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Original article

Haemocyte changes in honeybee (*Apis mellifera* L) artificially infected by *Pseudomonas aeruginosa*

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Summary — The changes in haemocytes of honeybees artificially infected with *Pseudomonas aeruginosa* ATCC 27014 *(P apiseptica)*, the bacterium known to cause septicaemia, were studied. It was found that the time of clinical manifestation did not significantly affect total and differential haemocyte counts. In the haemolymph of infected bees which showed no clinical symptoms the percentage of oval plasmatocytes was higher ($P \leq 0.05$), while the percentage of round plasmatocytes was lower ($P \leq 0.05$) 10 h after infection. The above changes were also observed in diseased bees independently of the time of clinical manifestation. There was no difference ($P > 0.05$) in the percentage of granulocytes and mitotically dividing haemocytes between diseased and control bees. The percentage of degenerated and/or dead haemocytes was higher in the haemolymph of diseased bees.

***Pseudomonas aeruginosa* / haemocyte count / experimental infection**

INTRODUCTION

Insect haemocytes are known to be capable of rapidly reducing the number of circulating foreign particles either by phagocytosis, nodule formation or encapsulation (Salt, 1970). These cellular responses have been shown to be accompanied by changes both in the number of circulating haemocytes and in the relative proportion of different haemocyte types in several insects (Wittig, 1966; Brehélin, 1982; Ratcliffe and Walters, 1983). In bees, changes in both total haemocyte count (THC) and differential haemocyte counts (DHC) were observed after injection of foreign particles (Van Steenkiste, 1988). Regarding bacterial infection, many papers in the literature deal with immunity acquired by bees following infection by non-pathogenic bacteria, while there are very few reports concerning pathogenic bacteria (Casteels *et al*, 1988, 1989, 1990; Van Steenkiste, 1988).

In this study, the bacterium *P apisepti-ca* was selected for infecting bees as it causes septicaemia (Burnside, 1928; Landerkin and Katznelson, 1959; Lan-gridge, 1963). The changes in THC, DHC, the percentage of degenerated and/or dead haemocytes and the percentage of the haemocytes in mitotic division after experimental infection by the bacterium *P apiseptica* were examined.

MATERIALS AND METHODS

Insects

Adult honeybees *(Apis mellifera macedonica)* < 24 h of age emerged in an incubator at 35 °C from healthy sealed brood. They were kept 50 per cage (Jacobs, 1977) for 9 d at 30 °C. The insects were treated, infected and their haemo-lymph withdrawn as described by Papadopou-lou-Karabela *et al* (1992). 4 900 bees were used for this experiment. Of these, 2 700 were infected, 1 200 were used as controls A and 1 000 as controls B.

Bacteria

P aeruginosa ATCC 27014 *(P apiseptica)* was obtained from the American Type Culture Col-lection. This strain was grown aerobically at 37 °C on blood–agar base (Starr *et al,* 1981). The number of viable cells was determined by dilution plating on blood–agar base.

For THC of 1 µl fresh haemolymph was placed in a micropipette and diluted 6-fold with Türk's dilution containing 2% EDTA (ethylene-diamino–tetraacetate) and 0.01% neutral red. Cells were counted in a Neubauer chamber haemocytometer under phase contrast optics. The above method is a modification of the method proposed by Van Steenkiste (1988).

For DHC measurement a smear was pre-pared and stained according to the method of May–Grünwald Giemsa as modified by Shapiro (1979) and Van Steenkiste (1988): 200 cells were counted on each smear. Degenerated and/ or dead (deg/dth) cells and cells in mitotic phase were also counted.

The THC, DHC and the percentage of deg/ dth haemocytes were estimated at varying times after infection from the 10th to 45th h. The bees in which symptoms appeared within a 5-h inter-val were classified in the same group.

Statistics

Differences between means were analysed by 1-way analysis of variance. In cases in which vari-ances were not equal, even after transformation to square root and arcsin, non-parametric tests were used. In our statistical analysis, we control the result at the significant level of $a = 0,05$.

RESULTS

A significant decrease in THC ($P \leq 0.05$) was observed in diseased bees 10–15 h after infection. In all other cases, no signifi-cant change in THC between diseased and control bees were found, or between controls A and B (table I).

According to the scheme of honeybee haemocyte classification as described by

Table I. Comparaison between diseased honey-bees, control A and B regarding total haemocyte count.

Time after infection (h)	*Diseased/ control A*	*Diseased/ control B*	*Control A/ control B*
10–15	*	–	–
15–20	–	–	–
20–25	–	–	–
25–30	–	–	–
30–35	–	–	–
35–40	–	-	–
40–45	–	-	–

* Statistically significant at level $a = 0.05$.

