Eicosanoid Actions in Insect Immunity

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Abstract

Insects express 3 lines of protection from infections and invasions. Their cuticles and peritrophic membranes are physical barriers. Infections and invasions are quickly recognized within insect bodies, and recognition launches 2 lines of innate immune reactions. Humoral reactions involve induced synthesis of antimicrobial peptides, the bacteriolytic enzyme lysozyme and activation of the prophenoloxidase system. Cellular immune reactions include phagocytosis, nodulation and encapsulation. These reactions entail direct interactions between circulating hemocytes and the invaders. Cellular immune reactions begin immediately after an invasion is detected while antimicrobial peptides typically appear in the hemolymph some hours after infection. Microaggregation is a step in the nodulation process, which is responsible for clearing the bulk of bacterial infections from circulation. Coordinated cellular actions lead to encapsulation of invaders, such as parasitoid eggs, that are very much larger than individual hemocytes. In this paper, we review the roles of eicosanoids as central mediators of insect immune reactions, particularly cellular reactions. We briefly describe insect immune functions, outline eicosanoid biosynthesis and treat eicosanoid actions in cellular immunity of insects. Eicosanoids act in several cellular defense functions,

including phagocytosis, microaggregation, nodulation, encapsulation, cell spreading and hemocyte migration toward a source of a bacterial peptide. We also describe our most recent work on the influence of one group of eicosanoids, prostaglandins, on gene expression in an established insect cell line.

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Introduction

Although estimates of insect species numbers have been very large, Novotny et al. [1] recently reduced the estimate to about 4–6 million species based on a consideration of host specificity of tropical herbivorous insects. The millions of insect species occupy virtually every known habitat (although there are only a few marine species) and all conceivable niches. Such diversity exposes insects to a very wide range of infectious agents, including viruses, bacteria, fungi, protozoans and parasites. Among the many adaptations that have ensured their long-term biological success, insects have evolved effective innate immune systems. Research into insect immunology is advanced by at least 3 powerful drivers. First, insects are interesting study subjects. Second, some research into insect immunology has revealed important insights into mammalian and human immunology. Third, insect immunity may be a useful physiological system for developing novel pest management strategies [2].

Three major systems of insect immunity are usually recognized. The outer surface of insects is composed of a single layer of cells arranged on a basement membrane and a multi-layered cuticle, which together make up the integument. The integument and peritrophic membrane (a semipermeable chitinous matrix thought to protect the midgut epithelium from invasions) lining most insect midguts form physical barriers to invading organisms. Once midgut cells or the hemocoel are invaded, the intruders are met with a robust innate immune system. Humoral immunity involves induced biosynthesis of antimicrobial peptides, the enzyme lysozyme and activation of the prophenoloxidase (PPO) system [3] and cellular immunity involves direct interactions between circulating hemocytes and invaders [4]. These cellular actions immediately follow an invasion, while most humoral reactions appear some hours after infection. Recently, Haine et al. [5] suggested that the humoral reactions serve a 'mop-up' role to eliminate intruders that survived the initial cellular defense reactions. Together, physical, humoral and cellular immune responses confer effective protection. To be sure, this oversimplifies a complex innate immune system. For example, hemocytes secrete proteins that enhance hemocyte-microbe binding. In this action, the opsonizing proteins effectively link humoral and cellular immunity [4].

Here, we briefly describe insect cellular immune functions and review research on roles of prostaglandins (PGs) and other lipid mediators as central signal moieties responsible for mediating and coordinating insect cellular immunity.

Insect Cellular Immunity

Descriptions of insect immune functions are general in nature. Insects first appeared on land during the Ordovician period (over 400 million years ago) and broad descriptions obviously cannot adequately treat the many variations in immune systems that have arisen during the lengthy course of insect evolution. While immunocompetence is crucial, it is also a biologically costly physiological function which can be reallocated to other physiological functions in some insect species [6]. Immune function has been traded off for the costs of foraging in bumble bees, *Bombus terrestris* [7], and for reproductive behavior in the mealworm beetle, *Tenebrio molitor* [8]. In honeybee, *Apis mellifera*, hemocyte numbers are reduced in young adult honeybee workers, in drones and in queens while the phenoloxidase component of humoral immu-

nity is maintained [9]. These comments are germane to our appreciation of insect immunity because they highlight a few of the many variations on the general theme.

