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The character and role of the bumble bee immune responses in protection against infections

Charakter i rola odpowiedzi immunologicznej trzmieli w obronie przed zakażeniami

Key words: bumble bees, immunity, lysozyme, apidaecins, immune response

Słowa kluczowe: trzmiele, odporność, lizozym, apidycyny, odpowiedź immunologiczna

Immunity of the bumble bee, like other holometabolous insects, depending on the character of immune responses and the role of immune reactions in body protection is defined as:

- No susceptibility of an organism to detrimental effects of microorganisms or parasites and their metabolites;
- Security of an organism against a particular disease;
- Capacity of a living organism to distinguish foreign material from self and to destroy, eliminate or neutralize by specific mechanisms that which is foreign, mainly saprophytes invading the body cavity (13).

The bumble bees defend themselves against invasion and colonization by foreign organisms due to the presence of the integument and gut barriers and by internal defense mechanism (21). Although insects lack the complexity of the vertebrate immune system (T and B lymphocytes and products of their activity such as immunoglobulins and lymphokines), they have developed alternative antimicrobial strategies that protect them well against infections. Among the mechanisms of insect resistance to diseases a key role play the immune responses, both cellular and cell-free in nature (16).

One of the most important characteristics of an immune system is discrimination of self from non-self (19). The bumble bees have a precise system of recognizing particulate and soluble molecules, including inorganic substances. The discrimination of self from non-self is realized by a process known as pattern recognition. Pattern recognition molecules are able to distinguish the highly conserved patterns for example lipopolysaccharide complex or peptidoglycan molecules in the case of bacteria, mannose, galactose or glucan residues in the case of fungi, that are unique to pathogens and are not usually found on the host cells (24). The capacity to recognize non-self is the first step towards inducing internal immune defense. The internal immune mechanisms of the bombus: clarifying of hemolymph from invaders by cellular and humoral defense reactions such as phagocytosis, nodule formation, and encapsulation (1, 9, 23). The stress conditions such as infection induce hypersynthesis of blood lysozyme and *de novo* synthesis of the inducible immune proteins which are the effector molecules of the induced protective immunity (6, 8, 17, 20, 22).

The induced protective immunity of insects:

- Develops quickly, in a few hours;
- Persists for a very short time, usually for 72-96 h, exceptionally for more than 144 h;
- Has unspecific character because it is directed to many foreign entities;
- Memory is not involved, and hence, there is a lack of secondary immune response;
- The inducible immune proteins of a low molecular weight (in the *Apoidea apidaecins*) are the effectors (3, 12, 20).

Immunologists use a number of key methods to evaluate the status of the immune system of insects. The most useful is the determination of the so-called "immunological profile". The immunological profile includes both natural and inducible immune responses. The differential hemocyte count, phagocytic index, and percent of hemocytes active in phagocytosis and the bactericidal activity of lysozyme-type in blood, characterize the intensity of the natural immune responses.

The inducible immune responses developed in the worker bumble bees after intrahaemocoelic injection of live bacterial cells – in our studies after intrahaemocoelic injection of 15,000 live cells of *Escherichia coli* D31 in 2 µl of the insect physiological salt saline for *Lepidoptera* (20).

The intensity of the inducible immune responses was evaluated after the induction of the immune system on the basis of: the level of apidaecins in blood, intensity of hypersynthesis of blood lysozyme, percent of blood cells active in phagocytic process and phagocytic index

The bumble hemocytes are capable of rapidly reducing the number of circulating foreign particles including microorganisms by phagocytosis. These cells are also active in nodule formation, encapsulation, blood clotting and wound healing, thus preventing the penetration of microorganisms into the body cavity via wounds.

In the hemocytic immune responses in bumble bees, like in other insect species, the most active are three types of hemocytes, called immunocytes: plasmatocytes, basophils, eosinophils (12, 14, 15). The immunocytes form more than 75% of the differential haemocyte count of the peripheral blood of the bumble bee.

