1994

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Miller, Jon S.; Nguyen, TuAnh; and Stanley-Samuelson, David W., "Eicosanoids mediate insect nodulation responses to bacterial infections" (1994). *Faculty Publications: Department of Entomology*. 219.  
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Eicosanoids mediate insect nodulation responses to bacterial infections

(cyclooxygenase/lipoxygenase/phospholipase A2/Manduca sexta/Serratia marcescens)

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Communicated by Wendell L. Roelofs, September 12, 1994 (received for review August 11, 1993)

ABSTRACT We propose that nodule formation is mediated by eicosanoids in insects. Nodulation is the temporally and quantitatively predominant cellular defense response to bacterial infection in insects and other invertebrates. Inhibition of eicosanoid biosynthesis in larvae of the tobacco hornworm Manduca sexta immediately prior to intrahemocoelic infections with the bacterium Serratia marcescens strongly reduced the nodulation response. Inhibition of eicosanoid biosynthesis also reduced formation of cellular aggregates at 1 hr postinfection, which indicates that eicosanoids mediate early stages of nodulation. Separate treatments with specific inhibitors of phospholipase A2, cyclooxygenase, and lipoxygenase reduced nodulation, which supports the view that nodule formation is a complex process involving prostaglandins and lipoxygenase products. The inhibitory effects of the phospholipase A2 inhibitor dexamethasone on nodulation were apparent by 1 hr after infection, and the effects increased, relative to controls, over 24 hr. The dexamethasone effects were expressed in a dose-dependent manner, and they were reversed by treating infected insects with eicosanoid-precursor polyunsaturated fatty acids. Treatments with the saturated fatty acid 16:0, which is not an eicosanoid precursor, did not reverse the dexamethasone effects on nodulation. These findings strongly support the identification of nodulation as a specific insect cellular defense mechanism that is mediated by eicosanoids.

Insects elaborate two broad categories of defense responses to bacterial infections, humoral and hemocytic (1–3). Humoral responses involve induced synthesis of antibacterial proteins such as cecropins (4 kDa), attacins (12–23 kDa), dipterins (8 kDa), and defensins (4 kDa) (3). The detergent properties of these antibacterial proteins disrupt bacterial cell membranes. Insects also synthesize lysozymes, enzymes that directly attack bacteria by hydrolyzing their peptidoglycan cell walls (4, 5). Hemocytic responses feature direct interactions between circulating hemocytes and bacteria. Specific cellular defense mechanisms include phagocytosis, nodulation, and encapsulation (1, 2).

We recently suggested that insect cellular immune responses to bacterial infections are mediated by eicosanoids (6). We observed the effects of pharmaceutical inhibitors of eicosanoid biosynthesis on the ability of the tobacco hornworm Manduca sexta to clear artificial infections from its circulation. Inhibition of total eicosanoid biosynthesis, with injections of the phospholipase A2 (PLA2) inhibitor dexamethasone, severely reduced the hornworm’s ability to clear injected bacteria from its hemolymph and increased larval mortality due to the bacterial infections. Inhibition of either of two specific eicosanoid biosynthesis pathways, cyclooxygenase and lipoxygenase, also reduced clearance of bacteria to a lesser extent compared to the effects of completely inhibiting eicosanoid biosynthesis. On the basis of these findings, we propose that eicosanoid products of the cyclooxygenase and lipoxygenase pathways are involved in insect immune responses to bacterial infections. Because most of our experiments were done during the first hour postinfection (PI), long before the appearance of antibacterial proteins in insect hemolymph, we suggested that eicosanoids mediate one or more hemocytic defense responses (6).

This suggestion opens a crucial question: which of the hemocytic defense mechanisms are mediated by eicosanoids? Because nodulation (see Fig. 1) is the quantitative major defense response (7), we hypothesized that nodulation is one of the cellular defense mechanisms that is mediated by eicosanoids in insects. In this paper we describe outcomes of experiments designed to test our hypothesis.

