., Lee S. Y., Cerenius L. and Söderhäll K. (2001) Properties of the prophenoloxidase activating enzyme of the fresh-

water crayfish, *Pacifastacus leniusculus*. Eur. J. Biochem. **268:** 895–902
29 Hall M., van Heusden M.C. and Söderhäll K. (1995) Identification of the major lipoproteins in crayfish hemolymph as proteins involved in immune recognition and clotting. Biochem. Biophys. Res. Com. **216:** 939–946
30 Yeh M. S., Huang C. J., Leu J. H., Lee Y. C. and Tsai I. H. (1999) Molecular cloning and characterization of a hemolymph clottable protein from tiger shrimp (*Penaeus monodon*). Eur. J. Biochem. **266:** 624–633
31 Komatsu M. and Andó S. (1998) A very-high-density lipoprotein with clotting ability from hemolymph of sand crayfish, *Ibacus ciliatus*. Biosci. Biotechnol. Biochem. **62:** 459–463
32 Venter J. C., Adams M. D., Myers E. W., Li P. W., Mural R. J., Sutton G. G. et al. (2001) The sequence of the human genome. Science **291:** 1304–1351
33 Kotani E., Yamakawa M., Iwamoto S., Tashiro M., Mori H., Sumida M. et al. (1995) Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von Willebrand factor. Biochim. Biophys. Acta **1260:** 245–258
34 Medzhitov R. and Janeway C. Jr (2000) Innate immune recognition: mechanisms and pathways. Immunol. Rev. **173:** 89–97
35 Nappi A. J. and Ottaviani E. (2000) Cytotoxicity and cytotoxic molecules in invertebrates. Bioessays **22:** 469–480
36 Lee S. Y., Wang R. and Söderhäll K. (2000) A lipopolysaccharide- and beta-1,3-glucan-binding protein from hemocytes of the freshwater crayfish *Pacifastacus leniusculus*. Purification, characterization and cDNA cloning. J. Biol. Chem. **275:** 1337–1343
37 Ma C. and Kanost M. R. (2000) A beta1,3-glucan recognition protein from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade. J. Biol. Chem. **275:** 7505–7514
38 Yu X. Q., Gan H. and Kanost M. R. (1999) Immulectin, an inducible C-type lectin from an insect, *Manduca sexta*, stimulates activation of plasma prophenol oxidase. Insect Biochem. Mol. Biol. **29:** 585–597
39 Yu X. Q. and Kanost M. R. (2000) Immulectin-2, a lipopolysaccharide-specific lectin from an insect, *Manduca sexta*, is induced in response to gram-negative bacteria. J. Biol. Chem. **275:** 37373–37381
40 Decker H. and Rimke T. (1998) *Tarantula* hemocyanin shows phenoloxidase activity. J. Biol. Chem. **273:** 25889–25892
41 Sugumuran M. and Kanost M. (1993) Regulation of insect hemolymph phenoloxidases. In: Parasites and Pathogens of Insects, pp. 317–342, Beckage N. E., Thompson S. N. and Federici B. A. (eds), San Diego, Academic Press
42 Leonard C., Ratcliffe N. A. and Rowley A. F. (1985) The role of prophenoloxidase activation in non-self recognition and phagocytosis by insect blood cells. J. Insect Physiol. **31:** 789–799
43 Aspan A., Huang T. S., Cerenius, L. and Söderhäll K. (1995) cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. Proc. Natl. Acad. Sci. USA **92:** 939–943
44 Hall M., Scott T., Sugumaran M., Söderhäll K. and Law J. H. (1995) Proenzyme of *Manduca sexta* phenol oxidase: purification, activation, substrate specificity of the active enzyme and molecular cloning. Proc. Natl. Acad. Sci. USA **92:** 7764–7768
45 Kawabata T., Yasuhara Y., Ochiai M., Matsuura S. and Ashida M. (1995) Molecular cloning of insect pro-phenol oxidase: a copper-containing protein homologous to arthropod hemocyanin. Proc. Natl. Acad. Sci. USA **92:** 7774–7778
46 Fujimoto K., Okino N., Kawabata S., Iwanaga S. and Ohnishi E. (1995) Nucleotide sequence of the cDNA encoding the proenzyme of phenol oxidase A1 of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **92:** 7769–7773
