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ORIGINAL ARTICLE / PEST CONTROL

Pyriproxyfen Inhibits Hemocytic Phagocytosis of the Beet Armyworm, *Spodoptera exigua*

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Abstract : The concept of innate immunity in insects which refers to the first line of host defense constitutes the humoral and cellular components which are involved in recognition and actively participate in the elimination of the intruding foreign micro- or macro-organisms. Several recent studies suggest that juvenile hormone (JH) modulates the cellular immune reactions in response to pathogen. In this study, pyriproxyfen (a JH agonist as an insect growth regulator) was tested in its any inhibitory effect on the immune reactions of the beet armyworm, *Spodoptera exigua*. To this end, five different hemocyte morphotypes of final instar *S. exigua* were identified by phase contrast microscopy. Plasmatocytes and granular cells, which constitute about 90% of the total hemocyte count, were prominently distinguished based on their basophilic/acidophilic nature using Giemsa stain. The role of pyriproxyfen on the functional ability of hemocytes was analyzed using FITC-labeled *Providencia vermicola* for the phagocytic potential of the hemocytes. Both granular cells and plasmatocytes exhibited phagocytosis behavior. Pyriproxyfen significantly inhibited the phagocytosis of both cell types, proposing its novel action as an immunosuppressant.

Key words : hemocytes, immune, phagocytosis, pyriproxyfen, *Spodoptera exigua*

INTRODUCTION

Insects, the most successful group among invertebrates which exist in a myriad of environment potential for infection by microorganisms and parasites, lack an acquired immune system, but have a well-developed innate immune response (Ratcliffe *et al.*, 1985). These insect immune reactions include both cellular and humoral origins, in which cellular responses depend on various hemocytic behaviors including phagocytosis, nodulation, and encapsulation, whereas humoral reactions mostly depend on the action of antimicrobial peptides (Gillespie *et al.*, 1997).

Phagocytosis is a widespread behavior exhibited by many protozoan species and all metazoan phyla and is well studied in mammalian macrophages (Aderem and Underhill, 1999). When a cognate receptor on the cell membrane binds to a target molecule, the process of phagocytosis is triggered by activating signaling cascades that regulate formation of a phagosome and target ingestion via an actin polymerization dependent mechanism (Foukas *et al.*, 1998). The phagosome in the cells fuses with lysosome, and the targets in the phagolysosomes are killed or degraded by reactive oxygen intermediates and reactive nitrogen intermediates (Nathan and Hibbs, 1991; Robinson and Badwey, 1994; Whitten and Ratcliffe, 1999).

Some endocrine factors are implicated in immune signal mediation which include eicosanoids (Stanley, 2000), biogenic amines (Baines *et al.*, 1992), and adipokinetic hormone (Goldsworthy *et al.*, 2003). Moreover, juvenile hormone (JH) and 20 hydroxyecdysone (20E) are also

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known to modulate insect immune responses (Franssens *et al.*, 2006), wherein, 20E stimulated differentiation of plasmatocytes in *Drosophila melanogaster* into actively phagocytic macrophages (Lanot *et al.*, 2001). A similar stimulatory effect of 20E was also observed with hemocytes of *Rhodnius prolixus*, in which azadirachtin, an ecdysteroid antagonist, inhibited phagocytosis, while the addition of 20E reversed the inhibitory action of azadirachtin (Figueiredo *et al.*, 2006). In comparison, JH injection suppressed hemocyte encapsulation response in *Tenebrio molitor* (Rantala *et al.*, 2003). The antagonistic action between JH and 20E was demonstrated in terms of plasmatocyte spreading behavior in *Pseudoplusia includens* in response to an insect cytokine (Clark *et al.*, 2005).

Pyriproxyfen (PYR) is a phenoxyphenyl compound mimicking JH action by inhibiting metamorphosis and stimulating reproduction (Kim *et al.*, 1999; Kawada *et al.*, 2006). PYR is actively inhibit larval to pupal metamorphosis of *Bombyx mori* (L.), resulting in dauer stage (Monconduit and Mauchamp, 1998; Kim *et al.*, 2004). The inhibitory link between insect immune reactions and JH led us to pose a hypothesis that PYR may inhibit hemocyte phagocytosis. To test our hypothesis, we needed to determine hemocyte types responsible for phagocytosis in the test insect, *Spodoptera exigua*. In turn, based on the phagocytotic hemocytes, we analyzed phagocytosis of *S. exigua* in response to PYR using fluorescent labeled bacteria.

