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The influence of *Pseudomonas aeruginosa* secreted virulence factors on hemocyte-mediated immune response of *Galleria mellonella*

Wpływ sekrecyjnych czynników wirulencji *Pseudomonas aeruginosa* na komórkowe mechanizmy odpowiedzi immunologicznej *Galleria mellonella*

SUMMARY

The influence of secreted virulence factors (SVF) of entomopathogenic strain of *Pseudomonas aeruginosa* on *Galleria mellonella* hemocytes-mediated immune response mechanisms was examined. The obtained results indicated that larvae injection with SVF adversely affected the morphology and consequently viability of hemocytes. Moreover, factors presented in culture supernatant lead to aberrant organization of the actin cytoskeleton. Within the time of SVF action, hemocytes preserved only the ability to adhere to the foreign surfaces in comparison to cells collected from naïve larvae, which formed pseudopodia and extensively spread. It seemed probable that cytoskeleton disruption led to overcoming hemocytes-mediated immune response mechanisms by entomopathogen. The phagocytosis in treated with SVF larvae was observed but hemocytes which engulfed bacteria became dead. Moreover, hemocytes ability to capsule formation was strongly inhibited in SVF-treated larvae.

STRESZCZENIE

Celem pracy było zbadanie wpływu sekrecyjnych czynników wirulencji entomopatogennego szczepu *Pseudomonas aeruginosa* na komórkowe mechanizmów odpowiedzi immunologicznej *Galleria mellonella*. Uzyskane wyniki wskazują, że iniekcja gąsienicom supernatantu, zawierającego sekrecyjne czynniki wirulencji powodowała zmiany morfologiczne hemocytów i w konsekwencji ich obumieranie. Poza tym obserwowano nieprawidłową organizację cytoszkieletu aktynowego. Hemocyty zachowywały jedynie zdolność do adhezji w porównaniu do hemocytów gąsienic zdrowych, które formowały wypustki i ulegały znacznemu rozpłaszczeniu na powierzchni ciała obcego.

Istnieje możliwość, że zmiany w organizacji cytoszkieletu pod wpływem sekrecyjnych czynników wirulencji są podstawą przełamywania komórkowych mechanizmów odpowiedzi immunologicznej *G. mellonella*. Zaobserwowano, że hemocyty, które sfagocytowały komórki bakteryjne były martwe. Poza tym zdolność hemocytów do enkapsulacji po działaniu seckrecyjnych czynników wirulencji była zahamowana.

Key words: hemocytes, phagocytosis, encapsulation, *Pseudomonas aeruginosa*, *Galleria mellonella*, virulence factors

INTRODUCTION

All living organisms are exposed to adverse environmental factors, including pathogenic bacteria, fungi, parasites etc. Thanks to correctly functioning immune system, the infection can be combated fast and without any damage of the host organism. In the evolution two kinds of immunity were developed: innate (non-specific) and acquired (specific) immunity. Innate immunity is characteristic of both vertebrates and invertebrates, whereas acquired mechanisms are typical only of vertebrates (Hoffman et al. 1999). Non-specific immunity is evolutionarily conserved in different groups of organisms (Salzet 2001).

Insects like other invertebrates depend only on the very effective innate mechanisms of immunity. During infection, pathogenic organisms have to overcome physiological and anatomical barriers to get into hemocel. At the site of the infection, local mechanisms of the immunity, namely coagulation, melanization and antimicrobial peptides synthesis are induced. They hinder the pathogens to disseminate. Some pathogens are able to overcome defence mechanisms and get into hemocel. Their cells stimulate systemic immunity, which is composed of cellular and humoral mechanisms. Cellular immunity is comprised of phagocytosis, encapsulation and nodulation, whereas humoral of melanization, coagulation, and synthesis of antimicrobial peptides, reactive oxygen and nitrogen intermediates (ROI, RNI), insect metalloproteinase inhibitors (IMPI). It should be underlined that these mechanisms cooperate and are dependent on each other (Iwanaga, Lee 2005; Lavine, Strand 2002).