47 Joliot A., Maizel A., Rosenberg D., Trembleau A., Dupas S., Volovitch M. et al. (1998) Identification of a signal sequence necessary for the unconventional secretion of Engrailed homeoprotein. Curr. Biol. **8:** 856–863
48 Prochiantz A. (2000) Messenger proteins: homeoproteins, TAT and others. Curr. Opin. Cell Biol. **12:** 400–406
49 Joliot A., Trembleau A., Raposo G., Calvet S., Volovitch M. and Prochiantz A. (1997) Association of Engrailed homeoproteins with vesicles presenting caveolae-like properties. Development **124:** 1865–1875
50 Csikos G., Molnar K., Borhegyi N. H., Talian G. C. and Sass M. (1999) Insect cuticle, an in vivo model of protein trafficking. J. Cell Sci. **112:** 2113–2124
51 Sass M., Kiss A. and Locke M. (1994) Integument and hemocyte peptides. J. Insect Physiol. **40:** 407–421
52 Ashida M., Ochiai M. and Niki T. (1988) Immunolocalization of prophenoloxidase among hemocytes of the silkworm, *Bombyx mori*. Tiss. Cell **20:** 599–610
53 Lai-Fook J. (1966) The repair of wounds in the integument of insects. J. Insect Physiol. **12:** 195–226
54 Gregoire C. (1974) Hemolymph coagulation. In: The Physiology of Insecta, vol. 5, pp. 309–360, Rockstein M. (ed.), Academic Press, New York
55 Lee K. M., Lee K. Y., Choi H. W., Cho M. Y., Kwon T. H., Kawabata S. et al. (2000) Activated phenoloxidase from *Tenebrio molitor* larvae enhances the synthesis of melanin by using a vitellogenin-like protein in the presence of dopamine. Eur. J. Biochem. **267:** 3695–3703
56 Brehélin M. (1979) Hemolymph coagulation in *Locusta migratoria*: evidence for a functional equivalent of fibrinogen. Comp. Biochem. Physiol. **62:** 329–334
57 Gellissen G. (1983) Lipophorin as the plasma coagulogen *in Locusta migratoria*. Naturwissenschaften **70:** 45–46
58 Barwig B. (1985) Isolation and characterization of plasma coagulation of the cockroach *Leocophaea maderae*. J. Comp. Physiol. **155:** 135–143
59 Duvic B. and Brehélin M. (1998) Two major proteins from locust plasma are involved in coagulation and are specifically precipitated by laminarin, a beta-1,3-glucan. Insect Biochem. Mol. Biol. **28:** 959–967
60 Geng C. and Dunn P. E. (1988) Hemostasis in larvae of *Manduca sexta*: formation of a fibrous coagulum by hemolymph proteins. Biochem. Biophys. Res. Com. **155:** 1060–1065
61 Jiang H. and Kanost M. R. (2000) The clip-domain family of serine proteinases in arthropods. Insect Biochem. Mol. Biol. **30:** 95–105
62 Huang T. S., Wang H., Lee S. Y., Johansson M. W., Söderhäll K. and Cerenius L. (2000) A cell adhesion protein from the crayfish *Pacifastacus leniusculus*, a serine proteinase homologue similar to *Drosophila* masquerade. J. Biol. Chem. **275:** 9996–10001
63 Hultmark D. (1994) Insect immunology. Ancient relationships. Nature **367:** 116–117
64 Murugasu-Oei B., Rodrigues V., Yang X. and Chia W. (1995) Masquerade: a novel secreted serine protease-like molecule is required for somatic muscle attachment in the *Drosophila* embryo. Genes Dev. **9:** 139–154
65 Lee S. Y. and Söderhäll K. (2001) Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*. J. Immunol. **166:** 7319–7326
66 Kwon T. H., Kim M. S., Choi H. W., Joo C. H., Cho M. Y. and Lee B. L. (2000) A masquerade-like serine proteinase homologue is necessary for phenoloxidase activity in the coleopteran insect, *Holotrichia diomphalia* larvae. Eur. J. Biochem. **267:** 6188–6196
67 Minnick M. F., Rupp R. A. and Spence K. D. (1986) A bacterial-induced lectin which triggers hemocyte coagulation in *Manduca sexta*. Biochem. Biophys. Res. Commun. **137:** 729–735

