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ABBREVIATIONS

AMPs	antimicrobial peptides
CCF	coelomic cytolytic factor
CHAPS	3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate
CPC	cetylpyridinium chloride
EALys	lysozyme of *Eisenia andrei*
EFAF	*Eisenia fetida andrei* factor
ER	endoplasmic reticulum
GlcNAc	*N*-acetylglucosamin
L-DOPA	L-β-3,4-dihydroxyphenylalanine
LPS	lipopolysaccharide
NAM	*N*-acetylmuramic acid
NF-κB	nuclear factor-κB
NO	nitric oxide
PAMPs	pathogen associated molecular patterns
PCR	polymerase chain reaction
PECs	peritoneal exudate cells
PO	phenoloxidase
PRR	pattern recognition-receptors
RACE	rapid amplification of cDNA ends
RT	reverse transcription
SDS	sodium dodecyl sulphate
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor

1. INTRODUCTION

The invertebrates are heterogeneous group of animals that remain relatively poorly understood from the immunological point of view in spite of the fact, that they have been an important experimental model since the very beginning of immunology.

All of the 30 major lineages of animals that we formally recognize with a phylum name, with one exception, belong exclusively to invertebrates. However, the one exception, the Chordata, is also partly comprised of invertebrates. Invertebrates often survive in very hostile environments, despite of lacking anticipatory, specific and lymphocyte-based immune system capable of clonal expansion, production of antibodies and memory. Their successful survival strategies are often based on a short life span combined with numerous offspring. Invertebrates also possess various physical and chemical barriers including mucus surrounding the body of many coelenterates, annelids, mollusks and protochordates or exoskeleton of coelenterates, molluscs, arthropods, echinoderms and protochordates. More importantly, invertebrates have developed a variety of defense mechanisms efficiently recognizing and responding to non-self substances including pattern recognition-receptors (PRRs), antimicrobial peptides (AMPs), RNA interference (RNAi), phagocytic cells, production of toxic oxygen and nitrogen metabolites and melanization pathway.

In the term of systematic biology, the earthworm family (Lumbricidae) is the largest member of the class Oligochaeta, phylum Annelida. There are over 3000 described earthworm species known worldwide and adapted to a wide range of soil habitats as well as freshwater lakes and streams. The earthworms are found in leaf litter, under stones and logs as well as in some arid areas, but most species prefer wetter, more heavily vegetated regions. Earthworms range in size from several millimeters to over one meter.

The earthworms are protostomian animals possessing a true coelomic cavity of mesenchymal origin. This cavity if filled with coelomic fluid containing free coelomocytes derived from the mesenchymal lining of the cavity. The coelomic cavity is metameric and the segments are separated by transversal septa. Each segment of the coelomic cavity is opened to the outer environment by a pair

of nephridia and by a dorsal pore. Annelids generally have a closed circulatory system, consisting of dorsal and ventral vessels and connecting capillaries. Earthworms are hermaphroditic, but two worms are required to mate and reproduce. The reproductive organs are in the clitellum, where the cocoons are formed to protect developing eggs.

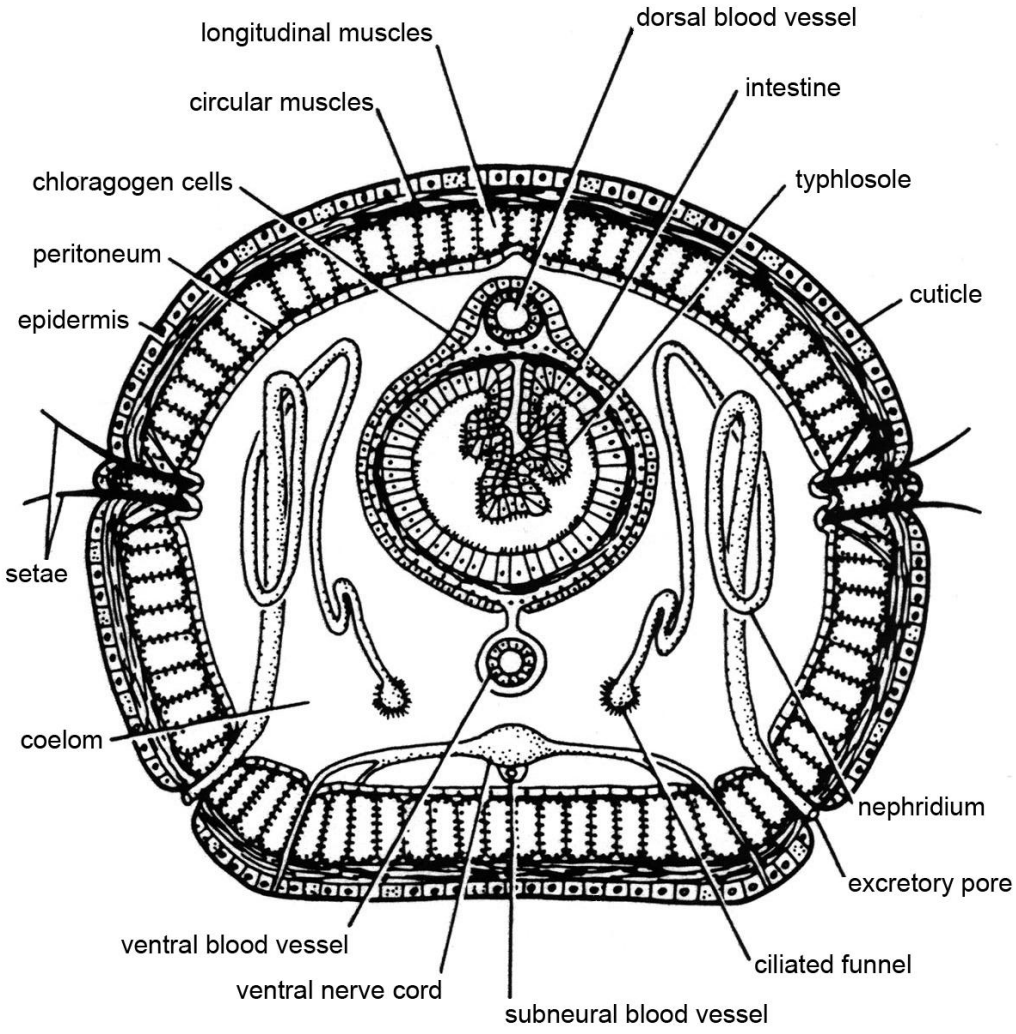

Fig. 1: Earthworm cross section (adopted from Biology of the Invertebrates, Pechenik, 1996).

The earthworms are beneficial in many different ways – they are important agents for the maintenance of soil fertility, they act as indicators of environmental quality and moreover they might be considered as a source of biologically active compounds with potential industrial and medical use. Actually, earthworm powder has been used in the Far East for thousands years as a traditional medicine to treat

various diseases. Currently, the therapeutic effect of earthworm active factors is being evaluated by a modern scientific approach. Some therapeutics containing fibrinolytic enzymes from *Lumbricus rubellus* earthworms (Mihara et al., 1991; Mihara et al., 1992; Nakajima et al., 1996; Nakajima et al., 2000) are already commercially available to support coagulation and fibrinolysis balance in the body and thus prevent or treat cardiac and cerebrovascular diseases (Boluoke® (lumbrokinase), Canada RNA Biochemical Inc.).

The earthworms are regarded as a model of comparative immunology since early 1960s when transplantation experiments were performed. American and French scientists reported that autologous transplants of earthworm body wall pieces were accepted in contrast to transplants between individual of another or even the same earthworm species (for review see (Cooper and Roch, 1994)). Moreover, a cell-mediated short-term memory was observed (Bailey et al., 1971). All these experiments proved the existence of self and non-self recognition in earthworms and initiated extensive studies of earthworm immune mechanisms.

2. DEFENSE OF EARTHWORMS

2.1. General defense mechanisms of earthworms

The first protective barrier of earthworms is represented by skin consisting of the epidermis and thin cuticle covering the entire body. The epidermis is formed by a single-layer epithelium of supporting cells, basal cells with an important role in wound healing and graft rejection and secretory cells secreting mucus containing mucopolysaccharide-lipid-protein complex (Alves et al., 1984; Bernaldo de Quiros and Benito, 1986) serving as a lubricant during locomotion and containing several antimicrobial factors (Valembois et al., 1984; Valembois et al., 1986). The cuticle contains mucopolysaccharides acting as an antimicrobial barrier (Rahemtulla and Lovtrup, 1974, 1975).