Hemocytes are cells presented in the hemolymph and specialized in cellular immunity. They are also engaged in foreign body recognition, antimicrobial peptides, proteins, RNI and ROI synthesis. Hemocytes participate in the initial steps of coagulation and melanization (Lavine, Strand 2002; Lavine et al. 2005; Shimabukuro et al. 1996). The most frequently described hemocyte types circulating in the hemolymph insects Lepidoptera are granulocytes (Gr), plasmatocytes (Pl), spherulocytes (Sph), and oenocytoids (Oe) (Ribeiro, Brehélin 2006). According to Ratcliffe et al. (1986), granulocytes constitute 48% and plasmatocytes 47% of all circulating hemocytes. While Sph and Oe 3% and 1%, respectively. Gr and Pl are the sole cells able to adhere to foreign bodies. This feature enables them to take part in phagocytosis, encapsulation and nodulation (*Galleria mellonella*). Oenocytoids contain precursor of the cytoplasmic form of phenol oxydase (melanins synthesis). They are non-adherent cells, like spherulocytes. The latter ones are probably involved in mucopolysaccharides transport (Ribeiro, Brehélin 2006).

Pseudomonas aeruginosa is a common Gram-negative bacillus. To this species belong both saprophytic and pathogenic strains (Mahajan-Miklos et al. 2000). Pathogenic strains are equipped with diverse virulence factors and mechanisms. In general they are divided into cell-surface and secreted virulence factors, type III secretion system and quorum-sensing (Kipnis et al. 2006). Their role in pathogenesis of human pathogenic strains has been examined in detail, while virulence mechanisms of entomopathogenic strains are known scantily.

Currently *G. mellonella* like *Drosophila melanogaster*, *Caenorhabditis elegans* or other invertebrate species are widely used as model organisms in research studies. Complex interactions

between innate and acquired immunity in vertebrates make studies under immunological system extremely difficult. This is why *G. mellonella,* devoid of innate immunity, has become a convenient model to study the pathogenesis and its interactions with innate immunity mechanisms of the host (Kavanagh, Reeves 2004; Scully, Bidochka 2006).

In this paper we presented the results of studies which were carried out to understand the relationships between secreted virulence factors of *P. aeruginosa* and *G. mellonella* hemocytes-mediated immune response.

MATERIAL AND METHODS

Biological species

The larvae of the greater wax moth *G. mellonella* L., (Lepidoptera: *Pyralidae*) were reared on a natural diet – honeybee nest debris at 30°C in the dark. Last instar larvae (7^{th}) of approximate 250–350 mg weight were used for all experiments.

P. aeruginosa strain ATCC 27853 was used in this study. The pyocyanin-producing strain is an isolate of moderate virulence to the 7^{th} instar larvae of *G. mellonella* (LD_{50} = 17 cells). In this study, *E. coli* K12, strain D31, LPS defective, streptomycin and ampicillin resistant (CGSC 5165) were also utilized. The bacterial cells were grown in nutrient broth for 24 h at 37°C.

Preparation of secreted virulence factors

Secreted virulence factors (SVF) were obtained from 24-hour bacterial culture *P. aeruginosa* by centrifugation at 8000 rpm for 10 min to pellet bacterial cells. Than supernatant was filter-sterilized (0.3 μm). SVF was stored at -20°C.

Larvae injection with secreted virulence factors

Before injection larvae were anesthetized on ice and surface was sterilized with ethanol. Into each larva 10 μl SVF was injected by puncturing larval abdomen with a sterile Hamilton syringe and 21-gauge needle. After the injection, the larvae were kept at 28°C in the dark on sterile Petri plates for 30 or 60 min. Naïve larvae were used as control. The hemolymph samples were obtained by puncturing larval abdomen with a sterile needle. The out-flowing hemolymph was immediately transferred into IPS (150 mM NaCl, 5 mM KCl, 0.1 M Tris-HCl, pH 6.9) /PTU (phenylthiourea) on microscopic slides to prevent melanization.

Morphological changes of hemocytes

Morphological changes of hemocytes were determined by means of contrast-phase microscope. All glassware were wiped with 70% ethanol before usage. Ten μl IPS/PTU was placed on microscopic slides. Then to each drop was added 10 μl of hemolymph collected from larvae 30 or 60 min., after SVF injection or from naïve larvae. The preparations were covered with coverslipe and examined at x200 and x400 magnification. In each group 4 larvae were used. Three independent experiments were carried out.

Spreading assays

The larvae were bled as described above. Hemolymph was collected from larvae 30 or 60 min. after SVF injection or from naïve larvae. Ten μl IPS/PTU was placed on microscopic slides. Then 10 μl hemolymph sample was applied to IPS/PTU. Preparations were kept in moist chamber by 30 min at 28°C to allow hemocytes to attach and spread. Unattached cells were rinsed gently three times with IPS. Hemocytes monolayers were observed under contrast-phase microscope at x400 magnification. In each group 4 larvae were used. Three independent experiments were carried out.