68 Finnerty C. M., Karplus P. A. and Granados R. R. (1999) The insect immune protein scolexin is a novel serine proteinase homolog. Protein Sci. **8:** 242–248

69 Kotani E., Yamakawa M., Iwamoto S., Tashiro M., Mori H., Sumida M. et al. (1995) Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von Willebrand factor. Biochim. Biophys. Acta **1260:** 245–258

70 Lander E. S., Linton L. M., Birren B., Nusbaum C., Zody M. C., Baldwin J. et al. (2001) Initial sequencing and analysis of the human genome. Nature **409:** 860–921

71 Theopold U., Rissler M., Fabbri M., Schmidt O. and Natori S. (1999) Insect glycobiology: a lectin multigene family in *Drosophila melanogaster*. Biochem. Biophys. Res. Com. **261:** 923–927

72 Khush R. S. and Lemaitre B. (2000) Genes that fight infection: what the *Drosophila* genome says about animal immunity. Trends Genet. **16:** 442–449

73 Albert S., Klaudiny J. and Simuth J. (1999) Newly discovered features of the updated sequence of royal jelly protein RJP57-1; longer repetitive region on C-terminus and homology to *Drosophila melanogaster yellow* protein. J. Apicult. Res. **35:** 63–68

74 Andersen S. O. (1985) Sclerotization and tanning of the cuticle. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 3, pp. 59–74, Kerkut G. A. and Gilbert L. I. (eds), Pergamon Press, Oxford

75 Dachary-Prigent J., Toti F., Satta N., Pasquet J. M., Uzan A. and Freyssinet J. M. et al. (1996) Physiopathological significance of catalytic phospholipids in the generation of thrombin. Sem. Thrombosis Hemostasis **22:** 157–164

76 Theopold U. and Schmidt O. (1997) *Helix pomatia* lectin and annexin V, two molecular probes for insect microparticles: possible involvement in hemolymph coagulation. J. Insect Physiol. **43:** 667–674

77 Bevers E. M., Comfurius P., Dekkers D. W. and Zwaal R. F. (1999) Lipid translocation across the plasma membrane of mammalian cells. Biochim. Biophys. Acta **1439:** 317–330

78 Daleke D. L. and Lyles J. V. (2000) Identification and purification of aminophospholipid flippases. Biochim. Biophys. Acta **1486:** 108–127

79 Rigotti A., Acton S. L. and Krieger M. (1995) The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. J. Biol. Chem. **270:** 16221–16224

80 Fadok V. A., Bratton D. L., Frasch S. C., Warner M. L. and Henson P. M. (1998) The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell. Death Differ. **5:** 551–562

81 Fadok V. A., Warner M. L., Bratton D. L. and Henson P. M. (1998) CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3). J. Immunol. **161:** 6250–6257

82 Franc N. C., Dimarcq J. L., Lagueux M., Hoffmann J. and Ezekowitz R. A. (1996) Croquemort, a novel *Drosophila* hemocyte/macrophage receptor that recognizes apoptotic cells. Immunity **4:** 431–443

83 Franc N. C., Heitzler P., Ezekowitz R. A. and White K. (1999) Requirement for croquemort in phagocytosis of apoptotic cells in *Drosophila*. Science **284:** 1991–1994

84 Pasquet J. M., Toti F., Nurden A. T. and Dacharyprigent J. (1996) Procoagulant activity and active calpain in platelet-derived microparticles. Thrombosis Res. **82:** 509–522

85 Horstman L. L. and Ahn Y. S. (1999) Platelet microparticles: a wide-angle perspective. Crit. Rev. Oncol. Hematol. **30:** 111–142

86 Diehl-Jones W. L., Mandat C. A., Whent G. and Downer R. G. H. (1996) Monoaminergic regulation of hemocyte activity. J. Insect Physiol. **42:** 13–19

87 Boman H. G. (1995) Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. **13:** 61–92

88 Maloy W. L. and Kari U. P. (1995) Structure-activity studies on magainins and other host defense peptides. Biopolymers **37:** 105–122

89 Rao L. V., Tait J. F. and Hoang A. D. (1992) Binding of annexin V to a human ovarian carcinoma cell line (OC-2008). Contrasting effects on cell surface factor VIIa/tissue factor activity and prothrombinase activity. Thrombosis Res. **67:** 517–531

90 Sugimura M., Donato R., Kakkar V. V. and Scully M. F. (1994) Annexin V as a probe of the contribution of anionic phospholipids to the procoagulant activity of tumour cell surfaces. Blood Coagul. Fibrinolysis **5:** 365–373

91 Lackie A. M. (1988) Haemocyte Behaviour. Adv. Insect Physiol. **21:** 85–178

92 Kutchan T. M., Hampp N., Lottspeich F., Beyreuther K. and Zenk M. H. (1988) The cDNA clone for strictosidine synthase from *Rauwolfia serpentina*. DNA sequence determination and expression in *Escherichia coli*. FEBS Lett. **237:** 40–44

93 Kutchan T. M. (1995) Alkaloid biosynthesis – the basis for metabolic engineering of medicinal plants. Plant Cell **7:** 1059–1070

94 Theopold U., Samakovlis C., Erdjument-Bromage H., Dillon N., Axelsson B., Schmidt O. et al. (1996) *Helix pomatia* lectin, an inducer of *Drosophila* immune response binds to hemomucin, a novel surface mucin. J. Biol. Chem. **271:** 12708–12715

