2004

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Jon S. Miller
Northern Illinois University, jsmiller@niu.edu

David W. Stanley
University of Nebraska-Lincoln

Published in *Comparative Biochemistry and Physiology Part A* 137:2 (2004), pp. 285–295;
doi:10.1016/j.cbpb.2003.10.003.

Submitted July 18, 2003; revised October 2, 2003; accepted October 3, 2003.

Lipopolysaccharide Evokes Microaggregation Reactions in Hemocytes Isolated from Tobacco Hornworms, *Manduca sexta*

Jon S. Miller[1] and David W. Stanley[2]

1. Department of Biological Sciences, Northern Illinois University, De Kalb, IL 60115, USA
2. Insect Biochemical Physiology Laboratory, University of Nebraska, Lincoln, NE 68583, USA

Corresponding author – Jon S. Miller, telephone 815-753-7828, email jsmiller@niu.edu

Abstract

Insect cellular immune reactions to bacterial infection include nodule formation. Eicosanoids mediate several cellular actions in the nodulation process, including formation of hemocyte microaggregates, an early step. In previous work, we reported that isolated hemocytes produce and secrete eicosanoids that influence hemocyte behavior in response to bacterial challenge. We also reported that microaggregate formation in response to challenge was mediated by prostaglandins (PGs), but not by products of the lipoxygenase (LOX) pathways. In this paper we describe experiments designed to test the idea that exposing isolated hemocytes to lipopolysaccharide (LPS) evokes formation of hemocyte microaggregates and this cellular action is mediated by PGs. Results show that isolated hemocyte preparations challenged with LPS formed more hemocyte microaggregates than unchallenged preparations (6.9×10^3 microaggregates/ml hemolymph vs. 2.5×10^3 microaggregates/ml hemolymph). LPS challenge stimulated formation of hemocyte microaggregates in a dose dependent manner. Experimental groups pretreated with cyclooxygenase inhibitors produced fewer hemocyte microaggregates in response to LPS challenge than untreated control groups. The formation of hemocyte microaggregates was not influenced by LOX inhibitors. Furthermore, the influence of dexamethasone was reversed by supplementing the experimental groups with the eicosanoid precursor fatty acid molecule, arachidonic acid and PGH_2. Palmitic acid, which is not substrate for eicosanoid biosynthesis, did not reverse the effects of dexamethasone on the formation of microaggregates. The LOX product 5(*S*)hydroperoxyeicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid also did not

reverse the effects of dexamethasone. These results are consistent with similar investigations performed with bacterial suspensions. We infer that isolated hemocyte preparations recognize and react to LPS by forming microaggregates and this reaction is mediated by PGs, but not products of the LOX pathway.

Keywords: eicosanoids, prostaglandins, cyclooxygenase, lipopolysaccharide, insect immunity, hemocyte microaggregates, *Manduca sexta*, in vitro

1. Introduction

Insect innate immune reactions to microbial infection are separable into humoral and cellular responses (Lavine and Strand, 2002). Humoral reactions involve induced biosynthesis of antibacterial proteins and enzymes, which appear in the hemolymph of infected insects approximately 6–12 h post-infection. Cellular defense responses, mainly phagocytosis, encapsulation, and nodulation, begin immediately as infection is detected. These cellular reactions, during which millions of hemocytes are depleted from circulation, are responsible for clearing the bulk of infecting microbes from insect hemolymph circulation (Horohov and Dunn, 1982). While the signaling pathways responsible for induced expression of antibacterial proteins are understood in considerable detail, much less is known about signal transduction mechanisms in insect cellular immunity.

On the basis of work with tobacco hornworms, *Manduca sexta*, it was hypothesized that insect cellular immune reactions responsible for clearing bacterial cells from circulation are mediated by eicosanoids (Stanley-Samuelson et al., 1991). Eicosanoids are oxygenated metabolites of certain C20 polyunsaturated fatty acids. Major groups of eicosanoids include prostaglandins (PGs; products of the cyclooxygenase (COX) pathways) and various products of lipoxygenase (LOX) pathways, all of which are detailed elsewhere (Stanley, 2000). With a view toward identifying specific cellular defense reactions which are mediated by eicosanoids, Miller et al. (1994) hypothesized that eicosanoids mediate microaggregation (an early phase in nodule formation) and nodulation reactions to bacterial infection. The outcomes of experiments with tobacco hornworms supported the hypothesis. Mandato et al. (1997) advanced this line with experiments on another insect, larvae of the wax moth, *Gallaria mellonella*. They found that other cellular defense actions, specifically cell spreading (a phase of nodulation) and phagocytosis, also depend on eicosanoids. This hypothesis has been tested in several insect species and the results of these tests have uniformly supported the idea, now formalized as the eicosanoid hypothesis (Howard and Stanley, 1999; Stanley, 2000; Stanley et al., 2002).