F-actin staining with phalloidin-FITC

Phalloidin-FITC was used to stain F-actin in hemocytes attached to the glass surface. Monolayers were prepared on 8-well microscopic slides (MP Biomedicals) as described above. Then hemocytes were fixed in 4% paraformaldehyde for 15 min. After three washes in IPS, cells were permeabilized in 0.5% (v/v) Triton X-100 for 25 min, washed three times in IPS and stained with phalloidin-FITC (1.0 µg/ml) for 30 min. Afterwards the dye was rinsed gently three times with IPS. The preparations were visualized using confocal microscope Axiovert 200 M at x200 and x400 magnification. All steps of staining were carried out at 28°C, in the dark and in moist chamber. All solutions were prepared in IPS. In each group 4 larvae were used. Three independent experiments were carried out.

Phagocytosis assays

In phagocytosis assays we used modified method according to Rohloff et al. (1994). As a test particles *E. coli* D31 labelled with FITC were used.

Preparation of FITC labelled bacteria

Briefly, *E. coli* were grown overnight in nutrient broth at 37°C. The bacteria were harvested by centrifugation (8000 rpm, 10 min, 4°C). Cell pellet was rinsed three times with PBS. Then bacterial cells were heat-killed by boiling for 30 min and two times rinsed with PBS. Finally pellet was suspended in carbonate-bicarbonate buffer pH 9.4 (0.2 M sodium carbonate, Na_2CO_3 and 0.2 M sodium bicarbonate, $NaHCO_3$). FITC was added to the final concentration 1 mg/ml. Next, bacteria were incubated for 30 min at 37°C on a rotary shaker (150 rpm). Afterwards, bacteria were rinsed two times in PBS and finally in IPS to remove excessive and unbound dye. The suspension was stored at -20°C.

***In vivo* phagocytosis assay**

Phagocytic activity of hemocytes were examined in larvae, 60 min. after SVF injection and in naïve larvae. For this purpose, FITC labelled *E. coli* ($5x10^3$ bacteria/larva/5µl) were injected into hemocel by means of Hamilton syringe. Larvae were kept in sterile Petri plates, for 30 min, at 28°C, in the dark. Next, hemolymph samples (10 µl/larva) were collected and mixed with IPS/PTU placed onto 8-well microscopic slides (MP Biomedicals). Preparations were incubated 30 min, at 28°C, in the dark moist chamber. Afterwards, monolayers were rinsed three times with IPS. Hemocytes were fixed in 4% paraformaldehyde for 15 min and washed three times in IPS. To quench the signal generated by non-phagocyted bacteria, hemocytes were treated with 0.2% Trypan Blue (in IPS). The dye was rinsed three times with IPS. Monolayers were observed using confocal microscope Axiovert 200 M at x200 and x400 magnification.

Encapsulation experiments

As a test particles dextrane beads – Sephadex G-200 were utilized. Ten % (w/v) suspension in IPS (5 µl per larva) was injected into hemocel, 60 min after SVF injection or into naïve larvae. Injection was done by means of Hamilton syringe. After beads injection, larvae were kept for 3 or 6 h in sterile Petri plates at 28°C, in the dark. Next, 10 µl of hemolymph samples/larva were collected and mixed with the same volume of IPS/PTU, placed onto microscopic slides. Capsule formation was examined under contrast-phase microscope, Olympus CX 40 at x200 magnification. In each group 5 larvae were used. Three independent experiments were carried out.

RESULTS AND DISCUSSION

There are many strategies evolved by parasites and pathogens which enable them to develop in host organisms. They include, among others, factors and mechanisms to avoid or overcome immune response mechanisms. In this study we examined the interactions between secreted virulence factors (SVF) of *P. aeruginosa* (presented in bacterial supernatant) and cellular immune response of *G. mellonella*. We determined morphological changes and spreading activity of hemocytes collected from larvae treated with bacterial supernatant.