MATERIALS AND METHODS

Organisms. Fifth-instar M. sexta larvae weighing 4–6 g were used in all experiments. Larvae were reared from eggs provided in weekly shipments by J. Buchner (Department of Agriculture/Agricultural Research Service Biosciences Research Laboratory, Fargo, ND). The larvae were reared on an artificial diet under the semisterile conditions developed by Dunn and his colleagues (7, 8).

Cultures of a nonpigmented strain of Serratia marcescens and nutrient broth (Difco) were purchased from Carolina Biological Supply. Bacteria were grown in 50 ml of nutrient broth in an environmental shaker at 37°C and 100 rpm. Bacteria were used in midlogarithmic or stationary phase at a dose of 2.5–7.0 × 10⁶ colony-forming units per ml (6).

Injections and Assays for Aggregate Formation and Nodulation. Test larvae were injected with either the PLA2 inhibitor dexamethasone [(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione]; the arachidonic acid analog 5,8,11,14-eicosatetraenoic acid (ETYA); one of the cyclooxygenase inhibitors indomethacin [1-(p-chlorobenzoyl)-5-methoxy-2-methyl-3-indolyl-acetic acid]; naproxen [4,6-(6-methoxy-2-naphthyl)propionic acid], ibuprofen [2-(4-isobutylphenyl)propionic acid], or piroxicam [3,4-dihydro-2-methyl-4-oxo-N-2-pyridyl-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide]; the dual cyclooxygenase and lipoxygenase inhibitor phenindone [1-phenyl-3-pyrazolinone]; or the 5- and 12-lipoxygenase inhibitor esculetin [6,7-dihydroxy-2H-chromen] (all inhibitors from Biomol, Plymouth Meeting, PA). In some experiments, larvae were also injected with arachidonic acid (5,8,11,14-eicosatetraenoic acid), eicosapentaenoic acid (5,8,11,14,17-eicosapentaenoic acid), or palmitic acid (hexadecanoic acid) (Sigma). Control larvae were injected with nutrient broth or 95% ethanol. All injections of pharmaceuticals were in a standard volume of 10 μl. The

Abbreviations: LSD, least significant difference; PI, postinfection; PLA2, phospholipase A2; PUFA, polyunsaturated fatty acid; ETYA, eicosatetraenoic acid.

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pharmaceutical products were injected at doses of 26 μg per larva, except in the dose–response experiments, and the fatty acids were injected at doses of 50 μg per larva. Larvae were surface sterilized by swabbing their surfaces with 95% ethanol. Bacteria (2.5–7.0 × 10⁷ colony-forming units per ml) were injected in 100-μl aliquots, using a 26-gauge 0.5-inch needle attached to a 1-ml syringe (Becton Dickinson). Drugs and control substances were injected in 10-μl aliquots into the opposite side of the larva using a Hamilton 701 syringe (Hamilton) (6).

Hemocyte aggregation (Fig. 1A) was assessed at 1 hr PI. Larvae were anesthetized by chilling on ice and then hemolymph was collected by pericardial puncture, using Teflon-lined needles as described by Horohov and Dunn (7). One drop of freely flowing hemolymph was applied to a Bright-Line hemacytometer (AO Instrument, Buffalo, NY). The number of cellular aggregates in each sample was estimated by direct observation of every field under phase-contrast optics. Total hemocyte count in the same samples was estimated by counting every field on the hemacytometer (7).

Nodulation (Fig. 1B) was assessed at selected times PI. Larvae were anesthetized by chilling on ice, and then the hemocoels were exposed. Melanized, dark nodules were counted under a stereomicroscope. After the initial counting, the alimentary canal was excised. Nodules in the previously unexposed areas and remaining internal tissues were then counted. The sizes of selected nodules were determined using an optical micrometer.

Background Control Experiments. Because the insects used in these experiments were reared under semisterile conditions, it was expected that the insects would have very few nodules before the treatments. To determine background nodulation, a total of 20 larvae were taken from culture, at various times in this project, and anesthetized on ice for 10 min; nodulation was asayed as just described. To assess the influence of injection wounds on nodule formation, nine larvae were treated by intrahemocoelic injection of a standard volume of ethanol. Nodulation was assessed 6 hr later.

