EICOSANOIDS MEDIATE NODULATION REACTIONS TO BACTERIAL INFECTIONS IN ADULTS OF THE AMERICAN COCKROACH, *PERIPLANETA AMERICANA* (L.)

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Abstract *Proc. ent. Soc. Ont.* 130: 97–108

Nodulation is the first, and qualitatively predominant, cellular defense reaction to bacterial infections in insects. We tested the hypothesis that eicosanoids mediate nodulation reactions to bacterial infections in adults of the American cockroach, *Periplaneta americana*. Treating experimental cockroaches with the eicosanoid biosynthesis inhibitor, dexamethasone, strongly impaired nodulation reactions to bacterial infections. The influence of dexamethasone was reversed by treating infected insects with arachidonic acid, an eicosanoid precursor. Several other eicosanoid biosynthesis inhibitors, including the lipoxygenase inhibitor, esculetin, and the cyclooxygenase inhibitors ibuprofen and naproxen, also impaired the ability of experimental cockroaches to form nodules in reaction to bacterial infections. The influence of two inhibitors, phenidone and esculetin, were expressed in a dose-dependent manner. These findings support the hypothesis that eicosanoids mediate cellular immune responses to bacterial infections in cockroaches, and more broadly, in insects.

Introduction

Hemocytic immune reactions to bacterial infections involve direct cellular interactions between circulating hemocytes and bacteria. Specific cellular defense mechanisms include phagocytosis, nodulation and encapsulation (Gupta 1986, 1991). While humoral and hemocytic immune reactions to bacterial infections are well documented, until recently there was virtually no information on the biochemical events responsible for mediating insect immune reactions. Drawing on the background of signal transduction systems in mammalian immunity, Stanley-Samuelson et al. (1991) suggested insect cellular immune reactions are mediated by eicosanoids. Eicosanoids are oxygenated metabolites of arachidonic acid and two other polyunsaturated fatty acids (Fig. 1). There are three major groups of eicosanoids. One includes the prostaglandins, another the epoxyeicosatrienoic acids. The third group is composed of the many lipoxygenase products. Eicosanoids are very well understood in the contexts of human and animal medicine, where they mediate many pathophysiological events, such as inflammation. Eicosanoids also are important for many actions in invertebrates, all detailed in recent reviews (Stanley-Samuelson 1994a,b; Stanley and Howard 1998; Stanley 1999).

In our initial investigations we determined that treating tobacco hornworms, *Manduca sexta*, with pharmaceutical inhibitors of eicosanoid biosynthesis rendered experimental hornworms unable to clear bacterial infections from hemolymph circulation. We inferred from these observations that

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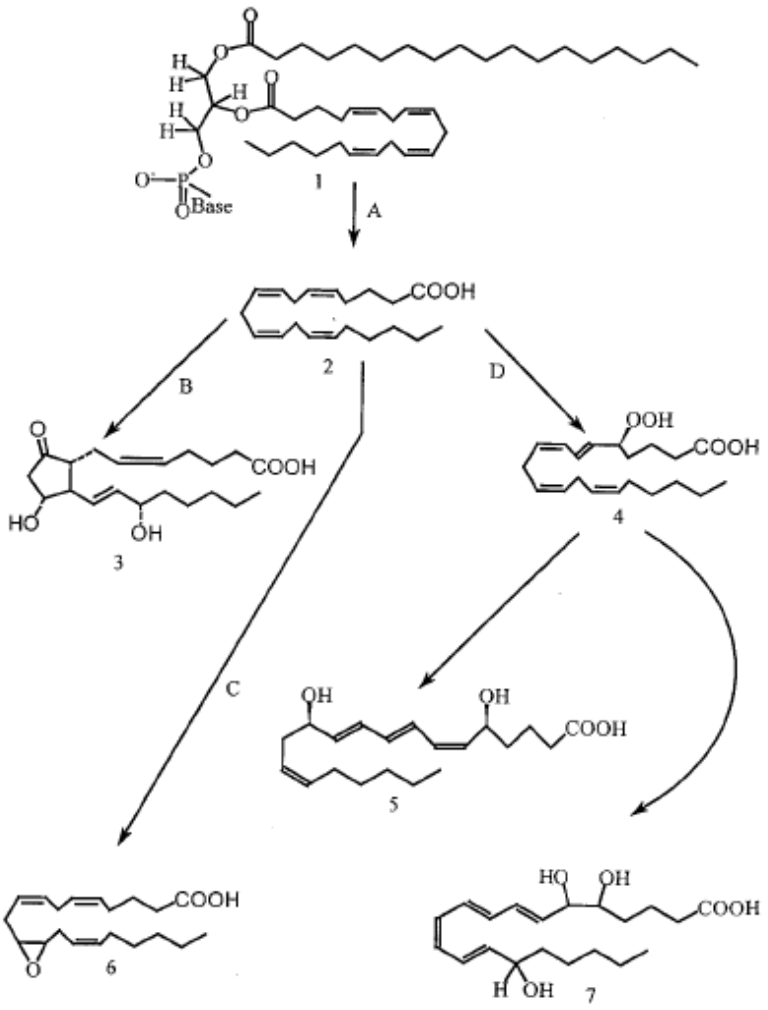