However, the coelomic fluid is not aseptic. The microorganisms passing through the epidermal barrier can enter the coelomic cavity mainly by the dorsal pores, but their growth is kept under control by various mechanisms. First, they can be excreted by nephridia (Cameron, 1932) or engulfed by cells of nephrostome or middle tube (Villaro et al., 1985). Second, the microorganisms

can be phagocytosed by free coelomocytes (see below) and the exhausted phagocytic cells are expelled through dorsal pores equipped with muscular sphincters controlling the exchange of material between the outer environment and the coelom (Cameron, 1932). Third, large foreign bodies, e.g. agglutinated bacteria or parasites are eliminated by encapsulation (Ratcliffe et al., 1985). This process begins by the recognition of foreign material, which cannot be due to its size engulfed. Within the first day, the foreign body is surrounded by free coelomocytes and after several days a dense capsule (often called brown body because of its melanin content) composed of flattened cells is formed. When the capsule is about 1-2 mm in diameter, its external cells lose its adhesiveness and consequently the capsule can migrate towards the posterior segments of the coelomic cavity where it is often eliminated by autotomy of caudal segments followed by wound healing (Keilin, 1925; Alonso-Bedate and Sequeros, 1985). It was documented in *E. fetida* earthworms that most brown bodies contain tissue wastes, agglutinated bacteria, gregarines or nematoda (Valembois et al., 1992).

2.2. Cellular defense mechanisms

The coelomic fluid of earthworms contains different types of cells named generally coelomocytes. The nomenclature of coelomocytes is based mainly on morphological and cytochemical criteria (Stein et al., 1977; Sima, 1994). Free chloragogen cells (eleocytes) have mainly accessory and nutritive functions and hyaline or granular amoebocytes represent effector immunocytes with strong phagocytic activity. However, this activity differs in both types of amoebocytes.

2.2.1. Phagocytosis

Phagocytosis represents an important defense mechanism in earthworms. Though the phagocytic properties of coelomocytes have been already mentioned by Metchnikoff in 1887, the first detailed description of this process was published by Cameron who described phagocytosis of numerous inert particles, cells and bacteria *in vitro* and *in vivo* (Cameron, 1932). His results were later confirmed in reports describing the uptake of bacteria (Dales and Kalac, 1992), yeast (Stein and Cooper, 1981), erythrocytes (Laulan et al., 1988) and inert particles (Stein et al., 1977; Bilej et al., 1990).

Phagocytosis begins with the recognition of non-self, which is followed by the engulfment and destruction of the particles. This activity, similarly to that of vertebrates, can be modulated by humoral components, opsonins, which coat the engulfed particle and thus promote its phagocytosis. It was proven that preincubation of both yeast and synthetic 2-hydroxyethylmethacrylate copolymer (HEMA) particles with the coelomic fluid significantly increased their engulfment (Bilej et al., 1990). Besides Laulan and his colleges described, that also mammalian opsonins, IgG immunoglobulin and C3b complement fragment enhance coelomocyte phagocytic activity, in contrast to IgM and C3d fragment, which did not affect this process (Laulan et al., 1988).

Engulfed material can be eliminated by several mechanisms – either by proteolytic and lysosomal enzymes or by an oxidative burst, which involves the production of highly reactive oxygen radicals. Oxygen radicals were detected both in the coelomic fluid and in chloragosomes of chloragogen cells (Valembois et al., 1991). However, they were not detected during the phagocytosis of inert synthetic particles (Bilej et al., 1991) suggesting the possible discrimination between antigenic and non-antigenic structures (Tuckova and Bilej, 1996).

The exhausted phagocytic cells can be eliminated by expulsion via dorsal pores, excretion by nephridia or by the encapsulation.

2.2.2. Transplantation immunity

The ability to recognize and respond to allografts as well as xenografts and, on the other hand, the ability to accept or not to destroy autografts was observed in some annelid species. This process begins like the reaction to injury. The first major change, which occurs after the healing of wound is, regardless on the graft origin, the accumulation of coelomocytes near the graft sites and their infiltration into the matrix. The response to the xenografts results in complete walling off the graft and its destruction by encapsulation reaction (Parry, 1978). The number of invading coelomocytes during the autograft transplantation is markedly lower (Cooper, 1970; Hostetter and Cooper, 1973) but the reaction seems to be more rapid. The maximum number of coelomocytes surrounding the allograft was detected within 24 hours, returning to the normal level by 72 hours. In contrast, the peak response to xenograft was observed on day 3 or 4 and normal levels are not reached

before day 7. The destruction of xenografts is completed approximately by day 17 after transplantation. If a second graft is transplanted at this time, an accelerated rejection within 6 or 7 days occurs. Moreover, the number of invading coelomocytes is 20 – 30 % higher. The increased number of coelomocytes during the re-transplantation is probably caused by an increased proliferating activity of mesenchymal lining of the coelomic cavity and the septa. These data suggest the existence of short-term and very limited memory which is based solely on cells as the transfer of either the coelomic fluid or other substances does not induce any accelerated reaction (Bailey et al., 1971; Hostetter and Cooper, 1973).

2.3. Humoral defense mechanisms

The coelomic fluid of annelids exhibits numerous biological activities involved in the defense mechanisms against invaders. It was documented that it contains various antimicrobial factors like lysozyme (Çotuk and Dales, 1984) and antimicrobial peptides (Cho et al., 1998; Wang et al., 2003; Liu et al., 2004; Tasiemski et al., 2007). Among the factors involved in *E. fetida* humoral immunity, particular attention has been devoted to cytolytic components secreted by coelomocytes into the coelomic cavity. The cytolytic activity of the coelomic fluid was originally demonstrated on vertebrate erythrocytes and the resulting effect was referred as hemolysis. The majority of the hemolysins identified so far show hemagglutination activity and, more interestingly, a spectrum of antibacterial and/or bacteriostatic activities against pathogenic soil bacteria (Valembois et al., 1986; Roch et al., 1991). Furthermore, it was observed that coelomic fluid lyses also some other eukaryotic cells than erythrocytes, namely chicken fibroblasts, guinea-pig polymorphonuclear cells, insect hemocytes and various tumor cell lines (Kauschke and Mohrig, 1987a; Bilej et al., 1995; Cooper et al., 1995). Hereinafter, individual components of humoral defense of coelomic fluid are described in detail.

2.3.1. Lysozyme

Lysozyme is a bacteriolytic enzyme that possesses the hydrolytic activity to specifically cleave β-1,4-glycosidic bonds between *N*-acetylglucosamin (GlcNAc) and *N*-acetylmuramic acid (NAM) of the peptidoglycan present in the bacterial cell

walls. Lysozyme thus efficiently protects its host against infections caused by Gram-positive bacteria (Jolles, 1996).

Lysozyme is a ubiquitous enzyme widely distributed within the animal and plant kingdoms (Jolles, 1996). Based on the differences in their structure, catalytic character and original source, lysozymes are classified into six groups: chicken-type lysozyme (c-lysozyme) present in many vertebrates and insects is the most extensively studied lysozyme, goose-type lysozyme (g-lysozyme) identified in many vertebrates including mammals, birds and fish (for review see Prager and Jolles (Prager and Jolles, 1996)), bacterial lysozyme (Holtje, 1996), phage lysozyme (Fastrez, 1996), plant lysozyme (Beintema and Terwisscha van Scheltinga, 1996) and cysteine–rich invertebrate type lysozyme (i-lysozyme). The existence of i-type lysozyme was proposed as early as in 1975 (Jolles and Jolles, 1975). Later on, i-lysozymes in various invertebrates were identified (Nilsen et al., 1999; Zavalova et al., 2000; Takeshita et al., 2003). The alignment and phylogenetic analyses using six bivalve lysozymes suggested that i-type lysozymes form a monophyletic family (Bachali et al., 2002).

Furthermore, it was described that coelomic fluid and the extract of coelomocytes of *E. fetida* earthworm contain a substance exhibiting lysozyme activity. Such an activity was reported to be low under normal conditions; however it was shown that the enzyme was released from coelomocytes following microbial infection as a part of defense mechanisms (Çotuk and Dales, 1984). Therefore, Köhlerova *et al.* investigated the modulation of lysozyme activity in *E. fetida* challenged with Gram-positive as well as Gram-negative bacteria. However, no major differences in the lysozyme activity in Gram-positive and Gram-negative bacteria infected worms were observed (Köhlerova et al., 2004). The lysozyme of *E. fetida* was purified from the homogenate of whole earthworm and the N-terminal sequence was determined (Ito et al., 1999). The sequence revealed a considerable homology with lysozyme from molluscs, echinoderms and the nematode *Coenorhabditis elegans*, while the homology to other known types of lysozymes was negligable.