It was observed in the preliminary studies that secreted virulence factors of *P. aeruginosa* caused morphological changes of *G. mellonella* hemocytes. Four types of hemocytes can be distinguished in the hemolymph collected from naïve

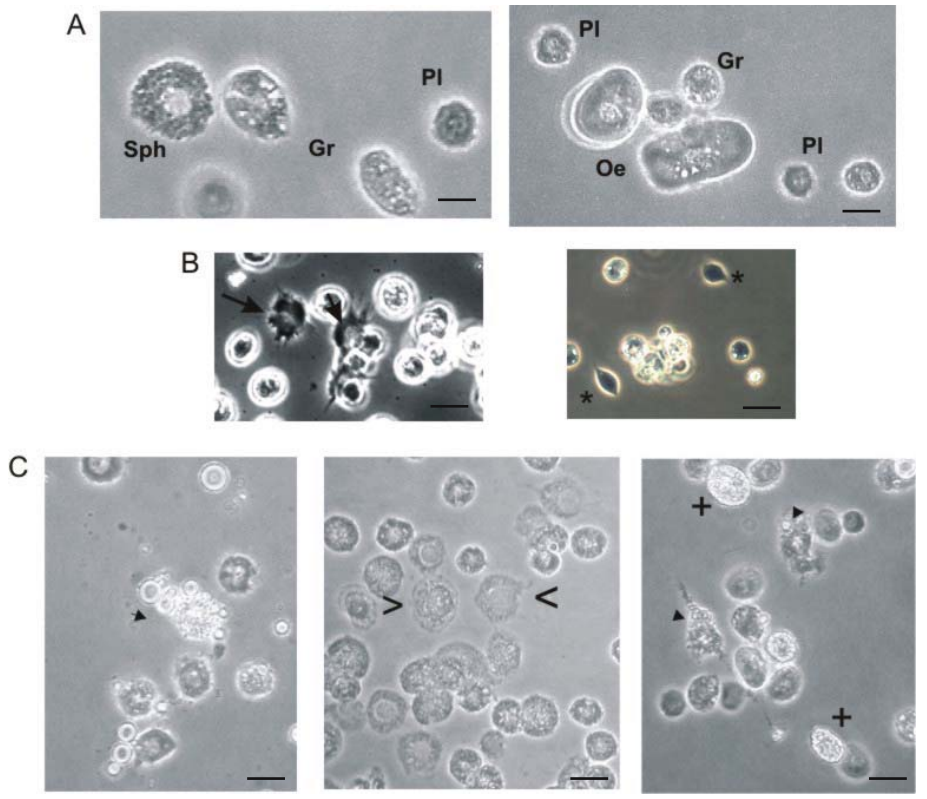

Fig. 1. Morphological changes of *G. mellonella* hemocytes treated with secreted virulence factors of *P. aeruginosa*. A – hemocytes from naïve larvae. B – hemocytes collected from larvae 30 min after SVF-injection. → cells with pseudopodia-like protrusions; * – cells with spindle shape. C – hemocytes collected from larvae 60 min after SVF-injection. ► – debris of hemocytes, cells with disintegrated membrane; <> – translucent, swollen hemocytes. + – refractive granulocytes. – 20 μm

larvae: plasmatocytes (Pl), granulocytes (Gr), oenocytoids (Oe), spherulocytes (Sph) (Fig. 1A). Plasmatocytes were oval or round cells and diameter around 8–20 μm. There were not observed any granules in the cytoplasm. Whereas granulocytes were slightly larger and mostly oval cells with numerous granules in the cytoplasm. Oenocytoids were the largest (25 μm) and rather regular in shape cells. Their characteristic feature was small, located peripherally nuclei and great amount of cytoplasm. Spherulocytes were rather rounded and containing a characteristic large inclusions (spherules) cells. Meanwhile, 30 min. after injection with SVF, the appearance of cells with spindle shape and characteristic, pseudopodia-like protrusions was observed (Fig. 1B). Whereas, 60 min. after treatment, Gr seemed to be more refractive and hemocytes, mainly Pl, formed aggregates. The appearance of characteristic, translucent, regularly round cells, which looked like swollen in comparison to the ones from naïve larvae was observed. Moreover, cells with disintegrated plasma membrane, so that only remnants of the nucleus and parts of the plasma membrane remained were observed (Fig. 1C).

These results could indicate that some factors produced and secreted by *P. aeruginosa* are cytotoxic for *G. mellonella* hemocytes. This kind of pathogenesis strategy (immunocompetent cells killing) is very common (Cai et al. 2004; Richards, Parkinson 2000; Zhang et al. 2005). For example, *Bombyx mori* hemocytes undergo apoptosis after infection with *Xenorhabdus* or *Photorhabdus* (Cho, Kim 2004). Many human pathogens have also developed mechanisms for immunocompetent cells killing, which enables them thriving and disseminating (Labbé, Saleh 2008).