FIGURE 1. An overview of 20:4n-6 metabolism as understood from mammalian physiology. Three polyunsaturated fatty acids, 20:3n-6, 20:4n-6 and 20:5n-3 are potential substrates for eicosanoid biosynthesis. Of these, metabolism of 20:4n-6 is most well studied. Chemical structures are denoted by numerals. **1** = a cellular phospholipid. **2** = hydrolyzed 20:4n-6. **3** = prostaglandin E_2. **4** = 5-hydroperoxyeicosatetraenoic acid. **5** = leukotriene B_4. **6** = 11,12-epoxyeicosatrienoic acid. **7** = lipoxin A. Capital letters indicate major enzyme systems responsible for eicosanoid biosynthesis. **A** = phospholipase A_2; **B** = cyclooxygenase and associated enzyme steps; **C** = cytochrome P_{450} epoxygenase; **D** = lipoxygenase.

some or all of the cellular defense reactions responsible for clearing bacterial infections from hemolymph are mediated by eicosanoids (Stanley-Samuelson et al. 1991). This was the first suggestion of a signal transduction system in invertebrate cellular immunity.

This work prompted more detailed experiments designed to determine which of the several cellular defense reactions depend on eicosanoid biosynthesis. Nodulation is an early insect cellular defense reaction responsible for clearing large numbers of bacterial cells from circulation during the first two hours of an infection (Horohov and Dunn 1983). Because nodulation is the predominant cellular reaction to bacterial infections, we hypothesized that eicosanoids mediate nodulation reactions to bacterial infections. We tested this idea by injecting hornworms with an eicosanoid biosynthesis inhibitor, then infecting them with bacteria. Compared to ethanol-treated controls, the experimental larvae produced far fewer nodules in response to similar bacterial challenges. We also showed that the influence of the eicosanoid biosynthesis inhibitor, dexamethasone, could be reversed by treating the experimental larvae with arachidonic acid, the immediate precursor of eicosanoids. We concluded that nodulation is one of the cellular immune responses to bacterial infections that is mediated by eicosanoids (Miller et al. 1994).

On the basis of these findings on a single lepidopteran species, we developed the hypothesis that eicosanoids mediate nodulation reactions to bacterial infections in most, if not all, insect species, now known as the eicosanoid hypothesis (Stanley and Howard 1998; Stanley 1999). Using similar experimental protocols, we have shown that nodulation reactions to bacterial infections depend on eicosanoid biosynthesis in the tenebrionid beetle, *Zophobas atratus* (Miller et al. 1996), in the silkworm, *Bombyx mori* (Stanley-Samuelson et al. 1997) and in two other moths, black cutworms, *Agrotis ipsilon* and true armyworms, *Pseudaletia unipuncta* (Jurenka et al. 1997). In related work, Mandato et al. (1997) found that cell spreading and prophenyloxidase activation, two distinct phases of nodulation, and phagocytosis are mediated by eicosanoids in waxmoths, *Galleria mellonella*. These findings support the eicosanoid hypothesis to the extent that several lepidopteran species represent the Class Insecta.