2.3.2. Antimicrobial peptides

Antimicrobial peptides have been reported to be involved in killing of microorganisms in both invertebrates and vertebrates. AMPs are abundant and diverse group of molecules produced by many tissues and cell types in a variety of plant, invertebrate and vertebrate species. Their amino acid composition, amphipathicity, cationic charge and size allow them to attach to and insert into membrane bilayers to form pores (Christensen et al., 1988; Lockey and Ourth, 1996). AMPs are generally considered to kill their microbial targets through insertion and damage/permeabilization of the cytoplasmic membranes of target cells (Jelinek and Kolusheva, 2005). However, recent observations suggest that a number of defense peptides may also interact with intracellular targets such as DNA and RNA, presumably interfering with their metabolic functions and thus leading to cell death (Brogden, 2005; Hale and Hancock, 2007). They can alter cytoplasmic membrane septum formation, inhibit cell wall synthesis; inhibit nucleic acid and protein synthesis; or inhibit enzymatic activity (Brogden, 2005).

Most AMPs of invertebrates were found in arthropods and only a limited number of bioactive peptides have been described in annelids until now. The antimicrobial peptide named lumbricin I was identified in *Lumbricus rubellus* (Cho et al., 1998). Lumbricin I is a proline-rich AMP showing *in vitro* antimicrobial activity against a broad spectrum of microorganisms without hemolytic activity. Furthermore, lumbricin I is constitutively expressed in adult animals and is not induced by bacterial infection.

A lumbricin I analog named PP-1 was found in the Asian earthworm *Pheretima tschiliensis* (Wang et al., 2003). PP-1 is synthesized only in the body wall and its localization in the mucus of the epidermis suggests its role in the mucosal defense. Furthermore, an antimicrobial short peptide OEP3121 of only five amino acids was found in *E. fetida* earthworm (Liu et al., 2004). In the marine polychaeta annelid *Arenicola marina*, two isoforms of AMPs arenicin containing a single disulfide bridge were isolated from coelomocytes and their membrane activity and possible selectivity toward bacterial cells was suggested (Ovchinnikova et al., 2004; Ovchinnikova et al., 2007; Ovchinnikova et al., 2008).

Recently, the AMP named hedistin was identified in the marine annelid *Nereis diversicolor* (Tasiemski et al., 2007). Hedistin is constitutively expressed

in circulating NK-like cells and the antimicrobial effect against a large spectrum of bacteria was shown. The primary sequence of hedistin includes in addition to C-terminal amidation increasing the net cationic charge and thus the electrostatic attraction to target membrane (Tossi et al., 2000; Shai, 2002) also bromotryptophan residues typical for marine organisms (Taylor et al., 2000; Shinnar et al., 2003; Uzzell et al., 2003). The presence of bromotryptophan could be the result of the adaptation of an enzymatic system in a specialized antimicrobial defense.

2.3.3. Coelomic cytolytic factor

A 42-kDa lectin named coelomic cytolytic factor (CCF) was isolated from the coelomic fluid of the earthworm *E. fetida* on the basis of experiments with tumor necrosis factor (TNF)-sensitive tumor L929 cell line (Bilej et al., 1995).

CCF acts in earthworm defense as a pattern recognition molecule (Beschin et al., 1998; Bilej et al., 2001). CCF binds, via lectin-like interactions, cell wall components of Gram-negative bacteria (O-antigen of lipopolysaccharide), Gram-positive bacteria (the peptidoglycan constituents muramic acid and muramyl dipeptide) and yeast (β-1,3-glucans and *N,N´*-diacetylchitobiose). Upon binding these microbial pathogen-associated molecular patterns (PAMPs), CCF triggers the activation of prophenoloxidase cascade (chapter 2.3.4), an important invertebrate defense mechanism resulting in the production of melanin (Johansson and Söderhäll, 1996; Beschin et al., 1998; Cerenius and Söderhäll, 2004).

Further, it was shown that CCF agglutinates both Gram-positive and Gram-negative bacteria (Beschin et al., 1998) and contributes to the opsonizing properties of the coelomic fluid, thereby providing an efficient mechanism for phagocytosis in earthworm defense reaction (Bilej et al., 1995). CCF is also involved in the cell-mediated cytotoxic reactions (Bilej et al., 1998) and potentiates the lytic activity of coelomic fluid against red blood cells (Bilej et al., 2000).

The broad recognition specifity of *E. fetida* CCF is based on the presence of two distinct lectin-like domains (Fig. 2) (Bilej et al., 2001). The first domain, localized in the central part of the molecule, has a homology with the polysaccharide-binding motif and glucanase motif of β-1,3-glucanases and other invertebrate defense molecules. This domain is implicated in interactions with

lipopolysaccharide and β-1,3-glucans. The C-terminal tryptophan-rich domain interacts with *N,N′*-diacetylchitobiose and peptidoglycan constituents.

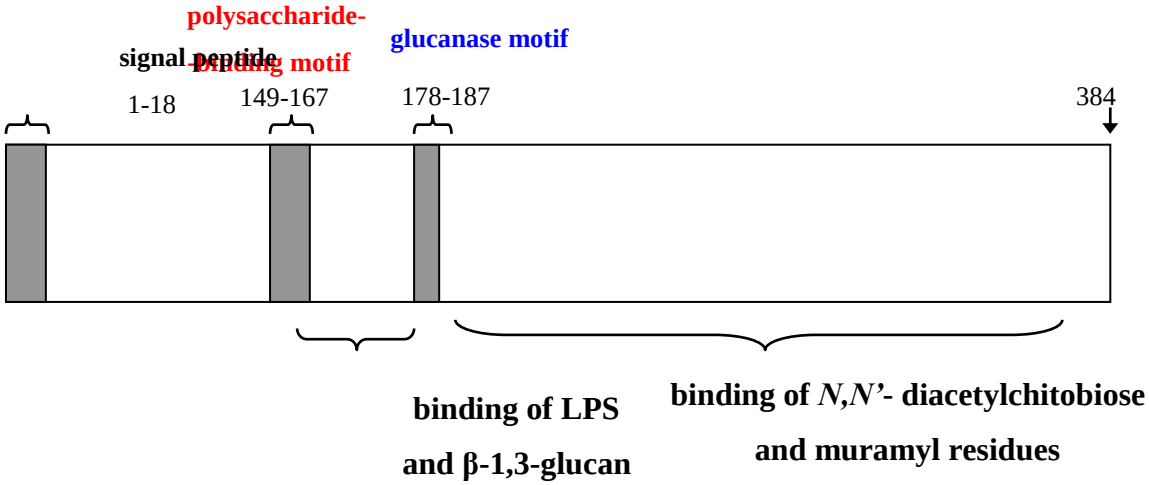

Fig. 2: Structure of *E. fetida* CCF.

CCF was originally described as a cytolytic protein that lyses TNF-sensitive tumor L929 cell line in protease-independent way and shows other functional analogies with the mammalian cytokine TNF. CCF is secreted by macrophage-like coelomocytes upon lipopolysaccharide stimulation (Bilej et al., 1998) while TNF is produced by macrophages (Aggarwal et al., 1985). Moreover, CCF is involved in the opsonizing properties of the earthworm coelomic fluid and similarly, TNF was reported to provide opsonin-like signals that mediate the attachment of bacteria to macrophages (Luo et al., 1993; Bilej et al., 1995). Both proteins bind *N,N′*-diacetylchitobiose and β-1,3-glucans via lectin-like interactions (Olson et al., 1996; Beschin et al., 1998). In addition, monoclonal antibodies elicited against lectin-like domain of TNF cross-react with CCF and, conversely, monoclonal antibody against CCF reacts with TNF without impairing the interaction of TNF with its receptor (Lucas et al., 1994; Magez et al., 1997). However, the lytic activity of CCF is not inhibited by anti-TNF antibody suggesting different mechanisms of TNF- and CCF-mediated lysis.

The *N,N′*-diacetylchitobiose lectin-like domain of TNF is involved in the killing of African and American trypanosomes (Lucas et al., 1994; Magez et al., 1997; Olivares Fontt et al., 1998) and so is the purified CCF as well

as coelomic fluid of *E. fetida* earthworms. This lytic effect of coelomic fluid and purified CCF on African *Trypanosoma brucei brucei* was completely inhibited in the presence of *N,N′*-diacetylchitobiose, anti-CCF monoclonal antibody and antibody against lectin-like domain of TNF (Beschin et al., 1999). In the case of American *Trypanosoma cruzi* the lytic activity was inhibited partially (Olivares Fontt et al., 2002).