To assess the effect of dexamethasone on nodulation in uninjected larvae, 26 μg of dexamethasone in 10 μl of ethanol was injected into 15 larvae. Nodulation was assessed 6 hr later. To control for the possibility that nutrient broth could stimulate nodulation, 14 larvae were similarly treated by intrahemocoelic injection of a standard volume (100 μl) of nutrient broth. Nodulation was assessed 6 hr later.

Time Course of Nodulation: Influence of Dexamethasone. Individuals in two groups of larvae were treated with 10 μl of ethanol or with 26 μg of dexamethasone in 10 μl of ethanol and then infected with _S. marcescens_ as described. At 1, 3, 4, 6, and 24 hr PI, subgroups of control and experimental larvae were anesthetized, and nodulation was assessed.

Dose–Response Relationship for Dexamethasone. Individuals in seven groups of larvae were treated with 10 μl of ethanol or with 2.6 × 10⁻⁷, 2.6 × 10⁻⁶, 2.6 × 10⁻⁵, 2.6 × 10⁻⁴, or 2.6 μg of dexamethasone in 10 μl of ethanol and then were infected with _S. marcescens_ as described. At 6 hr PI, the larvae were anesthetized, and nodulation was assessed.

Fatty Acid Rescue Experiments. Individuals in two groups of larvae were injected with either 10 μl of ethanol or 26 μg of dexamethasone in 10 μl of ethanol and then infected with _S. marcescens_ as described. Immediately after infection, the dexamethasone-treated larvae were divided into four subgroups. Individuals in each of three subgroups were treated with 50-μg injections of either arachidonic acid, eicosapentaenoic acid, or palmitic acid in 5 μl of ethanol. Individuals in the fourth subgroup were similarly treated with 5 μl of ethanol to control for the effects of the extra injection on nodulation. At 6 hr PI, the larvae were anesthetized, and nodulation was assessed.

Influence of Other Eicosanoid Biosynthesis Inhibitors on Nodulation. Individuals in groups of test larvae were injected with either one of the cyclooxygenase inhibitors (indomethacin, naproxin, ibuprofen, or piroxicam), the dual cyclooxygenase and lipoxygenase inhibitor phenidone, the lipoxygenase inhibitor esculolin, or the arachidonic acid analog ETYA, all in 10 μl of ethanol. Control larvae were injected with 10 μl of ethanol. Three to 10 min after injection of the inhibitors or ethanol, the larvae were infected with _S. marcescens_ as described. At 6 hr PI, the larvae were anesthetized, and nodulation was assessed.

Statistical Analyses. Significant treatment effects were confirmed for hemocyte aggregation by Student's _t_ test with _P_ = 0.10. Significant treatment effects were confirmed for all nodulation experiments by analysis of variance with _P_ ≤ 0.01. Where appropriate, significant differences among treatment means were determined by protected least significant difference (LSD).

RESULTS

Hemocyte Aggregation and Total Hemocyte Count. Table 1 displays the influence of dexamethasone on the number of

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**Fig. 1.** Photomicrographs of early and late phases of nodulation in response to bacterial infection. (A) Photomicrograph of hemocyte aggregation in response to bacterial infection. The cells and aggregates are shown against the background of the hemacytometer used in routine assessments at 1 hr PI using a Nikon Optiphot microscope equipped with phase-contrast optics and a Nikon FX-35A camera. Hemocyte aggregation is an early step in nodule formation. (B) Photomicrograph of melanized nodules in response to bacterial infection. The solid arrow points to one of several visible nodules, seen in situ against the background of larval fat body. This photograph was taken during routine assessment of nodulation at 6 hr PI using an Olympus SZ series stereomicroscope equipped with a SC 35 camera. (A, ×260; B, ×40; photographs by J.S.M.)
bacterial larva) were treated with ethanol or with vehicle. The ethanol-treated larvae in nodules of visible nodulation were 122 \times 10^{-4}, 10.62 \pm 1.52* 4**. The decreased recovery of hemocyte aggregates was reflected in significantly increased total hemocyte counts. The influence of dexamethasone on the number of hemocyte aggregates and on total hemocyte counts indicates that dexamethasone impacted visible nodule formation through its effects on hemocyte aggregation. In the following paragraphs, we describe the influence of probing eicosanoid biosynthesis on formation of visible nodules.