To consider a broader phylogenetic range of insect species, Miller et al. (1999) investigated the hypothesis in adults of a hemimetabolous insect, the cricket *Gryllus assimilis*. Similarly, Tunaz et al. (1999) conducted a parallel series of experiments with two species of 17-year periodical cicadas, *Magicicada septendecim* and *M. cassini*. Again, all experimental results support the hypothesis. On the basis of these findings, we suggested that eicosanoids mediate cellular immune reactions in hemimetabolous insects (Miller et al. 1999). However, this broader hypothesis is weakened because the eicosanoid hypothesis has been tested in only three hemimetabolous insect species. To address this shortcoming, we tested the hypothesis in adults of the American cockroach, *Periplaneta americana*, a hemimetabolous insect species representing the Orthopteroidea. Here we report that adults of this species form nodules in response to bacterial infections, and that the nodulation response is impaired by treating the cockroaches with eicosanoid biosynthesis inhibitors just prior to infection. In view of the widespread use of this species in insect immunity, these results facilitate future work on insect immunity.

Materials and Methods

Organisms

A culture of cockroaches, *P. americana*, was provided by Johnson and Johnson, Inc. All stages of the insects were maintained in a 50-gallon container partitioned with egg-crates and provisioned with dog chow and water. The containers were kept on laboratory benches at room temperature and exposed to a natural photoperiod. Male and female cockroaches were randomly used in all experiments.

Cultures of a non-pigmented strain of the bacterium *Serratia marcescens* and nutrient broth (Difco) were purchased from Carolina Biological Supply (Burlington, NC). Bacteria were grown in 50 ml of nutrient broth in an environmental shaker at 37°C and 100 rpm. Bacteria were grown to a titre of (10^3) colony forming units/ml, and used in stationary phase.

Injections and assays for nodulation

We followed the protocols formalized by Miller and Stanley (1998). Before injections, cockroaches were surface sterilized by swabbing their cuticle with 95% ethanol. We injected adult cockroaches with either the phospholipase A_2 (PLA_2) inhibitor dexamethasone {(11ß, 16)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-dione}, one of the cyclooxygenase inhibitors, ibuprofen {α -methyl-4 (2-methylpropyl) benzeneacetic acid}, indomethacin {1-P-(chlorobenzyl)-5-methoxy-2-methyl-3-indolyl-acetic acid}, naproxen {O-2-(6-methoxy-naphthyl) propionic acid}, or piroxicam {3,4-benzothiazine-3-carboxamide 1,1-dioxide}, the dual cyclooxygenase and lipoxygenase inhibitor phenidone {1-pheny-3-pryazolidinone}, or the 5- and 12-lipoxygenase inhibitor, esculetin {6,7-dihydroxycoumarin} (all inhibitors purchased from BioMol, Plymouth Meeting, PA). In rescue experiments, adults were injected also with arachidonic acid {5,8,11,14-eicosatetraenoic acid}, purchased from Sigma Chemical Co. (St. Louis, MO). Control insects were injected with 95% ethanol. Drugs and control substances were injected into the opposite side of the abdomen using a 10 µl Hamilton 701 syringe. All injections of pharmaceuticals were in a standard dosage of 26 µg in 3 µl of ethanol, except in dose-response experiments. The fatty acids were injected at dosages of 50 µg in 5 µl per adult cockroach.

Immediately after the drug injections, adult cockroaches were infected by injecting a standard bacterial dosage of 10^5 colony forming units/cockroach (Miller et al. 1994, 1996). Bacteria were injected in 10 µl aliquots, using a 26 gauge 0.5" needle attached to a 50 µl syringe (Hamilton, Reno, NV).

We assessed nodulation at selected times post infection (PI). We anesthetized cockroaches by chilling them on ice, then exposed their hemocoels. Melanized, brown nodules were counted under a stereo microscope at 60X. The nodules were distinct, and direct counting reliably reflected the extent on the nodulation response to infections (Miller et al. 1994, 1996; Miller and Stanley 1998). After the first counting, the alimentary canal was removed. Nodules in the previously unexposed areas and remaining internal tissues were then counted.

