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Haemocytes and protein changes in *Schistocerca gregaria* after infection with nucleopolyhedrovirus

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Innate immune response in insects is essential to resist the infections of pathogens. The present study investigates the antiviral immune response against intracellular baculovirus infection in the desert locust, *Schistocerca gregaria*. The desert locust was injected with nuclear polyhedral virus isolated from *Spodoptera littoralis* larvae collected from cotton and maize field in Giza governorates (*Sl*NPV). Haemolymph was perfused 24 h post injection. The hemolymph was taken from control and treated locusts to separate haemocytes from the supernatant plasma to characterize the reaction of the adult *S. gregaria* to the viral infection. Transmission electron microscope revealed that, infected granular plasmtocyte and prohaemocytes possessed phagocytic vacuoles. However, the haemocytes behavior against viral infections does not seem to be consistent. Conversely, the biochemical results demonstrated that the total number of protein bands of *S. gregaria* increased due to treatment with *Sl*NPV 24 h post injection. Analysis of haemolymph protein pattern of control (untreated adult) of *S. gregaria* detected 11 protein bands and 14 protein bands in treated haemolymph pattern. Three common protein bands between untreated and treated haemolymph protein patterns (15.7, 22 and 25 kDa), one protein band (kDa) was increased in its amount percentage due to treatment course 1.4 fold times, and two common protein bands with molecular weight (22 and 25 kDa) were decreased in amount percentage due to treatment course from by 2.4 and 1.04 folds, respectively. The present study provides the basis for supplementary studies of molecular mechanisms underlying baculovirus strategies that have evolved for the suppression or evasion of the antiviral immunity in infected cells.

Key words: Immunity, nuclear polyhedrosis virus, grasshopper.

INTRODUCTION

Insects get infected by a wide array of viruses some of which may be pathogenic to insects or transmitted to vertebrates by insects. The medical and economic importance of virus induced diseases highlights the need to understand the interaction between the pathogenic virus and the insect immune system. The study of insect defense mechanisms is of great practical importance in two contexts. First, the need to assess the

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ways by which insects might avoid destruction by biological control agents. Second, defense mechanisms play a critical but underestimated role in the complex relationships that exist between arthropod pests and its pathogens. A better understanding of these mechanisms may lead to methods for manipulating them to human advantage.

Nuclear polyhedrosis virus is a circular double stranded DNA virus belonging to the family baculoviridae. Baculoviruses, members of the family baculoviridae, include a diverse group of large enveloped DNA viruses that are specifically pathogenic to arthropods, including insects. Baculoviruses possess a large circular double-stranded DNA of approximately 80-180 kbp in size, which encoded 100-200 predicted proteins (Herniou et al., 2012).

Individual baculoviruses act together with different insect cell lines after entry into cells causing numerous types of abortive (non-productive) as well as productive infections (Castro et al., 1997; Morris and Miller, 1993; Shirata et al., 1999, 2004, 2010). The route of infection of baculovirus consists of several successive steps, starting from entry of the viruses into cells, morphogenesis and discharge of budded virus (BV), very late gene expression, until ending with the formation of occlusion bodies (OBs) which contain blocked virus (OV) which is a different phenotype from that of BV (Morris and Miller, 1993; Shirata et al., 1999).

Once pathogens break a surface barrier and gain the entry into insects, the second line of innate immunity at the cellular and molecular levels is activated in the haemocoel. When the immune response be successful, the pathogens were eliminated from the infected insects and prevented their propagation and spread. In contrast, pathogens have developed a variety of mechanisms to suppress or evade the activated innate immunity in the haemocoel. Since insects lack adaptive immunity as in vertebrates, innate immunity is essential for insects to survive infections of pathogens, including viruses. The cellular immune reaction of insects involves the recognition and subsequent phagocytosis or encapsulation of foreign bodies by the haemocytes (Salt, 1970). In case of cells infected with baculovirus, shutdown of global protein synthesis and apoptosis act as two major intracellular innate antiviral mechanisms (Clarke and Clem, 2003a; Clem, 2005; Thiem, 2009). Thus, baculoviruses and insects provide a good model system for studying the co- evolution of animals and their pathogens.

