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Effect of Eicosanoid Biosynthesis Inhibitors on the Immune Response of the Cotton Leaf Worm, *Spodoptera littoralis* (Boisd.) Infected with the Nematode, *Steinernema glaseri* (Rhabditida: Steinernematidae)

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ABSTRACT

Infection with the entomopathogenic nematode, *Steinernema glaseri* caused death to the Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.) larvae. Mortality of insect larvae increased when the nematodes were exposed to eicosanoid biosynthesis inhibitor dexamethasone. These effects were reversed when dexamethasone was used together with the eicosanoid precursor, arachidonic acid (AA), and other eicosanoid biosynthesis inhibitors (e.g. phenidone, ibuprofen, and indomethacin). *In vivo,* infection with *S. glaseri* infective juveniles enhanced nodule formation. Nodule numbers were reduced by dexamethasone, and restored by AA. In an *in vitro* experiment, incubation of the insect hemolymph with the nematode symbiotic bacteria, *Xenorahbdus nematophilus* raised numbers of both plasmtocytes and granulocytes in insect hemolymph and this was inhibited by dexamethasone, suggesting that dexamethasone acts directly on hemocytes formation. Although this inhibition was only partially reversed by arachidonic acid, we suggest that the *S. littoralis* immune response to insect pathogenic nematodes is normally modulated by physiological systems that include eicosanoid biosynthesis. These observations indicated that virulence of entomopathogenic nematode can be improved by compromising the insect host's immune system.

Key words: Arachidonic acid, Eicosanoids; insect immune system; Nodule formation; *Steinernema glaseri*; *Spodoptera littoralis*, *Xenorhabdus nematophilus*.

INTRODUCTION

The entomopathogenic nematodes (EPN) belong to both families Steinernematidae and Heterorhabditidae associated with their symbiotic bacteria *Xenorhabdus*, and *Photorhabdus,* respectively have been used commercially as biocontrol agents of economic insect pests (Gaugler, 2002). After the entrance of the infective juveniles (IJs) into the selected host insect, they release their symbiotic bacteria into the insect hemocoel where they grow causing septicemia and/or toxemia leading to host death (Hominick & Reid, 1990 and Georgis, 1992).

Eicosanoids have been implicated in the functioning or regulation of the insect immune system, especially in nodule formation; the major cellular immune response to bacterial infections (Horohov and Dunn and 1983; Shairra, 2007). Nodule formation occurs very quickly after immune challenge and involves hemocyte aggregation, during which a multicellular structure or nodule is formed (Ratcliffe and Rowley, 1979). During the process, a large number of bacteria become entrapped within the nodule, essentially isolating them from the insect hemocoel. Stanley-Samuelson *et al.* (1991) showed that eicosanoid biosynthesis inhibitors reduced the ability of the tobacco hornworm *Manduca sexta* to clear injected bacteria from the hemolymph and thus enhanced larval mortality caused by the septicemia. Miller *et al.* (1994) expanded on these findings to show that the impaired immune function caused by these inhibitors in *M. sexta* was due to inhibition of nodule formation. The physiological relevance of these results has been strengthened by the findings that both eicosanoids and the enzymes that synthesize them are present in *M. sexta* & *Schistocerca gregaria* tissues (Stanley-Samuelson *et al.*, 1991 and Shairra, 2007). Moreover, the discovery that various eicosanoids are biosynthesised in the fat body and hemocytes was of particular interest (Gadelhak *et al.*, 1995 and Stanley-Samuelson and Ogg, 1994). The formation of these compounds was inhibited by eicosanoid biosynthesis inhibitors (Jurenka *et al.*, 1997).