MATERIALS AND METHODS

Test insect

S. exigua larvae were reared on an artificial diet (Gho *et al.*, 1990) at 25 ± 1°C, under a photoperiod of 16:8 (L:D) h, in 60 ± 5% relative humidity. Only last instar larvae were used throughout this study.

Reagents

L-cysteine hydrochloride, glutaraldehyde, fluorescein isothiocyanate (FITC) and other chemicals used in this study were purchased from Sigma (St. Louis Missouri, USA). Anticoagulant buffer (ACB) was prepared, where 8 mg of L-cysteine hydrochloride was dissolved in 5 ml of TBS (50 mM Tris HCl, 100 mM dextrose, 5 mM KCl, 2.5 mM MgCl2 50 mM NaCl (pH 7.5; 300 mOsm) and the pH was adjusted to 7.5 using 0.1N NaOH. PYR[phenoxyphenoxy(*R,S*)-2-(2-pyridyloxy)propyl ether] (98% technical grade) was donated from Dong Bang Agrochemical Inc. (Seoul, Korea).

FITC labeled bacterial cells

A Gram negative bacterium, *Providencia vermicola*, was isolated from an entomopathogenic nematode (Yi *et al.*, 2007). The bacteria were cultured aerobically in Luria Broth (37°C, 18 h). After being harvested by centrifugation (3,000 x *g*, 20 min, 4°C), the cells were washed three times with 0.9% saline and then heat inactivated by holding the suspension at 70°C for 3 h. This heat inactivated *P. vermicola* was washed again by centrifugation (10,000 x *g*, 10 min, 4°C) and finally suspended as 1% (v/v) concentration and labeled with FITC by suspending in 1% FITC solution (carbonate-bicarbonate buffer: 0.2 M pH 9.0). Before use, this suspension was washed thrice in TBS and resuspended in TBS.

Hemolymph collection

The larvae of *S. exigua* were surface sterilized using cotton swab soaked in 70% ethanol. The proleg was cut with a pair of scissors and the hemolymph sample (~50 µl) was collected in 250 µl of ice-cold ACB and during hemolymph collection the tube was gently shaken to facilitate thorough mixing of hemolymph and ACB.

Phagocytosis assay

Four hemocyte monolayers were made using 50 µl of the above hemocyte suspension and left in moist chamber for 30 min for the hemocytes to settle, attach and spread. After 30 min, the first monolayer was assessed for the various hemocyte morphotypes. The second monolayer was overlaid with DMSO. The third and fourth monolayers were washed with TBS in order to remove plasma. Then, one monolayer was overlaid with FITC labeled *P. vermicola* suspended in buffer containing PYR (10^{-6} M final concentration) and the other monolayer was overlaid with *P. vermicola* suspended in buffer alone. These monolayers were held

