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ScienceDirect
Journal of Insect Physiology 54 (2008) 215–221
Journal of Insect Physiology
www.elsevier.com/locate/jinsphys

Eicosanoids mediate insect hemocyte migration

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Received 28 June 2007; received in revised form 11 September 2007; accepted 12 September 2007

Abstract

Hemocyte migration toward infection and wound sites is an essential component of insect defense reactions, although the biochemical signal mechanisms responsible for mediating migration in insect cells are not well understood. Here we report on the outcomes of experiments designed to test the hypotheses that (1) insect hemocytes are able to detect and migrate toward a source of N-formyl-Met-Leu-Phe (fMLP), the major chemotactic peptide from *Escherichia coli* and (2) that pharmaceutical modulation of eicosanoid biosynthesis inhibits hemocyte migration. We used primary hemocyte cultures prepared from fifth-instar tobacco hornworms, *Manduca sexta* in Boyden chambers to assess hemocyte migration toward buffer (negative control) and toward buffer amended with fMLP (positive control). Approximately 42% of negative control hemocytes migrated toward buffer and about 64% of positive control hemocytes migrated toward fMLP. Hemocyte migration was inhibited (by >40%) by treating hornworms with pharmaceutical modulators of cycloxygenase (COX), lipoxygenase and phospholipase A_2 (PLA_2) before preparing primary hemocyte cultures. The influence of the COX inhibitor, indomethacin, and the glucocorticoid, dexamethasone, which leads to inhibition of PLA_2, was expressed in a dose-dependent way. The influence of dexamethasone was reversed by injecting arachidonic acid (precursor to eicosanoid biosynthesis) into hornworms before preparing primary hemocyte cultures. The saturated fatty acid, palmitic acid, did not reverse the inhibitor effect. These findings support both our hypotheses, first that insect hemocytes can detect and respond to fMLP, and second, that insect hemocyte migration is mediated by eicosanoids.

Keywords: *Manduca sexta*; Insect immunity; Migration; Boyden chamber

1. Introduction

Insects express robust innate immune reactions to microbial, parasitic and wound challenges. Although the distinction is somewhat artificial, 2 broad categories of immune reactions are recognized, humoral and hemocytic (also called cellular). Humoral defense reactions, detected 6–12 h post-infection, involve induced biosynthesis of antimicrobial peptides and proteins. The intracellular signal transduction systems acting in humoral immune reactions have been extensively characterized (Hoffmann, 2003; Lemaitre and Hoffmann, 2007). Hemocytic reactions are characterized by direct interactions between host hemocytes and foreign invaders. Specific cellular defense reactions include phagocytosis, microaggregation, nodulation and, in the case of larger invaders, encapsulation (Lavine and Strand, 2002; Stanley and Miller, 2006). While cellular immune reactions are well described, far less information is available on the biochemical signaling systems responsible for mediating and coordinating them (Gillespie et al., 1997; Stanley, 2006).

We suggested eicosanoids mediate insect cellular immune reactions (Stanley-Samuelson et al., 1991; Miller et al., 1994). This idea launched two lines of research. On one hand, experiments with a broad sampling of insect taxa and developmental stages support the idea that eicosanoids mediate hemocytic immune reactions in all insect species that express cellular immunity (some do not, such as foraging honeybees [Bedick et al., 2001]). Similarly, experiments with various species of bacteria, fungi, parasitoids, protozoans and viruses indicate that

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0022-1910/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2007.09.004

eicosanoids act in immune protection against a phylogenetically wide range of challengers (Stanley, 2005, 2006; Stanley and Miller, 2006; Büyükgüzel et al., 2007; Durmus et al., 2007; Stanley and Shapiro, 2007). The outcomes of experiments by several research groups indicate that eicosanoids are crucial mediators of phagocytosis, microaggregation, cell spreading and nodulation reactions (reviewed in Stanley and Miller, 2006). Eicosanoids also mediate whole-animal behavioral fever reactions to infections in locusts, *Schistocerca gregaria*, and likely in other insect species as well (Bundey et al., 2003).