The eicosanoid hypothesis opens several lines of investigation into insect cellular immunity. One concerns the nature of the microbial cell components that insect immune tissues recognize as foreign. Howard et al. (1998) reported that the intensity of nodulation reactions, recorded as numbers of visible nodules, varies considerably according to the species of infecting bacteria. Because Gram-negative bacteria evoked intense nodulation reactions, it was suggested that the lipopolysaccharide (LPS) component of Gram-negative bacteria and certain algal species was responsible for evoking microaggregation and nodulation reactions. Bedick et al. (2000) reported that LPS challenge stimulated nodulation

reactions in larvae of the beetle *Zophobas atratus*. This was not an entirely surprising discovery because specific components of bacterial cell walls, including peptidoglycans (Kanost et al., 1988) and LPS (Charalambidis et al., 1995) were already known to stimulate defense reactions in insects. However, Bedick et al. (2000) also suggested that cellular reactions to LPS were mediated by eicosanoids. Hence, it would appear, as suggested by Lavine and Strand (2002) that insect immune surveillance systems recognize foreign infections and release various signals (including eicosanoids) which act to unleash the entire array of immune responses.

Many details of eicosanoid actions in insect immunity remained unclear. For one, the cellular source of eicosanoids responsible for mediating cellular defense reactions had not been identified. Biochemical work documented eicosanoid biosynthesis in the two main immunity-conferring tissues, fat body (Stanley-Samuelson and Ogg, 1994) and hemocytes (Gadelhak et al., 1995). Miller and Stanley (2001) reported that bacterial challenge stimulated isolated tobacco hornworm hemocyte preparations to biosynthesize and secrete eicosanoids that influence the behaviors of other hemocytes. This finding supported the idea that hemocytes per se are responsible for producing the eicosanoids that mediate cellular defense reactions. This work also uncovered an apparent paradox. Earlier research on the roles of eicosanoids in insect cellular immune reactions indicated that two major groups of eicosanoids, PGs and various LOX products, are crucial mediators of defense reactions. Miller and Stanley's (2001) work, contrarily, indicated that COX products, that is, PGs, but not LOX products, are responsible for mediating microaggregation reactions in isolated hemocyte preparations. The outcomes of further studies with additional pharmaceutical probes of the LOX pathways were consistent with this finding (Phelps et al., 2003).

The issue of identifying the specific eicosanoid biosynthesis pathways which act in insect immunity takes on additional interest with reports that eicosanoids mediate insect immune responses to pathogenic fungi. Dean et al. (2002) first suggested that products of the COX and LOX pathways mediate tobacco hornworm cellular reactions of the pathogenic fungus, *Metarhizium anisopliae*. The picture became murkier with a subsequent report, also on tobacco hornworms, showing that nodulation reactions to spores of the fungus *Beauveria bassiana* were mediated by LOX products but not by PGs (Lord et al., 2002). As now understood, the eicosanoid hypothesis stands on firm ground, however, it cannot be said with certainty which specific eicosanoids are responsible for signaling any given cellular defense action.

In this paper we report on experiments designed to test the idea that isolated hemocyte populations express a specific reaction, microaggregation, to challenge with a specific bacterial cell wall component, LPS. The microaggregation reactions are mediated by certain PGs, a subset of known COX products, but not by LOX products.

2. Materials and methods

2.1. Organisms

Eggs of the tobacco hornworm, *M. sexta*, were provided by Ms. Beverley Pagura (North Carolina State University). Larvae were reared on standard culture medium in individual cups under semisterile conditions developed by Dunn and Drake (1983). Hemocytes were

prepared from fifth instars, age 2–3 days. LPS was prepared from the bacterium, *Serratia marcescens*, as described by Bedick et al. (2000).

2.2. Reagents

Grace's Insect Medium and selected eicosanoid biosynthesis inhibitors, including the phospholipase A_2 inhibitor, dexamethasone w(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione], the COX inhibitors indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-3-indolyl-acetic acid], ibuprofen [2-(4-isobutylphenyl) propionic acid], naproxen [6-methoxy-α-methyl-2-naphthaleneacetic acid], the LOX inhibitors esculetin [6,7-dihydroxycoumarin] and baicalein [5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one], and the fatty acid, palmitic acid [hexadecanoic acid], were purchased from Sigma Chemical Company (St. Louis, Missouri). Arachidonic acid [5,8,11,14-eicosatetraenoic acid], PGH_2 and 5(*S*)-hydroperoxyeicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid (5(*S*)-HpETE) were purchased from Cayman Chemical (Ann Arbor, Michigan).