**Background Control Experiments.** The results of the control experiments are displayed in Table 2. No nodules were observed in larvae that were taken directly from the sterile rearing conditions. To examine the effects of injection on nodule formation, 10-μl doses of ethanol, the standard carrier vehicle for all pharmaceuticals, were injected into larvae. No nodules were observed in eight of nine larvae, and 4 nodules were observed in one larva. Dexamethasone treatments caused about 1 nodule per larva. Treatments with nutrient broth resulted in about 6 nodules per larva. Injections of standard dosages of *S. marcescens* resulted in about 121 nodules per larva. Throughout the course of this project, the sizes of selected nodules were estimated. A total of 762 nodules were sized, of which 98% were about 0.1 mm in diameter, 1% were 0.2 mm in diameter, and the remainder were 0.3–0.5 mm in diameter.

**Time Course of Nodulation.** Fig. 2 displays the time course of visible nodulation in two groups of larvae. Dexamethasone-treated larvae formed about 4 nodules at 1 hr PI, which increased to 30 at 6 hr PI and 42 nodules per larva at 24 hr PI. The ethanol-treated control larvae produced significantly more nodules at each time point, from 21 nodules at 1 hr PI to 122 at 6 hr PI and 148 nodules per larva at 24 hr PI (LSD, *P* ≤ 0.01). Nodulation was not substantially increased by 24 hr PI, and in all subsequent experiments nodulation was assessed at 6 hr PI.

**Dose–Response Curve for Dexamethasone.** The relationship between dexamethasone dose and number of nodules is shown in Fig. 3. Increased dexamethasone doses (0, 2.6 × 10^{-4}, 2.6 × 10^{-3}, 2.6 × 10^{-2}, 2.6, and 26.0 μg per larva) were associated with decreased nodulation in response to bacterial infections, from 134 to 30 nodules per larva. Mean nodulation was significantly related to dexamethasone dose (*y* = −3.04 *x* + 109, *R*² = 0.716, *F* = 15.96).

**Fatty Acid Rescue Experiments.** As we have just seen, dexamethasone treatments inhibited the ability of tobacco hornworm larvae to form nodules in response to bacterial infections. On the basis of the model that dexamethasone inhibits eicosanoid biosynthesis through its effects on PLA₂, we reasoned that injecting eicosanoid-precursor polyunsaturated fatty acids (PUFAs) into dexamethasone-treated, infected larvae should reverse the effects of dexamethasone on nodulation. To test this, larvae were treated in the following sequence: after injection with dexamethasone, larvae were infected with bacteria and then immediately treated with 50 μg of arachidonic acid, eicosapentaenoic acid, or palmitic acid in 5 μl of ethanol. To control for the possibility that the extra injection stimulated nodulation, some larvae were injected with 5 μl of ethanol. To control for the possibility that the extra injection stimulated nodulation, some larvae were examined for nodulation. Fig. 4 shows that the arachidonic acid rescued nodulation.

![Fig. 2](image.png)  
**Fig. 2.** Time course of *M. sexta* nodulation in response to intrahemocoelic infections with the pathogenic bacterium *S. marcescens*. Test larvae were first injected with dexamethasone (dashed line), and control larvae were first injected with ethanol (solid line). Within 3–10 min, both groups of insects were then intrahemocoelically infected with 5 × 10^5 bacterial cells. At the indicated times after infection, the insects were anesthetized on ice, and nodulation was assessed. Each point indicates the mean number of nodules found in each larva, and the error bars represent 1 SEM. The numbers in parentheses indicate the number of larvae.

![Fig. 3](image.png)  
**Fig. 3.** Dose–response curve for the effects of dexamethasone on nodule formation. *M. sexta* larvae were first injected with the indicated doses of dexamethasone and then intrahemocoelically infected with 5 × 10^5 cells of the bacterium *S. marcescens*. After 6 hr of incubation, the insects were anesthetized on ice, and nodulation was assessed. Each point indicates the mean number of nodules found in each larva, and the error bars represent 1 SEM. The numbers in parentheses indicate the number of larvae. The dashed line represents *y* = −3.04 *x* + 109.
cates that a second injection did not exert a significant effect on nodulation. Another eicosanoid-precursor PUFAs, eicosapentaenoic acid, similarly reversed the dexamethasone effects (Fig. 4). The rescue effects were specific to eicosanoid-precursor PUFAs, because palmitic acid (16:0), which cannot be desaturated to form C20 PUFAs, did not restore nodulation to control levels (LSD, \( P < 0.01 \)).