Furthermore, the capacity of lectin-like domain of TNF to induce a pH-dependent increase of membrane conductance resulting in membrane depolarization has been demonstrated in peritoneal macrophages and lung microvascular endothelial cells (Hribar et al., 1999). It was suggested that this effect is due to the interaction of lectin-like domain with amiloride-sensitive sodium ion channels (Van der Goot et al., 1999; Fukuda et al., 2001). Similarly, CCF was shown to activate amiloride-sensitive ion channels in lung endothelial cells and peritoneal macrophages via its C-terminal lectin-like domain (Bloc et al., 2002).

Importantly, despite their functional analogies, CCF and TNF have neither gene nor amino acid sequence homology, which indicates their different evolutionary origin (Beschin et al., 1999).

2.3.4. Prophenoloxidase cascade

The prophenoloxidase cascade (Fig.3) represents one of the most important defense mechanism in many invertebrates. Following the recognition of microbial cell wall components such as lipopolysaccharide, peptidoglycan and β-1,3-glucans, proteinases cleave inactive prophenoloxidase (proPO) into its active form, phenoloxidase (PO) (Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004). Conversion of proPO to its active state is achieved by proteolytic cleavage that depends on a cascade of serine proteinases, so called prophenoloxidase-activating enzymes, and other factors. The resulting PO catalyses both the *o*-hydroxylation of monophenols and the oxidation of diphenols to quinones. Consequently the quinones non-enzymatically polymerize to melanin (Ashida and Yamazaki, 1990; Söderhäll et al., 1994). Melanin, as a final product of a proPO cascade, has fungistatic, bacteriostatic and antiviral properties and together with the intermediates is involved in the innate immune response of certain invertebrates, especially

arthropods. Melanin also serves as a structural component in wound healing and is important for encapsulation of foreign materials.

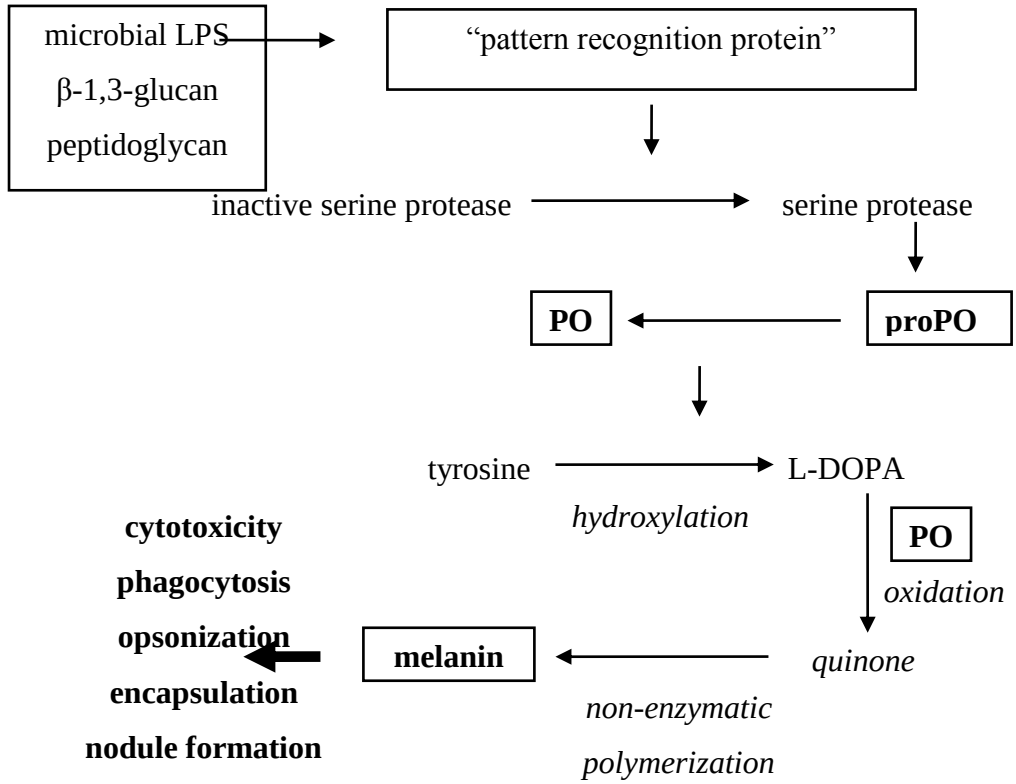

Fig. 3: Prophenoloxidase activating cascade. The recognition of PAMPs leads to the cleavage of inactive prophenoloxidase to its active state phenoloxidase. Active enzyme catalyzes both hydroxylation and oxidation of phenols to quinones, which are subsequently polymerized to melanin. Melanin exerts various biological activities important in defense reactions.

ProPO as well as PO have been isolated and characterized in many invertebrate species. Their molecular weight varies between 70 and 90 kDa and all of the enzymes contain two functional copper-binding sites. The primary structure of these sites is highly conserved among invertebrates as well as the peptide motif GCGEQNM, which is present in α2-macroglobulins in both invertebrates and vertebrates (Spycher et al., 1987; Hall et al., 1989), in the vertebrate complement proteins C3 and C4 (Dodds and Law, 1998) and in the complement-related proteins of some invertebrates (Marino et al., 2002; Dishaw et al., 2005).

The finding of phenoloxidase activity in the coelomic fluid of *E. fetida* proved the presence of phenoloxidase cascade in annelids (Seymour et al., 1993; Beschin et al., 1998). It was documented that proPO cascade of *E. fetida* is directly activated by Gram-negative bacteria and yeast, while Gram-positive bacteria have to be preincubated with lysozyme to activate the cascade (Bilej et al., 2001). As mentioned above, it was documented that the activation of proPO cascade is triggered by pattern recognition receptor present in the coelomic fluid of *E. fetida*, by CCF, upon the recognition of microbial PAMPs (Beschin et al., 1998; Bilej et al., 2001).

2.3.5. Hemolytic molecules

The coelomic fluid of *E. fetida* earthworms exhibits numerous biological activities including hemolytic activity. The majority of the proteins with hemolytic activity identified so far show bactericidal and/or bactreriostatic activities against pathogenic soil bacteria (Valembois et al., 1986; Roch et al., 1991). Therefore, the biological relevance of the *E. fetida* cytolytic and agglutinating system consists partly in growth inhibition of the potential pathogens living in manure and possessing antigens common with red blood cells.

First described hemolytic proteins isolated from coelomic fluid were characterized as 40 and 45 kDa glycoproteins secreted by chloragocytes and eleocytes and named EFAF (*Eisenia fetida andrei* factor), that differ in the extent of glycosylation (Roch et al., 1981) and constitute a polymorphic system. 45 kDa protein was shown to be encoded by one nonpolymorphic gene, while 40 kDa protein is encoded by a gene having four alleles representing four isoforms with pI of 6.3, 6.2, 5.95 and 5.9. Each individual earthworm possesses either two or three isoforms of the 40 kDa molecule (Roch, 1979; Roch et al., 1987). In addition to hemolytic activity, these molecules also agglutinate red blood cells (Valembois et al., 1984) and may participate in the cytotoxic activity of the coelomic fluid (Kauschke and Mohrig, 1987b).

Later, the EFAFs were characterized at the molecular level and named fetidins. Their amino acid sequence comprises N-glycosylation site and a peroxidase motif. Accordingly, fetidins display peroxidase activity. Fetidins polymerize upon binding sphingomyelin, which is a major lipid constituent of plasma membrane

of most mammalian cells, generating 10 - nm open channels trough the lipid bilayer (Roch et al., 1989). Moreover, they display antibacterial activity against both Gram-positive and Gram-negative bacteria (Hirigoyenberry et al., 1992), particularly on strains pathogenic for earthworms (Valembois et al., 1986; Roch et al., 1991). In addition to antibacterial activity, fetidins may also mediate opsonization (Sinkora et al., 1993), and they contribute to the clotting of the coelomic fluid (Valembois et al., 1988).

Independently, a 41 kDa hemolytic protein, which is produced by large coelomocytes and causes the contraction of rat vascular smooth muscles, was characterized and named lysenin. Simultaneously, two 42 kDa lysenin-related proteins with weak contraction activity were identified (Sekizawa et al., 1996; Sekizawa et al., 1997). Lysenin displays a high amino acid sequence homology with lysenin-related proteins and moreover with fetidins, suggesting a close relationship between these hemolytic molecules. Lysenin specifically recognizes sphingomyelin in the target membranes, forms oligomers and subsequently pores of about 3 nm in diameter (Yamaji et al., 1998; Yamaji-Hasegawa et al., 2003).