2.3. Hemolymph collection and preparation

Second and third day fifth-stadium hornworms were anesthetized by chilling on ice for 15 min, then surface sterilized by swabbing their exteriors with 95% ethanol. Hemolymph (≈ 500 μl/larva) was collected by the pericardial puncture procedure described by Horohov and Dunn (1982). Briefly, a 20-gauge sterile, siliconized needle was inserted anteriorly at the thoracic abdominal junction such that the needle penetrated into the pericardial sinus. Freely dripping hemolymph was collected into chilled, sterile polypropylene 1.5-ml centrifuge tubes charged with 500 ml of cold Grace's Insect Medium. The hemolymph was gently mixed with Grace's Insect Medium by inverting the test tube several times. The hemocyte preparations were immediately used in each experiment.

2.4. In vitro assay for hemocyte microaggregate formation

A sterile 96-well (250 μl per well), flat bottom, polystyrene, micro-titer plate was used for each experiment (Becton Dickenson, Lincoln, NJ). Each well was preloaded with 70 μl cold Grace's Insect Medium amended with 2 μl ethanol or a selected pharmaceutical and/or fatty acid, PGH_2 or 5(*S*)-HpETE dissolved in 2 μl ethanol. Then 20 μl of hemolymph suspension (≈ 6.4×10^5 cells) was added to each well. The hemocyte preparations were then challenged by adding 20 μg LPS (standard dosage) dissolved in 5 μl Grace's Insect Medium. The micro-titer plate was incubated for selected time periods, specified in Section 3, at 26°C on a rotary shaker at 100 rpm. After the selected incubation period, 20 ml of the hemocyte preparation was applied to a hemacytometer. After a 3-min settling period, a cover slip was applied and the number of hemocyte microaggregates (operationally defined as a cluster of five or more cells) for each sample was determined by direct counting with phase contrast optics.

Test preparations were treated with the PLA_2 inhibitor, dexamethasone, or one of the COX inhibitors indomethacin, ibuprofen, or naproxen or the 5- and 12-LOX inhibitor, esculetin or 5-LOX inhibitor, baicalein dissolved in ethanol. In some experiments, test preparations were also treated with arachidonic acid, palmitic acid, PGH_2 or 5(*S*)-HpETE dissolved in ethanol. Test preparations were amended with pharmaceutical products at a

final concentration of 100 μM. Fatty acids were administered at dosages of 0.2 μg in 2 μl ethanol per well preparation (final concentration equals 50 μM). PGH_2 and 5(*S*)-HpETE were administered at dosages of 0.2 μg in 2 μl ethanol per well preparation (final concentration equals 50 μM).

2.5. Control experiments

Negative and positive controls were prepared with each experimental group. To assess the response in unchallenged preparations (negative control), preparations were treated with 2 μl ethanol (drug vehicle) but were not exposed to LPS challenge. Positive control preparations were treated with 2 μl ethanol, then challenged with 20 μg of LPS in 5 μl of Grace's Insect Medium. After 15 min of incubation the number of microaggregates was assessed as described.

2.6. Dose response for LPS

Hemocyte preparations were divided into seven groups and separately treated with 0.0, 0.1, 0.5, 1.0, 2.0, 20, and 200 μg of LPS dissolved in 5 μl of Grace's Insect Medium. The preparations were then allowed to incubate for 15 min, after which the number of microaggregates was assessed as described.

2.7. Time course of hemocyte microaggregation: influence of dexamethasone

Hemocyte preparations were divided into three groups. One group (negative control) was treated with 2 μl of ethanol. The second group was treated with 2 μl of ethanol and the third group was treated with dexamethasone in 2 μl of ethanol (100 μM final concentration). The second and third groups were then challenged with 20 μg of LPS in 5 μl of Grace's Insect Medium. Plates were incubated for 5, 15, 30, 45 and 60 min post challenge. At each time point, the number of microaggregates was assessed as described.

2.8. Influence of other eicosanoid biosynthesis inhibitors on hemocyte microaggregation

Hemocyte preparations were set up as described, then divided into eight groups. One group was treated with 2 μl ethanol only (negative control). A second group was first treated with 2 μl ethanol then inoculated with 20 μg LPS (positive control). The other groups were individually treated with the PLA_2 inhibitor, dexamethasone, or one of the COX inhibitors indomethacin, ibuprofen, or naproxen, or the LOX inhibitors esculetin or baicalein, dissolved in ethanol (final concentration equals 100 μM). Then 20 μg LPS in 5 μl Grace's Insect Medium was introduced into each experimental preparation and incubated for 15 min post challenge. After the incubation period the number of microaggregates was assessed as described.