Phagocytosis is a key mechanism for the host's defense system and tissue remodelling. It has been known now that phagocytosis in insects consists of several steps which include chemotaxis, activation of receptors on the haemocyte membrane, attachment of the microorganisms to the membrane receptors, engulfment, formation of phagolysosome and killing and digestion of invading microorganisms (23). Glycosylated protein and lipoprotein receptor molecules function during non-opsonin mediated lectinophagocytosis and opsonophagocytosis of microbes coated with exogenous lectins. The multifunctional prophenoloxidase cascade (proPO cascade) generates opsonic intermediates that assist in the initial recognition and attachment phases of phagocytosis (2). Killing of the microorganisms trapped in phagolysosomes may result from oxygen-independent mechanisms, oxygen-dependent mechanisms (superoxide anions and hydrogen hydroxide) and most probably by nitric acid pathway (1, 9).

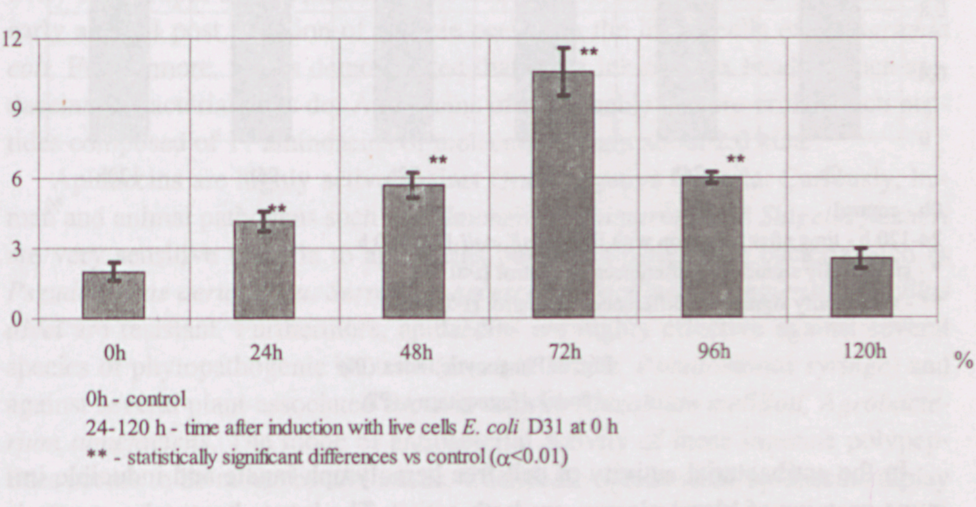
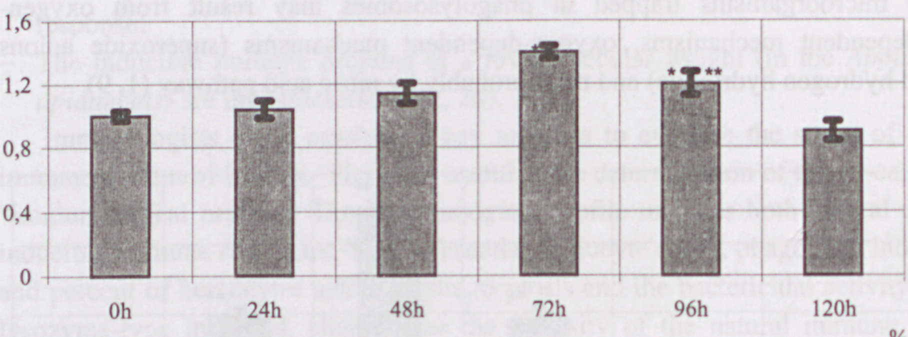


Fig. 1. Percent of hemocytes active in phagocytosis
Odsetek hemocytów zaangażowanych w fagocytozie

The percent of hemocytes participating in phagocytosis increases significantly after infection or artificial injection of bacteria into the body cavity of the bumble bee. At the peak that is noted after 72 h since bacterial induction more than 10% of blood hemocytes is active in phagocytosis. After 120 h since the induction, the number of phagocytic cells decreases to the initial level (before induction) (Fig. 1).

Several factors including temperature, humidity, toxicants and drugs, may have adverse effects on phagocytosis. Moreover, phagocytic activity depends on the bacterial species, concentration of bacteria in the blood, and the diameter and shape of the foreign particles. Phagocytosis in the bumble bees may be also stimulated by the use of immunostimulators, for example chitin derivatives.

Phagocytic index (PI), the number of bacterial cells (*Serratia marcescens*) inside a phagocyte multiplied by 100 and subtracted by the number of hemocytes phagocytosing, was high in the workers of the *Bombus terrestris* – 1.0. The PI increased steadily after the bacterial induction or infection. The peak of the index was noted after 72 h since infection. The index returned to the initial value after 120 h since induction (Fig. 2).