The basic feature of hemocytes for mediating cellular immunity mechanisms, like phagocytosis or encapsulation is their ability to attach and adhere to foreign surfaces. This starts with pseudopodia formation. After that cells start to spread on foreign surfaces. Adhesive and spreading ability of the cells is based on the cytoskeleton rearrangement. Some pathogens evolved virulence mechanisms which enable them to affect cytoskeleton.

The preliminary studies revealed that SVF *P. aeruginosa* was not only cytotoxic for hemocytes but also caused evident changes in the cell shape. Therefore, further studies were aimed at examining whether secreted virulence factors of *P. aeruginosa* can be involved in immunity overcoming by disruption of normal cytoskeletal organization. Hemolymph samples collected from naïve larvae or 30 and 60 min after SVF-injection were applied onto microscopic slides, which acted as foreign surfaces for hemocytes. Their ability to adhere and spread were examined under contrast-phase microscope. In further studies phalloidin-FITC was used to stain F-actin filaments. These preparations were observed under confocal microscope.

Hemocytes from control larvae showed strong ability to adhere and spread on the foreign surface. After adhering, Pl started to form numerous pseudopodia

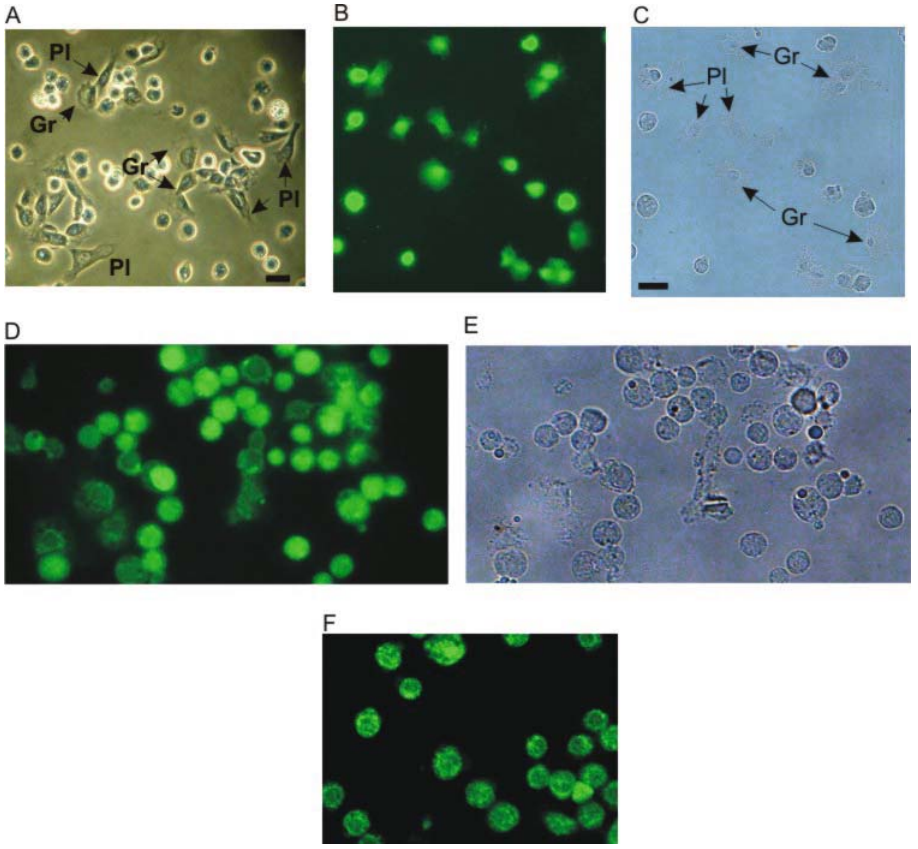

Fig. 2. The *G. mellonella* hemocytes ability to adhere and spread onto foreign surfaces after treatment with secreted virulence factors of *P. aeruginosa*. A, B, C – hemocytes from naïve larvae. D, E, F – hemocytes collected from larvae 30 and 60 min after SVF-injection. Pl – plasmatocytes; Gr – granulocytes. A – photograph was taken under contrast-phase microscope; B, D, F – under confocal microscope after F-actin staining with FITC-phalloidin; C, D – photographs present the same field as B and D, respectively. ─ 20 μm