2.9. Arachidonic acid and prostagladin H_2 rescue experiments

Negative and positive control groups were set up as previously described. Experimental preparations were first treated with dexamethasone in 2 μl ethanol and allowed to incubate for 15 min, then inoculated with 20 μg LPS in 5 μl of Grace's Insect Medium. Immediately after challenge, the dexamethasone-treated preparations were divided into five subgroups. Preparations in the first dexamethasone-treated subgroup received no further

treatment. Preparations in the second subgroup were treated with the eicosanoid precursor fatty acid arachidonic acid. Preparations in the third subgroup were treated with palmitic acid, which is not substrate for eicosanoid biosynthesis. Preparations in the fourth subgroup were treated with PGH_2. Preparations in the fifth subgroup were treated with the LOX product 5(*S*)-HpETE. The preparations were then incubated for 15 min. After incubation, the number of microaggregates was assessed as described.

2.10. Statistical analyses

Significant treatment effects were confirmed for in vitro hemocyte microaggregates by analysis of variance with $P \leq 0.05$. Where appropriate, significant differences among treatment means were determined by protected least significant differences (LSD).

3. Results

3.1. Control experiments

Formation of microaggregates may be influenced by a number of factors, including the necessary handling involved with setting up in vitro hemocyte preparations. We considered the possibility of adventitious microaggregate formation by conducting routine control experiments. Negative control experiments were meant to reveal the background numbers of microaggregates in untreated hemocyte preparations. It can be seen from the data presented in table 1 that negative control preparations uniformly yielded approximately 3×10^3 microaggregates/ml hemolymph. Positive control experiments were designed to assess hemocyte preparation capacity for forming microaggregates. The data in table 1 indicate that routine preparations treated with a standard dose of LPS (20 μg in 5 μl of Grace's Insect Medium) produced approximately 7×10^3 microaggregates/ml hemolymph. The mean number of microaggregates was significantly different between negative and positive control preparations (LSD, $P < 0.05$).

Table 1. Outcomes of control experiments

Control treatment	Number of microaggregates
Negative controls ($n = 32$)	2.56 ± 0.40
Positive controls ($n = 27$)	6.94 ± 0.44

Hemocyte preparations were treated with 2 μl of ethanol, then divided into two groups. One group was allowed to incubate without any further treatment, while the other group was challenged with a standard dose of LPS. Both groups were allowed to incubate for 15 min, then the number of microaggregates was assessed. The values ($\times 10^3$) represent the mean number of microaggregates/ml hemolymph ± standard error of the mean (S.E.M.). The mean number of microaggregates was significantly different between negative and positive control preparations (LSD, $P < 0.05$).

3.2. Dose response for LPS

The relationship between LPS dose and the number of microaggregates is shown in figure 1. Increasing doses of LPS were associated with increased numbers of observed microaggregates, from just less than 3×10^3 microaggregates/ml hemolymph with 0.0 μg LPS, to approximately 9×10^3 microaggregates/ml hemolymph with 2.0 μg LPS, and approximately 12×10^3 microaggregates/ml hemolymph with 200.0 μg LPS per preparation at 15-min incubation. Mean number of microaggregates was significantly related to LPS.