Control Experiments

To determine the level of the background nodulation in our cockroach colonies, we conducted several control experiments. To record the nodulation in unchallenged adults, 4 cockroaches were taken from culture at various times in this project. We anesthetized cockroaches on ice for 10 minutes, then assessed nodulation. To determine the influence of injection wounds on nodule formation, 4 adults were injected with a standard volume of ethanol, the drug vehicle. Nodulation was assessed 10 h later, following the same protocol. To assess the effect of dexamethasone on nodulation in unchallenged cockroaches, a standard dosage of dexamethasone in 3 µl ethanol was injected into 4 cockroaches. Nodulation was assessed by standard methods 10 h later. Finally, we tested the possibility that nutrient broth could stimulate nodulation by injecting 10 µl of broth into 4 cockroaches. Nodulation was assessed by standard methods 10 h later.

Time course of nodulation: Influence of dexamethasone

Individuals in two groups of cockroaches were injected with 10 µl of ethanol or with 26 µg of dexamethasone in 3 µl of ethanol. The cockroaches were immediately injected with bacteria as

described. At 2, 4, 8, 10, 12, 16 and 24 hours PI, sub-groups of control and experimental insects were anesthetized, and nodulation was assessed.

Dose-response curves for phenidone and esculetin

Individuals in six groups of cockroaches were injected with 10 µl of ethanol, or 0.0026, 0.026, 0.26, 2.6, 26 µg of phenidone in 3 µl ethanol, then infected with a standard dosage of bacteria. At 10 h PI, the cockroaches were anesthetized, and nodulation was assessed.

We used another drug, esculetin, to determine a dose-response curve for a specific lipoxygenase inhibitor, which has not been carried out for any invertebrate. Individuals in 3 groups of cockroaches were injected with 10 µl ethanol, or with 0.026 and 26 µg of esculetin in 3 µl of ethanol, then injected with a standard dosage of bacteria. After 10 h PI, the cockroaches were anesthetized, and nodulation was assessed.

Fatty acid rescue experiment

Individuals in two groups of adult cockroaches were injected with either 10 µl ethanol or 26 µg of dexamethasone in 3 µl of ethanol and then infected with bacteria as described. Immediately after infection, the dexamethasone-treated cockroaches were divided into two sub-groups. Individuals in one sub-group were treated with 50 µg arachidonic acid in 2 µl of ethanol. Another sub-group was treated with 5 µl of ethanol to control for the effects of the extra injection on nodulation. At 10 h PI, cockroaches were anesthetized and nodulation assessed.

Influence of other eicosanoid biosynthesis inhibitors on nodulation

We divided cockroaches into groups and injected individuals in each group with either one of the cyclooxygenase inhibitors indomethacin, naproxen, ibuprofen or piroxicam, the dual cyclooxygenase and lipoxygenase inhibitor phenidone, or the lipoxygenase inhibitor esculetin, all in standard dosages of 26 µg in 3 µl of ethanol. Control insects were injected with 10 µl of ethanol. Following injections, the cockroaches were infected with a standard dosage of bacteria as described. At 10 h PI, the cockroaches were anesthetized and nodulation was assessed.

Results

Control experiments

Table 1 displays the results of control experiments. We recorded approximately 5 nodules/cockroach (n=4 cockroaches) in untreated insects taken directly from the culture. Similarly, we observed approximately 5 nodules/insect in cockroaches injected with ethanol, and in cockroaches injected with dexamethasone. Injections with nutrient broth resulted in approximately 7 nodules/insect. By comparison, challenges with standard dosages of *S. marcescens* resulted in approximately 45 nodules per adult.

Time course of nodulation

The time course of visible nodule formation in two groups cockroaches, experimentals and controls, is shown in Figure 2. Dexamethasone-treated adults formed approximately 10 nodules/insect at 2 h PI, which increased to 23 at 10 h PI, whereas the ethanol-treated control adults produced significantly more nodules at each time, from 15 nodules at 2 h PI to 63 at 10 h PI (LSD, $p<0.001$). The numbers of nodules did not increase in cockroaches incubated longer than 10 h PI, and in all subsequent experiments nodulation was assessed at 10 h PI.

TABLE I. Outcomes of background control experiments. Cockroaches, *Periplaneta americana*, were treated as specified in the left column, and nodulation was assessed at 10 hours post-treatment as described in Materials and Methods.

