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Hemocyte Quantitative Changes in *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) Larvae Infected by AgMNPV

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ABSTRACT

The initial effects of the infection by AgMNPV in the total and differential counts of the hemocytes in Anticarsia gemmatalis (Lepidoptera: Noctuidae) *larvae were studied. The total number of the hemocytes did not decrease in infected larvae, as it occurred in non infected larvae. In infected larvae, the hemocyte types showed the following frequencies: plasmatocytes - 47.8%, esferulocytes - 25.9%, granulocytes - 15.8%, oenocytoids - 7.2%, prohemocytes - 2.8%, vermicytes - 0,5%. Only the percentage of the granulocytes was different among infected and non infected larvae, indicating that these cells responded quickly to the initial viral infection. These results showed the effective role of the hemocytes in the response of the A. gemmatalis to the infection by AgMNPV. The comprehension of the immunological mechanisms of this insect is an important tool to understand its biological control.*

Key words: Velvetbean caterpillar, viral infection, cellular defense, DHC, THC

INTRODUCTION

Insects use the immunity system to counteract infections by a diverse array of pathogens (Janeway and Medzhitov, 2002; Ratcliffe and Whitten, 2004; Nappi and Christensen, 2005). This system presents humoral and cellular defenses. The humoral defenses include direct and indirect pathogen recognition; extracellular and intracellular signal transduction citotoxic effect responses. The cellular defense is promoted through the interaction of different hemocytes (Beutler, 2004; Gupta et al., 2005). The hemocytes are cells that circulate in the hemolymph, providing quick and efficient response against pathogens that invade the hemocoel. The cellular defense refers to hemocyte-mediated immune responses, as phagocytosis, nodulation and encapsulation (Lavine and Strand, 2002).

The velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, is the major destructive lepidopteran pest of soybean due to the frequent foliar damages that it causes to the crop (Moscardi, 1993; Hoffmann-Campo et al., 2003). A program was established in Brazil since early 1980's to use an *A. gemmatalis* multicapsid nucleopolyhedrovirus (AgMNPV) as a microbial insecticide to control the insect instead of chemical insecticides. This virus is highly specific and able to escape efficiently from the defense mechanisms of the host larvae, consisting of an excellent agent of biological control (Moscardi, 1999).

The success of the immune response depends on the role of the hemocytes in this process (Russo et

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al., 2001). In the hemolymph of *A. gemmatalis* larvae, Andrade et al. (2003) identified six types of hemocytes: plasmatocytes (PL), granulocytes (GR), prohemocytes (PR), spherulocytes (SP), oenocytoids (OE) and vermicytes (VE). Although the biology of this pest has received a lot of attention, little is known about the immune system of *A. gemmatalis*, especially in infected insects. The alterations in the number of the hemocytes in response to the entomopathogens in hemolymph and consequently the role of these cells in defense mechanisms are so far not characterized. This work aims to study the role of the hemocytes in antiviral immunity through the quantitative alterations in these cells in *A. gemmatalis* larvae infected by the AgMNPV.

MATERIALS AND METHODS

The *A. gemmatalis* larvae, provided by Embrapa Soja, Londrina-PR, Brazil, were kept under laboratory-controlled conditions, according to Hoffmann-Campo et al. (1985). The insects were taken to the Laboratory of Histology/Universidade Estadual de Londrina, and kept in room temperature during the experiment.

Larvae of 4^{th} instar (7^{th} to 11^{th} day of development) were divided into two groups: a group consisting of infected larvae and a control group of non-infected larvae. Both groups were kept on plain artificial diet (Hoffmann-Campo et al., 1985) and to the infected larvae, were use modified diet, in which were incorporated 4,860 AgMNPV occlusion bodies/ml of diet. On each day, the larvae of the experimental group were infected through contact with this modified diet for 2 hours before the extraction of the hemolymph. The larvae were anesthetized and immobilized by low temperature (0ºC, 5 min) and cleaned in 70% alcohol. The hemolymph was collected through delicate puncture with a hematological needle in the abdominal region of the insect. The drops of hemolymph were collected with Pasteur pipettes, previously rinsed in Anticoagulant Solution for Insect (ASI) (Leonard et al., 1985), and kept for the Total Hemocytes Count (THC) to determine the total number of circulating hemocytes per μL and the Differential Hemocytes Count (DHC) to determine the percentage of each cell type. These quantifications were carried out in 15 larvae per day of larval development (7^{th} to 11^{th} day).

The THC was performed in not diluted hemolymph analyzed in a Neubauer modified chamber.

For the DHC, the hemolymph was diluted in ASI. We counted an allotment of 200 cells for each larva in phase-contrast microscope.

For statistical analysis, it was used the Kruskal-Wallis test complemented by Dunn ($p<0,05$) and the Mann-Whitney test, according to Zar (1999).