At the same time, another 38 kDa hemolytic protein named eiseniapore was identified in the coelomic fluid of *E. fetida* earthworm. It was shown that eiseniapore functionally requires sphingomyelin or galactosylceramide to bind and lyse erythrocytes (Lange et al., 1997). Eiseniapore was documented to form hexamers, which induce the formation of pores with inner and outer diameters of 3 nm and 10nm, respectively (Lassalle et al., 1993; Lange et al., 1997). Eiseniapore seems to be associated in the coelomic fluid with a natural inhibitor named eiseniapore-regulating protein (Mohrig et al., 1997).

Eue et al. described three hemolytic proteins in the coelomic fluid of *E. fetida*, H_1 (46 kDa), H_2 (43 kDa) and H_3 (40 kDa). Each hemolysin consists of several isoforms with pI between 5.1 and 6.2 and they share structural similarities. Despite the cross-reactivity of the monospecific antisera, hemolysins functionally differ. H3 was demonstrated to be a bifunctional protein that can lyse and agglutinate erythrocytes (Eue et al., 1998). Later on, Koenig et al. isolated and analyzed two hemolytic proteins from cell lysate (CL_{39} and CL_{41}) and three hemolytic proteins from coelomic fluid (H_1, H_2 and H_3) of *E. fetida*. Using mass spectrometry and bioinformatics the identity of CL_{39} with fetidin and CL_{41} with lysenin was

demonstrated. Moreover, it was described that H_{1-3} proteins share sequence components with fetidin, but they seem to be glycosylated (Koenig et al., 2003).

2.3.6. Calreticulin

Calreticulin is a highly conserved calcium-binding protein affecting many cellular processes inside and outside of the endoplasmic reticulum (ER). It participates in the regulation of Ca^{2+} homeostasis, acts as a chaperone and modulates gene transcription, integrin-mediated cell signalling as well as cell adhesion. Moreover, calreticulin was shown to be important for defense mechanisms. It is required for stress response (Park et al., 2001; Goo et al., 2005) and is involved in phagocytosis (Asgari and Schmidt, 2003; Kuraishi et al., 2007).

Calreticulin was first isolated from sarcoplasmic reticulum of rabbit muscle (Ostwald and MacLennan, 1974) and the isolation of cDNA followed (Fliegel et al., 1989; Smith and Koch, 1989). Later, it was identified in ER of variety of vertebrates, invertebrates and higher plants (Michalak et al., 1998). It is a 46 kDa protein containing three structural and functional domains – a highly conserved globular N-domain, central proline-rich P-domain which binds Ca^{2+} with high affinity and an acidic C-domain containing high-capacity Ca^{2+}-binding site (Baksh and Michalak, 1991).

More interestingly, Kauschke *et al.* found peptides homologous to calreticulin in the perforin-like lytic fraction of coelomocyte lysate of *E. fetida* earthworms (Kauschke et al., 2001).

3. AIMS

The general aim of this thesis was to characterize various factors involved in innate immunity of earthworms. The defense molecules chosen for our experiments were described in detail on a molecular level. Besides, the functional role of these molecules in earthworm immunity and/or their modulation during immune response was examined.

1. Lysozyme

Lysozyme is a ubiquitous enzyme described in many organisms catalyzing the hydrolysis of the β-1,4-glycosidic bonds present in peptidoglycan of the bacterial cell walls and thus protecting hosts against infection from invading microorganisms. Both the lysozyme activity of the coelomic fluid and the N-terminal sequence of the lysozyme isolated from coelomocyte extract were described previously. However, the complete sequence of any earthworm lysozyme was not determined until now. Therefore we wanted **to characterize the lysozyme of *E. andrei* (formerly *E. fetida andrei*) earthworm both structurally and functionally. Molecular characterization of lysozyme provides a new tool for monitoring of innate immunity in earthworms.**

2. CCF

Previously, it was described that coelomic fluid of *E. fetida* earthworm contains the pattern-recognition protein named coelomic cytolytic factor (CCF) recognizing the components of microbial cell walls. Upon binding these PAMPs, CCF triggers the activation of the prophenoloxidase defense cascade, resulting in the production of antimicrobial and cytotoxic molecules.

CCF was shown to be present also in coelomic fluid of another earthworm species, *Lumbricus terrestris*. Therefore **we decided to elucidate the presence of CCF-like molecules also in other earthworms from *Lumbricidae* family and, potentially, to characterize their primary sequence and to compare the biological properties of these molecules.**

CCF was described to share functional analogies based on the similar saccharide recognition specificity with mammalian cytokine, tumor necrosis factor (TNF). Both TNF and CCF were shown to induce an increase of membrane

conductance in some mammalian cells resulting in membrane depolarization. **We investigated whether the CCF interaction with peritoneal macrophages and subsequent membrane depolarization results in the activation of these cells.**

3. Calreticulin

Calreticulin is a molecule participating in many cellular processes – Ca^{2+} homeostasis, chaperoning function, modulation of gene transcription and so on. Moreover, the role of calreticulin in the defense mechanisms was described in both vertebrates and invertebrates. Therefore we wanted **to prove the presence of calreticulin in the coleomic fluid of *E. fetida* earthworms, characterize the primary sequence and determine the expression in different organs. In addition, we attempted to show the phylogenetic relationship of this molecule.**

4. Hemolytic molecules

The coelomic fluid of the earthworms has been reported to contain a variety of proteins causing the lysis of red blood cells. All hemolytic molecules described so far share biochemical analogies, possess similar molecular mass, pI and ability to bind sphingomyelin and to form pores in lipid membranes. Therefore **we attempted to clarify the relationship between two of these molecules – lysenin and fetidin and to determine the level of their expression in coelomocytes of individual *E. fetida* earthworms.**

5. Prophenoloxidase

The prophenoloxidase-activating system is a sensitive non-self recognizing cascade triggered by components of microbial cell walls. This cascade is one of the most important defense mechanism in many invertebrates. The finding of prophenoloxidase activity in the coelomic fluid of *E. fetida* suggests the presence of prophenoloxidase cascade in annelids. However, no direct evidence has been demonstrated. Hence **we wanted to prove the presence of the prophenoloxidase cascade as a defense mechanism in *E. fetida* earthworms.**

4. RESULTS

4.1. Characterization of lysozyme from *E. andrei* earthworms

Lysozyme is a ubiquitous enzyme widely distributed within the animal and plant kingdoms. It possesses the hydrolytic activity to specifically cleave β-1,4-glycosidic bonds between *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (NAM) of the peptidoglycan present in the bacterial cell walls and thus efficiently protects the host against infections caused by Gram-positive bacteria (Jolles, 1996).

The lysozyme activity of coelomic fluid of *Eisenia* earthworms was first reported more than twenty years ago (Çotuk and Dales, 1984). Moreover, Ito et al. determined the N-terminal sequence of *E. fetida* lysozyme and showed its high homology with other known invertebrate lysozymes (Ito et al., 1999). However, the complete sequence of any earthworm lysozyme has not been determined until now.

In our study, we report on structural and functional characterization of lysozyme of *E. andrei* (formerly *E. fetida andrei*) earthworms (EALys).

Lysozyme cDNA fragment of *E. andrei* was initially isolated by PCR amplification using degenerated primers designed on the basis of conserved regions of known invertebrate lysozyme sequences. The resulting fragment of about 230 bp was cloned and subsequent sequencing revealed a high homology with other described i-type lysozymes. In order to assemble the full length cDNA sequence, rapid amplifications of the 5´and 3´ cDNA ends were performed. The full length cDNA of EALys has an open reading frame coding 160 amino acids. The primary sequence of the protein contains twelve conserved cystein residues, three amino acid residues potentially important for the lysozyme activity and five amino acid residues probably involved in substrate fixation. In addition, the N-terminal 16 amino acid residues long signal peptide was predicted.

Further characterization of EALys was based on successful expression of recombinant *E. andrei* lysozyme (rEALys). We documented that rEALys possesses, in addition to its lysozyme activity, also isopeptidase activity (the hydrolysis of ε-(γ-Glu)-Lys cross-links between Glu and Lys in a stabilized fibrin and peptidoglycan in bacterial cell walls). We evaluated the influence of pH, ionic strength and temperature on both lysozyme and isopeptidase activity.

The difference in optimum pH for lysozyme and isopeptidase activity of rEALys was observed. This finding may indicate that these activities are executed by independent active sites within the molecule. Regarding the influence of ionic strength for lysozyme activity, the optimum activity was achieved at approximately I=0.01, while no effect of ionic strength was observed on isopeptidase activity. Furthermore, both isopeptidase and lysozyme activity reach their maximum at 37 ºC. Notably, the kinetics of lysozyme and isopeptidase activity was also different. The lysozyme activity was detected almost immediately and the maximum was reached within 30 minutes, the isopeptidase activity was very slow – the maximum was reached only after 48 hours.