Van Steenkiste (1988), in our stained smears 6 types of haemocytes were classified based on their morphology: round plasmatocyte (PL_1); oval plasmatocyte (PL_3); fusiform plasmatocyte (PL_4); transitional form of plasmatocyte (PL_2); granulocyte (GR); coagulocyte (CO). PL_1, PL_2, PL_3 and PL_4 are plasmatocyte subtypes this division being based on their shape, chromatin structure, visibility of the plasma membrane, amount of cytoplasm and presence of granules within the cytoplasm. PL_2 haemocytes have characteristics between those of PL_1 and PL_3. Our study showed that the round and oval plasmatocytes comprised the greatest percentage of the total cell population in bee haemolymph (figs 1, 2).

A significant increase in the percentage of PL_3 and a decrease in the percentage of PL_1 was observed in infected bees which showed no clinical symptoms 10 h after infection. Similar observations were made in diseased bees throughout the entire observation period (figs 1, 2). The CO percentage rose ($P \leq 0.05$) in diseased bees 10–15 h after infection, while the PL_2 percentage decreased ($P \leq 0.05$), 25–45 h after infection. No significant change of GR and PL_4 percentages between diseased and control bees was found. The time of clinical manifestation had no effect on total and differential haemocyte counts.

The types of degenerated or dead haemocytes were PL_1, PL_3 and PL_2. An increase was observed in the percentages of deg/dth haemocytes in diseased bees which was significant in PL_1 and PL_3 15–45 h after infection and in PL_2 30–35 and 40–45 h after infection.

Mitosis was confined to PL_1 and PL_3. No significant differences in haemocytic mitosis between diseased and control bees was observed.

DISCUSSION

In this study it is interesting to note that there was a great variation in the number of circulating haemocytes and in the proportion of the different haemocyte types, although the bees were homogeneous as far as age and origin were concerned. Similar results have obtained by other researchers for bees and other insects (Wittig, 1966; Van Steenkiste, 1988).

The reduction in PL_1 percentage is probably the result of degeneration or death and also transformation into PL_3. The possibility of transformations among bee haemocytes was reported by Wille and Vecchi (1966). The increase in the

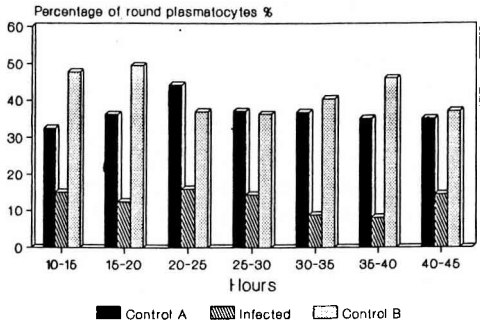

Fig 1. Relative amounts (%) of round plasmatocytes.

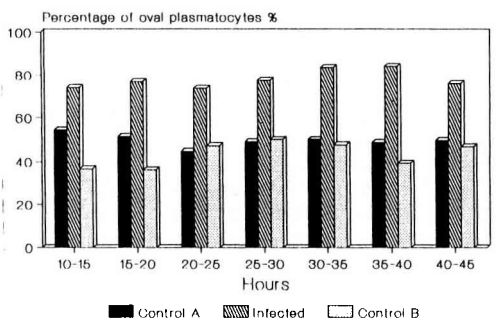

Fig 2. Relative amounts (%) of oval plasmatocytes.

percentage of deg/dth haemocytes in diseased bees could be due to the production of toxic factors. Haemocyte death has also been observed in the larvae of *Galleria mellonella* after the injection of toxic proteases produced by the bacterium *P aeruginosa* (Madziara-Borusiewicz and Lysenko, 1971). In bees haemocyte death is also reported after addition of the bacterium *Serratia marcescens* to haemocyte culture (Van Steenkiste, 1988).

The increase in the percentage of PL_3 may be due to the transformation of PL_1 to PL_3 and probably to the release of these haemocytes from the tissues. Sessile haemocytes might be mobilized in bees and other insects in response to injection of inert particles or bacteria (Brehélin, 1982; Ratcliffe and Walters, 1983; Van Steenkiste, 1988). This increase could not be attributed to increased cellular division since no significant increase in the percentage of mitotically dividing PL_3 was manifested. Also the presence of haemopoetic organs, which may release haemocytes into the circulation, has not been found in honeybees.

In contrast to these results, Wille and Vecchi (1974) did not report any change in the proportion of the different haemocyte types in bees infected by bacteria or protozoa, and collected at random with or without clinical symptoms. However, similar changes concerning the percentages of PL_3 and PL_1 were observed in bees infected by *Nosema apis*. The percentage of PL_2 in diseased bees was reduced in a similar manner to PL_1.

No significant changes in the percentage of GR in bees diseased by *P apiseptica* were found. According to Van Steenkiste (1988) the number of GR significantly increased after injection of inert substances which are easily phagocytosed by these cells, while phagocytosis of viable bacteria is rarely observed.

