Identification of haemocytes in the haemolymph of desert locust, *Schistocerca gregaria* and their activity against *Bacillus thuringiensis israelensis*

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ABSTRACT

This study aimed to understand the role played by haemocytes of *Schistocerca gregaria* adults in response to injection with *Bacillus thuringiensis israelensis* (Bti) into the hemocoel. Light microscopy recognized four types of haemocytes namely: prohaemocytes (PRs), plsamatocytes (PLs), granular cells (GRs) and coagulocytes (COs). Different responses were observed in the haemocyte populations at different time intervals (6, 12 24 and 48 h) following injection with a sublethal dose of bacteria; (1) Pathological consequences in the haemocytes including variation in the cell volume, vacuolization in the cytoplasm, distortion of the cell membrane and pycnosis in the nuclei, (2) Various changes in the differential haemocyte counts (DHCs), the most important is a decrease in the numbers of GRs and COs accompanied with an increase in PLs and PRs, (3) A marked decrease in the total haemocyte counts (THCs) at most time periods. (4) Phagocytosis was shown during all periods post-injection with peak at 6 h, and PLs were the essential phagocytic cell type. (5) A significant increase in nodule counts at all time intervals post-injection was observed above controls, with the peak was at 12 h post-injection.

Keywords: *Schistocerca gregaria*, *Bacillus thuringiensis israelensis*, haemocytes, DHCs, THCs, phagocytosis, nodule formation.

INTRODUCTION

Blood cells (haemocytes) of insects, like vertebrate leukocytes are a mixture of cell types with different morphological and biological functions. It is difficult to classify insect haemocytes, according to the invertebrate white blood cell classification scheme, into well-defined ontogenetic classes due to cell diversity as well as limited knowledge of their development and differentiation (Ribeiro and Brehélin, 2006). Therefore, the classification of haemocytes in insects is a subject of controversy, and the terminology used to designate each cell type often differs from one species to another. However, several literatures reviewed the haemocytes in insects and suggested that there are some similarities between most cell types and their functions, in the different insect species (Price and Ratcliffe, 1974; Rowley and Ratcliffe, 1981; Gupta, 1985; Brehélin and Zachary, 1986; Lavine and Strand, 2002). However, it was evident that there are no comparative investigations dealing with the ultrastructure and functional aspects.

Insect haemolymph is highly effective in eliminating invaders from the hemocoel by means of an internal defense system consisting of cellular components and humoral mechanisms that provide highly effective protection against invading microorganisms and interact cooperatively to destroy non-self-elements (Jiang et al., 2010). These defensive reactions were observed against pathogens, parasites, and other foreign bodies that have entered the hemocoel (Ottaviani, 2005). Cellular defense mechanisms are accomplished by...
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haemocytes, which have the ability to distinguish non-self-factors, and to mediate phagocytosis, encapsulation, wound repair and coagulation (Kanost, 2008). Cellular defenses occur immediately upon contact with the foreign invader (Cartoon and Nabi, 1997). Hence, much attention is needed in describing the role of haemocytes in defense mechanisms. Total and differential haemocyte counts, and the pathological consequences of haemocytes, are important for comparative work.

The desert locust, *Schistocerca gregaria* (Forskal) represents a relatively important group of plant-feeding insects with strong immune responses against bacteria (Mishrif and Barakat, 2002; Barakat *et al.*, 2002; Mo’men *et al.*, 2010). This locust was used in this study as a laboratory model for studies of the immune response against the challenge with a potential biological control agent, *Bacillus thuringiensis* var. *israelensis* (*Bti*). The current study forms part of a broad study aimed at eliciting information about *Schistocerca* immunity. It aims to characterize these cellular elements morphologically, using Giemsa-stained smears under light microscopy. It also aims to quantify, through differential and total counts of haemocytes and a description of their interactions with the invading bacteria. A better understanding of these mechanisms may lead to ways to manipulate them for the benefit of humans. Indeed, the phenomena that will be discussed in this regard are fundamental to the biological control of insect pests.