Treatment	Nodules per adult (Mean ± SEM)
None	4.7 ± 1.4
Inject drug vehicle	7.0 ± 2.5
Inject dexamethasone	5.2 ± 2.7
Inject nutrient broth	7.0 ± 2.3
Inject bacteria in broth	48.2 ± 4.6

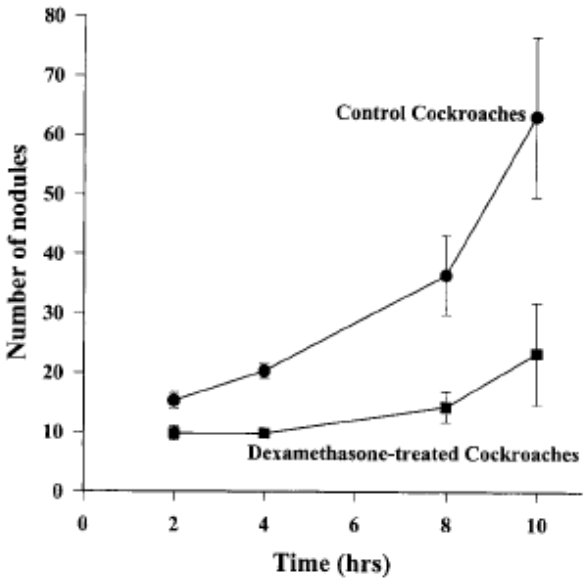

FIGURE 2. Time course of nodulation in adult cockroaches, *Periplaneta americana*, in response to intrahemocoelic infections with the pathogenic bacterium, *Serratia marcescens*. Each point indicates the mean number of nodules found in each insect (n=6 individuals), and the error bars represent 1 SEM.

Dose-response curves for phenidone and esculetin

The influence of phenidone and esculetin on nodulation in response to bacterial infections was expressed in a dose-dependent manner. Nodulation declined from approximately 46 nodules/cockroach in ethanol-treated control cockroaches, to approximately 13 nodules/cockroach in cockroaches treated with the highest phenidone dosage (Fig. 3) and to approximately 10 nodules/

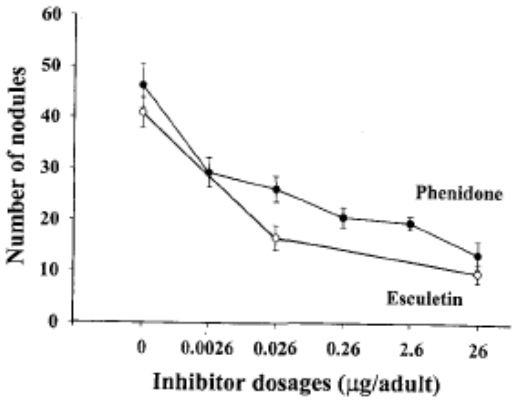

FIGURE 3. Dose-response curves for the influence of phenidone and esculetin on nodule formation in adults of *Periplaneta americana*. Each point indicates the mean number of nodules found in each insect (n=6 individuals) and the error bars represent 1 SEM.

cockroach in cockroaches treated with highest esculetin dosage (Fig. 3). For both pharmaceuticals, intermediate dosages produced intermediate nodulation reactions, approximately 15 to 30 nodules/cockroach.

Influence of other eicosanoid biosynthesis inhibitors on nodulation

We considered the influence of seven pharmaceutical inhibitors of eicosanoid biosynthesis on nodulation in response to bacterial infections. As can be seen in Figure 4, compared to control (EtOH) cockroaches, all experimental cockroaches exhibited significantly reduced nodulation in response to bacterial infections (LSD, $p<0.001$). We did not obtain significant differences among the effects of individual inhibitors on nodulation.

Fatty acid rescue experiment

According to the evidence taken from research in mammalian physiology, dexamethasone, as one of its actions, inhibits eicosanoid biosynthesis through its effect on PLA_2 (Fig. 1). If this is true, then injecting the eicosanoid-precursor polyunsaturated fatty acid, arachidonic acid, into dexamethasone-treated infected adults should reverse the effects of dexamethasone on nodulation. To test this idea, we proceeded as follows: after injection with dexamethasone, adults were infected with bacteria and then immediately treated with arachidonic acid. To control for the influence of the third injection on nodulation, an additional control group of cockroaches was injected with ethanol. As can be seen in Figure 5, arachidonic acid treatments reversed the effects of dexamethasone on nodulation ($p<0.05$). The ethanol-injected control cockroaches yielded approximately 48 nodules/cockroach and dexamethasone-treated cockroaches approximately 17 nodules/cockroach, both in line with expectation. The arachidonic acid-treated cockroaches produced approximately 70 nodules per cockroach, also in line with expectation for control animals. The second control group, injected with a second dose of ethanol, yielded approximately 14 nodules/cockroach.

