RESPONSE OF THE APOIDEA TO FUNGAL INFECTIONS

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Summary

*The survival of the bee depends on the successful defense against microbial invaders, parasites and predators. The honey bee immune system, like other species of holometabolous insects, depends on two main categories of defense reactions: the cell-mediated responses such as phagocytosis and encapsulation of foreign objects and cell-free defense mechanisms represented by the antimicrobial immune proteins. Phagocytosis and encapsulation are the most common mechanisms in bees against entomopathogenic fungi. The hygienic behaviour, antimicrobial secretions of worker bees and protective barriers of the body coverings forming the effective thresholds and protecting the bee haemocoel against fungal invasions are supported by the haemocyte-mediated immune responses in defense against mycotic infections. The protection of the bee colony to fungi is realized by hygienic behaviour and secretions but individuals are protected by body coverings and cellular immune reactions. Neither the lysozyme nor the inducible immune proteins of the bee act against fungal invaders. The antifungal immune peptides such as drosomycin in the fruitfly (*Drosophila melanogaster*), thanatin in the bug (*Podissus maculiventris*), mietchnikowins and metalnikowins that exhibit activity against both bacteria and fungi, have not been detected in the naïve and infected honeybees.*

Introduction

Fungi are common saprophytes of bees and combs. Most of the fungi collected by bees are unable to become established within the bee and the bee hive. However, some of the fungal species: *Ascosphaera apis*, *Aspergillus* sp., *Aureobasidium pullulans*, *Trichoderma lignorum*, *Mucor hiemalis*, *Rhizopus* and yeasts *Torulopsis* are considered to be the honey bee pathogens.

In the honeybee fungi initiate infection by a spore germination. The developing invasive hyphae penetrate the cuticle mechanically and enzymatically, enter the bee body cavity where they rapidly develop and overgrown the internal organs. The invasion may sometimes start from ingested fungal spores that germinate in the intestines. Physical, chemical and biological stress factors, mainly temperature and high humidity, environment pollution, pesticide poisonings, parasite invasions, attacks of predators, are the factors predisposing to the development of fungal infection in bees. They all can reduce the resistance pattern of the insect organism to mycoses by compromising the immune system and by impairment the protective barriers of the body coverings, alimentary tract and tracheae (GLIŃSKI and JAROSZ, 2001).

Fungal toxins released by fungi, for example, aflatoxins produced by *Aspergillus flavus*, act directly on the central nervous system of the bee. By affecting the endocrine system and most probably the internal defence system of the invaded individuals, they decrease the resistance in the bees to mycotic infection. The outcome of infection depends upon the genetic potential of the pathogen to grow rapidly, utilizing host body constituents for nutrition, production of cuticle-degrading enzymes to penetrate anatomical protective thresholds of insect body, and to resist the host immune mechanisms. Death of an insect may result from mechanical and enzymatic damage to tissues affected by mycelium, abnormal function of organs, mechanical disturbances of blood circulation, toxic action on the host bee. Competition for food between growing fungus and the infected bee organism can not be excluded in pathogenesis of mycoses (GLIŃSKI and JAROSZ, 2000; GOCHNAUER and MARGETTS, 1979).

Protective barriers of the honeybee against mycotic invasion

Various immune mechanisms can operate in the honeybee in protection to fungal infections. The best known are those active in chalkbrood and stonebrood. Among them, the most important are protective barriers of the cuticle, tracheal system and intestines (BARR and SHOPE, 1975; ORIHEL, 1975). In internal defense, non-degradable materials and large parasites are encapsulated by a large mass of haemocytes that serve as a barrier between the haemocoel and the object. Bee haemocytes may also directly kill bacteria, fungal spores and other small foreign molecules in phagocytic process (GÖTZ, 1986). Neither lysozyme nor the inducible haemolymph antibacterial proteins seem able to inhibit or kill fungal spores or mycelia in the invaded bee. Hygienic behaviour is important in resistance of the bee colony to chalkbrood and stonebrood (GILLIAM et al., 1983; SOUTHWICK, 1994).

The impermeable and hard cuticle, the biochemical environment of the midgut juice, its peritrophic membrane together with tracheal system form mechanical and physiological barrier effectively protecting the bee's body cavity against fungal invasion. Fungal spores, fragments of mycelia occurring on the body surface of the bee are removed mechanically with the sloughing of the epidermis. The antifungal activity of the cuticle results from the presence of waxes and unsaturated fatty acids impregnating the cuticle or present on its surface. Only yeasts and moulds that produce chitinase can actively penetrate the cuticular lining of the body and then enter to the haemocoel. The cuticle damaged mechanically or enzymatically by growing

hyphae also allow bacteria to enter body cavity and develop fatal septicaemias (GLIŃSKI and JAROSZ, 2000; GLIŃSKI and KOSTRO, 2001).