RESULTS AND DISCUSSION

In general, the total number of hemocytes in *A. gemmatalis* infected larvae (Table 1) did not show significant alterations during the days of development. However, in non-infected larvae the THC showed a significant decreasing in the number of hemocytes during the larval development (Andrade et al., 2003). Thus, it suggests that presence of virus in the insect hemolymph after two hours of the infection, caused alterations in the total hemocyte number, keeping high levels during the studied period.

According Rosenberger and Jones (1960), the presence of pathogens in the hemocoel of the insects can activate their defense system, causing alterations in the total number of hemocytes. However, there is no consensus in associating the defense response to the decrease or the increase in the total number of these cells. Some authors, as Ratcliffe et al. (1985), Morton et al. (1987) and Rivers et al. (2002), reported that the presence of pathogens would cause a decrease in the number of circulating hemocytes so as to make the infection successful. This decrease is related to the nodule formation and encapsulation around the invaders, as well as the degranulation of some cell types. In contrast to that, Richards and Edwards (1999) and Russo et al. (2001) reported that the presence of pathogens in the hemocoel stimulated the hemopoiesis, increasing the number of the cells in the hemolymph. Thus, the values in the total number of hemocytes in *A. gemmatalis* infected larvae can be the indicatives of activation of the defense mechanisms of the insect against the AgMNPV, promoting faster response to the viral infection.

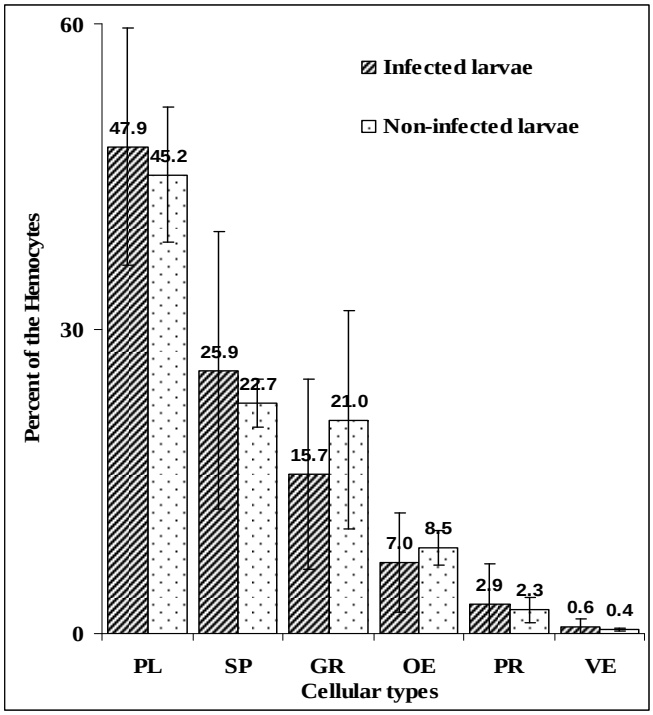

Figure 1 - Differential Hemocyte Count (DHC) of *A. gemmatalis* hemolymph, in infected larvae with AgMNPV and non-infected larvae. Values represent the means of the counted cells per development day (7^{th} to 11^{th}) ± standard deviation. PL: plasmatocytes; SP: spherulocytes; GR: granulocytes; OE: oenocytoids; PR: prohemocytes; VE: vermicytes.

Table 1 - Total Hemocyte Count (THC) of *Anticarsia gemmatalis* hemolymph, in infected larvae and non-infected larvae with AgMNPV.

Development days	Total Hemocytes Count (cells/µl of hemolymph)[1]	
	Infected larvae[2,3]	Non-infected larve[2,3]
7	16226.7 ± 4054.8 a A	18773.3 ± 5502.3 a A
8	18243.3± 3757.4 a A	20480.0 ± 6150.4 a A
9	15910.0 ± 4523.0 a A	13363.3 ± 2520.9 b A
10	15096.7 ± 3288.7 a A	7630.0 ± 5125.5 c B
11	13353.3 ± 4370.1 a A	9923.3 ± 3278.0 bc B

[1] Means ± standard deviation.
[2]Means followed by different minuscule letters in the columns indicate significant variation in the number of cells between the development days, determined by Kruskal Wallis test ($p<0.05$).
[3] Means followed by different capital letters in the lines indicate significant variation in the number of cells between the NIL and IL, determined by Mann Whitney test ($p<0.05$).

The values obtained for the DHC (Fig. 1) allowed the quantification of the six cellular types in *A. gemmatalis* infected larvae, with the relative frequency of these cells determined as PL>SP>GR>OE>PR>VE.

Both PL and GR are the main hemocytes involved in immunological defense mechanisms on insects. According to Gupta (1979), the PL activation and mobilization in nodules and capsules lead to a reduction in their percentage in relation to the other circulating cells. However, in *A. gemmatalis*, the infection with AgMNPV did not affect the

number of PL and its value was high (47.9%) and constant during the period of larval development, similar to the observed in control group.