and long lamellipodia. Next, this type of hemocytes extensively spread on the microscopic slides, become spindle- or fibroblastic-shape cells. Whereas Gr, which adhered to foreign surface, form many pseudopodia and then spread so that the cytoplasm encircled the cell. In Pl and Gr which adhered and spread extensively, actin formed visible contractile fibers (Fig. 2A, B, C). Factors presented in SVF caused changes in pseudopodia formation and spreading ability of hemocytes. Thirty min after injection, hemocytes adhered to the glass and formed pseudopodia but were not able to spread. In these cells contractile fibers were not observed. Whereas, hemocytes collected 60 min after SVF treatment were able only to adhere onto foreign surface, without pseudopodia formation and spreading. They

had rounded configuration. Sometimes these cells were translucent and stained with phalloidin-FITC uniformly (no visible fibers) (Fig. 2D, E). In some cells, patches of actin stained with phalloidin-FITC were observed (Fig. 2F). Described above changes indicated that *P. aeruginosa* produced and secreted some factors which can affect normal actin organization of hemocytes. It is well established that toxins, like ExoS, ExoT and ExoY of *P. aeruginosa* are able to disrupt cytoskeleton normal organization. It is probable that these toxins are produced by tested strain of entomopathogen and they are responsible for observed changes (Kipnis et al. 2006).

To test whether aberrant actin arrangement, as a result of *P. aeruginosa* secreted virulence factors action, affects hemocytes-mediated immune response, ability of cells to the phagocytosis and encapsulation was examined.

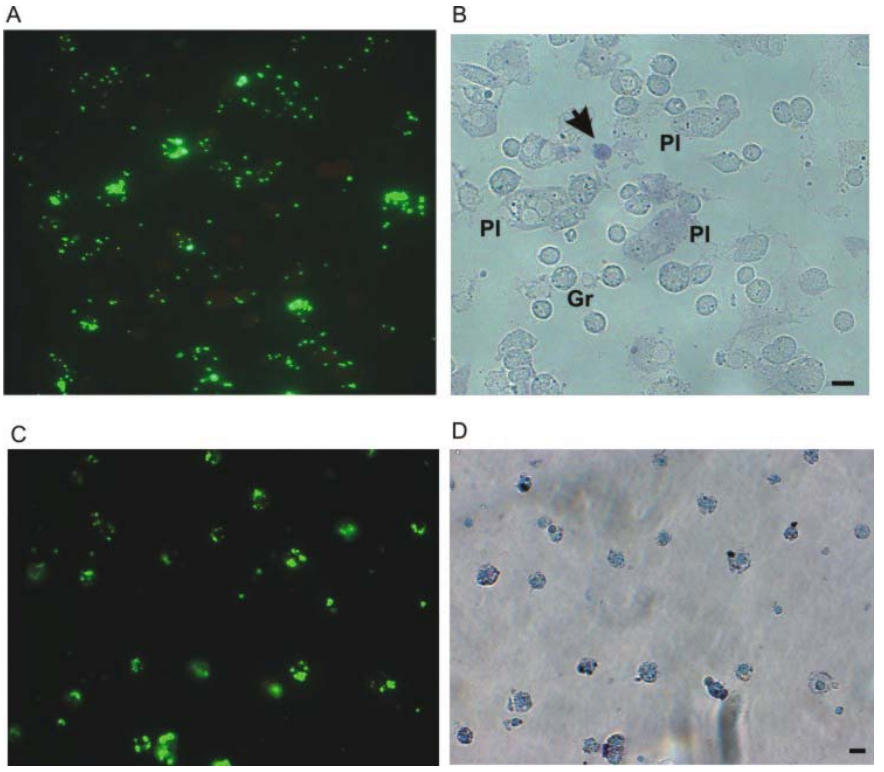

Fig. 3. The bacterial cells phagocytosis by *G. mellonella* hemocytes: A, B – in naïve larvae; C, D – in larvae 60 min after SVF-injection and subsequent *E. coli*-FITC injection. A, C – photographs were taken under confocal microscope; B, D – photographs present the same field as A and C, respectively. Photographs taken under contrast-phase microscope. Phagocytosed bacterial cells present green fluorescence. Dead hemocytes were stained blue by Trypan Blue (B, D). Pl – plasmatocytes; Gr – granulocytes; → dead hemocytes from naïve larvae. ─ 20 μm