The chitinous lining of the fore- and hindgut is an adequate protective mechanical barrier for ingested microbes, excluding the chitinase producers. The midgut, however, is completely devoid of an chitinous lining and, therefore it is potentially the most vulnerable part of the alimentary canal from which microbes can penetrate into the haemocoel.

The biochemical environment of the midgut juice prevents the growth and multiplication of many bacterial species. Antimicrobial substances such as phytonicides, volatile essences present in ingested food can destroy bacterial and fungal invaders. Competition for food between gut bacteria and fungi could efficiently eliminate the massive doses of fungal spores from the gut. The role of a cellular, gelatinous peritrophic membrane in protecting the midgut epithelium from mechanical and chemical damage by growing mycelium can not be excluded entirely. The epithelium and muscle layers of the intestines form barriers that restrict penetration of mycelium from the gut lumen into the haemocoel. The relatively low humidity in the tracheae is as important in restricting germination of spores and growth of fungus in the bee respiratory tract. Nevertheless, infections with heavy doses of spores or infections caused by highly pathogenic fungal species destroy the anatomical and physiological barrier of the honeybee (GLIŃSKI and JAROSZ, 1995a, b). Fungi enter into the haemolymph and cause severe deleterious effects in invaded brood and bees. *Asosphaera apis* and *Aspergillus flavus* infect brood through the alimentary canal or via cuticle abrasions. In adult bees, the intestines are important portal of entry for *Aspergillus*.

The antimicrobial activity of honey, nectar and pollen is a important factor in the colony that inhibits the development of many saprophytic bacteria and fungi in stored food, and that could destroy some pathogenic microorganisms (BURGETT, 1978). The acidity, osmotic pressure and production and accumulation of hydrogen peroxide is responsible for this effect in honey and nectar (WHITE and SUBERS, 1963). Honey as a hyperosmotic medium may kill many living cells, except those of osmophilic fungi and bacteria.

Secretions from honeybee exocrine glands contain biologically significant components. The hypopharyngeal gland secretions of young workers contain proteins to be bacteriostatic and bactericidal to a wide range of bacterial species (ROSE and BRIGGS, 1969). At least, two bacterial inhibitors are identified in royal jelly: 10-hydroxy-2-decenoic acid and glucose oxidase. It can also inhibit or delay the growth of many fungi, for example *A. apis*.

Propolis that is a highly complex mixture of waxes, resins, balsams, oils and a small amount of pollen form a part of antimicrobial defense of the bee colony. Flavanones together with flavones, caffeic acid and its esters are considered to be responsible for antibacterial action of propolis (GREENEWAY et al., 1990). It is quite possible that fungi of plant origin and from animal sources, polluting environment and contaminating pollen sources and water gathered by bees are inhibited by biologically active compound of propolis.

Behavioural resistance to fungal infections in honeybees

Hygienic behaviour can be characterized by the rapid detection of sick and dead brood by worker bees, removal of dead insects from the colony, and the thorough cleaning of the cell of honey comb. Worker bees groom their own bodies and those of other bees, maintain the hygiene of the nest and remove debris from the hive. This hygienic activity is important in the resistance to chalkbrood and stone brood. The adults remove the mummified larvae using their mandibles and carry the larvae away from the nest. Bees that have no means of removing the pathogenic fungi from the gut and the body hair subsequently reinfect susceptible larvae when feeding them or pass on infections fungal spores to other adults to the colony (SOUTHWICK, 1994). Resistance is supported by an ability of some worker bees to filter ingested spores and mycelial fragments from the proventriculus. Inhibitors in the glandular-produced brood food are strong antibacterial and antifungal agents.

There are at least two mechanisms of behavioural resistance, both are genetic in nature. Hygienic behaviour is believed to be controlled by two recessive genes, one for uncapping diseased brood, and one for the removal of mummy (TAUBER, 1992). The expression of hygienic behaviour depends on the strength of the bee colony. When colony size is reduced by removing frames of brood and associated bees, hygienic activity is depressed in hygienic colonies but there is no effect in nonhygienic colonies. The expression of hygienic behaviour is also altered by adding hygienic or nonhygienic bees to colony, and by the colony composition. TAUBER (1992) has stated that all bees with hygienic behaviour tested to chalkbrood were resistant. SOUTHWICK (1994), however, has suggested that there is not straight-forward correlation between hygienic behaviour and resistance to chalkbrood. The chalkbrood infected colonies showed a weak correlation with hygienic behaviour.