Desert locusts, *Schistocerca gregaria* (Forskal), are the most dangerous of locust species. Under favorable environmental conditions, a few solitary individuals can dramatically multiply, form large swarms able to migrate great distances, and therefore, threaten agriculture (Cressman, 1999). Most studies on insect innate immunity have focused on bacterial and fungal infections (Ferrandon et al., 2007; Kanost et al., 2004; Lemaitre and Hoffmann, 2007).

Nuclear polyhedrosis viruses are considered to be limited in their host range to one species or genus of insects. The narrow host range that characterizes the baculoviruses is regarded as a great advantage over chemical pesticides (Bensimon et al., 1987). They succeeded to transmit *Spodoptera littoralis* nucleopolyhedrovirus to infect *S. gregaria* with dark cheeks disease.

The challenge in the future lies in the implementation of control strategies that protect food security with minimal environmental costs. Therefore, in the current investigation, we address the antiviral immunity in baculovirus-infected desert locust, *S. gregaria*, and determine the humoral antiviral immune response to provide a basis for further molecular mechanisms underlying baculovirus strategies that have evolved for the suppression or evasion of the antiviral immunity in infected cells.

MATERIALS AND METHODS

Colonization of *S. gregaria*

The desert locust *S. gregaria*, was obtained from the Locust and Grasshopper Research Department, Plant Protection Research Institute, Agricultural Research Center, Egypt. Locusts were reared in wooden cages at 32 ± 2°C, 50- 60% relative humidity RH) and 16 h day light in the facilities of Entomology Department, Ain Shams University. The locusts were fed on daily fresh clover plant, *Trifolium repens* on daily bases. Packed moist sterilized sand in suitable glass containers about 7 cm in diameter and 10 cm deep were used for egg-laying.

Virus preparation, injection and sampling haemolymph

Nuclear polyhedral virus isolate was obtained from *S. littoralis* larvae collected from cotton and maize field in Giza governorates. The larvae were fed on castor bean leaf (*Ricinus communis*), after the symptoms of the viral infection appeared in the infected larvae were placed into 1.5 ml 0.1% Sodium dodecyl sulphate (SDS) to homogenize; the homogenate was centrifuged for 100 *g* for 5-10 s and the supernatant was removed into clean tube to centrifuge at 2500 *g* for 5 min. To pellet the virus, the supernatant was discarded, and resuspended in 1.5 ml of distilled water and centrifuged at 2500 – 5000 *g* for 5 min. Finally, the supernatant was discarded and the pellet was suspended into a small volume of distilled water. The concentration of the virus was measured by counting under hemocytometer and stored at -20°C until use.

Stimulation to insect immune response

Injections and sampling of haemolymph

Trials were prepared by injecting each adult grasshopper with 10 µl of 10^7 PIB/ml of SlNPV final concentration using a 10 µl of Hamilton micro-syringe (Miranpuri and Khachatourians, 1993). Locusts were injected with 10 µl viral suspension into the insect's haemocoel by inserting the needle in the last coxal corium. Control insects were injected only with equivalent volume of sterile distilled