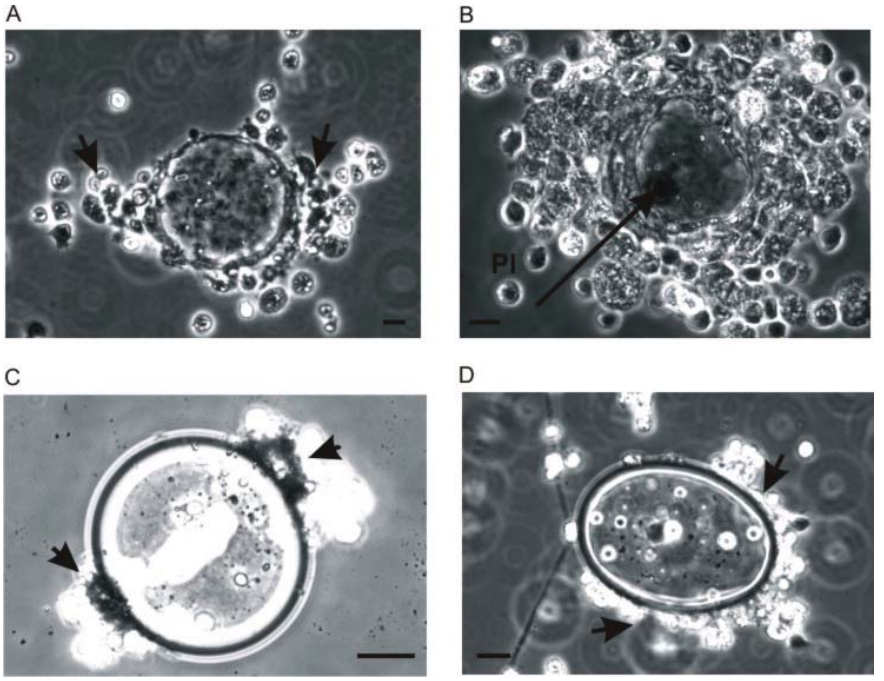

Fig. 4. Dextrane beads encapsulation by *G. mellonella* hemocytes: A, B – in naïve larvae. C, D – in larvae 60 min after SVF-injection and subsequent injection with beads. Pl – plasmatocytes; A, C, D – → hemocytes adhered to the dextrane beads; B – → dextrane beads surrounded by multiple layers of hemocytes and melanized on the surface. Photographs were taken under contrast-phase microscope. ─ 20 μm

To examine phagocytic activity of hemocytes, naïve and SVF-treated larvae were injected with FITC labelled *E. coli* D31 cells. Afterwards, hemocytes were examined under confocal microscope (to check phagocytosis) and contrast-phase microscope (to check hemocytes viability). In the case of hemocytes from naïve larvae, both Pl and Gr phagocyted bacterial cells and hemocytes were viable (Fig. 3A, B). Whereas larvae treatment with SVF caused hemocytes death. Despite this, hemocytes phagocyted bacterial cells (Fig. 3C, D).

To test hemocytes ability to capsule formation, naïve and SVF-treated larvae were injected with dextrane beads suspension. Hemolymph samples were examined under contrast-phase microscope for capsules presence. In the hemolymph collected from naïve larvae, almost each observed bead was encapsulated. The test particles were surrounded by different numbers of hemocytes layers, composed mainly of Gr. The formed structures were sometimes melanized on the surface (Fig. 4A, B), whereas in the hemolymph of treated larvae collected 3 h after beads injection test particles were hardly surrounded by hemocytes. Only minute quan-

tity of cells adhered to the foreign surface (Fig. 4C). Only in the samples collected 6 h after beads injection, test particles were surrounded by more cells, but their number was still smaller than that observed in the case of naïve larvae (Fig. 4D).

Overcoming the hemocytes-mediated immune response mechanism by cytoskeleton disruption was also documented for other insect pathogens or parasites. For example, secondary metabolites (mycotoxins) produced by entomopathogenic fungus *Metarhizium anisopliae* or venom from endoparasitic wasp *Pimpla hypochondriaca* or parastoid species *Pteromalus puparum* and *Nasonia vitripennis* adversely affect cytoskeleton and, consequently, cellular immunity (Cai et al. 2004; Richards, Parkinson 2000; Vey et al. 2002; Vilcinskas et al. 1999; Vilcinskas et al. 1997; Zhang et al. 2005).

REFERENCES

Cai J., Ye G.-y., Hu C. 2004. Parasitism of *Pieris rapae* (Lepidoptera: *Pieridae)* by a pupal endoparastoid, *Pteromalus puparum* (Hymenoptera: *Pteromalidae*): effects of parasitization and venom on host hemocytes. J. Insect Physiol. 50, 315–322.