**MATERIALS AND METHODS**

1. **Experimental design**

The desert locust, *S. gregaria* were reared according to methods of Hassanein (1965). The insects were divided into groups and maintained at 30±2°C, 60-0% RH under a photoperiod of 16:8 (Light: Dark) and were fed on an artificial diet as a dry mixture of: bran, 2; dried whole milk, 2; wheat, 2; dried brewer’s yeast, 1 (parts by volume); plus a small quantity of fresh clover leaves. Adults (20-30 individuals, 2-4 days after the final molt) were used and injected with 10 ul of *B. thuringiensis israelensis* (*Bti*) adjusted as a concentration of 3×10⁴ CFU/ml into the hemocoel. A similar number of adults were injected with equivalent volumes of saline solution (0.5% NaCl) and were considered as controls. Bacteria-injected locusts after 6, 12, 24 and 48 h along with saline-injected controls were weighed individually, and then submerged in hot water bath at 60°C for 2-5 min then, the locusts were allowed to dry on paper towel. The heat-killed insects were amputated at the hind coxa and gentle pressure was applied to the thorax until a drop of haemolymph appeared at the point of amputation. Haemolymph from two individual insects was never combined.

2. **Description differential count of haemocytes:**

According to Arnold and Hinks (1979), drops of haemolymph were smeared onto clean glass slides. The smears were allowed to dry for 1-2 min, and then fixed for 2 min with drops of absolute methyl alcohol. Fixed cells were then stained with Giemsa’s solution (diluted 1:20 in distilled water) for 20 min. The slides were washed several times with distilled water. The stained smears were air-dried, mounted in Canada balsam and examined microscopically. Cell-shape, diameter, nuclear-cytoplasmic ratio, cytoplasmic inclusions, vacuoles and other characteristics were determined and used for the classification of haemocytes using the classification scheme of Brehélin and Zachary (1986). Various types of haemocytes were differentially counted by examining ~100 cells per slide. 10 slides, prepared from 10 locusts, were used for each count. The percentages of haemocyte types was calculated as follows:

\[
% = \left( \frac{\text{No. of each haemocyte}}{\text{Total No. of haemocytes examined}} \right) \times 100
\]
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3. Determination of total haemocyte count:

The oozed haemolymph was collected directly into Thoma-white blood cell diluting pipette to the mark 0.5. Diluting solution (NaCl- 4.65 g, KCl- 0.15 g, CaCl₂- 0.11 g, crystal violet- 0.05 g and acetic acid- 1.25 ml/liter distilled water) was taken up to the mark 11 on the pipette (dilution is 20). The mixture was dispensed to both chambers of the counting slide (the chamber depth is 1.0 mm). Duplicate counts were made with each sample to check counting error. The count was repeated for at least 10 times. If either the cells clumped or uneven distribution (a difference of more than 20 cells between any two squares) of haemocytes occurred in the chamber, the preparation was discarded. The number of haemocytes per cubic millimeter was calculated according to the formula of Jones (1962):

\[ THC = \frac{\text{No. of haemocytes counted} \times \text{dilution} \times \text{chamber depth}}{\text{No. of 1 mm squares counted}} \]

4. Estimation of haemocyte defense reactions:

4.1. Phagocytosis:

The Giemsa stained blood smears were prepared after 6, 12, 24, and, 48 h post bacteria injection. For each sample, 100 cells were examined by light microscopy. In each preparation the percentage of haemocytes that had phagocytosed bacteria was calculated according to the method described by Rowley and Ratcliffe (1980).

4.2. Nodule formation:

The method described by Gunnarsson and Lackie (1985) was used to determine the numbers of nodules. Injected insects, after 6, 12, 24 and 48 h were dissected, examined under a binocular dissecting microscope and the number of nodules per insect was counted. The general appearance and size (small < 40 μm, medium 40–100 μm, large > 100 μm) of the nodules were also noted.

5. Statistical analysis:

Data were expressed as mean ± standard error (SE). Levels of significance for differences of means were determined using Student’s *t*-test for paired samples. The level of significance for each experiment was set at P < 0.05 or P<0.01 and was corrected using Bonferroni’s method for multiple comparisons.

RESULTS

1. Haemocyte morphology:

On the basis of morphological characteristics by using light microscopy, four main haemocyte types have been found in *S. gregaria* adult haemolymph (Fig. 1). These are: prohaemocytes (PRs), plasmacytocytes (PLs), granular cells (GRs) and coagulocytes (COs). The PRs were small round cells with large nuclei (Fig. 1, A). PLs were abundant, ovoid (Fig. 1. B), but commonly spindle (Fig. 1, B) in shape and the nucleus occupied about half of the cell volume. COs were round or ovoid in shape with small and acentric nuclei (Fig. 1, C). GRs were also abundant, often round in shape and the nucleus was spherical and centric in position (Fig. 1, D1, D2).