**Influence of Other Eicosanoid Biosynthesis Inhibitors on Nodulation.** To dissect the possible roles of cyclooxygenase and lipoxygenase pathways in nodulation, larvae were treated with standard doses of four cyclooxygenase inhibitors (indomethacin, naproxin, ibuprofen, or piroxicam), the dual cyclooxygenase and lipoxygenase inhibitor phenidone, the 5- and 12-lipoxygenase inhibitor esculetin, or the arachidonate analog ETYA and then artificially infected with bacteria. Fig. 5 shows that compared to control larvae, all test larvae exhibited significantly reduced nodulation in response to bacterial infections (LSD, \( P < 0.01 \)). There were no significant differences among the effects of individual inhibitors on nodulation.

**DISCUSSION**

The data described here permit us to identify nodulation as one of the specific cellular defense responses that is mediated by eicosanoids in insects. This insight is supported by the following points. First, the dexamethasone effect on nodulation began within the first hour PI and increased relative to control animals throughout the time course of the experiments. Second, the dexamethasone effects on nodule formation were expressed in a dose-dependent manner. Third, the influence of dexamethasone was reversed by treating the larvae with free eicosanoid-precursor fatty acids. Finally, inhibition of eicosanoid biosynthesis with specific inhibitors of PLA2, cyclooxygenase, and lipoxygenase significantly reduced nodulation in infected hornworms. Nodules are easily visible in tobacco hornworms because they are darkly melanized, and it is not immediately clear whether eicosanoids mediate aggregation of hemocytes, which leads to nodulation, or if eicosanoids mediate the melanization reactions, which would make nodules easily visible. Because significantly reduced numbers of circulating aggregates were recovered from dexamethasone-treated larvae at 1 hr PI, we postulate that eicosanoids mediate early steps in nodule formation. This idea is also supported by the influence of dexamethasone on total hemocyte counts in infected larvae. In their work on tobacco hornworm larvae, Geng and Dunn (9) found that bacterial infections elicited decreased circulating hemocyte numbers, presumably due to invading hemocytes in nodule formation. In our experiments, ethanol-treated larvae yielded larger numbers of cellular aggregates and smaller total hemocyte counts, whereas dexamethasone-treated larvae yielded fewer cellular aggregates and greater total hemocyte counts. Again, while not ruling out the possible influence of eicosanoids on melanization reactions, we propose that eicosanoids mediate the nodulation response to bacterial infections in insects. The significance of this insight lies in a broad understanding of immunological defenses in invertebrates: nodulation is a primary means of responding to bacterial infections in arthropods and in other phyla. To the extent that immune responses in tobacco hornworms model invertebrate immunity, eicosanoid biosynthesis is a fundamental and crucial step in the mediation of cellular defense reactions.

Because the melanization responses to injection wounds can produce the appearance of nodulation, we conducted experiments designed to control for background nodulation (Table 2). To minimize background nodulation, the tobacco hornworms were reared in the semisterile culture conditions developed by Dunn and colleagues (7, 8). No nodules were present.
seen in insects taken directly from our culture. Injections of the drug vehicle, of dexamethasone, and of sterile nutrient broth uniformly resulted in negligible background nodulation (Table 2). These data indicate that the nodulation we observed was a specific response to bacterial infections and not a reflection of the insects’ background health.

The experimental treatments could result in widely varying nodule sizes that might obscure accurate nodulation assessments. We recorded the sizes of over 750 nodules, 98% of which were about 0.1 mm in diameter. The uniform nodule sizes suggest that our assessments accurately index the nodulation response to bacterial infections.