Haemocyte-mediated antifungal immune responses

Antifungal activity of insect haemolymph includes haemocyte mediated immune responses and cellfree immunity. Phagocytosis and encapsulation are two common types of defense reactions in the honeybee against invading fungal pathogens. These cellular immune reactions have been shown to be

accompanied by changes both in the number of circulating haemocytes and in the relative proportions of different haemocyte types in the blood (HINK, 1970). In general, the infection of the haemocoel initiates a premature differentiation of haemocytes and their migration towards chemotactic stimulus. Phagocytosis predominantes when the body cavity is exposed to small numbers of bacteria or fungal spores. In the final stage of phagocytic process, the engulfed spores or small fragments of fungal mycelium are digested in a phagolysosome that is formed by the combination of a lysosome with a phagosome. The lysosomal hydrolytic enzymes that destroy bacteria in some instances act against engulfed fungous material. Most probably, plasmatocytes and granular cells active in phagocytosis of bacterial cells can ingest and destroy fungi in phagocytic process. The role of the phenoloxidase system, melanins cannot be excluded in phagocytosis of insect pathogenic fungi.

Encapsulation consists of the formation of a capsule-like envelope around foreign objects with a diameter more than 10 µm that cannot be phagocytized by a single cell. Encapsulation is the most effective haemocyte-mediated immune response in protection of insect haemocoel in fungal infections. In general, the capsule is formed by attaching blood cells, mainly granular cells and plasmatocytes. The granulocytes release haemotactic factors which attract to plasmatocytes to form the outer layer of the capsule around the encapsulated fungus. In some cases, melanin in melanotic crusts is deposited in the wall of the capsule.

Immune peptides of antimicrobial action

Neither lysozyme nor inducible antimicrobial peptides or small proteins of the honey bee possess antifungal activity. Lysozyme, N-acetylmuramylhydrolase, is commonly found in haemolymph of several orders of insects. Lysozyme attacks primarily Gram positive bacteria, although some exceptions, for example, Gram negative bacteria such as mutants of *Escherichia coli*. Haemolymph of normal bees contains low levels of lysozyme. In larval honey bees and in adult worker bees it ranges from 5 to 25 µg/ml, and in pupae from 5-10 µg/ml of haemolymph (MOHRIG and MESSNER, 1968; GÖTZ and TRENCZEK, 1991). The activity of bee lysozyme drastically increased during infection.

The honey bee generate several groups of humoral immune factors to resist microbial infections. The apidaecin-family peptides represent a large group of inducible small (about 2.0 kDa) proline-rich immune peptides of antibacterial activity against plant-associated, phytopathogenic and enteric bacteria (CASTEELS et al., 1989, 1993; CASTEELS-JOSSON et al., 1993). They are most prominent component of the honey bee's inducible humoral defence against bacterial invasions. Antibacterial action of apidaecins is supported in honey bee defense by abaecin (CASTEELS et al., 1990) and hymenoptaecin (CASTEELS et al., 1993). Abaecin is a large inducible proline-rich peptide (4.0 kDa) of a moderate effect on both Gram negative and Gram positive bacteria. Hymenoptaecin is a glycine-rich small protein (10 kDa) with bactericidal activity for Gram negative and Gram positive bacteria.

In addition to the production of antibacterial peptides, the fat body of infected insects synthesizes cyclic molecules with antifungal activity and other immune entities of activity directed against both bacteria and fungi (BULET et al., 1996; GLIŃSKI and JAROSZ, 1998).

Two cyclic antifungal peptides have been characterized so far, drosomycin from the fruitfly *Drosophila melanogaster* and thanatin from the bug *Podisus maculiventris.* Both peptides have a potent activity against phytopathogenic and human pathogenic filamentous fungi (FEHLBAUM et al., 1994). Drosomycin of 44 amino acid residues with 8 cysteines engaged in the formation of four intramolecular disulfide bridges exhibits activity against a wide range of phytopathogenic and human pathogenic fungi, but it is inactive against bacteria (FLYG et al., 1987). Thanatin, a 21 residue inductible immune peptide with a single disulfide bridge forming a C-terminal loop of 8 residues, is active against Gram positive and Gram negative bacteria and against phytopathogens and fungous human invaders (BULET et al., 1996). At least, two inductible proline-rich peptides metalnikowins from *Palomera prasina* and *Drosophila* (BULET et al., 1996) and mietchnikowins from *Drosophila melanogaster* (LEVASCHINA et al., 1995) act both on bacteria and fungi.

Concluding comments

Obviously, the haemocyte mediated defense mechanisms, lysozyme and the inducible immune peptides offer the honey bee a very impressive set of mechanisms protecting well the insect against bacterial invaders. Usually, these defense responses have a broad activity spectrum directed against a large variety of bacteria. Phagocytosis and encapsulation are a key element in the immune defense of the honey bee to fungal infections. Up to now, neither antifungal immune peptides such as drosomycin in fruitfly (*Drosophila melanogaster*), thanatin in the bug (*Podissus maculiventris*) nor mietchnikowins and metalnikowins that exhibit activity against both bacteria and fungi have been found in the honey bee defence against *Ascosphaera apis* and aspergilli. Under the above circumstances it is reasonable to conclude that hygienic behaviour, antimicrobial entities secreted by workers and protective barriers of the body coverings form the effective threshold protecting bee's organism against mycotic invasions. Finally, the protection of bees to mycotic diseases is realized by the neural-immune-endocrine network.

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