Insect cellular immune functions include phagocytosis, nodulation and encapsulation. Phagocytosis, the internalization and separate killing of microbes, is probably the oldest defense mechanism functioning in defense against microbes. In *Amoeba* and *Dictyostelium*, phagocytosis acts in feeding and in defense [10]. Multicellular animals feature dedicated, sometimes called 'professional', phagocytes. Lepidopteran granulocytes are professional phagocytic cells while in *Drosophila*, plasmatocytes are the main phagocyte. Phagocytosis is a complex process requiring expression of large numbers of genes. In their work, with a cell line established from *Drosophila* (S2 cells), Stroschein-Stevenson et al. [11] identified 184 genes required for effective phagocytosis of *Candida albicans*. In *Dictyostelium*, 443 genes are differentially regulated by phagocytosis [12]. The involvement of these many genes speaks to the complexity of phagocytosis.

Insects respond to large bacterial infections by nodule formation (fig. 1), the quantitatively predominant response to bacterial infections [13]. Nodulation begins with entrapment of bacterial cells by granule-containing hemocytes. The granule-containing cells undergo degranulation, which releases proinflammatory chemicals. Hemocytes with their entrapped bacterial cells form microaggregates (fig. 2) that grow by joining with additional cells to form nodules. The nodulation process is completed by attaching layers of flattened phagocytes to the mature nodule. The last stage is a melanization process, leaving darkened, easily visible nodules attached to the inner sides of the body wall or various organs. Invading microbial cells are topologically removed from circulation by effectively forming an impermeable wall between the nodule and the remainder of the organism. Insects respond to invasion by organisms larger than bacterial cells, such as parasites, by encapsulating them within multiple hemocytes.

Insects also express additional forms of cellular immunity. Autophagy is a process of sequestering and breaking down cellular organelles. Autophagy acts in a variety of functions, including control of infections at the intracellular level [14]. Some insect cells resist viral infections by apoptosis, a process of programmed cell death [15]. Insects also express an RNA-silencing pathway in which small RNAs are produced from double-stranded RNA. The small, interfering RNAs (iRNAs) operate to silence viral genes [16]. Viral suppression by iRNAs exerts sufficient selection power that some viruses have evolved suppressors of iRNA silencing [16].

Fig. 1. Nodulation reactions to bacterial infection in fifth instar tobacco hornworms, *M. sexta*. **a** An immunologically naïve hornworm. **b** A hornworm following challenge by injecting the bacterium, *S. marcescens*, into the hemocoel. At 4 h after injection, the hemocoel was exposed for photography. The nodules (arrows) in this photograph are 0.1 mm in diameter. Photo by J.S.M.

Research into insect immune surveillance and the signal transduction systems responsible for mediating humoral defense response to immune challenge has yielded impressive advances, including insights into cellular recognition of various microbial taxa, and discovery of the Toll and immune deficiency (*imd*) pathways [3]. Compared with the advanced knowledge of humoral immunity, there is far less information on the biochemical signaling systems involved in mediating and coordinating cellular actions in insect immunity [17]. Biogenic amines influence hemocyte activities, including enhancing

Fig. 2. Microaggregation reactions to bacterial infection in fifth instar tobacco hornworms, *M. sexta*. Hornworms were challenged by injecting the bacterium, *S. marcescens*, into the hemocoel. At selected times after injection, hemolymph was withdrawn, diluted with buffer and placed on a microscope slide for observation and photography. A photomicrograph of a large microaggregate, taken 1 h after injection. The cells in these photographs range from 10 to 12 μ m. Photo by J.S.M.

phagocytosis and nodulation in cockroach hemocytes [18] and transiently increasing rates of plasmatocyte migration [19]. Various cytokines, including active forms of Spaetzle and plasmatocytes spreading peptide, also act in insect cellular immunity [4]. Drawing on the biomedical literature, we hypothesized that PGs and other eicosanoids serve as central mediators of insect immunity.