In order to evaluate the changes of mRNA levels in the coelomocytes of earthworms infected with *Bacillus subtilis* or *Escherichia coli*, we followed the EALys gene expression up to two days after bacterial challenge and compared the mRNA levels of infected and uninfected worms. The results suggest the involvement of EALys in protection against infections caused not only by Gram-positive, but also by Gram-negative bacteria.

To sum up, we proved the presence of lysozyme in *E. andrei* earthworm and identified it as a novel lysozyme belonging to invertebrate type family. Further, we characterized this molecule both structurally and functionally. Our data suggest that both enzymatic activities described in EALys might play a significant role in the defense against both Gram-positive and Gram-negative bacteria.

Josková R., Šilerová M., Procházková P., Bilej M.: Structural and functional characterization of a novel invertebrate-type lysozyme from *Eisenia andrei* earthworm. Dev. Comp. Immunol. 33: 932-938 (2009).

4.2. Characterization of CCF-like molecules in different Lumbricid species

A 42-kDa cytolytic protein named CCF (for coelomic cytolytic factor) was isolated from the coelomic fluid of *E. fetida* and later on characterized as a pattern recognition molecule (Bilej et al., 1995; Beschin et al., 1998). Upon binding microbial cell wall antigens such as the O-antigen of the lipopolysaccharide (LPS) on the surface of Gram-negative bacteria, peptidoglycan constituents of Gram-positive bacteria or *N,N´*-diacetylchitobiose and β-1,3-glucans of yeast, CCF triggers the activation of prophenoloxidase cascade, an important invertebrate defense mechanism resulting in the production of melanin exerting potent antimicrobial and cytotoxic activities. Moreover, CCF was found to contribute to the cytolytic as well as trypanolytic activity of the coelomic fluid (Bilej et al., 1995; Olivares Fontt et al., 2002).

The broad recognition specifity of *E. fetida* CCF is based on the presence of two distinct lectin-like domains - domain localized in the central part of molecule sharing homology with the polysaccharide motif and glucanase motif of β-1,3-glucanases and C-terminal domain interacting with *N,N´*-diacetylchitobiose and peptidoglycan components (Bilej et al., 2001).

The aim of this study was to characterize CCF-based biological properties of coelomic fluids of other Lumbricid species – *Aporrectodea caliginosa*, *Aporrectodea icterica*, *Aporrectodea longa*, *Aporrectodea rosea*, *Dendrobaena veneta*, *Lumbricus rubellus* and *Lumbricus terrestris* and to link them with their taxonomical classification, taking into account the microbial environment they are subjected to.

Using western blot analysis we detected a 42-kDa protein in coelomic fluids of all tested species cross-reacting with 12C9, a monoclonal antibody elicited against *E. fetida* CCF. Knowing that CCF of *E. fetida* is involved in cytolytic and trypanolytic activities of the coelomic fluid, we tested the lysis of TNF-sensitive tumor cell line L929 and *Trypanosoma brucei brucei* parasites caused by the coelomic fluids of other Lumbricid species. However, we were not able to detect neither cytolytic nor trypanolytic activity of these coelomic fluids.

Since it was documented that upon recognition of various microbial compounds CCF of *E. fetida* triggers the activation of prophenoloxidase cascade, we tested coelomic fluids of other earthworm species to do so upon binding either laminarin (poly(β-1,3-Glc)) or *N,N´*-diacetylchitobiose. All coelomic fluids are able to activate the proPO cascade in the presence of laminarin, whereas only the coelomic fluid of *E. fetida* triggers the cascade in the presence of *N,N´*-diacetylchitobiose suggesting a broader pattern recognition specificity.

The cDNA encoding CCF-like molecules of all eight earthworm species was sequenced and deduced amino acid sequences revealed 80 – 90 % identity. Very high level of homology was found in the central part of these molecules, which is considered to be capable of binding β-1,3-glucans, which is in coincidence with the ability of CCF and CCF-like molecules to bind laminarin and initiate the activation of proPO cascade. The C-terminal part of CCF-like molecules was found to be the most variable supporting the fact that *N,N´*-diacetylchitobiose can be recognized only by *E. fetida* CCF.

The phylogenic analysis of CCF and CCF-like molecules revealed that CCF-like molecules from the genus *Lumbricus* fall together into a well-supported group having the same progenitor, whereas CCF-like molecules of the genus *Aporrectodea* are more heterogenous and form a paraphyletic group. *E. fetida* CCF is closely related to the CCF-like molecule of *D. veneta* although its saccharide recognition specificity differs.

As the composition of microflora is dependent on a vast number of environmental factors such as water, temperature, pH, mineral composition and substrate availability, each tested earthworm group living in a different part of the soil is thus subjected to the different microbial conditions. The highest microbial activity is found in the places with the most abundant organic matter. It is therefore obvious that *E. fetida* living in decaying organic matter, in compost and in mold appears to be the best equipped to resist microbial activity, as reflected by the broader CCF pattern recognition specificity.

To sum up, we have identified the presence of CCF-like molecules in seven earthworm species, described their pattern recognition specificity and suggested the influence of microbial environment on the recognition repertoire of CCF and CCF-like molecules.

Šilerová M., Procházková P., Josková R., Josens G., Beschin A., De Baetselier P., Bilej M.: Comparative study of the CCF-like pattern recognition protein in different Lumbricid species. Dev. Comp. Immunol. 30: 765 – 771 (2006).

4.3. Interaction of an invertebrate defense lectin with vertebrate cells

Coelomic cytolytic factor (CCF), a pattern recognition molecule of *E. fetida* earthworm, was originally isolated as a 42 kDa protein responsible for proteinase-independent lysis of some TNF-sensitive cell lines (Bilej et al., 1995). CCF was shown to share functional analogies with mammalian cytokine tumor necrosis factor (TNF) on the basis of the similar saccharide recognition specificity. Besides CCF was described to exhibit more TNF-like features. Namely, CCF expression is up-regulated in macrophage-like coelomocytes upon LPS stimulation (Bilej et al., 1998) while TNF is produced by macrophages (Carswell et al., 1975), both CCF and TNF were suggested to interact with various pathogens via saccharide recognition (Luo et al., 1993; Beschin et al., 1998) and to lyse African and American trypanosomes via a similar lectin-like activity with *N,N´*-diacetylchitobiose specificity (Lucas et al., 1994; Beschin et al., 1999; Olivares Fontt et al., 2002). Importantly, despite their functional analogies, CCF and TNF have neither gene nor amino acid sequence homology, which indicates that they have a different evolutionary origin (Beschin et al., 1999).

Moreover, TNF has been shown to interact with amiloride-sensitive ion channels on the surface of vertebrate cells via its *N,N´*-diacetylchitobiose lectin-like domain and to induce a pH-dependent increase of membrane conductance resulting in membrane depolarization (Hribar et al., 1999; Van der Goot et al., 1999; Fukuda et al., 2001). Similarly, CCF was found to activate amiloride-sensitive ion channels in lung endothelial cells and peritoneal macrophages in a TNFR-independent manner via its *N,N´*-diacetylchitobiose lectin-like domain (Bloc et al., 2002).

In the present study, we investigated the interaction of CCF with the membrane of non-elicited adherent peritoneal cells (PECs) and their subsequent activation.

The *in vitro* activation of non-elicited PECs isolated from wild-type and TNFR (1 and 2) knock out mice resulted in the production of TNF, IL-6 and nitric oxide (NO). In the case of PECs isolated from TNF knock out mice, CCF triggered lower production of IL-6 and NO than in PECs from wild type and TNFR KO mice, suggesting the cumulative effect of CCF and TNF on IL-6 and NO release.

In agreement with previous patch-clamp experiments (Bloc et al., 2002), CCF caused a membrane depolarization in PECs that was evidenced using the fluorescent anionic dye bis-oxonol in fluorescent microscopy and flow cytometry. CCF-induced depolarization was inhibited by *N,N′*-diacetylchitobiose and amiloride confirming a lectin-like saccharide interaction with amiloride-sensitive sodium ion channels.

To further address the role of sodium channels in macrophage activation, three amiloride-related molecules were tested – amiloride (inhibitor of epithelial sodium channels and Na^+/H^+ exchangers), benzamil and phenamil (amiloride derivatives with higher specificity to the cell type and type of the channel). The most potent inhibitor was found to be phenamil, causing already 50 % of inhibition of TNF, IL-6 and NO production at the lowest concentrations of CCF tested. Furthermore, the PCR analysis revealed the absence of expression of α, β and γ subunits of epithelial sodium ion channels in PECs and, on the other hand, the presence of Na^+/H^+ exchanger expression. These data indicate the involvement of these exchangers in macrophage activation. Moreover, the production of cytokines and NO was inhibited by *N,N′*-diacetylchitobiose confirming the involvement of lectin-like domain of CCF.