Many other insect species, from several different orders have been studied in an attempt to generalize the finding that eicosanoids mediate nodulation during infection (Jurenka *et al.*, 1997; Ayaad *et al.*, 2008; Stanley *et al.*, 1999 and Stanley-Samuelson *et al.*, 1997). However, almost all previous work has been concerned with immune responses provoked by the bacteria. The study of Mandato *et al.* (1997) is an exception in using silica microspheres, while Bedick *et al.* (2000) used the bacterial cell wall component, lipopolysaccharide (LPS) to elicit nodule formation. Also, Carton *et al.* (2002) suggested that encapsulation (a similar response to nodulation) of parasitoid eggs in Drosophila is also mediated by eicosanoids.

In this study, we used the entomopathogenic nematodes *S. glaseri* which infects a wide range of host insects (Akhurst and Bedding, 1986). The effects of eicosanoid biosynthesis inhibitors on *S.*

littoralis immune response toward *S. glaseri* were studied. It is an attempt to provide evidence that larvae treated with these inhibitors become more susceptible to nematode infection, and to show that this immune impairment is correlated with suppression of nodule formation.

MATERIALS AND METHODS

I. Experimental insects: *S. littoralis* larvae were reared on castor leaves, *Ricinus communis* according to Ibrahim (1974). Newly molted sixth instar larvae were utilized in all experiments.

II. Nematodes: Imported nematode species, *S. glaseri* was provided by Dr. El-Sadawy, National Research Center, Giza, Egypt. For mass culturing of the used nematode isolates, last instar larvae of both the greater wax moth, *Galleria mellonella* L. and of the cotton leaf worm, *S. littoralis* were used as insect hosts according to Shamseldean (1994).

III. Pharmacological agents: unsaturated fatty acid, arachidonic acid (AA), [C_{20}: 4n-6 (5, 8, 11, 14-eicosatetraenoic acid)], the cyclooxygenase inhibitor, indomethacin (Indo) (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid). The dual cyclooxygenase/ lipoxygenase inhibitors, ibuprofen (Ibu) (a-methyl-4-[isobutyl]-phenylacetic acid), and phenidone (Phen) (1-phenyl-3-pyrazolidone), or the phospholipase A_2 inhibitor, dexamethasone (Dex), each inhibitor is dissolved in 2µl of 95% ethanol. They all are obtained from Sigma Chemical Co., St Louis, MO, USA.

IV. Susceptibility of *S. littoralis* larvae to the nematode, *S. glaseri*: 6th instar larvae were divided into five groups. They were chilled at 4°C for 15 minutes prior to surface swabbing with 95% ethanol. Six insect larvae of each group were then injected with *S. glaseri* suspensions of (1, 2, 3, 4 and 5 IJs / 2 µl of distilled water). Injection was carried out using a 30 gauge sterile, siliconized needle fitted to a 50µl syringe (Hamilton, Reno, NV) calibrated to deliver a volume of 2µl. This injection method allows standardizing the level of infection (i. e., the exact number of IJs/ larva). The needle was carefully inserted between the fifth and sixth abdominal segment. Preliminary tests showed that this method reduced insect bleeding. Four replicates of each dose were carried out. The number of cadavers was recorded, 6, 12, 24 & 48 hours post-injection and the percentage of mortality was calculated to estimate the LD_{25} values. To assess the response in unchallenged individuals, negative controls (intact) were included. The positive control individuals were injected with 2µl distilled water. The experimental treatments were incubated at 25 ± 2°C.

V. Effect of dexamethasone and arachidonic acid on mortality of *S. littoralis* larvae: insect larvae were injected with a dose of (LD_{25} = 3-4 IJs) infective juveniles of *S. glaseri* suspended in 2 µl of distilled water. Control larvae were injected with 2 µl of distilled water and/or 95% ethanol. All pharmacological agents were dissolved in 95% ethanol and were injected into test larvae in 2µl aliquots. Each larva received 4 µg of eicosanoid biosynthesis inhibitor. AA was injected at a dose of 7 µg per larva together with dexamethasone. The larvae were left for 15 minutes after nematodes injection to release its symbiotic bacteria *Xenorhabdus nematophilus* within the larval hemocoel, then the drugs was injected into the opposite side of the body. Groups of control larvae were injected only with the inhibitors to determine whether the injected drugs had any visible detrimental effects on treated insect larvae. After injection, larvae were held without any food for 24 hours. Then they were fed on castor leaves and maintained under normal rearing conditions. Insect larvae were examined daily to observe symptoms of nematode infection and/or death. Mortality was recorded when dead insect larvae were unable to move back when placed on their dorsal side and were unable to respond to poking.