A Sketch of Eicosanoids

The chemical structures and biosynthetic pathways of PGs and other eicosanoids are detailed elsewhere [20, 21]. Here, we provide a context with a sketch of eicosanoid biosynthesis (fig. 3). Eicosanoids are biosynthesized by enzymatic oxygenation of arachidonic acid (AA) or 2 other C20 polyunsaturated fatty acids. Because most of the mammalian eicosanoids are derived from AA, eicosanoid biosynthesis is often taken to be synonymous with 'arachidonate metabolism'. Eicosanoid biosynthesis begins with hydrolysis of AA from the *sn*-2 position of cellular phospholipids, catalyzed by a cellular phospholipase A₂ (PLA₂) [22]. The hydrolyzed AA can potentially enter 3 oxygenation pathways, cyclooxygenase (COX; yields PGs), lipoxygenase (LOX; yields a large range of

Fig. 3. Eicosanoids are synthesized from fatty acids, mainly from AA (20:4n-6) released upon cell stimulation from membrane phospholipids. AA is liberated from the membrane phospholipids via activation of a PLA₂. The free fatty acids are subsequently metabolized via two enzymic pathways: (1) the COX pathway, forming PGs, thromboxanes or prostacyclins, and (2) the various LOX pathways, forming leukotrienes, lipoxins, hepoxilins and hydro(pero)xy- and hydroxy-fatty acids. These pathways can be specifically inhibited (shown by dashed lines) using appropriate pharmaceutical probes. The glucocorticoid and other compounds inhibit PLA₂. Indomethacin, ibuprofen and other over-the-counter analgesics reduce PG production by competitive inhibition of COX activity. Esculetin and other compounds specifically inhibit LOX activity (Stanley, 2000).

products) and cytochrome P₄₅₀-‘epoxygenase’ (yields epoxyeicosatrienoic acids). Most work on insect systems has focused on COX products, the PGs. Whereas at least 2 and possibly more forms of COX are known from studies on mammalian systems, we do not yet know how many COX forms are expressed in insects. This is because the genes responsible for encoding COX or COX-like enzymes have not been reported. This will likely change in the near future as the *Drosophila Pxt* is most likely a COX-like gene [23].

Because the products of these pathways exert potent biological actions in humans, a great deal of research has led to the discovery of pharmaceutical products that selectively inhibit specific enzymes in the pathways. Many of these compounds are routinely used in biomedical applications, such as ameliorating pain. In mammalian systems, dexamethasone (Dex) is a glucocorticoid that acts

by increasing the expression of the PLA₂-binding protein annexin A1, which inhibits eicosanoid production by blocking PLA₂ activity [24]. Annexin A1 acts in several aspects of cell biology [25] and Dex does not exclusively inhibit PLA₂-dependent eicosanoid biosynthesis. Nonetheless, the inhibitory influence of Dex in insect immune systems is uniformly reversed by AA treatments, from which it is inferred that the Dex actions observed in experiments with insect systems are linked to eicosanoid biosynthesis [20].

There is substantial evidence on the presence and biological actions of PGs and other eicosanoids in invertebrates [20]. Among insects, PGs or PG biosynthesis have been detected in cricket reproductive tissues [26], tobacco hornworm midgut [27] and in insect immune tissues, hemocytes [28] and fat body [29, 30]. Hemolymph PG titers and hemocyte PLA₂ activity increase following bacterial infection [31, 32]. Hence, eicosanoid system elements are present and visibly dynamic during infection in insect immune tissues. The verisimilitude of the work on the presence of PGs in insect tissues was established with full mass spectra of PGs [27, 31]. While we have enough solid evidence of PGs and other eicosanoids in insects and other invertebrates, a major shortcoming is that the full spectrum of eicosanoids (the eicosanomics) produced in any invertebrate tissue awaits elucidation.

Eicosanoids Mediate Insect Cellular Immune Reactions to Bacterial Challenge

Many lipid mediators, including PGs, leukotrienes, platelet-activating factor, lysophosphatidic acid and endocannabinoids, act in human immune functions [33]. Based on this background, Stanley-Samuelson et al. [34] hypothesized that eicosanoids act in insect immunity. The experimental results strongly supported the idea that eicosanoids mediate one or more cellular reactions to bacterial infections. Insights into specific cellular actions came with the report that eicosanoids mediate insect nodulation responses to bacterial infections [35]. These two investigations with tobacco hornworms were the first experiments on eicosanoid actions in invertebrate immune systems and they led to the issue of whether eicosanoids similarly act in nodulation reactions in other insect species. This question was tested in a series of similar exercises with over 22 other species, summarized elsewhere [36–38]. These species make up a sampling of the class Insecta, and all results support the view that PGs and other eicosanoids are key mediators of insect cellular

immunity. As mentioned above, this generalization probably holds for insects that express cellular immune functions, recognizing that some do not.