0h - control

24-120 h - time after induction with live cells*E. coli* D31 at 0 h

* - statistically significant differences vs control ($\alpha<0.05$)

** - statistically significant differences vs control ($\alpha<0.01$)

Fig. 2. Phagocytic index (PI)
Indeks fagocytarny (PI)

In the antibacterial activity of cell-free hemolymph innate and inducible immune proteins of blood plasma, are both active. The innate humoral response is represented by lysozyme, the inducible immune response by apidaecins (13).

Lysozyme, a basic protein of a molecular weight of about 1.5 kDa, which attacks in insects mainly Gram-positive bacteria, was the first antibacterial factor purified from insect hemolymph (17). The low innate level of lysozyme, about 5 μg/ml of hemolymph in the worker bumble bee, increased drastically following infection with bacteria and under stress conditions. Lysozyme seems to be a primitive entity of humoral antibacterial immunity. The highest level of this enzyme was noted after 24 h since infection. The enhanced activity of lysozyme together with hemocytic defense reactions reduces the risk of generalized bacterial infection during the first 24–48 h after the penetration of bacteria in the insect body cavity.

Induction of immune response in higher insects shows characteristics of the innate response in mammals: recognition of genetic microbial epitopes, synthesis of antibiotic peptides at the site of insult and in specialized tissues, and gross physiological adaptation. Inducible (acquired) immunity appears after infection or induction with biotic and abiotic objects that disturb the physiological activity of an insect and is associated with *de novo synthesis* in the fat body of immune proteins. The main function of immune proteins in *Apoidea* defense seems to be protection of haemocoel against infections caused by saprophytic bacteria that are ubiquitously present in the niche of the living insects.

Among the insect immune proteins, there are biochemically well characterized the cecropin-family peptides and attacins in *Lepidoptera* (3), diptericins in *Diptera* (18), insect defensins (4) and apidaecins of the honey bee and other *Apoidea* (5-8, 11).

The antibacterial activity of apidaecins could tentatively be classified to activity of cecropin-like substances. The antibacterial activity could be detected as early as 8-24 post injection of bumble bees with the living cells of *Escherichia coli*. Furthermore, it was demonstrated that India ink or latex beads induce apidaecins as bacterial cells do. Apidaecins of the bumble bee are proline rich peptides composed of 17 aminoacids of molecular weight about 2.0 kDa.

Apidaecins are highly active against Gram-negative bacteria. Curiously, human and animal pathogens such as *Salmonella typhimurium* and *Shigella flexneri* are very sensitive bacteria to apidaecins but insect-pathogenic bacteria such as *Pseudomonas aeruginosa*, *Serratia marcescens, Bacillus thuringiensis, Bacillus alvei* are resistant. Furthermore, apidaecins are highly effective against several species of phytopathogenic bacteria (*Erwinia salicis, Pseudomonas syringe*) and against several plant-associated bacteria such as *Rhizobium melliloti, Agrobacterium tumefaciens*. The mode of antibacterial activity of these immune polypeptides seems to be rather bacteriostatic. Chemically synthesized apidaecins diplay similar bacteriostatic activity as their natural counterparts (5-8, 11).

Probably, the apidaecins do not disturb cellular membranes. They do not kill bacteria immediately by a lytic action but rather interfere with cell propagation. No activity could be demonstrated against phospholipid layers. Therefore, similar to cecropins, eucaryotic organisms are totally resistant to the action of apidaecins.

It can be hypothesized that the marked activity of apidaecins against enteric and plant-pathogenic bacteria has developed in *Apis mellifera* and in the bumble bees as a defense mechanism directed against the microorganisms present commonly in the environment. Bees and bumble bees that visit flowers and collect water are highly exposed to human and plant-associated bacteria, contaminating plant and water sources.

Apideacins are the most prominent component of the bumble bee's inducible defense against bacterial invasion. A sharp increase of apidaecin transcript levels occur 4-6 h after infection, followed by a steady rise of several more hours. Peak concentration of apidaecins in the bumble hemolymph is within 48-72 h post-infection, and then the concentration of the apidaecin compounds declines in blood gradually. In the bumble bee the activity of apidaecins disappeared completely after 144 h since the induction (Fig. 3).