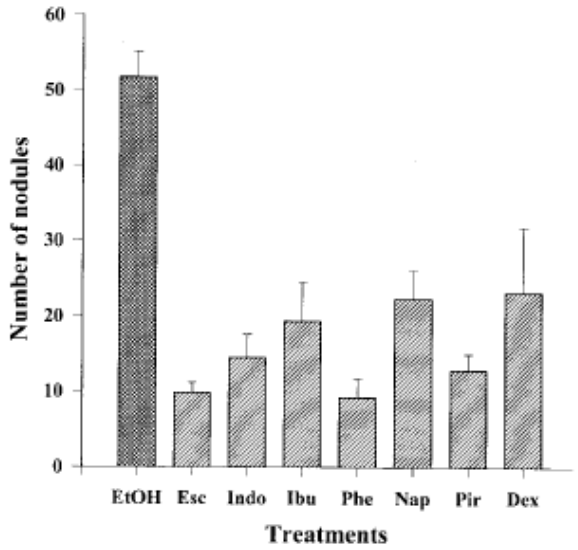

FIGURE 4. Effect of treating *Periplaneta americana* adults with individual eicosanoid biosynthesis inhibitors on nodule formation in response to intrahemocoelic infections with the insect pathogen, *Serratia marcescens*. Test insects were first injected with 26 µg of either dexamethasone (Dex; PLA_2 inhibitor), esculetin (Esc; lipoxygenase inhibitor), indomethacin (Indo), ibuprofen (Ibu), naproxen (Nap), or piroxicam (Pir), all cyclooxygenase inhibitors, phenidone (Phe; dual cyclooxygenase/lipoxygenase inhibitor). Control insects were first injected with ethanol (EtOH). The height of the histogram bars represents the mean number of nodules found in each insect (n=6 individuals) and the error bars represent 1 SEM. Histogram bars with the same fill pattern are not significantly different from each other (LSD, $p<0.01$).

Discussion

In this paper we report the outcomes of experiments designed to test the eicosanoid hypothesis in the American cockroach, *P. americana*. The results of all experiments support the hypothesis. First, treating experimental cockroaches with dexamethasone prior to infecting them with bacteria significantly reduced nodulation at all points in the time course experiments. Second, the influence of two eicosanoid biosynthesis inhibitors, phenidone and esculetin, on nodulation was expressed in a dose-dependent manner. Third, seven different eicosanoid biosynthesis inhibitors significantly reduced nodulation when compared to control treatments. Finally, the influence of dexamethasone on nodulation was reversed by treating infected cockroaches with arachidonic acid, an eicosanoid precursor polyunsaturated fatty acid. Taken together, these four separate lines of evidence strongly support the overall hypothesis.

The results of the time course experiment indicate that cockroaches treated with dexamethasone produced significantly fewer nodules than the control cockroaches at all time points in the experiment. We infer from this finding that inhibition of eicosanoid biosynthesis influences the cellular events involved in nodulation early in the infection process, and continues to exert a negative influence for many hours PI.

population sizes account for differences in nodulation capacity, then it should not be surprising to record considerable differences among insect species in nodulation responses to similar infection challenges.

We conducted two independent dose-response studies for the influence of eicosanoid biosynthesis inhibitors on nodulation in response to similar bacterial challenges. Dose-response relationships are basic to physiological research, and the approximately linear negative relationships we obtained for phenidone and esculetin strongly support the idea that eicosanoids mediate nodulation reactions to bacterial infections in the American cockroach.