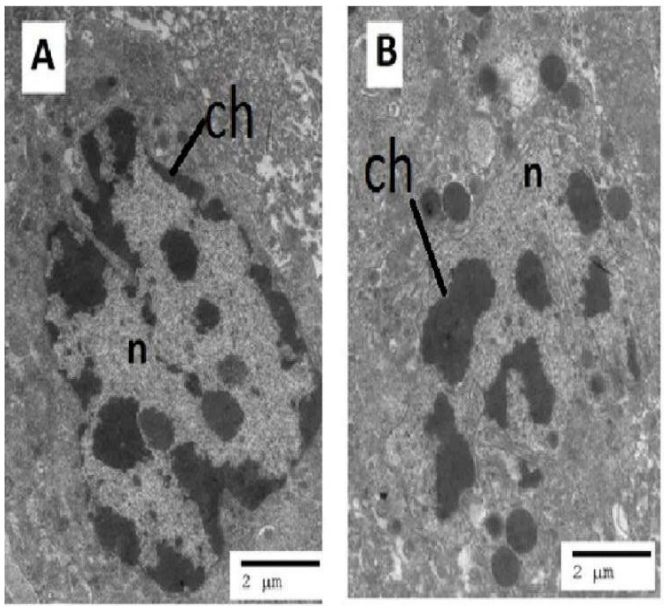

Figure 1 (a, b) . Normal haemocytes of adult *S. gregaria*, showing nucleus (n) with chromatin (ch).

water. Samples of haemolymph were taken from locusts without cooling or anesthesia at 24 h post-injection. The insects were amputated at the hind coax with fine scissors; gentile pressure was applied on the thorax until the haemolymph appeared and obtained with fine–tipped calibrated glass capillary, according to Hoffmann (1980). The perfused haemolymph was centrifuged at 12000 rpm for 15 min and the plasma stored at -20°C to separate haemocytes from the supernatant plasma for using in cellular and molecular study.

Transmission electron microscopy (TEM)

To characterize the cellular antiviral immune response in infected adult *S. gregaria*, intra- thoracic haemolymph was obtained from 5-10 locusts via micropipette.

The haemocytes pellets were separated from plasma. Five drops of 3% gluteraldehyde was added to the haemocytes for 2 h, during which a precipitate was formed. The supernatant was eliminated from the samples, which were washed with 5 drops of PBS-sucrose three times for 10 min. Samples were further fixed with 2% osmium tetroxide for 3h, after which they were washed three times with PBS-sucrose. Samples were then gradually dehydrated with increasing concentrations of alcohol: 10 to 90% for 10 min. Finally, for 30 min in each sample of three changes of 100% alcohol. After this, 100% alcohol and propylene oxide were added in proportions of 2:1, 1:1, and 1:2, finalized with 100% propylene oxide; each stage of these changes in proportion lasted 30 min. Epon 812 propylene was added in proportions of 2:1, 1:1, and 1:2, with changes in proportions every 30 min. With 100% Epon 812, the samples were left open for 4 h to evaporate the solvent. The samples were then polymerized at 60ºC for 24 h. From each polymerized block, semi-fine sections were made using Reichert Om U3 ultra-microtome and transferred to pioloform-treated 200-mesh grids. The sections were examined under SEO PEM-100TEM. Cell types were photographed with transmission electron microscope (TEM) at the facility of Military Medical Academy, Cairo, Egypt.

Protein electrophoresis

To determine the humoral antiviral immune response, the hemolymph of the normal and infected adult locusts was perfused. SDS PAGE gel electrophoresis was used by adding droplets to a tube containing 20 ml of gel buffer [2% sodium dodycel sulphate (SDS), 60 mM Tris Cl pH 6.8, 10% glycerol and 50% protease inhibitor cocktail (one tablet/50 ml, Boehringer Mannheim, Indianapolis, IN)]. The perfused hemolymph was resolved in SDS polyacrylamide gel electrophoresis (SDS-PAGE). SDS PAGE was performed at 200 V, for approximately 3 h. Gel was stained using Brilliant Blue R-250 and followed by distaining process. Images of the gel were captured and analyzed using specialized software; Gel-Pro Analyzer (Media Cybernetics, NT, USA).

RESULTS

Transmission electron microscopy (TEM)

The reaction of the adult *S. gregaria* to the viral infection was characterized by injecting insects with 10 µl of 10^7 PIB/ml of Giza *S*lNPV, and perfuses the hemolymph 24 h later. Ultrathin sections cut through normal and infected haemocytes adult locust revealed a significant changes in the blood cells compared to the normal (Figures 1 and 2). In the normal haemocytes, the nucleus occupies a large size of the whole cell with definite patches of chromatins (Figures 1). However, after 24 h post injection, the haemocytes displayed a phagocytic activity with being able to internalize virus particles inside cytoplasmic vacuoles, particularly in virus-phagocytosing cells (Figure 2b, 2c). Also, some of the infected haemocytes showed an extended protrusion filled with virus particles inside the cytoplasmic vacuoles, seemingly (Figure 2).