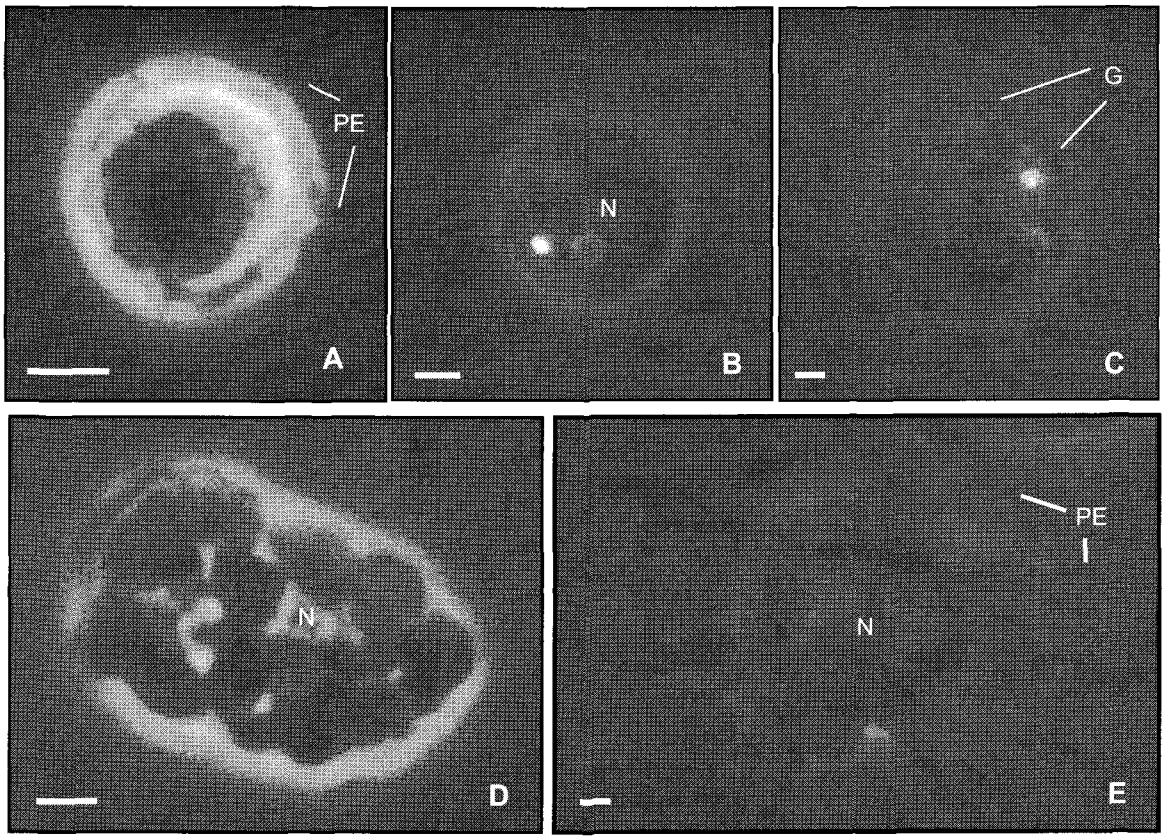

Fig. 1. Phase-contrast photomicrograph of five different hemocyte morphotypes found in the hemolymph of the fifth instar *Spodoptera exigua* collected using cysteine.HCl as anticoagulant. (A) prohemocyte, (B) oenocytoid, (C) granular cell, (D) spherule cell, and (E) plasmatocyte. Scale bar = 10 μm. PE: pseudopodial extensions, N: nucleus, G: cytoplasmic granules.

in moist chamber up to 30 min. Then overlaid with 1% trypan blue dye solution in order to quench the non-phagocytosed bacterial cells and fixed using 1.5% glutaraldehyde solution. After 5 min of fixation, these slides were observed under confocal microscope (Olympus, Tokyo, Japan) in fluorescence mode at 400x. Phagocytosed hemocytes were counted and expressed as per cent phagocytic cells based on 200~250 cells each slide.

RESULTS AND DISCUSSION

Hemolymph coagulation, which is a continuous process, is initiated by alterations taking rapidly in a category of labile hemocytes, followed by the development of islands of coagulation in the surrounding plasma (Gregoire, 1974; Theopold and Schmidt, 1997). The anticoagulant, cyteine.HCl, used in this study prevented this coagulation probably due to its reducing activity to antagonize quinine melanization catalyzed by the action of phenoloxidase (Madanagopal and Kim, 2006). In this condition, we identified five distinct hemocyte morphotypes in *S. exigua*, namely pro-hemocytes, oenocytoids, granular cells, spherule cells, and plasmatocytes, based on their morphological characteristics under phase-contrast microscope (Fig. 1) as have been characterized in selected species using antibody and genetic markers (Chain *et al.*, 1992; Mullet *et al.*, 1993; Willott *et al.*, 1994; Strand and Johnson, 1996; Gardiner and Strand, 1999; Lebestky *et al.*, 2000). Unlike in other lepidopterans, all the five hemocytes adhered on the glass surface thus enabling their identification. Prohemocytes were small cells which did not show extensive spreading, but well attached on the glass surface with short pseudopodial extensions (Fig. 1A). Oenocytoids were round cells with large nucleo-cytoplasmic ratio. (Fig. 1B). Granular cells constitute large number of granules, which were phase