Due to *Bti*-injection into the hemocoel of adult locusts, certain pathological consequences were observed in the haemocytes. The PLs, GRs and COs showed great variation in the cell volume, vacuolization in the cytoplasm, distortion of the cell membrane and nucleus (Fig. 2). Vacuolization in the cytoplasm (Fig. 2, A1), distortion of the cell membrane and variation in the cell volume (Fig. 2, A2), some nuclei appeared lost part of their chromatin materials and showed vacuoles in their nucleoplasia (Fig. 2, B) or even binucleated (Fig. 2, C), some cells became lysed (Fig. 2, D1) and the cytoplasm may be eroded, ruptured, and the cytoplasmic contents were extruded (Fig. 2, D2).
Fig. 1: Normal haemocytes of S. gregaria, examined by light microscopy.  
(A): Prohaemocyte; (B1,2): Plasmatocytes; (C): Coagulocyte; (D1,2): Granulocyte.  
Bar=10µm

Fig. 2: Infected haemocytes of S. gregaria, examined by light microscopy.  
(A1,2): distortion of the cell membrane, (B): nuclei loss part of their chromatin material, (C): bilobed nuclei, (D1,2): vacuolization in the cytoplasm. Bar= 10µm

2. Differential haemocyte counts (DHCs):

Percentages of different haemocyte types of S. gregaria were estimated and tabulated in Table (1). The percentages of PRs, PLs, GRs and COs in un-injected locusts were 16.30 ± 1.51, 52.30 ± 2.66, 13.2 ± 0.58 and 18.2 ± 0.90, respectively. In Bti-injected locusts, the percentage of PRs was significantly increased (P<0.05) at 6, 12 and 24 h post-injection, then returned to the original level at 48 h. The percentage of PLs was increased significantly at all periods post-injection. The GRs and COs were decreased significantly (P<0.05) at all times post-injection, compared with water injected insects.
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Table 1: Differential haemocyte counts (%) of *S. gregaria* adults determined at different time intervals post-injection with *Bti*.

| Hours post-treatment | Prohaemocytes (PRs) | Plasmatocytes (PLs) | Granular cells (GRs) | Coagulocytes (COs) |
|----------------------|---------------------|---------------------|---------------------|---------------------|
|                      | Control             | Treated             | Control             | Treated             | Control             | Treated             |
| 6                    | 13.9 ±0.55          | 17.3 ±2.5*          | 50.60 ±1.69         | 57.6 ±2.1*          | 15.0 ±0.6           | 12.1 ±0.93*         | 20.5 ±1.29          | 14.0 ±1.4*          |
| 12                   | 14.2 ±1.16          | 17.9 ±1.76*         | 51.8 ±1.46          | 61.0 ±0.5*          | 13.2 ±0.97          | 11.0 ±0.51*         | 15.4 ±1.21          | 10.1 ±0.4*          |
| 24                   | 15.4 ±1.11          | 16.0 ±0.54*         | 53.4 ±1.08          | 58.46 ±3.71*        | 15.0 ±0.7           | 13.54 ±1.30*        | 16.20 ±0.9          | 12.0 ±0.51*         |
| 48                   | 16.8 ±0.86          | 16.66 ±0.32         | 53.4 ±1.9           | 58.0 ±5.8*          | 13.8 ±0.8           | 11.78 ±3.8*         | 16.0 ±0.7           | 11.56 ±0.8*         |
| Untreated            | 16.30 ± 1.51        | 52.30 ± 2.66        | 13.2 ± 0.58         | 18.2 ± 0.90         |

n = 10 replicates.

*Significant differences between the untreated and the controls when P≤ 0.01 and between the controls and the bacterium-injected insects when P≤ 0.025 based on Bonferoni correction.

3. Total haemocyte counts (THCs):

The mean THC value of un-injected locusts was 9529.5±597.35 cells/mm$^3$. The THCs of *Bti*-injected locusts showed significant decrease (P<0.01) at all times post-injection compared with controls (Fig. 3).

![Fig. 3: Total haemocyte counts (x 10$^3$ cells/mm$^3$) of *S. gregaria* adults determined at different time intervals post-injection with *Bti*.](image)

4. Phagocytic activity:

Results revealed that, phagocytosis was observed during all time intervals examined, the peak was at 12 h post-injection. PLs were the essential phagocytic cells (Fig. 4), while the other haemocyte types didn't participate in the phagocytosis reaction. During the course of phagocytosis, phagocytic cells (PLs) showed morphological characteristics which were different from their typical morphology. Phagocytosis was shown to be composed of a sequence of events: contact and attachment (Fig. 4.1), activation of pseudopodia (Figs. 4.2), cell-cell aggregation (Figs. 4.3), engulfment (Figs. 4.4 & 5), and then vacuole formation (Figs. 4.6).
Fig. 4: Phagocytic events caused by PLs towards bacteria injected.
(1): Attachment; (2): Activation of pseudopodia; (3): Cell-cell aggregation; (4,5): Engulfment of bacilli; (6): vacuole formation. Bar = 10 µm.