95 Bennett M. R. (1998) Monoaminergic synapses and schizophrenia: 45 years of neuroleptics. J. Psychopharmacol. **12:** 289–304

96 Carlsson A. (1976) The contribution of drug research to investigating the nature of endogenous depression. Pharmakopsychiatr. Neuropsychopharmakol. **9:** 2–10

97 Kinuthia W., Li D., Schmidt O. and Theopold U. (1999) Is the surface of endoparasitic wasp eggs and larvae covered by a limited coagulation reaction? J. Insect Physiol. **45:** 501–506

98 Fabbri M., Delp G., Schmidt O. and Theopold U. (2000) Animal and plant members of a gene family with similarity to alkaloid- synthesizing enzymes. Biochem. Biophys. Res. Commun. **271:** 191–196

99 Nappi A. J. and Silvers M. J. (1986) *Drosophila* blood cell surface changes in response to stimulation by dopamine in vitro. Dev. Comp. Immunol. **10:** 437–442

100 Baines D., DeSantis T. and Downer R. G. H. (1992) Octopamine and 5-hydroxytryptamine enhance the phagocytic activity and nodule formation activities of cockroach (*Periplaneta americana*) haemocytes. J. Insect Physiol. **38:** 905–914

101 Kim M. H., Joo C. H., Cho M. Y., Kwon T. H., Lee K. M., Natori S. et al. (2000) Bacterial-injection-induced synthesis of N-beta-alanyldopamine and dopa decarboxylase in the hemolymph of a coleopteran insect, *Tenebrio molitor* larvae. Eur. J. Biochem. **267:** 2599–2608

102 Wright T. R. F. (1996) The Wilhelmine E. Key 1992 invitational lecture. Phenotypic analysis of the dopa decarboxylase gene cluster mutants in *Drosophila melanogaster*. J. Heredity **87:** 175–190

103 Wang D. and Marsh J. L. (1995) Developmental regulation of the alpha methyldopa hypersensitive gene of *Drosophila melanogaster.* Dev. Biol. **168:** 598–612

104 de Jong W. W., Leunissen J. A. and Voorter C. E. (1993) Evolution of the alpha-crystallin/small heat-shock protein family. Mol. Biol. Evol. **10:** 103–126

105 MacRae T. H. (2000) Structure and function of small heat shock/alpha-crystallin proteins: established concepts and emerging ideas. Cell. Mol. Life Sci. **57:** 899–913

106 Theopold U., Dorian C. and Schmidt O. (2001) Changes in glycosylation during *Drosophila* development. The influence

of ecdysone on hemomucin isoforms. Ins. Biochem. Mol. Biol. **31:** 189–197
107 Wright L. G., Chen T., Thummel C. S. and Guild G. M. (1996) Molecular characterization of the 71E late puff in *Drosophila melanogaster* reveals a family of novel genes. J. Mol. Biol. **255:** 387–400
108 Lackie A. M. (1986) Transplantation immunity in arthropods: is immunorecognition merely wound-healing? In: Immunity in Invertebrates, pp. 125–138, Brehélin M. (ed), Springer, Berlin
109 Sugiyama H. and Natori S. (1991) A novel mechanism of *Sarcophaga* lectin gene expression. Possible involvement of the sulfhydryl group of a fat-body protein. Eur. J. Biochem. **200:** 495–500
110 Matzinger P. (1998) An innate sense of danger. Semin. Immunol. **10:** 399–415
111 Gallucci S. and Matzinger P. (2001) Danger signals: SOS to the immune system. Curr. Opin. Immunol. **13:** 114–119
112 Anderson C. C. and Matzinger P. (2000) Danger: the view from the bottom of the cliff. Semin. Immunol. **12:** 231–238
113 Ashida M. and Yamazaki H. I. (1990) Biochemistry of the phenoloxidase system in insects: with special reference to its activation. In: Molting and Metamorphosis, pp. 239–265, Ohnishi E. and Ishizaki H. (eds), Springer, Berlin
114 Söderhäll K. and Häll L. (1984) Lipopolysaccharide-induced activation of prophenoloxidase activating system in crayfish hemocyte lysate. Biochim. Biophys. Acta **797:** 99–104
115 Nagai T. and Kawabata S. (2000) A link between blood coagulation and prophenol oxidase activation in arthropod host defense. J. Biol. Chem. **275:** 29264–29267
116 Decker H., Ryan M., Jaenicke E. and Terwilliger N. (2001) SDS-induced phenoloxidase activity of hemocyanins from *Limulus polyphemus*, *Eurypelma californicum*, and *Cancer magister*. J. Biol. Chem. **276:** 17796–17799