Beyond the work described here, several other laboratories have investigated the influence of eicosanoids in insect immunity. Mandato et al. [39] investigated eicosanoid actions in 3 discrete cellular processes within the overall nodulation reaction of the waxmoth larvae, *Galleria mellonella*, reporting that eicosanoids mediate phagocytosis, cell spreading and PPO activation. Morishima et al. [40] suggested that eicosanoids mediate induction of the genes for cecropin and lysozyme in fat body of the silkworm, *Bombyx mori*. This work marks the recognition of a newly-discovered eicosanoid action in invertebrate immunity, made all the more interesting in light of the recent discovery of a functional coupling between eicosanoid biosynthetic pathways and the imd pathway in *Drosophila* [41]. We foresee that the roles of eicosanoids in immune signaling will ultimately be placed in the context of the many other signaling moieties operative in insect immunity.

Eicosanoids Act in Immune Reactions to All or Most Invaders

Fungi

Dean et al. [42] found that eicosanoids also mediate cellular defenses to the pathogenic fungi *Metarhizium anisopliae* in tobacco hornworms. Treating hornworms with inhibitors of eicosanoid biosynthesis led to increased larvae mortality, which was reversed by coinjections of the PG precursor, AA. Hornworms also produce nodules following fungal infection, and the nodulation response was reduced in experimentally treated hornworms. Again, the treatment effect was reversed by AA treatment. Tobacco hornworms also form nodules in reaction to blastospores of another fungal pathogen, *Beauveria bassiana* [43]. This work revealed that products of the LOX, but not COX, pathways acted in nodulation reactions to *B. bassiana*. The significance of this finding lies in the identification of a specific group of eicosanoids that act in an immune response.

Many infected insects are able to generate a behavioral fever by selecting postures or microhabitats to increase their body temperature. For example, house flies, *Musca domestica*, select warmer zones in a thermal gradient following infection with the fungus *Entomophthora muscae*, creating a heat therapy to ameliorate the pathogenic effects of *E. muscae* infection [44]. Behavioral fevers are

sufficiently efficacious that they have practical implications in the application of pathogenic fungus in locust biocontrol programs [45]. Adult males of the locust *Schistocerca gregaria* generate febrile responses to infection by the fungus *M. anisopliae* and by the bacterium *Serratia marcescens* [46]. Bunday et al. [46] found the fever induced by laminarin is mediated by eicosanoids. We infer from these findings that eicosanoids are involved in at least 2 lines of defense against fungal infection, the cellular defense of nodulation and the febrile defense of increasing host body temperatures to a nonpermissive level for the fungus.

We note that eicosanoids, particularly PGs, influence behaviors in vertebrates as well as in insects. These behaviors take place mostly in the context of reproduction [20] and lie outside the scope of this paper.

Protozoans

Beyond fungal infections, the blood-sucking blood, *Rhodnius prolixus*, is commonly infected by the protozoan, *Trypanosoma rangeli*, which is pathogenic to the insect but not to humans. Garcia et al. [47] reported that eicosanoids mediate immune defense reactions to *T. rangeli*. This group also recognized the actions of another lipid mediator, platelet-activating factor, in *R. prolixus* hemocyte microaggregation reactions to *T. rangeli* infection [48]. In later work, they noted that both eicosanoids and platelet-activating factor act in *R. prolixus* hemocyte phagocytosis [49]. Their work is important because it expands the range of lipid mediators known to act in insect immunity.

Parasitoids

Many insects are infected by small parasitoid wasps of the families Braconidae and Ichneumonidae, each of which includes thousands of species. Females deposit their eggs on or in the bodies of host insects, often lepidopteran caterpillars. The venom and viruses deposited in the host often inhibit host development and immunity, allowing the parasitoids to hatch and grow by consuming the host. Host insects defend themselves from parasitoid infection by hemocytic encapsulation of the eggs. Carton and his colleagues [50] demonstrated that Dex inhibits cellular immune reactions to parasitoid eggs. They used the *Drosophila melanogaster*/*Leptopilina boulardi* host-parasite system, which included a strain of *D. melanogaster* resistant to *L. boulardi* infection. This work supports the idea that eicosanoids act in insect cellular encapsulation of metazoan parasites.