The linear relationship between logarithmically increasing dexamethasone doses and decreasing nodulation and also reversing the dexamethasone effects on nodulation with eicosanoid-precursor PUFAs (Figs. 3 and 4) powerfully argue that eicosanoids mediate the insect nodulation response to bacterial infections. The dexamethasone effects on eicosanoid biosynthesis are thought to act at the level of PLA2, which catalyzes hydrolysis of PUFAs from cellular phospholipids. If dexamethasone exerts its effects by inhibiting PLA2 in M. sexta, then subsequent treatments with eicosanoid-precursor PUFAs should reverse the dexamethasone effects. Fig. 4 shows that two eicosanoid-precursor PUFAs, arachidonic acid and eicosapentaenoic acid, restored nodulation in dexamethasone-treated, infected hornworms. An apparent restoration of nodulation could have resulted from nonspecific effects, such as additional handling, the extra injection required to administer the fatty acids after infecting the larvae, or a pharmacological effect of the fatty acids. However, handling and injecting 5 µl of ethanol into dexamethasone-treated, infected larvae did not increase nodulation. Similarly, injecting palmitic acid, which cannot lead to eicosanoid biosynthesis, appeared to slightly increase nodulation, but did not restore nodulation to the level seen in control larvae or eicosanoid-precursor PUFA-treated larvae. These experiments support the idea that eicosanoids mediate the nodulation response to bacterial infections in insects.

Fig. 5 indicates that a wide range of eicosanoid biosynthesis inhibitors effectively reduced nodulation in infected larvae. The point to offer is that various eicosanoids, including cyclooxygenase and lipoxygenase products, may be involved in a number of specific cellular events in the complete nodulation process. Inhibition of any one or more of the eicosanoid-mediated steps may negatively impact the overall nodule formation. If this is so, it would not be surprising that inhibition of either of the major eicosanoid-biosynthetic pathways would similarly inhibit nodulation.

A major difficulty with using pharmaceutical inhibitors to probe the possible roles of eicosanoids in physiological systems in invertebrates is the possibility of nonspecific or nonphysiological effects (10). We have addressed this issue by arguing from cases in which the inhibitors we used have been shown to have similar pharmacological actions in invertebrates and in mammals (6). Moreover, we recently obtained biochemical evidence that very low levels of naproxin and indomethacin (0.001 µM) strongly inhibited cyclooxygenase activity in fatty body and hemocyte preparations (11–13). Use of inhibitors to probe these pathways also rests upon the assumption that the inhibitory compounds reach the target tissues in vivo. We also have learned that one inhibitor, indomethacin, is rapidly accumulated from heat-molymph circulation by tobacco hornworm tissues, including hemocytes and fat body (J.S.M. and D.W.S.-S., unpublished results). Considerably more work on the pharmacology and the quantitative influence of putative eicosanoid biosynthesis inhibitors on in vivo eicosanoid titers in insects is required before these compounds can be completely accepted as probes to assess the influence of eicosanoids in insect physiology (10). In the meantime, drawing on the evidence just cited, we propose that the compounds used in our experiments express their effects through inhibition of eicosanoid biosynthesis.

Cyclooxygenase and lipoxygenase products are crucial mediators of many aspects of mammalian physiology, including cellular host defense actions. For example, cyclooxygenase products have profound effects on macrophage locomotion, cell shape changes, and phagocytosis (14); and lipoxygenase products mediate chemotaxis, chemokinesis, and adherence responses of neutrophils (15). Coupled with the background of mammalian studies, our findings with tobacco hornworms allow us to propose that eicosanoids mediate cellular defenses in vertebrates and invertebrates. This idea is also supported by recent findings on blood cells from the crab Carcinus maenas. These cells biosynthesize eicosanoids, including cyclooxygenase and lipoxygenase products (16), which may similarly mediate defense responses.

We thank Dr. Leon Higley for his expert help with the statistical analyses and for critical comments on this paper. We also thank Dr. J. Buckner (Fargo, ND) for regular shipments of M. sexta eggs. This paper is no. 10475, Nebraska Agricultural Research Division, and contribution no. 837 of the Department of Entomology. This work was supported by National Institutes of Health Grant AI 31509 to D.W.S.-S.