VI. *In vivo* effect of dexamethasone and arachidonic acid on nodule formation: Nodule formation was determined in sixth instar *S. littoralis* larvae when injected with 3-4 IJs of *S. glaseri* nematodes, or co-injected with dexamethasone as mentioned above. Nodule formation was assessed after 12 hours. Insects were immobilized on ice for 30 minutes before dissection in 1% (w/v) NaCl solution saturated with phenylthiourea (which prevented general post-dissection melanization). Melanized, dark nodules within the hemocoel were counted using a stereomicroscope.

VII. *In vitro* effects of some pharmacological inhibitors and nematode *S. glaseri* on hemocytes count: Both plasmatocytes and granulocytes were *in-vitro* counted as an index of nodule formation. Many protocols were applied and tested Miller and Stanley (2001). Larvae were chilled on ice for 5 minutes and the area around the dorsal horn was disinfected with 70% ethanol. This technique also cleaned the cuticle from any deposits which

could contaminate the hemolymph. Differential hemocyte counts in the nematode-injected larvae were assessed at a given time (12 hours post-injection). Larvae were anesthetized by chilling on ice and hemolymph was collected by pericardial puncture using Teflon-lined needles (Gunnarsson & Lackie, 1985). Ten microliters of hemolymph were collected, mixed with 80µl of diluting solution (NaCl, 4.65g; KCl, 0.15g; $CaCl_2$; 0.11g; crystal violet; 0.05g and acetic acid; 1.25ml /liter distilled water). Twenty µl of diluted hemolymph were applied to a hemocytometer (AO instrument Co., Buffalo, NY). The hemocytes in each sample were counted in four large fields of the hemocytometer using phase-contrast optics. The control injected with same amount of 95% ethanol was also tested.

VIII. Statistical analysis: In the present study, data presented in percentage values and were normalized using arcsine transformation values of LD_{25} using a software package "Ldp-line" a copyright by Ehab, M. Bakr, Plant Protection Research Institute, ARC, Giza, Egypt and Finney, 1952. Data analyses were made using the software package "COSTAT", a product of Cohort Software Inc., Berkeley, California. Significant treatment effects were identified by one way ANOVA, computer program, copyright©, 1989 (H. S. Motulsky, version 1.0, Dr. Schouest) UC Riverside, serial # 8901685).

RESULTS AND DISCUSSION

Susceptibility of *S. littoralis* larvae injected with the nematode, *S. glaseri* is depicted in Fig. 1. Statistical analysis of the data revealed that, the LD_{25} values were 5, 4, 3, 2 and 1 IJs / larvae after 6, 12, 24 and 48 hours post-injections, respectively. Significant differences ($P<0.05$) were obtained considering the LD_{25} values between the 6h and those of 12, 24 and 48 hours values post-injection as indicated by the non-overlapping of the corresponding 95% confidence limits. Additionally, the nematode infective juveniles induced percentage mortality of *S. littoralis* larvae reaching 100% at 48 hours post- injection with the high dose of 5 IJs/ larvae. At the low doses of 1, 2 & 3 IJs/larvae, the percentage mortality reached 40, 50 & 80 % at 48 hours post- injection, respectively (Fig. 1). In contrast, the percentage mortality of the desert locust, *Schistocerca gregaria* nymphs differed significantly ($P < 0.05$) when the injected dose increased, an injected dose of 5 IJs/insect of *Heterorahabditis indica* (RM_1) strain was sufficient to give 20% mortality at 12 hours post injection (Shairra, 2007).