Fig. 4. Photomicrograph of plasmatocyte from untreated larvae after spreading on a glass cover slip for 60 min. Digital images of the plasmatocytes were analyzed using ImageJ software (freeware from Howard Hughes Medical Institute). The line indicates the method of measuring the length (L) and the corresponding width (W). Photo by J.S.M.

Viruses

Eicosanoids are involved in insect cellular immune reactions to bacteria, fungi, protozoans and parasitoids, from which we might generalize that eicosanoids function in all cellular defense functions. However, most recently, we considered the possibility that eicosanoids also act in insect defense reactions to viral infections. We infer from the outcomes of experiments with *G. mellonella* and with *Pimpla turionellae* that eicosanoids are involved in nodulation reactions to viral infections [51, 52]. We also reported that treating larvae of the gypsy moth, *Lymantria dispar*, with various inhibitors of eicosanoid biosynthesis significantly increased the susceptibility of the larvae to mortality due to a baculovirus infection [53].

Eicosanoids and PPO Activation

Taken together, the results of experiments with bacteria, fungi, a protozoan, a parasitoid wasp and 2 viruses support the view that eicosanoids are central mediators of insect immune responses to invasion, including the whole animal febrile response, microaggregation, nodulation and possibly some aspects of humoral immunity, including PPO activation and expression of some antimicrobial peptides. Data from several groups indicate that eicosanoid signaling is involved in PPO activation in

some insects, but not in others. PPO activation is mediated by eicosanoids, in *G. mellonella* [39], *R. prolixus* [47], but not in *Locusta migratoria* [54] nor in fungal-challenged *Manduca sexta* [43].

Shrestha and Kim [55] reported a new role for eicosanoids in PPO activation in beet armyworms, *Spodoptera exigua*. In lepidopterans, PPO is expressed in a specific cell type, oenocytoids, i.e. crystal cells in *Drosophila* [56], from which it is released by cell rupture. The *S. exigua* work showed that PGs, but not LOX products, mediate activation of the beet armyworm PPO by inducing the rupture of oenocytoids to facilitate the release of PPO [55]. This work confirmed the cellular source of PPO and provided an insight into how PGs act in PPO activation. It is an important paper because it also raises the question of whether PGs similarly act in the posttranscriptional activation of PPO in other insect species. This may help understand why eicosanoids appear to act in PPO activation in some insect species, but not others.

Eicosanoids Mediate Specific Cell Actions in vitro

We investigated eicosanoid actions on in vitro hemocyte preparations. The infection-stimulated elevation in hemolymph PG titers [31] raised questions about the source of the mediating PGs. The fat body and hemocytes were prime candidates for infection-stimulated PG biosynthesis. Reasoning that the hemocytes were the source, we recorded infection-stimulated microaggregations in in vitro hemocyte preparations isolated from tobacco hornworms [57]. We concluded that hemocytes are competent to produce and secrete the eicosanoids required to mediate microaggregation reactions to bacterial challenge.

Mandato et al. [39] noted that eicosanoids influence phagocytosis and cell spreading in their work on wax moths, *G. mellonella*. Cell spreading is a phase of nodulation, and Miller [58] considered this using plasmatocytes from tobacco hornworms, *M. sexta* (fig. 4). This work identified plasmatocyte elongation as a specific eicosanoid-mediated cell action within the nodulation process. The importance of this work is that it leads to more detailed linkages between PGs and individual cell actions.

Aside from microaggregation and cell spreading, eicosanoids mediate hemocyte migration toward a source of N-formyl-Met-Leu-Phe (fMLP), the major chemotactic peptide from *Escherichia coli* [59]. We used primary hemocyte cultures prepared from tobacco hornworms to

assess hemocyte migration toward buffer (negative control) and toward buffer amended with fMLP (positive control). The work was conducted in Boyden chambers, in which cells are separated from potential attractants by a membrane. Hemocytes move through pores in the membrane toward the source of an attractant. In our work, fMLP stimulated migration in positive control preparations. Hemocyte migration was inhibited by pretreating hornworms with pharmaceutical modulators of COX, LOX and PLA₂ before preparing primary hemocyte cultures. This work reveals 2 key points in insect cellular immunity. First, insect hemocytes migrate toward a source of fMLP. This implies that insect hemocytes express fMLP receptors, perhaps analogous to the 2 fMLP receptors in many mammalian cells. Second, eicosanoids act in the complex cellular process of migration.