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Résumé — Modification des hémocytes d'abeille (*Apis mellifera* L) infectés artificiellement par *Pseudomonas aeruginosa*. L'étude porte sur les changements concernant le nombre total d'hémocytes (THC), la proportion relative des divers types d'hémocytes (DHC), le pourcentage d'hémocytes dégénérés et/ou morts, et le pourcentage d'hémocytes en cours de mitose chez des abeilles infectées artificiellement par la bactérie *Pseudomonas aeruginosa* ATCC 27014 *(P apiseptica)*. Des abeilles âgées de 9 j ont été maintenues en cagettes depuis leur émergence et infectées par immersion dans 20 ml d'une suspension de bactéries à la concentration d'environ 5×10^9 CFU par ml de solution saline normale. Le THC a été compté à l'aide d'un hémocytomètre de Neubauer après dilution avec la solution de Türk contenant 2% d'EDTA et 0,01% de rouge neutre. La DHC a été calculée sur des frottis colorés par la méthode de May-Grünwald Giemsa modifiée par Shapiro (1979) et Van Steenkiste (1988). Sur chaque frottis, 200 cellules ont été comptées. Les cellules dégénérées et/ou mortes et celles en cours de mitose ont également été dénombrées.

On n'a pas observé de changement significatif ($P \leq 0,05$) dans le THC des abeilles malades pendant presque toute la durée de l'expérience (tableau I). Dix heures après l'infection, le pourcentage de plasmatocytes ovales s'est accru ($P \leq 0,05$) et celui des plasmatocytes ronds a diminué ($P \leq 0,05$) dans l'hémolymphe des abeilles infectées et ne présentant pas de symptômes cliniques. Ces mêmes change-

ments ont été observés chez les abeilles malades (figs 1, 2). La diminution du pourcentage de plasmatocytes ronds est probablement le résultat de leur dégénérescence et/ou de leur mort et aussi de leur transformation en plasmatocytes ovales. L'augmentation du pourcentage de ces derniers est due à cette transformation et vraisemblablement aussi à leur libération à partir des tissus. On n'a pas noté de changement ($P > 0,05$) concernant le pourcentage des granulocytes et des hémocytes en cours de division mitotique chez les abeilles malades. Chez ces dernières, on a parfois observé une augmentation significative du pourcentage d'hémocytes dégénérés et/ou morts.

***Pseudomonas aeruginosa* / hémocyte / hémogramme / septicémie / réponse immune**

Zusammenfassung — Die Veränderungen in der Haemolymphe von Honigbienen nach künstlicher Infektion mit *Pseudomonas aeruginosa*. Diese Untersuchung befaßt sich mit den Veränderungen ander gesamten Zahl von Hämozyten (THC), unterschiedlichen Zahl von Hämozyten (DHC), dem Prozentsatz von degenierte/tote Hämozyten und dem Prozentsatz von Hämozyten in mitotischer Teilung bei Bienen nach experimenteller Infektion mit dem Bakterium *Pseudomonas aeruginosa* ATCC 27014 *(P apiseptica)*, dem häufigsten Erreger der bakteriellen Septikämie. Neun Tage alte Bienen wurden seit ihrem Schlüpfen in Käfigen gehalten. Die Tiere wurden durch Eintauchen in 20 ml einer Bakterien-Aufschwemmung von einer Konzentration zu etwa 5 x 10^9 CFU pro ml normaler Kochsalzlösung infiziert. Die Zahl zirkulierender Hämocyten wurde nach Verdünnung mit Türk's Lösung (mit 2% EDTA und 0,01% Neutralrot) in einem Neubauer-Hämozytometer bestimmt. Der relative Anteil der verschiedenen Typen von Hämozyten wurde in einem nach May-Grünwald-Giemsa in der Modifikation von Shapiro (1979) und Van Steenkiste (1988) gefärbten Ausstrich gezählt. In jedem Ausstrich wurden 200 Zellen vollständig ausgezählt. Degenerierte oder/und tote Zellen sowie Zellen im Zustand der Mitose wurden in diesem Versuch ebenfalls gezählt.

Fast während der gesamten Dauer des Versuches wurde keine signifikante Veränderung der Hämozytenzahl beobachtet (Tabelle I). Wie die Beobachtung ergab, war die Zahl der ovalen Plasmatozyten 10 Stunden nach der Infektion in der Hämolymphe von Bienen ohne klinische Symptome erhöht ($P \leq 0,05$), die Zahl der runden jedoch verringert ($P \leq 0,05$). Diese Veränderungen wurden auch in kranken Bienen gefunden (Abb 1, 2).

Die Verringerung des Anteils runder Plasmatozyten ist wahrscheinlich die Folge ihrer Degeneration oder/und Absterbens und auch ihrer Umformung zu ovalen Plasmatozyten. Die Vermehrung ovaler Plasmatozyten könnte auf diese Umformung und auch auf ihre Freisetzung aus dem Gewebe zurückgehen. Bei den kranken Bienen waren keine Veränderungen im Prozentsatz der Granulozyten und der in mitotischer Teilung befindlichen Hämozyten festzustellen ($P > 0,05$). Bei ihnen war jedoch manchmal ein signifikanter Anstieg degenerierter oder/und toter Hämozyten zu finden.

Septikämie / *Pseudomonas aeruginosa* / Hämozyten

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