Protein electrophoresis

The analysis of protein content extracted from hemolymph of the adult *S. gregaria* revealed an increase in the total number of protein bands in the infected hemolymph when compared with control (Figure 3). Analysis of hemolymph protein pattern of control detected 11 protein bands of size which ranged from 11.5 to 155.6 kDa. However, 14 protein bands were detected in the infected hemolymph that ranged from 13.4 to 96.4 kDa. The new protein bands of infected in comparison to control were 11 protein bands with different amount percentage as shown in Table 1. In addition, eight protein bands disappeared due to treatment with *S*lNPV (11.5, 45.4, 57, 62.5, 69, 82.9, 105.3, and 155.6 kDa) .We observed three common protein bands between control and treated specimens with 10^7 µl of Giza *S*lNPV, one of them (15.7 kDa) showed an increase in the amount percentage by 1.4 fold from 16.9 to 24 due to treatment course. The other two common protein bands with molecular weight (22 and 25 kDa) decreased in amount percentage (from

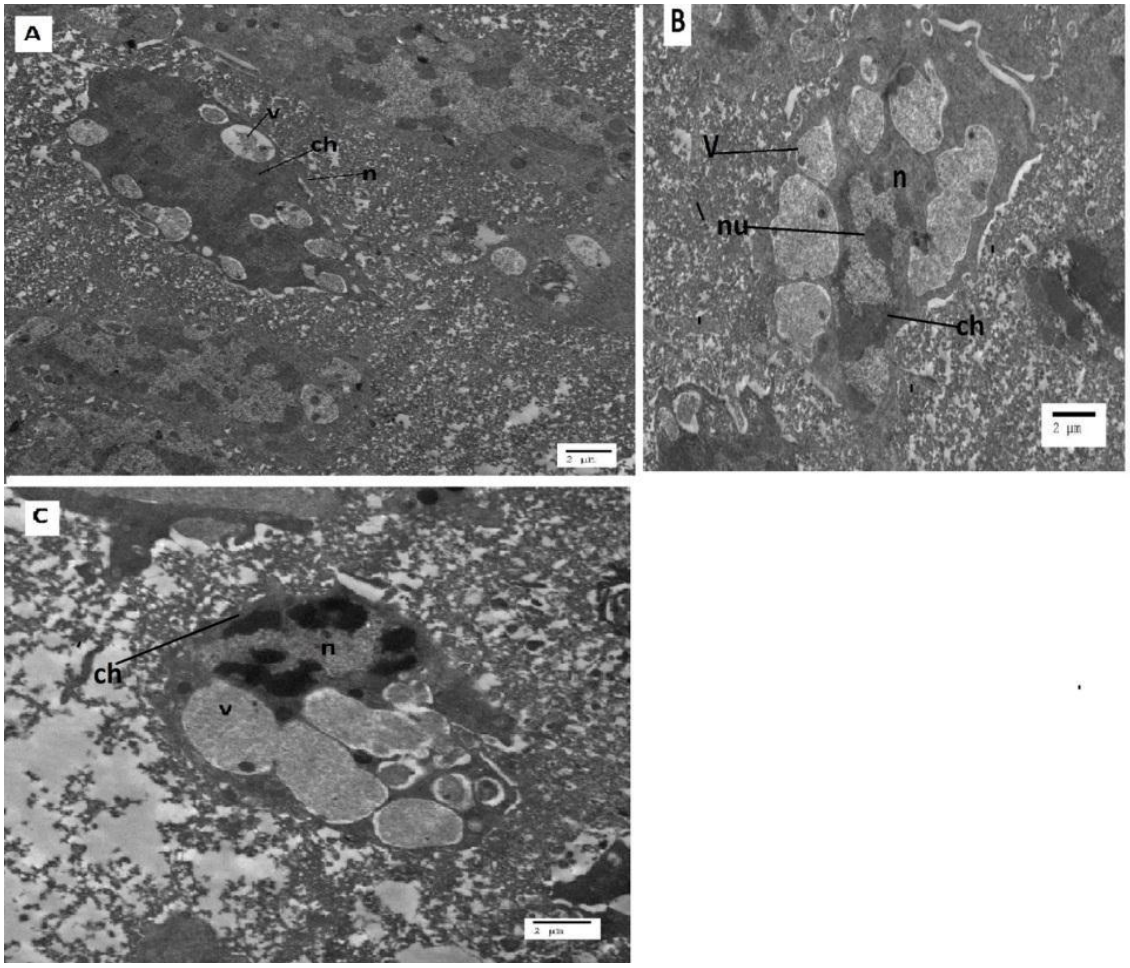

Figure 2 (A, B and C). Haemocytes showing cytoplasmic vacuoles (V). ch =chromatin; n= nucleus; nu= nucleolus.

6.4 to 2.6 amount %, and from 4.9 to 4.7 amount %) by 2.4 and 1.04 folds, respectively.

DISCUSSION

Little is known about insect defense against virus infection; neither cell-mediated nor humoral immunity has been demonstrated against virus infection in insects (Narayanan, 1997).

Recently, advances have been made which demonstrate viral resistance and the ability of an insect to clear viral pathogens (Briese, 1986).

In our work, the haemocytes behavior against viral infections does not seem to be consistent. The hemocytes can have different roles, from actively spreading the baculoviruses, via avoiding being infected, to actively clearing the haemolymph from the viruses. The first description of an effective immune response in insects against viral infection was a study on *Helicoverpa zea* (Lepidoptera: Noctuidae) infected with NPV (Washburn et al., 1996). The haemocytes appeared in aggregation form around midgut-associated tracheae infected by baculovirus. The baculovirus-infected cells were then encapsulated by haemocytes and thereafter removed from the haemocoel.

At the molecular level, there are many studies on the change in the electrophoretic pattern of haemolymph proteins of larvae infected with the nuclear polyhedrosis virus (Watanabe, 1986). In some unsuccessful infections with baculovirus, it is clear that both cellular and viral protein synthesis are dramatically reduced and completely blocked in late infection (Ikeda et al., 2012). This explanation may give a reason for not appearance of which may be the reason of reduction of total protein however, the observed increase in total protein at 24 hpost infection could be due to the interference of the viral protein with the protein of the infected cells. Also, post- infection duration could be not enough shutdown global protein synthesis and may need increased time after the infection 48 or 72 h. The mechanism of shutdown of