Mechanisms of PG Action in Insect Cells

The biomedical literature provides impressively detailed information on the mechanisms of PG actions in mammalian cells [60, 61]. Many PGs act by way of G-protein-coupled rhodopsin class receptors. In the case of PGE, for example, there are 4 receptor subtypes, EP1–EP4 [60]. The cellular actions mediated by these receptors influence homeostatic physiology, such as induction of fever [62] – true also for insects, as noted earlier [46] – and smooth muscle contraction (or relaxation) [63]. PGs also influence gene expression in many mammalian systems. PGE₂ stimulates expression of the prolactin gene in the leukemic cell line Jurkat via EP3 and EP4 receptors [64]. PGs also act in malignancy growth and metastasis [65]. The potential influence of PGs on mammalian gene expression is emphasized by the revelation of a nuclear PG signaling system in which EP3 receptors are localized in the nuclear membrane. These nuclear receptors influence gene expression via several mechanisms, including Erk-MAP kinase-dependent pathways and nuclear factor- κ B activation [66]. Overall, PGs can influence immediate cell events regulating homeostasis and long-term changes in gene expression in mammalian cells.

Unlike the biomedical background, we have virtually no information on eicosanoid actions in insect cells. Nonetheless, we infer from the work by Morishima et al. [40] that eicosanoids act in induction of genes encoding cecropin B and the enzyme lysozyme. Similarly, with respect to a *Drosophila* model, Yajima et al. [41] showed that lipopolysaccharide-stimulated induction of the gene encoding the antibacterial protein, dipterecin, is mediated

by the imd pathway, but not by the Toll pathway. In a screen of inhibitory compounds, they found that inhibitors of PLA₂ repressed lipopolysaccharide-stimulated gene expression. The authors concluded that there is a functional coupling between the imd pathway and pathways responsible for eicosanoid biosynthesis. Based on these findings, we generated the hypothesis that one mode of PG action in insect cells is the ability to influence gene expression.

We tested our hypothesis by treating an established cell line (BCIRL-HzAM1) with PGA₁ and, separately, with PGE₁, following what has become a fairly routine proteomic protocol [67]. The PG treatments resulted in substantial changes in the expression of 30 proteins, some of which are relevant to host defense or cell function. For examples, PGE₁ treatments resulted in a 1,700-fold expression increase for a heat-shock protein 70 and a 4-fold expression increase for a mitochondrial processing peptidase. Similar PGA₁ treatments resulted in a 2-fold increase in expression of an Mn superoxide dismutase. Genes encoding many of the 30 proteins were downregulated by PG treatments. The PG influence on gene expression was transiently expressed as the influence was generally high at 12-hour posttreatment and gone by 24 h. Overall, this work supports our hypothesis that PGs influence gene expression in insect cells. We expect this work will ultimately help identify specific genes whose products serve directly in defense reactions against immune challenges.

Conclusions

It is now approximately 17 years since the first suggestion that eicosanoids mediate insect immune reactions to bacterial infection. Since then, the biochemistry of eicosanoid biosynthesis has been characterized in immunity-conferring tissues, the eicosanoid hypothesis has been supported by experiments with a phylogenetically wide range of insect species, and a wide range of infecting agents and several specific eicosanoid-mediated cellular actions have been identified. In research on the biochemical modes of PG actions, the influence of PGs on gene expression in insect cells has been recorded, linking PG actions to specific proteins. Very recent work indicates that the *Drosophila* *Pxt* may be a gene encoding an insect COX, and this opens new possibilities for experimentation. The number of known biomolecules acting in insect immunity is increasing and this will also open possibilities to investigate the interaction of these different moi-

eties in immune reactions. For example, PGs and the protein Noduler, a rapidly expressed binding protein [68], are both required for nodulation and if either is inhibited, nodulation is reduced. Are eicosanoids involved in the expression of nodular reaction? Continued research into eicosanoid actions in insect immunity holds promise for a great deal of discovery.

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