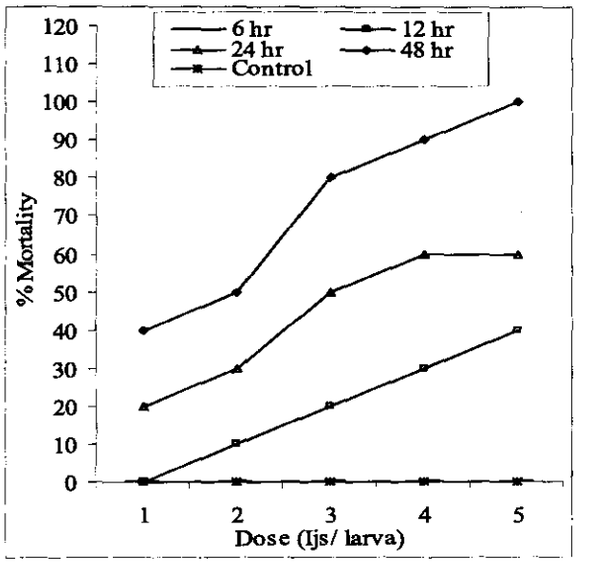

Fig. (1): Dose-response curve of the nematode *S. glaseri* (IJs) injected into *S. littoralis* larvae at different time intervals. Each point indicates the mean mortality percentage of insect larvae.

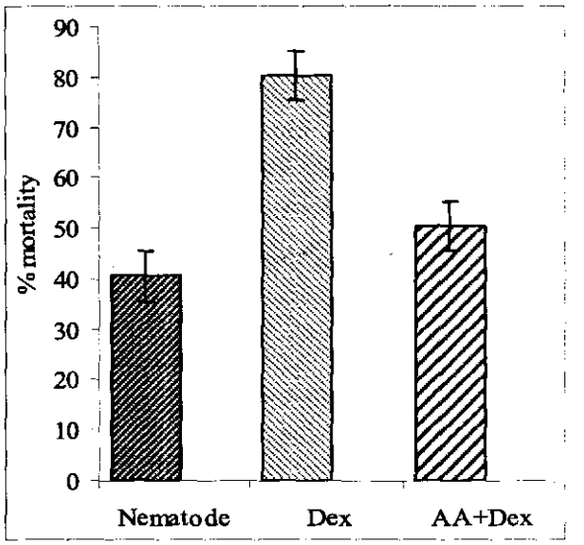

Fig. (2): *Spodotera littoralis* larvae injected with the infective juveniles of *S. glaseri* or co-injected with dexamethasone (Dex) or arachidonic acid with dexamethasone (AA+Dex). Error bars represent 1 SEM.

Dexamethasone was tested on the mortality of cotton leaf worm larvae injected with different doses of *S. glaseri* infective juveniles to determine the appropriate dose for the mortality assay (Fig. 2). Host mortality was dependent on nematode dose with high doses causing earlier mortality. An injected dose of 3-4 nematode infective juveniles was sufficient to kill the insects over an extended period adequate to test the effects of dexamethasone. Dexamethasone strongly enhanced the nematode killing competence (Fig. 2). This potential was significantly reversed when AA was co-injected with dexamethasone. There was no significant difference between the mortality of the control