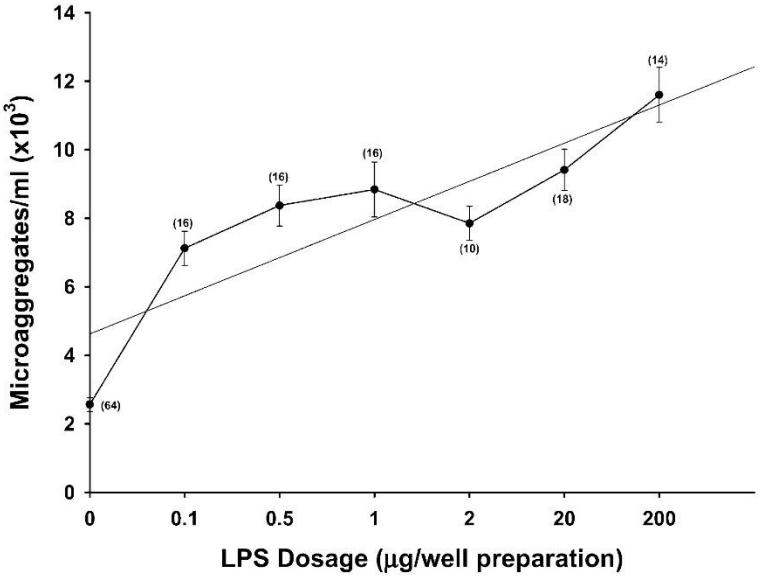

Figure 1. The influence of LPS dose on numbers of microaggregates formed by hemocyte preparations isolated from the tobacco hornworm, *M. sexta*. The hemocyte preparations were inoculated with the indicated doses of LPS (purified from the bacterium *S. marcescens*) indicated and allowed to incubate for 15 min. Hemolymph samples were applied to a hemacytometer. Numbers of hemocyte microaggregates were determined by direct counting. The points indicate the mean number of microaggregates/ml hemolymph ($\times 10^3$) and error bars indicate 1 S.E.M. The numbers in parentheses represent the value of *n*.

3.3. Time course of hemocyte microaggregations: influence of dexamethasone

The time course of microaggregation in three groups of hemocyte preparations is displayed in figure 2. Ethanol-treated (no LPS) hemocyte preparations yielded approximately 3×10^3 microaggregates/ml hemolymph at 5 min, which increased to approximately 6×10^3 microaggregates/ml hemolymph at 60 min. Isolated hemocyte preparations treated with LPS yielded significantly more microaggregates, approximately 7×10^3 microaggregates/ml hemolymph at 5 min post challenge and increased to approximately 12×10^3 microaggregates/ml hemolymph at 60 min post challenge. Isolated hemocyte preparations pretreated with dexamethasone then inoculated with LPS yielded approximately 3×10^3 microaggregates/ml hemolymph at 5 min post challenge and nearly 3×10^3 microaggregates/ml hemolymph at 60 min post challenge.

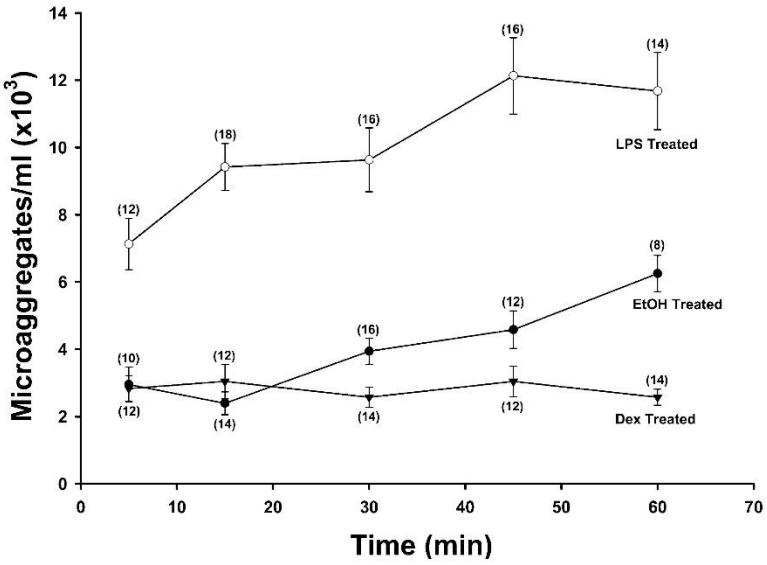

Figure 2. Time course of microaggregation in *M. sexta* hemocyte preparations in response to LPS challenge. Experimental hemocyte preparations (solid triangles) were treated with dexamethasone (Dex-treated), then challenged with LPS. Positive control preparations (open circles) were challenged with LPS. Negative control preparations (solid circles) were treated with the drug vehicle, ethanol, but were not exposed to LPS challenged. At the indicated times post challenge, microaggregation was assessed by direct counting under phase contrast optics. Each point indicates the mean number of microaggregates/ml hemolymph (× 10^3) in each hemocyte preparation and the error bars represent 1 S.E.M. The numbers in parentheses represent the value of *n*.

3.4. Influence of other eicosanoid biosynthesis inhibitors on hemocyte microaggregation

To dissect the possible roles of COX and LOX pathways in microaggregate formation, hemocyte preparations were treated with either standard doses of the COX inhibitors indomethacin, ibuprofen, naproxen or the 5-and 12-LOX inhibitor esculetin or with the 5-LOX inhibitor baicalein. The data in figure 3 indicate that isolated hemocyte preparations treated with COX inhibitors yielded significantly fewer microaggregates than positive control preparations, on par with negative control preparations. Microaggregate formation was not influenced in hemocyte preparations treated with the LOX inhibitors, esculetin and baicalein. There were no significant differences among the effects of individual COX inhibitors on microaggregation.