In the infected larvae, the GR represented only 15.7% of the circulating cells, while in the non-infected larvae the frequency of this cell was 23% (Andrade et al., 2003). The difference of these values suggests that the initial viral infection is responsible for the alterations in the population of the GR. According Gliński and Kostro (2001), some viruses of a low virulence infecting the insects are killed by hemocytes whereas heavy infections or virulent strains can replicate and kill specific types of hemocytes involved in antiviral defensive reactions. This would be justified to the lower frequency of the GR in infected larvae. Our data is discordant to the one presented by Shapiro (1979) in ample literature review, in which was related the increase in GR percentile in response to viral diseases. However, this fact was not shown in *A. gemmatalis* infected larvae. The difference between our data and the ones presented by Shapiro (1979) are probably due to the long time of post-infection considered by this author.

For the SP, our results were similar to the 20% presented by Bombonato and Gregorio (1995), Falleiros et al. (2003), and Kurihara et al. (1992) in other insects. Different functions are attributed to the SP, as: tecidual remodeling, substance transport and synthesis (Ratcliffe et al. 1985), what could justify the absence of differences between non-infected and infected *A. gemmatalis* larvae.

The PR showed a low rate of occurrence in *A. gemmatalis*, in agreement with Chiang et al. (1988), Bombonato and Gregório (1995), Falleiros et al. (2003), and in disagreement with Silva et al. (2002), who reported the frequency of 38% for this cell type in *Anastrepha obliqua.* As the results obtained in infected larvae and non-infected larvae (Andrade et al., 2003) were similar, we can suggest that this cellular type is not directly involved in the immune responses to the AgMNPV.

In general, the low values presented by OE in infected and non-infected larvae were similar to the low frequency of these cells described in other insects, as *Galleria mellonella* (Shapiro, 1979), *Spodoptera litura* (Kurihara et al,. 1992), *D. saccharalis* (Barduco et al. 1988; Bombonato and Gregorio, 1995; Falleiros et al., 2003), *Lacanobia oleracea* (Richards and Edwards, 1999). Our results are similar to those observed by Silva et al. (2002), who reported the absence of quantitative alterations of OE in response to pathogens in the hemocoel of *A. obliqua* larvae and are discordant to Bauer et al. (1998), who reported the increase of this cellular type in larvae of *P. brassicae* parasited by *Cotesia glomerata*. However, this species is a parasitoid and we worked with a virus that might have led to the different results. It is known that the OE cells are involved in the production of prophenoloxidase (Lavine and Strand, 2002), an enzyme that actively participates in the mechanisms of defense in insects (Crossley, 1979). However, in the period of infection used, we could not detect alteration in the frequency of the OE in infected *A. gemmatalis* larvae compared to non-infected larvae, suggesting that these cells are not directly involved in the processes of immunological defense against the AgMNPV or that its participation occurs in the posterior stages of immunological responses.

The VE has been confirmed as a rare type of hemocyte. Our results were similar to the ones reported by Bombonato and Gregório (1995) and Falleiros et al. (2003), who found values lower than 5% for VE in the hemolymph of *D. saccharalis* larvae, and to ones observed by Kurihara et al. (1992) in *S. litura* larvae, where these cells represented less than 1% of the hemocytes. The average percentages of VE observed in infected larvae were similar in non-infected larvae suggesting that these cells do not have an important role in the defense mechanisms of *A. gemmatalis* larvae against the AgMNPV.

In this study, we can conclude through DCH analysis, that the GR are involved in a fast defense response to the presence of the virus in the hemolymph after two hours of infection.

This work can be used as in additional tool to help in the investigation of the role of the different cell types on the defense mechanisms of the *A. gemmatalis* larvae. They may also contribute to the improvement of the techniques used in the control of this pest, specially the biological control that occurs when the virus successfully deceives the defense system of the host.

RESUMO

Os efeitos iniciais da infecção por AgMNPV nas contagens total e diferencial dos hemócitos em *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) foram estudados. O número total de hemócitos não diminuiu nas larvas infectadas, como ocorreu nas

larvas não infectadas. Nas larvas infectadas, os tipos de hemócitos apresentaram as seguintes freqüências: plasmatócitos - 47,8%, esferulócitos - 25,9%, granulócitos - 15,8%, oenocitóides - 7,2%, prohemócitos - 2,8%, vermiformes - 0,5%. Apenas a porcentagem de granulócitos foi diferente entre larvas infectadas e não infectadas, indicando que estas células responderam rapidamente à infecção viral inicial. Estes resultados mostraram o papel efetivo que dos hemócitos na resposta de *A. gemmatalis* à infecção por AgMNPV. A compreensão dos mecanismos imunológicos deste inseto é uma ferramenta importante para compreender seu controle biológico.

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