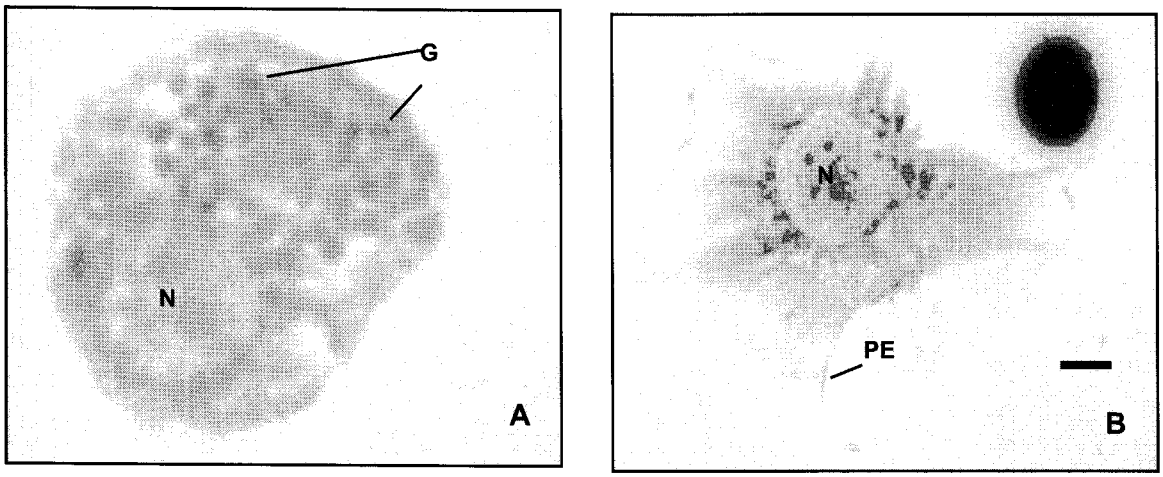

Fig. 2. Bright field photomicrograph of (A) granular cell and (B) plasmatocyte of *Spodoptera exigua* stained with Giemsa stain. Scale bar = 10 μm. N: nucleus, G: cytoplasmic granules, PE: pseudopodial extensions.

dark after their extensive spreading (Fig. 1C). Spherule cells, which are suggested to transport cuticular components (Sass *et al.*, 1994), contained large globular structures in their cytoplasm, while their nucleus became discernible after spreading (Fig. 1D). Plasmatocytes were the cells that readily attached on the glass surface and immediately started to put forth prominent filopodial and pseudopodial extensions and with time their nucleus and cytoplasmic areas were distinct (Fig, 1E). Based on the cytochemical analyses using Giemsa stain, the nuclei of granular and plasmatocytes stained dark blue to purple (Fig. 2). The granules within the cytoplasm of granular cells stained purple or basophilic in nature thereby indicating the presence of mainly acidic substances (Humason, 1972) within these granules. Plasmatocytes also possessed the similar granules, but few in numbers. The granules of the plasmatocytes contained substances which were less acidic in nature because they were stained in blue color with Giemsa.

Incubation of hemocytes with FITC-labeled *P. vermicola* in the absence of PYR showed a phagocytic rate of 78 ± 3% (Fig. 3). All phagocytotic cells were granular cells and plasmatocytes, in which granular cells appeared to be more active (data not shown). In *S. exigua*, phagocytosis is inhibited by phospholipase A_2 inhibitor, indicating that it is mediated by eicosanoids (Shrestha and Kim, 2007). This also suggests that target recognition signal by a specific cognate receptor relays via eicosanoids to neighboring hemocytes, which in turn activates phagocytosis. Internalization of the bacteria involves several factors such as attachment to hemocyte surface (for example, integrin receptors) by the action of phenoloxidase and cytoskeletal rearrangement (Foukas *et al.*, 1998).

On the other hand, this frequency of phagocytic activity was significantly reduced ($t = 33.072$; $df = 4$; $P < 0.0001$) in the presence of PYR (Fig. 4). The results presented here clearly showed that the FITC-labeled bacterial cells with trypan blue as a quenching dye can