Insect microaggregation, nodulation and wound reactions necessarily involve migration to guide hemocytes toward developing microaggregates and nodules as well as toward wound sites. Migration is a fundamental property of prokaryotic and eukaryotic, including insect, cells (Baker et al., 2006; Jin and Hereld, 2006). We hypothesized that insect hemocytes are able to detect and migrate toward a source of N-formyl-Met-Leu-Phe (fMLP), the major chemotactic peptide from *Escherichia coli* (Marasco et al., 1984) and that pharmaceutical modulation of eicosanoid biosynthesis inhibits hemocyte migration. Here we report on the outcomes of experiments designed to test our hypothesis.

2. Materials and methods

2.1. Organisms

Eggs of the tobacco hornworm, *M. sexta*, were provided by Ms. Beverley Pagura (North Carolina State University Insectary). We reared the hornworms on standard culture medium in individual cups under semi-sterile conditions developed by Dunn and Drake (1983). Second and third day, fifth instar hornworms were used in all experiments.

2.2. Reagents

We purchased formyl-methionyl-leucyl-phenylalanine (fMLP) from the Sigma Chemical Company (St. Louis, MO). Dulbecco's phosphate buffered saline (dPBS) was purchased from Gibco (Invitrogen Corporation, Carlsbad, CA). Selected eicosanoid biosynthesis inhibitors, including the glucocorticoid, dexamethasone [(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1, 4-diene-3,20-dione], the cyclooxygenase (COX) inhibitors indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-3-indolyl-acetic acid], ibuprofen [2-(4-isobutylphenyl) propionic acid], naproxen [6-methoxy-a-methyl-2-naphthaleneacitic acid], the lipoxygenase (LOX) inhibitors esculetin [6,7-dihydroxycoumarin], caffaic acid [3,4-dihydroxycinnamic acid], and the fatty acid, palmitic acid [hexadecanoic acid], were purchased from Sigma Chemical Company (St. Louis, MO). Arachidonic acid [AA; 5,8,11,14-eicosatetraenoic acid] was purchased from Cayman Chemical (Ann Arbor, MI).

2.3. Injections and primary hemocyte preparations

Hornworms were anesthetized by chilling on ice for 15 min, then surface sterilized by swabbing their exteriors with 95% ethanol (EtOH). Injections were performed as described in Miller and Stanley (1998). Briefly, for each injection, a syringe needle was inserted into the intersegmental suture between and just above the last two spiracles, then moved forward into the immediate anterior segment, keeping the needle parallel to the body wall to avoid injuring the alimentary canal. The plunger was then depressed, and the needle withdrawn slowly to ensure that hemolymph did not leak out of the insect.

Control larvae were injected with 10 µl EtOH. Experimental larvae were injected with one of these pharmaceutical compounds: the glucocorticoid dexamethasone, one of the COX inhibitors indomethacin, ibuprofen, naproxen, or one of the LOX inhibitors esculetin or caffaic acid dissolved in EtOH. In some experiments, dexamethasone-treated larvae were given an additional injection of arachidonic acid or palmitic acid dissolved in EtOH. Treated larvae were held at room temperature (ca. 22 °C) for 30 min post-injection.

Hemolymph was collected by the pericardial puncture procedure described by Horohov and Dunn (1982). 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 (approximately 500 µl) was collected into a chilled, sterile polypropylene 1.5 ml centrifuge tubes preloaded with 500 µl of cold dPBS. The hemolymph was gently mixed with dPBS by inverting the test tube several times, the number of hemocytes determined on a hemacytometer and hemocyte concentrations were adjusted to 2.5×10^4 cells/ml. The hemolymph suspensions are primary hemocyte cultures and were immediately used in experiments.

Viability of the hemocytes was assessed by visual examination during counting on a hemacytometer and was periodically confirmed by the Trypan blue exclusion test as described elsewhere (Miller and Stanley, 2001).

2.4. Hemocyte migration assay

Hemocyte migration assays were performed in a Boyden chamber (Boyden, 1962; Fig. 1) with a polycarbonate membrane (8 µm pore size, selected after preliminary experiments with 3 and 5 µm pore sizes NeuroProbes, Gaithersburg, MD). The migration stimulus, fMLP, was dissolved in dPBS (final concentration 10^{-6} M) and added to the bottom wells of the chamber. After laying the membrane atop the lower half of the chamber, hemocyte preparations (20 µl/well) were added to the top wells of the Boyden chamber. The charged Boyden chamber was held for 1 h at room temperature (ca. 22 °C). Following incubation, the membrane was removed from the chamber. The upper surface of the membrane was gently scraped to