5. Nodule formation:
Injection of S. gregaria with saline (control) or bacteria resulted in the formation of melanized nodules which were found attached to a variety of tissues including the fat body, the alimentary canal and the body wall. Identification of nodules was normally relatively easy due to their size and their melanized appearance. Nodulation of Bti cells showed that they began at 6 h after injection and this response increased gradually with a peak at 24 h. There was a significant increase (P<0.01) in the number of nodules above controls in all observation periods post-injection. The small and medium-sized nodules were superior at these times (Fig. 5).

Fig. 5: Dissected adult S. gregaria showed the intensive formation of different sized nodules by haemocytes 24 h post-Bti-injection. (A): normal (uninjected) insect; (B): saline-injected insect; and (C) Bti-injected insect; (S): small nodules; (M): medium nodules; (L): large nodules. Bar = 10 mm.

DISCUSSION
Insect haemocytes are key components of immune system and they defend against foreign bodies via innate immune responses. Haemocyte stability is influenced by their immediate environment. The use of a buffer system that eliminates any aberrant effects is a prerequisite for studies of haemocytes (Miranpuri, et al., 1991).
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Before describing the alterations of the blood picture during the course of Bti-infection into S. gregaria, it is necessary to describe the types of haemocytes. The morphological description of the identified haemocytes (PRs, PLs, GRs and COs) in the present study are much related to the description of Gupta (1985), Barakat et al. (2002) and Mo-men et al. (2010) in S. gregaria, and of Price and Ratcliffe (1974), Brehêlin et al. (1978) Brehêlin and Zachary (1983), Sharma et al. (2003) and Barakat et al. (2016; 2017) in other insects.

Results also revealed that there are great differences in percentages and numbers of different haemocyte types after bacterial injection. GRs and COs decreased in numbers and PRs, PLs increased. This increase may be due to liberation of sedentary haemocytes attached to the organs. Similar explanation was given by Shapiro (1968) on Galleria mellonella larvae. Mori (1979) returned this increase to the transformation of PRs (stem cells) into PLs which are needed in cellular defense reactions (phagocytosis, nodule formation).

On the other hand, the decrease in GRs may be attributed to their involvement in cellular defense reactions (phagocytosis, nodule formation). These findings are supported by the observations of Lia-Fook (1968) on Rodnius prollexus after wounding, Horohov and Dunn (1982) on Manduca sexta larvae following injection of bacteria, and Ayaad et al. (2001) on Parasarcophaga surcoupfi larvae infected with the nematode, Heterohabditis bacteriophora. Another explanation states that toxins secreted by injected bacteria cause lysis of haemocytes or induce programmed cell death (Sussman, 1952).

The decrease of COs. at all experimental periods post-injection may be due to the great demand of COs into the site of injection to form a temporary closure or may be rupture to initiate the coagulation in nodule formation (Cherbas, 1973). There is an evidence indicating that the COs type may be the first cells to contact the foreign surface and may then lyse releasing granule contents (Lackie et al., 1985). They contain high level of lysozyme and phenoloxidase that are of potential importance in melanization (Ashida and Dohke, 1980; Jiang et al., 1997; Lavine et al., 2005). Consequently, changes of COs proportions following bacterial injection may be attributed to the beginning of humoral activity or the active transformation of granulocytes into COs as proposed by Gupta (1985).

Various abnormal conditions may profoundly affect the THCs such as wounding, extreme low or high temperature and infection (Shapiro, 1979). The observed decrease in THCs after bacterial-injection may be due to the involvement of haemocytes in phagocytosis and nodule formation which always accompanied with the death of defensive haemocytes (Gunnarsson, 1988), or may be due to the action of the released toxins and its lytic effect on the haemocoel and degradation of haemocytes (Faye, 1978 and Gregore and Bowen, 1998). Similar observations were also reported by Hoffmann et al. (1974) on Locustamigratoria, Abu El-Magd et al. (1994) on Spodoptera littoralis and Barakat et al. (2016; 2017) on Apis mellifera larvae after injection with bacteria. Contrarily, these results disagree with Abu El-Magd (1992) on L. migratoria may be due to species and technique differences.