In addition, the intracellular pathway involved in CCF-mediated PECs activation was evaluated. We detected the inhibition of cytokine and NO production by the inhibitor MG132, blocking the I-κB degradation and thus NF-κB activation.

In summary, we propose the model of macrophage activation where CCF binds via its C-terminal lectin-like domain to an Na^+/H^+ exchanger or an Na^+/H^+ exchanger-associated molecule. The resulting membrane depolarization leads to NF-κB activation and subsequent production of TNF, IL-6 and NO. In turn, the secreted TNF interacts mainly via its *N,N′*-diacetylchitobiose lectin-like domain with the ion channel/ion channel-related structure, or classically via TNFR1 or TNFR2 on the macrophage surface, thus increasing the activation signal provided by the lectin domains of CCF and TNF.

Bilej M., Josková R., Van den Bergh R., Procházková P., Šilerová M., Ameloot P., De Baetselier P., Beschin A.: An invertebrate TNF functional analogue activates macrophages via lectin-saccharide interaction with ion channels. Int. Immunol. 18(12): 1663-70 (2006).

4.4. Characterization of calreticulin in *E. fetida* earthworms

Calreticulin is a highly conserved calcium-binding protein affecting many cellular processes inside and outside of endoplasmic reticulum (ER). First it was isolated as Ca^{2+}-binding protein from sarcoplasmic reticulum of rabbit muscle (Ostwald and MacLennan, 1974), where it participates in the regulation of Ca^{2+} homeostasis (Michalak et al., 2002; Trombetta, 2003). Besides, calreticulin acts as a main lectin-like chaperone necessary for the maturation of glycoproteins in the eukaryotic cells (Helenius et al., 1997; Michalak et al., 1999; Johnson et al., 2001), modulates gene transcription (Helenius et al., 1997), integrin-mediated cell signaling as well as cell adhesion. Importantly, calreticulin controls osmotic lysis mediated by perforin (Fraser et al., 2000).

Calreticulin is a 46 kDa protein containing N-terminal signal peptide, C-terminal KDEL/HDEL ER retrieval signal and three functional and structural domains – highly conserved globular N-domain, central proline-rich P-domain with high affinity Ca^{2+}-binding site and acidic C-domain containing high capacity Ca^{2+}-binding site (Baksh and Michalak, 1991).

The aim of our study was to characterize the sequence of calreticulin in *E. fetida* earthworm, to determine the expression of this molecule in different organs and to show the phylogenetic relationships with calreticulin molecules of other species.

To assign the sequence of *E. fetida* calreticulin, we designed the set of degenerated primers based on known invertebrate calreticulin nucleotide sequences. Subsequent RT-PCR reaction with these primers followed by sequencing resulted in an approximately 700 bp fragment homological to other known calreticulin molecules. In order to assemble the full length cDNA sequence, RACE amplifications of 5´and 3´cDNA ends was performed. The resulting cDNA and deduced amino acid sequence identified the molecule as a member of highly conserved calreticulin family with 18 amino acids long signal peptide, ER retention KDEL signal and central P-domain containing two sets of amino acid repeats.

Furthermore, we found that *E. fetida* calreticulin shares significant homology (65 – 75 %) with other invertebrate calreticulin molecules. To gain more insight into the relationship of calreticulin molecules, their amino acid sequences were subjected

to phylogenetic analysis, which suggests the common origin of *E. fetida* calreticulin and that of the mollusk *Aplysia californica*.

In order to detect the localization of calreticulin in coelomocytes and on transversal as well as longitudinal cryosections of *E. fetida*, we used a polyclonal rabbit anti-calreticulin antibody. The labelling was detected in the mesenchymal lining of the coelomic cavity, i.e. a site from where the coelomocytes are derived and proliferate, and in granular intracellular bodies of certain coelomocytes.

Moreover both *in situ* hybridization and real-time PCR revealed calreticulin expression in various cells and tissues of *E. fetida*. Dominant expression was detected in the epidermis, neurons of ventral nerve cord and in sperm cells, but also in the epithelial cells of the intestinal tract, in the cells of body wall muscles, in the cells of mesenchymal lining of the coelomic cavity and in coelomocytes.

To sum up, we proved the presence of calreticulin in *E. fetida* earthworms and specified the sequence of calreticulin-coding cDNA. Phylogenetic analysis revealed the common origin of *E. fetida* calreticulin and calreticulin of molluscs. The expression of calreticulin was detected in various tissues using immunohistochemistry, *in situ* hybridization and real-time PCR.

Šilerová M., Kauschke E., Procházková P., Josková R., Tučková L., Bilej M.: Characterization, molecular cloning and localization of calreticulin in *Eisenia fetida* earthworms. Gene 397: 169-177 (2007).

4.5. Relationship between hemolytic molecules in *E. fetida* earthworms

The coelomic fluid of *E. fetida* earthworm exhibits numerous biological activities including hemolytic activity. The proteins responsible for the lysis of red blood cells represent a heterogenous set of molecules including *Eisenia fetida andrei* factor (EFAF; (Roch et al., 1981; Roch, 1984)), fetidin (Lassegues et al., 1997; Milochau et al., 1997), lysenin (Sekizawa et al., 1996; Sekizawa et al., 1997), eiseniapore (Lange et al., 1997) and hemolysins isolated either from the coelomic fluid (H_1, H_2, H_3) or from the cell lysate (CL_{39} and CL_{41}) (Eue et al., 1998; Koenig et al., 2003). All hemolytic molecules described so far share biochemical analogies - similar molecular mass around 40 kDa, similar pI and the ability to bind sphingomyelin and create pores in target lipid membranes. These findings indicate them to be the same or similar molecules.

The aim of our study was to clarify whether fetidin and lysenin are either protein isoforms encoded by different alleles of one gene, or products of two related genes.

Both cDNA and amino acid sequences of fetidin and lysenin display high homology with differences in 5´-UTR. On the basis of these differences, we designed two sets of primers, specific either for fetidin or for lysenin. However, using lysenin-specific primers in PCR we amplified also fetidin. Subsequent sequencing revealed errors in published 5´-UTR sequence of fetidin. According to the obtained sequences a new set of primers based on the presence of different deletions in the sequence of both molecules was designed. The combination of these specific primers together with stringent temperature in PCR reaction provided a reliable tool for amplifying the desired sequence only. Using these primers we proved the presence of fetidin and lysenin mRNA in the coelomocytes of individual *E. fetida* earthworms. Moreover, PCR reactions with *E. fetida* genomic DNA resulted in amplification of both molecules, suggesting that these molecules are encoded by two distinct genes.

In order to quantify the mRNA levels of lysenin and fetidin in individual earthworms, real-time PCR experiments with fetidin- and lysenin-specific primers were performed. We found out that while the expression of fetidin is similar in all

individuals, the expression of lysenin strongly varies. The expression of lysenin was up to 26 times higher in some individuals than in others.

To address the relationships between hemolytic activity and the expression of fetidin and lysenin, coelomic fluids of individual earthworms were separated in native polyacrylamide gel and the gels were subsequently applied on sheep erythrocyte suspensions embedded in agarose. After several hours of incubation, four hemolytic patterns were observed. In parallel, the hemolytic activity of individual coelomic fluids was quantified by the incubation with erythrocyte suspension. The distinct hemolytic patterns correlated with differences in the level of hemolytic activity of the coelomic fluids. All animals exhibiting higher hemolytic activity share the same hemolytic pattern consisting of several bands, reflecting the presence of more hemolytic proteins in the coelomic fluids of these earthworms.

Moreover, all fetidin- and lysenin-specific primer sets were tested in PCR reactions with cDNAs obtained from other earthworm species (*Lumbricus terrestris, Lumbricus rubellus, Aporrectodea caliginosa, Aporrectodea icterica, Aporrectodea longa, Aporrectodea rosea, Dendrobaena veneta*). However, no PCR amplification occurred in these species, which is in compliance with the fact, that coelomic fluids of these species do not show hemolytic activity. These finding suggests a unique occurrence of these hemolytic molecules in *E. fetida* earthworms.

In conclusion, we documented that fetidin and lysenin are encoded by two distinct genes with high homology and that their expression differed in individual earthworms.

Procházková P., Šilerová M., Felsberg J., Josková R., Beschin A., De Baetselier P., Bilej M.: Relationship between hemolytic molecules in *Eisenia fetida* earthworms. Dev. Comp. Immunol. 30: 381-392 (2006).