Cho S., Kim Y. 2004. Hemocytes apoptosis induced by entomopathogenic bacteria, *Xenorhabdus* and *Photorhabdus* in *Bombyx mori*. J. Asia-Pacif. Entomol. 7, 195–200.

Hoffmann J. A., Kafatos F. C., Janeway C., Ezekowitz R. A. 1999. Phylogenetic perspective in innate immunity. Science 284, 1313–1318.

Iwanaga S., Lee B. L. 2005. Recent advances in the innate immunity of invertebrate animals. J. Biochem. Mol. Biol. 38,128–150.

Kavanagh K., Reeves E. P. 2004. Exploiting the potential of insects for *in vivo* pathogenicity testing of microbial pathogens. FEMS Microbiol. Rev. 28, 101–112.

Kipnis E., Sawa T., Wiener-Kronish J. 2006. Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis. Méd. Mal. Infect. 36, 78–91.

Labbé K., Saleh M. 2008. Cell death in the host response to infection. Cell Death Differ. 15, 1339–1349.

Lavine M. D., Strand M. R. 2002. Insect hemocytes and their role in immunity. Insect. Biochem. Biol. 32,1295–1309.

Lavine M. D., Chen G., Strand M. R. 2005. Immune challenge differentially affects transcript abundance of three antimicrobial peptides in hemocytes from the moth *Pseudoplusia inculdens*. Insect. Biochem. Mol. Biol. 35, 1335–1346.

Mahajan-Miklos S., Rahme L. G., Ausubel F. M. 2000. Elucidating the molecular mechanisms of bacterial viturlence using non-mammalian hosts. Mol. Microbiol. 37, 981–988.

Ratcliffe N. A., Mead G. P., Renwrantz L. R. 1986. Insect haemocyte separation – an essential prerequisite to progress in understanding insect cellular immunity. In: M. Brehelin (Ed.), Immunity in Invertebrates. Springer-Verlag, Berlin Heidelberg, 3–11.

Ribeiro C., Brehélin M. 2006. Insect hemocytes: What type of cell is that? J. Insect. Physiol. 52, 417–429.

Richards E. H., Parkinson N. M. 2000. Venom from the endoparasitic wasp *Pimpla hypochondriaca* adversely affects the morphology, viability, and immune function of hemocytes from larvae of the tomato moth, *Lacanobia oleracea*. J. Invertebr. Pathol. 76, 33–42.

Rohloff L. H., Wiesner A., Gotz P. 1994. A fluorescence assay demonstrating stimulation of phagocytosis by haemolymph molecules of *Galleria mellonella*. J. Insect Physiol. 40, 1045–1049.

Salzet M. 2001. Vertebrate innate immunity resemebls a mosaic of invertebrate immune responses. Trends Immunol. 22, 285–288.

Scully L. R., Bidochka M. J. 2006. Developing insect models for the study of current and emerging human pathogens. FEMS Microbiol. Lett. 263, 1–9.

Shimabukuro M., Xu J., Sugiyama M., Taini K., Okuda K. K., Kato Y., Yamamoto M., Chowdhury S., Choi S. K., Miyanoshita A., Debnath N. C., Yamakawa M. 1996. Signal transduction for cecropin B gene expression in adherent hemocytes of silkworm, *Bombyx mori* (Lepidoptera: *Bombycidae*). Appl. Entomol. Zool. 31, 135–143.

Vey A., Matha V., Dumas Ch. 2002. Effects of the peptide mycotoxin destruxin E on insect haemocytes and on dynamics and efficiency of the multicellular immune reaction. J. Invertebr. Pathol. 80, 177–187.

Vilcinskas A., Jegorov A., Landa Z., Götz P., Matha V. 1999. Effects of beaverolide L and cyclosporin A on humoral and cellular immune response of the greater wax month, *Galleria mellonella*. Comp. Biochem. Physiol. (C) 122, 83–92.

Vilcinskas A., Matha V., Götz P. 1997. Effects of the entomopathogenic fungus *Metarhizium anisopliae* and its secondary metabolites on morphology and cytoskeleton of plasmatocytes isolated from the greater wax month, *Galleria mellonella*. J. Insect. Physiol. 43,1149–1159.

Zhang Z., Ye G.-y., Cai J., Hu C. 2005. Comparative venom toxicity between *Pteromalus puparum* and *Nasonia vitripennis* (Hymenoptera: *Pteromalidae*) toward the hemocytes of their natural hosts, non-target insects and cultured insect cells. Toxicon. 46, 337–349.