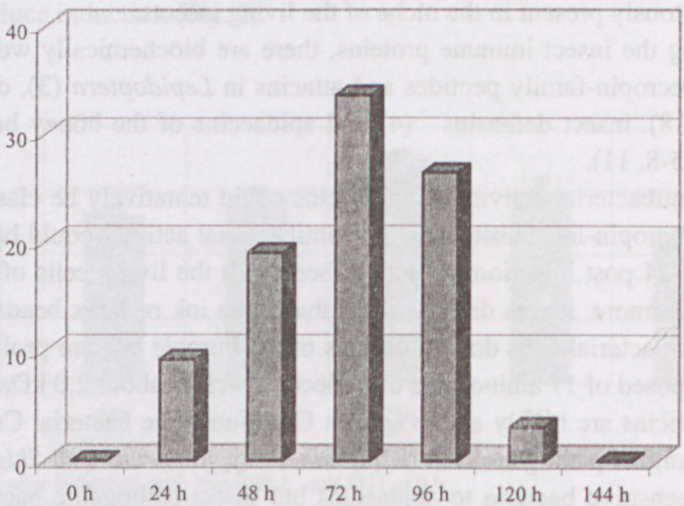


Fig. 3. Level of apidaecins (mean value, μg of synthetic cecropin A *Hyalophora cecropia*/ml)
Poziom apidycyn (wartość średnia w μg cekropiny A *Hyalophora cecropia*/ml)

1. In the worker bumble bee, *Bombus terrestris*, the defense against microorganisms involves an array of different hemocytic and cell-free mechanisms operating in the hemolymph.

2. Phagocytosis and the humoral antimicrobial peptides, lysozyme and apidaecins, play a significant role in the defense of the bumble bee hemocoel against invading bacteria.

REFERENCES

1. Arakawa T.: Superoxide generation *in vitro* in lepidopteran larval haemolymph. J. Insect. Physiol. **40,** 165, 1994.
2. Ashida M., Dohke K.: Activation of prophenyloxidase by the activating enzyme of the silkworm, *Bombyx mori*. Insect Biochem. **10**, 37, 1980.
3. Boman H. G., Hultmark D.: Cell-free immunity in insects. Ann. Rev. Microbiol. **41,** 103, 1987.
4. Bulet P., Hoffman N. D., Hetru C.: Antimicrobial peptides-polypeptides from insects.: biochemical aspects. Cooperation in Science and Techniques, Action – 819 Entomopathogenic Nematodes Workshop, Univ. of Azores, Ponta Delgada, March 18-22, 1996, 1-14.
5. Casteels P., Ampe C., Jacobs F., Tempst P.: Functional and chemical characterisation of hymenoptaecin, an antibacterial peptide that is infection-inducible in the honeybee (*Apis mellifera*). J. Biol. Chem. **268**, 7044, 1993.
6. Casteels P. R., Ampe C., Jacob F., Vaeck M., Tempst P.: Apidaecins: antibacterial peptides from honeybees. European Molecular Biology Organization Journal. **8**, 2387, 1989.
7. Casteels R., Ampe C., Riviere L., van Damme J., Elicone C., Fleming M., Jacobs F., Tempst P.: Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (*Apis mellifera*). Eur. J. Biochem. **187**, 381, 1990.
8. Casteels-Josson K., Capaci T., Casteels P. R., Tempst P.: Apidaecin multipeptide precursor structure.: Putative mechanisms for amplification of the insect antibacterial response. European Molecular Biology Organization Journal **12**, 1569, 1993.
9. Choi S. K., Choi H. K., Kadono-Okuda K., Tainiai K., Kato Y., Yamamoto M.: Occurrence of novel types of nitric acid synthase in the silkworm, *Bombyx mori*. Biochem. Biophys. Res. Comm. **207**, 452, 1995.
10. Engström A., Engström P., Tao Z., Carlsson A., Bennich H.: Insect immunity.: The primary structure of the antibacterial protein attacin F and its relation to two native attacins from *Hyalophora cecropia*. European Molecular Biology Organization Journal **3**, 2119, 1984.
11. Gliński Z, Jarosz J.: Novel antibacterial insect immune peptides and proteins. Folia Veterinaria (Kosice) **42**, 33, 1998 .
12. Gliński Z., Jarosz J., Młynarska M.: Odporność przeciwzakaźna trzmieli. Medycyna Wet. **55**, 800, 1999.
13. Gliński Z., Kostro K.: Key stones in insect immunity. Central Eur. J. Immunol. **26,** 43, 2001.
14. Gupta A.P.: Cellular elements in the hemolymph. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology. Integument, Respiration and Circulation. G.A. Kerkut, L. I. Gilbert (eds), Vol. 3. Pergamon Press, New York, 1985, 401-451.
15. Gupta A. P.: Hemocyte types.: Their structure, synonymies, interrelationships, and taxonomic significance. In: Insect Hemocytes. A. P. Gupta (ed.), Cambridge University Press, Cambridge 1979, 85-127.
16. Hoffmann J. A., Richert J. M., Hetru C.: Innate immunity in higher insects. Current Opinion in Immunology **8**, 8, 1996.