larvae (injected only with ethanol) and dexamethasone/AA injected ($P > 0.05$). The idea that eicosanoids are important in insect immune system was discovered by Stanley-Samuelson *et al.* (1991). It was shown that inhibition of eicosanoid biosynthesis reduced the clearance of the pathogenic bacterium *Serratia marcescens* from the hemolymph of *Manduca sexta* larvae that raised mortality of the infected insects. It was also shown in the same work that larvae of *M. sexta* contain all the C-20 polyunsaturated fatty acids were capable of converting arachidonic acid (AA) into several eicosanoid metabolites. In contrast, Miller *et al.*, (1994) reported also that dexamethasone and other eicosanoid biosynthesis inhibitors inhibited nodule formation during artificial infections of *S. marcescens* in *M. sexta* and this effect was reversed by AA. This work is providing the basis for what is now called the eicosanoid hypothesis formalized by Stanley, 2000, which proposes that endogenous eicosanoids mediate nodulation reactions to bacterial challenge in insects. Various authors depended on such work, and there is now much evidence of the same general type to show that eicosanoids may mediate innate immune responses to bacteria in a wide range of insects. However, the question of whether eicosanoids also modulate defense reactions directed against fungi and nematodes has so far not been addressed.

Effects of eicosanoid biosynthesis inhibitors on different haemocyte count of *S. littoralis* larvae were studied. The formation of both the plasmatocytes and granulocytes as an immune reaction towards the injection of the nematode *S. glaseri* IJs and the released bacteria *X. nematophilus* was also determined. Data obtained showed that, reduction in cell numbers of both plasmatocytes and granulocytes was evident after nematode infection in insects (Table 1). There was a positive relationship between exposure time and the increase in hemocyte numbers. Differential hemocyte count of infected larvae injected with various eicosanoid biosynthesis inhibitors was taken after 12 hours post injection. All eicosanoid biosynthesis inhibitors caused a significant increase ($P<0.05$) in numbers of plasmatocytes and granulocytes when exposure time increased, in the infected larvae, compared with the uninfected controls (Table 1). Thus, in all cases, numbers of plasmatocytes and granulocytes of *S. littoralis* infected larvae injected with indomethacin, ibuprofen, phenidone, esculetin, or dexamethasone increased significantly more than the corresponding plasmatocytes and granulocytes of the control (only ethanol injected). Injection of any of these inhibitors alone (without nematode) had no effects on the growth rate of the larvae relative to controls (unpublished data).

Table (1): The percentage of plasmatocytes (P) and granulocytes (G) of *S. littoralis* larvae at different times post-injection with some pharmacological inhibitors and infective juveniles of the nematode, *S. glaseri*

Treatment		% Hemocyte types (mean ± S.E.) Post injection time in hours		
		3	6	12
Control	P***	16.1± 3.1[a]	17.5± 0.2[a]	29.2± 2.2[b]
	G***	24.9 ± 1.0[a]	39.4 ± 0.3[b]	48.4 ± 1.0[c]
Nematode IJs only	P	25.3± 2.4[a]	35.3±1.3[b]	43.2± 0.4[b]
	G	12.7± 2.1[a]	39.5± 0.9[b]	84.8± 2.3[c]
Ethanol	P	20.2± 4.4[a]	35.3± 2.3[b]	32.5± 2.4[b]
	G	45.2± 0.7[a]	38.6± 8.4[b]	55.3±0.9[c]
Dexamethasone	P	40.1 ±0.1[a]	31.2 ± 1.2[b]	35.5 ± 0.3[b]
	G	58.2 ± 4.1[a]	42.7± 2.3[b]	54.8 ± 1.2[a]
Ibuprofen	P	32.46 ± 2.3[a]	29.1 ± 2.1[a]	41.5 ± 2.3[b]
	G	43.3 ± 1.3[a]	46.5± 2.3[a]	48.2 ± 2.1[a]
Indomethacin	P	34.1 ± 0.3[a]	48.2 ± 1.4[b]	40.6 ± 2.0[b]
	G	60.1 ± 0.3[a]	52.4± 0.5[b]	41 ± 1.2[c]
Phenidone	P	32.4± 0.5[a]	30.2± 1.3[a]	45.1± 1.2[b]
	G	51.1± 2.2[a]	48.3 ± 1.5[a]	63.3± 0.4[b]

*Means followed by the same letter in the same column of the parameter are not significantly different ($P > 0.05$).
**All tested larvae were pre-injected with the nematode Infective Juveniles (IJs). Control larvae were injected only with ethanol.
*** P. = Plasmatocytes, G. = Granulocytes.