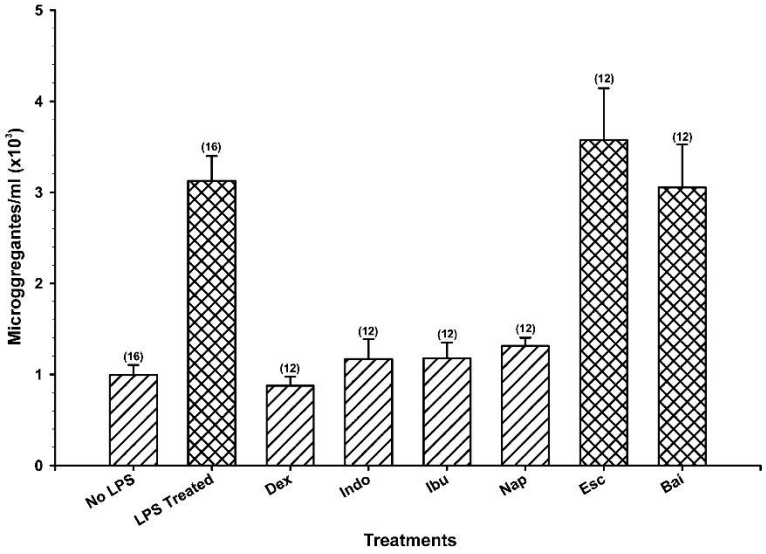

Figure 3. The influence of individual eicosanoid biosynthesis inhibitors on microaggregation in response to LPS challenge in hemocyte preparations isolated from the tobacco hornworm, *M. sexta*. Test preparations were first treated with either dexamethasone (Dex), or indomethacin (Indo), naproxen (Nap), or esculetin (Esc), or Baicalein (Bai). Control preparations were treated with ethanol (EtOH). Then all preparations were challenged with LPS. At 15 min post challenge, microaggregation was assessed. The height of the histogram bars represents the mean number of microaggregates/ml hemolymph ($\times 10^3$). The error bars represent 1 S.E.M. Histogram bars with the same fill pattern are not significantly different from each other (LSD, $P < 0.05$). The numbers in parentheses represent the value of n.

3.5. Rescue experiments

In reaction to LPS challenge, dexamethasone-treated hemocyte preparations produced fewer microaggregates than ethanol-treated control preparations. On the model that dexamethasone inhibits eicosanoid biosynthesis through its inhibitory effect on PLA_2, we reasoned that supplementing dexamethasone-treated preparations with arachidonic acid, an eicosanoid precursor, would reverse the influence of dexamethasone on microaggregate formation. Figure 4 shows that control hemocyte preparations treated with the drug vehicle ethanol, but not LPS, produced less than 2×10^3 microaggregates/ml hemolymph. Hemocyte preparations challenged with LPS produced significantly more microaggregates/ml hemolymph, approximately 5×10^3 microaggregates/ml hemolymph. As seen previously, hemocyte preparations treated with dexamethasone then challenged with LPS produced approximately 2×10^3 microaggregates/ml hemolymph, on par with the negative control hemocyte preparations. Arachidonic acid reversed the influence of dexamethasone on microaggregate formation. Hemocyte preparations treated with dexamethasone, challenged with LPS and then treated with arachidonic acid yielded approximately 6×10^3 microaggregates/ml hemolymph, similar to positive control preparations. Similarly, PGH_2

reversed the influence of dexamethasone. Hemocyte preparations treated with dexamethasone, challenged with LPS and then treated with PGH_2 yielded approximately 4×10^3 microaggregates/ml hemolymph, similar to positive control preparations. Hemocyte preparations treated with dexamethasone, challenged with LPS and then treated with palmitic acid produced less than 3×10^3 microaggregates/ml hemolymph, not significantly different from negative control hemocyte preparations. Finally, hemocyte preparations treated with dexamethasone, challenged with LPS and then treated with the LOX product 5-HpETE produced approximately 2.5×10^2 microaggregates/ml hemolymph, not significantly different from negative control hemocyte preparations.