Generally, the THC is positively correlated with the rate of phagocytosis and nodule formation. The total number of haemocytes is likely to reflect the capability of immune system to deal with pathogens by phagocytosis. These findings were in agreement with the results of Hoffmann et al. (1974), Cheung et al.(1978), Abu El-Magd (1992), Gillespie et al.(1997) and Meshrif and Barakat (2002) on S. gregaria.

There is a considerable disagreement about the cell type mainly responsible for phagocytosis in insects. The present results indicated that the PLs possess the strongest endocytotic capabilities. This was supported by the concept of Ratcliffe and Rowley (1979) who, reported that the PLs are the most important phagocytic cells. These results agreed with other authors (Sharma et al., 1986; Mitro, 1994; Tojo et al., 2000, Barakat et al., 2002; 2016 and 2017) who reported that the predominant cells involved in phagocytosis are PLs, followed by GRs. Whereas, Brehêlin and Zachary (1986) believed that the GRs may be the
main effective cells. These differences probably result from the large variations occurring in haemocyte types even between closely related species.

Nodule formation reaction is a type of cellular defense reactions, which is a multi-cellular haemocytic aggregation. This may entrap a large number of bacteria in an extracellular material when phagocytosis is inadequate to face those (Franssens et al., 2005). Nodule formation was observed within few hours post-injection with Bti as well as saline solution into the adults of S. gregaria, but the reaction was weaker comparable to bacteria. These results are in accordance with the results of Gunnarsson and Lackie (1985), Tackle (1988) and Abu El-Magd (1992) on S. gregaria and Rahmet-Alla and Rowley (1989) on Leucophaea maderae; Barakat et al. (2016; 2017) on A. mellifera.

The small number of nodules seen in saline-injected S. gregaria probably resulted from an incomplete stimulation of the cellular reaction due to the absence of any antigenic materials, which would provoke cell breakdown and encapsulation. Similar explanation was reported by Ratcliffe and Gagen (1977) on G. mellonella larvae.

Bacteria entrapped in nodules come to one of the three ends: (1) destroyed by digestion (Bucher, 1959), (2) endure the reaction and persist alive throughout metamorphosis (Cameron, 1934), (3) pathogenic to the blood cells, probably prevent the full development of nodules or multiply in spite the reaction took place (Bucher, 1959; Walters and Ratcliffe, 1981 and 1983).

Conclusion

In conclusion, the results of this study indicated that, despite the strong immunity demonstrated by the adult S. gregaria, Bti can be used as an environmentally friendly alternative to synthetic chemical insecticides, and promising against orthopteran pests through its upsetting interference with insect haemocytes.

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تحذير الخلايا الدموية في دم في الجراد الصحراوي

Schistocerca gregaria

Bacillus thuringiensis israelensis

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المستخلص

هدفت هذه الدراسة إلى فهم الدور الذي تلعبه الخلايا الدموية داخل التجويف الدموي. تم التعرف بواسطة Bacillus thuringiensis israelensis في الاستجابة للحقن بـ gregaria الفحص المبكر الضوئي على أربعة أنواع من الخلايا الدموية وهي: الخلايا الأولية، والخلايا الثنائية، والخلايا الحبيبية وخلايا التخثر. لوحظت استجابات مختلفة في مجموعات خلايا الدم على فترات زمنية مختلفة (6، 12 و 24 و 48 ساعة).

بعد الحقن بجرعة جرعة من البكتيريا: (1) تأثيرات المرضية في خلايا الدم بما في ذلك التباين في حجم الخلية والتغذية في السوبلزام، وتشوه غشاء الخلية والتضخم في النواة: (2) التغييرات المختلفة في تعداد خلايا الدم التناوبية، والأهم هو انخفاض في عدد الخلايا الحبيبية وخلايا التخثر المصحوبة بزيادة في الخلايا الأولية والخلايا الثنائية، (3) انخفاض ملحوظ في إجمالي عدد خلايا الدم في معظم الفترات الزمنية، (4) ظهرت عملية البلعامة خلال جميع فترات ما بعد الحقن مع ذروة في 6 ساعات، وكانت الخلايا الثنائية هي الخلايا البلعامية الأساسية. (5) لوحظت زيادة كبيرة في تعداد العقيدات في جميع الفواصل الزمنية بعد الحقن فوق مجموعات التحكم، وكانت الذروة عند 12 ساعة بعد الحقن.