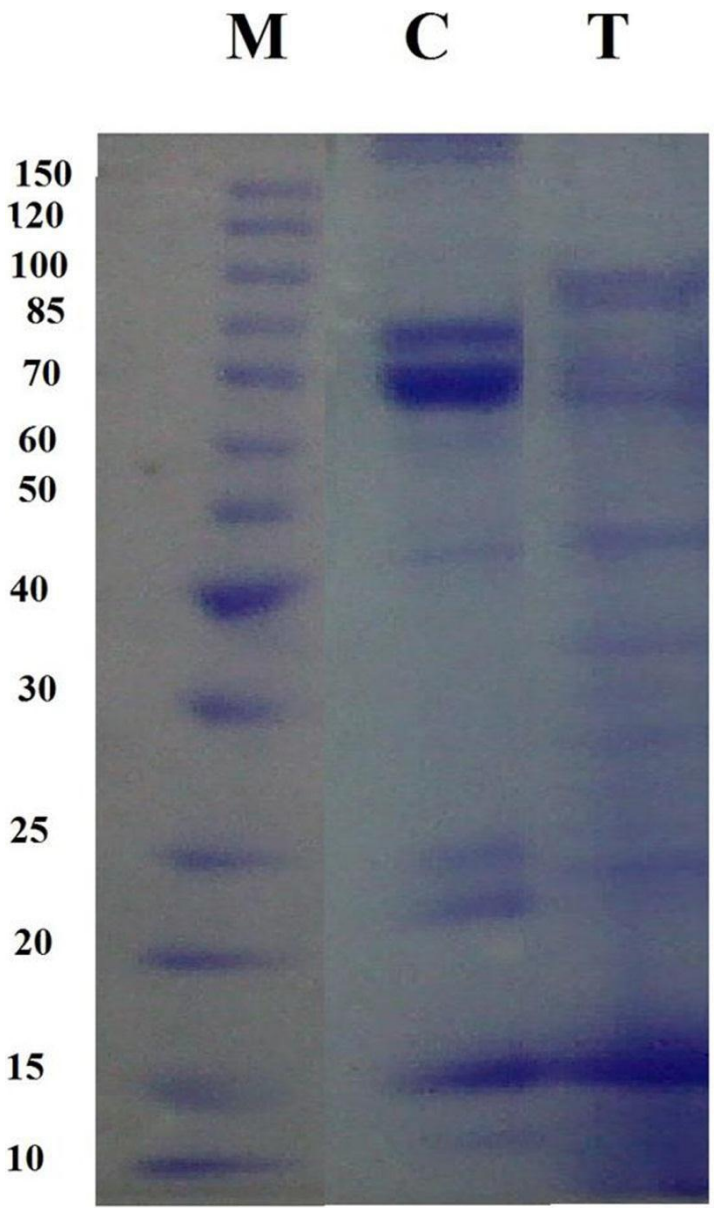

Figure 3. Photograph illustration of electrophoretic hemolymph protein patterns of infected and control adults of *S. gregaria.* M = protein marker (kDa). Lane C= control. Lanes T= Haemolymph protein patterns after infection with 10 µl of 10^7 of *Sl*NPV.

global protein synthesis is generally observed in the late infection with baculovirus and could be distinguished from the shutdown of the host cell protein synthesis (Schultz and Friesen, 2009; Schultz et al., 2009; Xue et al., 2012) as found in *Lymantria dispar* cell line infected with *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) in which the protein synthesis is globally shut down at late stages of infection.

Conversely, Sarma et al. (1994) found an observable decrease in protein content in NPV infected silkworm larvae, called “hypoproteinemia”. The authors attributed the reduction of hemolymph protein to the decrease of protein synthesis. Finally, the incompatibility between cellular response and protein analysis in our study could be related to that abortive infection which included in virus replication or from activation of innate antiviral responses, and leads to apoptosis and global protein synthesis shutdown in infected cells (Ikeda et al., 2013).

Finally, it could be concluded that this work is considered as a preliminary trial to predict cellular and molecular mechanisms of innate immune response of one of the most dangerous species insect, locusts, towards baculovirus infection. Better understanding of

Table 1. Monitoring, amount percentage and relative fragmentation of haemolymph protein fractions of infected and control adults of *S. gregaria* expressed as molecular weight.

Lane	Marker (mol.w.)	Lane C (mol.w.)	Lane T (mol.w.)	Lane C	Lane T
				Amount %	Amount %
1	150	155.6		0.8	
2	120				
3	100	105.3	96.4	0.68	3.1
4	85	82.9	91.3	9	1.9
5	70	69	73.4	22.4	3.7
6		62.5	67.1	2.3	4.1
7	60	57		0.9	
8					
9	50	45.4	46.9	2.5	3.1
10			43.3		0.8
11	40		35.5		2.3
12			31.5		2.3
13	30		29		4.1
14			25.9		3.1
15	25	25	25	4.9	4.7
16		22	22	6.4	2.65
17	20				
18					
19	15	15.7	15.7	16.9	24.1
20			13.4		6.5
21	10	11.5		8.4	
Sum	71.9			75.6	67.15
In lane	100			100	100

M= Protein marker (kDa). Lane C= control. Lanes T = treated hemolymph protein patterns with 10 µl of 10^7 of *Sl*NPV

humoral defense mechanism and molecular basis against viruses will provide necessary information for better management and modify host range properties of insect viruses in the future.

Conflict of interests

The authors did not declare any conflict of interest.

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