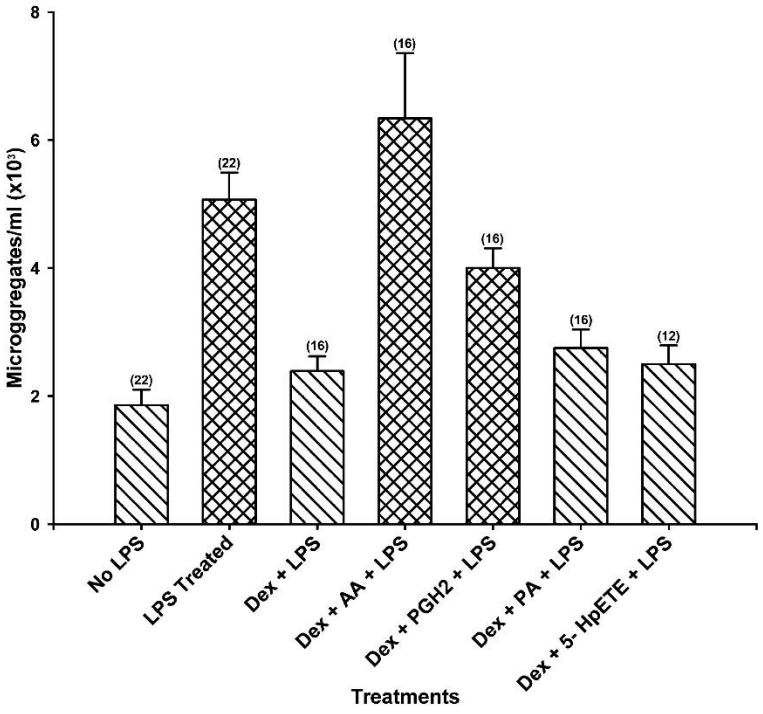

Figure 4. Arachidonic acid and PGH_2 reversed the effects of dexamethasone on microaggregate formation. Tobacco hornworm hemocyte preparations were treated with ethanol (EtOH) or dexamethasone (Dex), then challenged with LPS. Immediately after LPS challenge, dexamethasone-treated test preparations were treated with arachidonic acid (Dex + AA), PGH_2 (Dex + PGH_2) or palmitic acid (Dex + PA). At 15 min post challenge, the number of microaggregates was assessed. The height of the histogram bars represent the mean number of microaggregates/ml hemolymph ($\times 10^3$) and error bars represent 1 S.E.M. Histogram bars with the same fill pattern are not significantly different from each other (LSD, $P < 0.05$). The numbers in parentheses represent the value of n.

4. Discussion

The data reported in this paper indicate that isolated hemocyte preparations are competent to recognize and react to LPS challenge. The data also support the hypothesis that microaggregation reactions in isolated hemocyte preparations are mediated by certain PGs, but not by products of the LOX pathway. Three points of evidence support the hypothesis. First, isolated hemocyte preparations challenged with LPS formed more hemocyte microaggregates than unchallenged preparations. Second, LPS stimulated microaggregate formation in a dose dependent manner. Third, isolated hemocyte preparations pretreated with the PLA_2 inhibitor, dexamethasone, or one of the COX inhibitors, indomethacin, ibuprofen or naproxen produced fewer hemocyte microaggregates than control preparations. The 5-and 12-LOX inhibitors esculetin and 5-LOX inhibitor baicalein did not influence the formation of hemocyte microaggregates in this system. Finally, the effects of dexamethasone were reversed by treating experimental groups with the eicosanoid precursor fatty acid, arachidonic acid. Moreover, treating experimental groups with the PG endoperoxide PGH_2 reversed the influence of dexamethasone. Palmitic acid, which is not a substrate for eicosanoid biosynthesis, did not reverse the influence of dexamethasone on hemocyte microaggregation. Similarly, the LOX product 5(*S*)-HpETE did not reverse the influence of dexamethasone.

The results of several lines of experiments support the view that certain PGs, but not LOX products, are responsible for mediating hemocyte microaggregation reactions to bacterial challenge. Miller and Stanley (2001) found that inhibitors of PLA_2, the first step in eicosanoid biosynthesis, and various COX inhibitors impaired microaggregation reactions to bacterial challenge in isolated hemocyte preparations. Similar experiments with the 5- and 12-LOX inhibitor, esculetin, did not influence microaggregation reactions to bacterial challenge. Because esculetin inhibited nodulation reactions to bacterial challenge in intact tobacco hornworms, Phelps et al. (2003) investigated the influence of other LOX inhibitors in isolated hemocyte preparations. They reported that none of the LOX inhibitors impaired microaggregation. From these data, it would appear that, at least with respect to challenge with whole bacteria, products of the COX, but not LOX, pathways are the key microaggregation mediators.

This study reveals new detail on the roles of eicosanoids in microaggregation reactions. First, isolated hemocyte preparations are competent to recognize purified LPS, a specific chemical associated with Gram-negative bacteria and a few algae (Bedick et al., 2000). Second, the microaggregation reaction to LPS is mediated by PGs. Third, the reversal of the dexamethasone influence on microaggregation by treatments with the PG endoperoxide PGH_2 yields new understanding on which PGs influence microaggregation reactions.