The present research had three main objectives: 1. investigate whether eicosanoid biosynthesis inhibitors could impair the ability of the insect to defend itself against a nematode pathogen, 2. assess the importance of eicosanoid biosynthesis in nodule formation as an immune reaction against a nematode pathogen, and 3. explore whether eicosanoid biosynthesis inhibitors have a direct effect on insect hemocyte responses to the nematode infective juveniles of *S. glaseri*. Taking into consideration that nematodes are entomopathogenic whether the juvenils were applied topically which is the normal route of natural infection or given internally by injection into the hemocoel. In the present study, direct injection of *S. glaseri* IJs was practiced to investigate the role of the insect's cellular immune response against the nematodes. If infected juveniles are applied topically, penetration of the host cuticle must be considered, independent of cellular immune processes. Moreover, since nematode penetration takes some time and each infective juvenile penetrates within different time intervals, it would be difficult to judge when to administer the pharmacological agents following inoculation. Thus, the experiments concerned with the role of eicosanoids in the immune responses to nematode within the hemolymph, and don't address the question of whether these agents mediate defense responses to nematode during penetration of the insect's host. Injection of *S. littoralis* with *S. glaseri* caused stop feeding and eventually death. These

symptoms depend on the dose of juveniles injected. As the first objective was to determine whether eicosanoid biosynthesis inhibitors caused increased susceptibility of the insect to the nematodes, the results were unequivocal. Infected larvae given dexamethasone died significantly sooner than those given the carrier (ethanol) alone. This effect was completely abolished by the co-addition of arachidonic acid, an eicosanoid precursor. Previous studies, using bacterial pathogens have reported similar findings, in which dexamethasone enhances the insect's susceptibility to the bacteria (Connick *et al.*, 2001; Park and Kim, 2000 and Stanley-Samuelson *et al.*, 1991). The present work seems to be the first one to show that dexamethasone also increases the susceptibility of an insect to an entomopathogenic nematode. Ideally, the effects of pure eicosanoids on the insect immune response to the nematodes should be tested. Unfortunately, at the present time this is not possible because the identities of the relevant endogenous eicosanoids are unknown, and eicosanoids are unlikely to have a sufficiently *in vivo* long half-life to be useful in experimental work described in this study. Both indomethacin and esculetin showed specifically to inhibit eicosanoid biosynthesis in *M. sexta* tissues (Gadelhak *et al.*, 1995 and Stanley-Samuelson and Ogg, 1994).

In vivo effect of dexamethasone on nodule formation is shown in figure 3. Nodule formation in *S. littoralis* was found to be an important part of the defense response towards *S. glaseri*. A large number of nodules (220 nodules) were *in vivo* formed in response to the injection of 3-4 *S. glaseri* infective juveniles (Fig. 3). When dexamethasone was co-injected with *S. glaseri* nematode, number of formed nodules was strongly reduced to only 67 nodules compared with higher numbers of nodules in the control levels ($P < 0:05$). The suppressive effect of dexamethasone was significantly evident ($P < 0:05$) by the co-administration of AA with dexamethasone (only 86 nodules). Nodule formation by insects as a response to bacterial infection is now well discussed in the literature (e. g. Horohov and Dunn, 1983; Ratcliffe and Rowley, 1979 and Ratcliffe and Walters, 1983). The present research work illustrated that nodule formation is also an important response to the entomopathogenic nematodes and this immune response is modulated by eicosanoids. To live and flourish in its insect host, a successful entomopathogen must escape the vigilance of the insects' immune system especially during early stages of infection. Given the importance of eicosanoid signaling to the insect immune system, interference with eicosanoid metabolism would seem to be a sensible strategy, and thus a potentially important virulence factor for an entomopathogen. We predict that at least some entomopathogens will inhibit eicosanoid signaling in their insect hosts.

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