17. J o l l è s J., S c h o e n t g e n F., C r o i z i e r G., C r o i z i e r L., J o l l è s P.: Insect lysozymes from three species of *Lepidoptera*: their structural relatedness to the C (chicken) type lysozyme. J. Biol. Evol. **14**, 309, 1979.

18. K e p p i E., Z a c h a r y D., R o b e r t s o n M., H o f f m a n n D., H o f f m a n n J.A.: Induced antibacterial proteins in the haemolymph of *Phormia terranovae* (*Diptera*). Insect Biochemistry **16**, 395, 1986.

19. L a c k i e A. M.: Immune recognition in insects. Dev. Comp. Immunol. **5**, 191, 1981.

20. M ł y n a r s k a M.: Kształtowanie się profilu imunologicznego u robotnic *Bombus terrestris (Apidae)*. Dys. dokt. Wydział Medycyny Wet. AR, Lublin 1999.

21. O r i h e l T. C.: The peritrophic membrane.: Its role as a barrier to infection of the arthropod host. In: K. Maramorosch, R. E. Shope (eds), Invertebrate Immunity. Academic Press, New York, 1975, 67-73.

22. R e e s J. A., M o n i a t t e M., B u l e t P.: Novel antibacterial peptides isolated from European bumblebee, *Bombus pascuorum* (*Hymenoptera, Apoidea*). Insect Biochem. Molec. Biol. **5**, 513, 1997.

23. S ö d e r h ä l l K.: Prophenyloxidase activating system and melanization – a recognition mechanism of arthropods? A review. Dev. Comp. Immunol. **6**, 60, 1982.

24. S c h m i d t O. I., F a y e I, L i n d s t r o m - D i n n e t z I., S u n S.C.: Specific immune recognition of insect hemolin. Dev. Comp. Imunol. **17**, 195, 1993.

STRESZCZENIE

W przeciwieństwie do układu immunologicznego kręgowców opartego o immunoglobuliny, owady nie mają układu immunoglobulin. Niemniej jednak dysponują one skutecznymi mechanizmami odporności adaptacyjnej na zakażenia drobnoustrojami, ataki pasożytów i drapieżców. Układ immunologiczny trzmieli, podobnie jak innych gatunków owadów holometabolicznych, dysponuje dwoma kategoriami odczynów obronnych: komórkowymi odczynami (reprezentowanymi przez fagocytozę i tworzenie otoczek) oraz odczynami humoralnymi (reprezentowanymi przez polipeptydy i białka odpornościowe). Odporność humoralna to odczyny wrodzone, takie jak aktywność bakteriolityczna hemolimfy typu lizozymu, układ fenoloksydazy, oraz odczyny nabyte związane głównie z apidycynami. Induktorem odporności nabytej są czynniki (bakterie, wirusy, grzyby, niektóre związki chemiczne), które zaburzają integralność ciała owada. Odporność indukowana jest następstwem *de novo* syntezy w ciele tłuszczowym specyficznego odpornościowego RNA i białek rybosomalnych i pojawienia się w jamie ciała owada polipeptydów i białek odpornościowych (*immune proteins*).

W następstwie infekcji ma miejsce hipersynteza lizozymu, przy czym jego poziom zwiększa się około 100-krotnie. Apidycyny trzmiela o masie około 2,0 kDa zawierają w cząsteczce dużą ilość reszt proliny. Ich aktywność jest skierowana przeciwko bakteriom jelitowym kręgowców, bakteriom patogennym dla roślin i drobnoustrojom związanym symbiotycznie z roślinami.