Due to their importance in human medicine, many different inhibitors of eicosanoid biosynthesis are available. Some, such as aspirin and ibuprofen, are available as analgesic drugs for relief of minor pains, while many others are not yet approved for use in humans. These compounds, which we refer to with the general term "eicosanoid biosynthesis inhibitors", exert different actions in cellular eicosanoid biosynthesis. For example, dexamethasone inhibits PLA_2. Dexamethasone exerts other actions, as well, including influence on gene expression. Several compounds specifically inhibit cyclooxygenase, the first step in prostaglandin biosynthesis (Fig. 1), while esculetin is a specific inhibitor of 5- and 12-lipoxygenases. Our experiments with different inhibitors showed that all seven of the compounds we tested resulted in similar reductions in nodulation. The observation that separate experiments with inhibitors of cyclooxygenase and lipoxygenase pathways similarly retarded nodulation in adult cockroaches indicate that cyclooxygenase and lipoxygenase products act in nodule formation. We interpret this finding in terms of the nodulation process. Nodulation results from complex cellular physiology which involves many separate cellular actions. Inhibiting one or more of the eicosanoid-mediated steps would be registered as reductions in the overall nodulation process. It follows that eicosanoids mediate more than one cellular step in nodulation.

The results with seven different inhibitors are subject to a slightly more subtle interpretation. Indomethacin, ibuprofen, naproxen and piroxicam are all cyclooxygenase inhibitors; however, they inhibit mammalian cyclooxygenases in slightly different ways. Moreover, phenidone is a dual lipoxygenase/ cyclooxygenase inhibitor. Again, this compound acts in a slightly different way from the other cyclooxygenase inhibitors. Hence, the observation that all of these compounds yielded similar results indicates that their actions are exerted through a common mechanism, specifically, inhibition of cyclooxygenase.

The results of the rescue experiments strongly support our hypothesis that eicosanoids mediate nodulation in American cockroaches. Dexamethasone is thought to act by inhibiting PLA_2, the enzyme responsible for releasing arachidonic acid from cellular phospholipids. This is the first and rate-limiting step in eicosanoid biosynthesis. Dexamethasone inhibits eicosanoid biosynthesis by inhibiting release of substrate from cellular phospholipids, which in effect withholds substrate from cyclooxygenase and other eicosanoid biosynthesizing enzymes. If this is so, then providing arachidonic acid to the immunity-conferring cells within the cockroaches would be expected to reverse the influence of dexamethasone on nodulation. Indeed, the arachidonic acid treatments restored the cockroaches' ability to produce nodules in response to bacterial infections.

Stanley-Samuelson (1994a) indicated that experiments with eicosanoid biosynthesis inhibitors were based, in part, on the assumption that the experimental insects were competent to biosynthesize eicosanoids. The presence of eicosanoid precursor polyunsaturated fatty acids and the enzymes responsible for eicosanoid biosynthesis have been documented in American cockroaches. Stanley-Samuelson and Pipa (1984) determined the presence of three eicosanoid precursor polyunsaturated fatty acids in several specific tissues from adult American cockroaches. Also, Jurenka et al. (1986) documented the presence of eicosanoid biosynthesizing enzymes in fat body of adult cockroaches. Hence, a basic assumption of this line of experimentation has been met.

While the data reported here represents males and females, we considered the possibility that each gender may express different reactions to bacterial infections. We recorded the sex and mass of each adult in all experiments; however, statistical analysis showed no differences in the nodulation reactions to bacterial infections in American cockroaches due to gender or mass.

Our background control experiments indicate that the nodules we recorded were due to the experimental treatments, and not to adventitious infections. Cockroaches taken directly from the colony had a low background of nodulation. The inject treatments, similarly, did not influence the low background of nodulation. Hence, the experimental protocols allow a physiological interpretation of the data: inhibition of eicosanoid biosynthesis impairs cockroach immunity.

Finally, the hypothesis that eicosanoids mediate cellular immunity in cockroaches takes on broader significance in mechanisms of signal transduction in invertebrate immunology. While eicosanoids were the first biochemical signal moieties discovered in insect cellular immune reactions (Stanley-Samuelson et al. 1991; Stanley 1999), they are not the sole mediators of cellular immune reactions in insects, nor other invertebrates. Downer and his colleagues have shown that biogenic amines are involved in hemocytic reactions, including phagocytosis, nodulation, and cellular movements (Baines et al. 1992; Baines and Downer 1994; Diehl-Jones et al. 1996). These works suggest that eicosanoids and amines are essential in overall cellular defense processes. Certainly, other, still unknown, biochemicals will be found to exert important actions in invertebrate cell defense reactions.

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