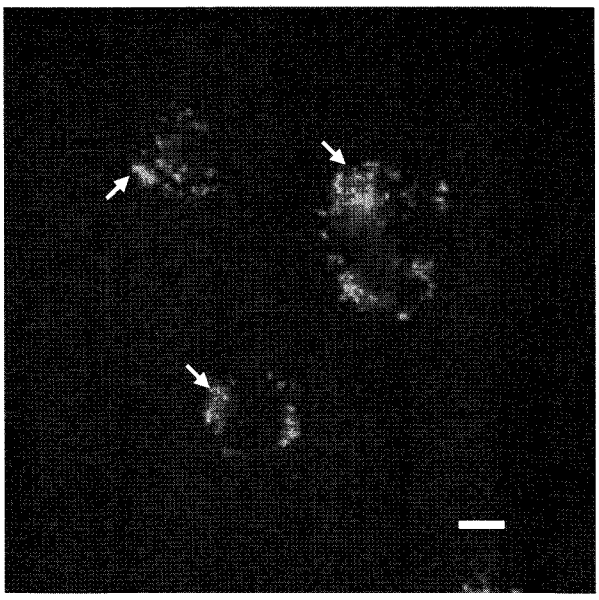

Fig. 3. Photomicrograph of *Spodoptera exigua* hemocyte monolayers after phagocytosis of FITC labeled *Providencia* vermicola observed under Olympus Confocal Microscope. Arrows indicate only ingested *P. vermicola* with bright fluorescence after quenching with trypan blue. Scale bar = 10 μm.

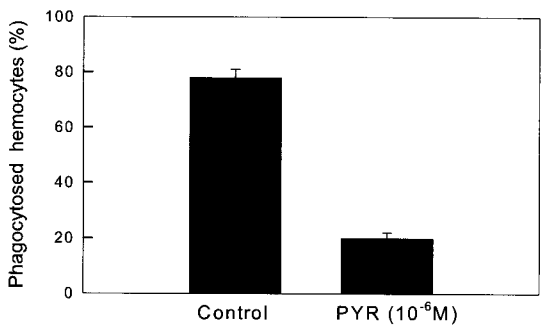

Fig. 4. *In vitro* phagocytic activity of *Spodoptera exigua* hemocytes against FITC-labeled *Providencia vermicola* in the presence or absence (control) of pyriproxyfen ('PYR', 10^{-6} M). Vertical bars represent means (±SD) of phagocytosis rates of three independent determinations. The difference in phagocytosis rates between the control and experimental was statistically significant at Type I error = 0.05 (LSD test).

be used for the unambiguous determination of phagocytic activity in *S. exigua*. PYR also inhibited hemocyte spreading in *Plutella xylostella*, which resulted in significant immunosuppression (Kwon and Kim, 2007). The inhibitory mechanism of PYR remains unclear. However, several previous reports (described in Introduction) support the inhibitory effect of PYR due to its JH agonistic effect (Kawada *et al.*, 2006). Considering the effect of JH to stimulate ovarian follicle cell patency by decreasing its volume through dehydration (Kim *et al.*, 1999; Sevala and Davey, 1989), PYR may inhibit spreading behaviors accompanying phagocytosis by stimulating Na^+-K^+ pump to reduce cell volume. This hypothesis needs to be explored to understand JH effect on hemocytes. To the best our knowledge, the inhibitory effect of PYR on phagocytosis by hemocytes is unknown and further studies has to be performed.

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파밤나방(*Spodoptera exigua*)의 혈구세포 식균반응에 대한 피리프록시펜의 억제효과

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요약 : 외래 병원체 침입에 대해서 방어기작으로서 곤충이 보이는 선천성 면역작용은 세포성 및 체액성 면역반응을 포함하며, 이는 비자기 인식 후 유기된다. 최근 여러 연구는 유약호르몬이 외래 물질에 반응한 세포성 면역작용을 조절한다고 제시하고 있다. 본 연구는 유약호르몬 동력제로서 곤충생장조절제인 피리프록시펜을 이용하여 이 약제가 가지는 면역억제작용을 파밤나방(*Spodoptera exigua*)을 대상으로 분석하였다. 이를 위해 본 연구는 위상차현미경을 이용하여 파밤나방 최종령 유충으로부터 5가지 형태의 서로 다른 혈구세포를 동정하였다. 이 가운데 과립혈구와 부정형혈구는 전체 혈구의 90% 이상을 차지하며, Giemsa 염색법에 의해 이들 상호간에 뚜렷한 형태적 구분이 가능했다. 유약호르몬 동력제인 피리프록시펜의 혈구세포의 식균작용에 미치는 영향이 FITC로 표지된 세균(*Providencia vermicola*)을 이용하여 분석하였다. 과립혈구와 부정형혈구는 활발한 식균작용을 보였다. 피리프록시펜은 현격하게 이들 두 혈구세포의 식균작용을 억제시켰다. 본 연구는 면역억제자로서 피리프록시펜의 새로운 기능을 제시하고 있다.

Key words : 혈구세포, 면역, 식균작용, 피리프록시펜, 파밤나방