The actions of 20:4n–6 (arachidonic acid) and PGH_2 in reversing the influence of dexamethasone are best understood in terms of the biochemistry of PG biosynthesis, drawn from Stanley (2000). PG biosynthesis begins with release of 20:4n–6 from membrane phospholipids, usually by action of a cellular PLA_2. The free 20:4n–6 is substrate for metabolism via of the COX and LOX pathways. COX is thought to be a constitutively active enzyme with two distinct catalytic activities. First, a COX activity catalyzes formation of PGG_2. Second, a peroxidase activity catalyzes a two-electron reduction of PGG_2 to form PGH_2. The

PGH_2 is then substrate for a range of cell- and tissue-specific enzymes responsible for converting PGH_2 into several biologically active PGs, including PGD_2, PGE_2, and $PGF_{2\alpha}$. Other biologically active PGs include PGA_2 and Δ^{12}-PGJ_2. These products are formed by nonenzymatic rearrangements of the primary PGs and are thought to act through cellular mechanisms, which are fundamentally different from the actions of PGD_2, PGE_2, and $PGF_{2\alpha}$. In mammalian cells PGA_2 is formed from PGE_2, however, Stanley-Samuelson and Ogg (1994) found that PGE_2 is not converted into PGA_2 in tobacco hornworm preparations. It appears that PGH_2 is converted into PGD_2, PGE_2, and $PGF_{2\alpha}$ in insect systems. We infer that one or a mixture of these three primary PGs are responsible for mediating microaggregate reactions to LPS challenge in isolated hemocyte preparations.

Free 20:4n–6 can also enter a range of LOX pathways. Gadelhak et al. (1995) characterized eicosanoid biosynthesis in tobacco hornworm hemocytes, which revealed that, unlike fat body, a LOX product, tentatively identified as 15-hydroxyeicosatetraenoic acid, was the predominant eicosanoid produced by hemocytes taken from immunologically naive hornworms. While hemocytes certainly produce one or more LOX products, these products do not act in microaggregation reactions to bacterial challenge in isolated hemocyte preparations (Miller and Stanley, 2001; Phelps et al., 2003), nor do they act in reactions to purified LPS.

Pretreating intact insects and isolated hemocyte populations with various inhibitors of eicosanoid biosynthesis impairs cellular reactions to immune challenge (Miller et al., 1994; Stanley et al., 2002). It may be inferred from this observation that the specific eicosanoids responsible for mediating cellular immune reactions are produced after an immune challenge is registered within an infected insect. Indeed, Jurenka et al. (1999) reported that injecting true armyworms with bacteria led to substantial increases in hemolymph titers of $PGF_{2\alpha}$ and several other eicosanoids that are yet to be identified. More recently, Tunaz et al. (2003) reported that bacterial challenge stimulated increased PLA_2 activity in hemocytes from tobacco hornworms. The increased PLA_2 activity began approximately 30 s post-infection and lasted approximately 5 min. Yajima et al. (2003) also recorded bacterial-stimulated, transient increases in PLA_2 activity in the fly *Sarchphaga peregrina*. Hence, we infer that immune challenge provokes increased eicosanoid biosynthesis.

The precise roles of eicosanoids in insect immunity remain to be elucidated. However, we now have a couple of points of meaningful information. First, PGs and various LOX products are important in complete nodulation reactions to fungal, bacterial, and LPS challenge in whole insects (Miller et al., 1994; Bedick et al., 2000; Stanley et al., 2002). Second, eicosanoids of unknown origin mediate cellular reactions to parasitoid eggs in *Drosophila* larvae (Carton et al., 2002). Third, recent information suggests that eicosanoids also act in humoral immunity to bacterial challenge. Morishima et al. (1997) reported that indomethacin inhibited expression of genes for cecropin and for lysozyme in two *Bombyx mori* preparations, intact larvae and isolated fat body. Yajima et al. (2003) discovered functional linkages between eicosanoid biosynthesis and activation of the immune deficiency pathway in *Drosophila*. Finally, as suggested here, certain PGs, but not LOX products, are responsible for mediating microaggregation reactions to LPS challenge in isolated hemocytes.

Acknowledgments – Thanks to Dr. Ted Pardy for his gift of purified LPS. This work was supported by the Agricultural Research Division, University of Nebraska–Lincoln and by the Department of Biological Sciences, Northern Illinois University. A contribution of the University of Nebraska Agricultural Research Division, Journal Series No. 14310.

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