4.6. Prophenoloxidase activity in coelomic fluid of *E. fetida* earthworms

Prophenoloxidase cascade represents one of the most important defense mechanism in many invertebrates. Generally, upon recognition of polysaccharides of microbial cell walls, serine proteinases cleave inactive proPO to its active state phenoloxidase, catalyzing the subsequent reactions resulting in production of melanin. Melanin and its precursors involved in the prophenoloxidase cascade exert strong antimicrobial properties and a wide range of other biological activities (phagocytosis, opsonization, wound healing).

ProPO activity was evidenced both in protostomians and deuterostomians. However; suggestive evidence for the existence of the proPO activation cascade in annelids was limited to description of melanization reactions and formation of brown bodies in polychaetes and oligochaetes (Porchet-Hennere and M'Berry, 1987; Valembois et al., 1992). More recently, presence of proPO activity was indicated by Beschin et al. (Beschin et al., 1998) by finding that the incubation of coelomic fluid with various components of microbial cell walls results in the production of L-DOPA (L-β-3,4-dihydroxyphenylalanine), a substrate of PO in *E. fetida* earthworms.

In our study, we observed that coelomic fluid causes the spontaneous oxidation of L-DOPA after eight hours of incubation. However, in the presence of an activator (LPS, β-1,3-glucan), the L-DOPA oxidation started already after two hours and reached the maximum within six to ten hours of incubation. In comparison with the data of PO activity in arthropods, where this activity can be observed within several minutes, our results suggest very low level of PO in earthworms.

To determine the specificity of activation of proPO cascade, we used five different substrates (L-DOPA, dopamine, N-acyldopamine, 4-methylcatechol and tyrosine) and three non-specific activators (sodium dodecyl sulphate (SDS), cetylpyridinium chloride (CPC) and 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)). The highest activation was obtained by combining CPC as an activator and L-DOPA as a substrate.

To evaluate the observed PO activity, coelomic fluid content was separated in native PAGE and incubated with L-DOPA as a substrate and CPC as an activator.

Under these conditions only one band showing phenoloxidase activity could be observed. This activity was completely blocked when the coelomic fluid was either boiled before the separation or preincubated with irreversible proteinase inhibitor Pefabloc. This suggests an enzymatic nature of the compound exhibiting PO activity.

Bands showing oxidation were cut from the native gel, electroeluted and separated in SDS-PAGE. The eluted material revealed a high molecular weight band formed probably by precipitated material, a strong band of approximately 90 kDa and a few weak bands of molecular weight lower than 40 kDa. Further, the 90 kDa band was subjected to amino acid sequence analysis. Edman degradation revealed only an eight amino acids long N-terminal peptide sequence. This sequence revealed homology with the endogenous inhibitor of phenoloxidase from housefly *Musca domestica*. The sequencing of four internal peptides showed partial homologies with the sequences of phenoloxidase and hemocyanin of different invertebrate species.

To sum up, these data suggest the presence of phenoloxidase and its inhibitors in the coelomic fluid of *E. fetida* earthworms, but the level of PO activity is lower than in other invertebrates and the PO activation is slower.

Procházková P., Šilerová M., Stijlemans B., Dieu M., Halada P., Josková R., Beschin A., De Baetselier P., Bilej M.: Evidence for proteins involved in prophenoloxidase cascade in *Eisenia fetida* earthworms. J. Comp. Physiol. [B]. 176: 581-587 (2006).

5. CONCLUSIONS AND DISCUSSION

Invertebrates are widely distributed animals. They can be found in almost any kind of habitat. Their expansion and survival depend on successful defense mechanisms against various kinds of microorganisms and parasites. It is therefore clear that invertebrates must have very efficient means of recognizing and combating potentially harmful microorganisms.

The defense mechanisms of earthworms were studied over the past four decades. It became apparent that the earthworms, as well as other invertebrates, lack specific immunoglobulins, lymphocytes or other features of the adaptive immune system described in vertebrates, but possess innate defense components. A natural immunity is formed by anatomic, physical and chemical protective barriers, efficient cellular mechanisms – phagocytosis of invading microorganisms, encapsulation of large foreign bodies, clotting and coagulation responses as well as a broad range of antimicrobial factors – lysozyme, antimicrobial peptides, molecules involved in prophenoloxidase cascade, proteases and cytolytic and haemolytic proteins.

In this study, we focused on the detail description of some defense molecules involved in innate immunity of earthworms – lysozyme, CCF, calreticulin, two hemolytic molecules fetidin and lysenin and the molecule of prophenoloxidase.

Previously the lysozyme activity was observed in coelomic fluid as well as in extract of coelomocytes of *Eisenia* earthworms (Çotuk and Dales, 1984). Later, the active protein was isolated and partially sequenced (Ito et al., 1999). In our study we proved the presence of lysozyme (EALys) in *E. andrei* (formerly *E. fetida andrei*) earthworms and identified it as a novel member of invertebrate type family of lysozymes. We characterized the molecule both structurally and functionally. The amino acid sequence of EALys revealed the presence of N-terminal signal peptide, high amount of cystein residues typical for i-type lysozymes and amino acid residues important for the substrate fixation and lysozyme activity. Further we documented the lysozyme (hydrolysis of β-1,4-glycosidic bonds between GlcNAc and NAM of peptidoglycan) and isopeptidase (hydrolysis of ε-(γ-Glu)-Lys cross-links between Glu and Lys in a stabilized fibrin and peptidoglycan) activities and evaluated the influence of pH, ionic strength and temperature for both activities. These activities can provide a useful tool for monitoring of innate immunity

in earthworms. Moreover we followed the changes of mRNA levels in the coelomocytes of earthworms infected by *B. subtilis* and *E. coli* and suggested the involvement of EALys in protection against infections by both Gram-positive and Gram-negative bacteria.

Coelomic fluid of *E. fetida* earthworm was previously described to contain 42 kDa lectin named coelomic cytolytic factor (CCF) acting as a pattern-recognition receptor (Bilej et al., 1995; Beschin et al., 1998; Bilej et al., 2001). We characterized CCF-based biological properties of coelomic fluids of other Lumbricid species – *Aporrectodea caliginosa*, *Aporrectodea icterica*, *Aporrectodea longa*, *Aporrectodea rosea*, *Dendrobaena veneta*, *Lumbricus rubellus* and *Lumbricus terrestris.* CCF-like molecules of these species were described to share 80 – 90 % homology with *E. fetida* CCF. Very high level of homology was found in the central part of these molecules, which is considered to bind β-1,3-glucans, whereas the C-terminal part responsible for binding *N,N´*-diacetylchitobiose and peptidoglycan components was found to be most variable. These data are in coincidence with the finding that *E. fetida* CCF is the only one capable of binding *N,N´*-diacetylchitobiose on the surface of various cells and inducing lysis of TNF-sensitive cell lines and *Trypanosoma* parasites. We suggest that the broad recognition repertoire of *E. fetida* CCF reflects a particular microbial environment this species lives in.

Furthermore, CCF of *E. fetida* was found to activate amiloride-sensitive ion channels in lung endothelial cells and peritoneal macrophages in a TNFR-independent manner via its *N,N´*-diacetylchitobiose lectin-like domain (Bloc et al., 2002). We investigated the interaction of CCF with the membrane of non-elicited adherent peritoneal cells (PECs) and their subsequent activation. On the basis of our results we proposed the model of macrophage activation where CCF binds to a Na^+/H^+ exchanger or a Na^+/H^+ exchanger-associated molecule. The resulting membrane depolarization leads to NF-κB activation and subsequent production of TNF, IL-6 and NO. In turn, the secreted TNF interacts mainly via its *N,N´*-diacetylchitobiose lectin-like domain with the ion channel or ion channel-related structure, or classically via TNFR1/TNFR2 on the macrophage surface thus increasing the activation signal provided by CCF. This artificial setting of macrophage activation triggered by an invertebrate defense molecule mimicking the lectin-like activity of TNF may reveal an ancient mechanism of cell activation

that has evolved in parallel with the receptor-based network currently prevailing vertebrates.

Another molecule possibly involved in innate immunity of earthworms we focused on was calreticulin. This molecule was described in perforin-like lytic fraction of coelomocyte lysate of *E. fetida* earthworms (Kauschke et al., 2001). In our experiments the molecule of calreticulin of *E. fetida* was described in detail. It was identified as a member of highly conserved calreticulin family with 18 amino acids long signal peptide, ER retention KDEL signal and central P-domain containing two sets of amino acid repeats. The phylogenetic analysis of calreticulin molecules suggested the common origin of *E. fetida* calreticulin and calreticulins of molluscs. Moreover, both *in situ* hybridization and real-time PCR revealed calreticulin expression in various cells and tissues of *E. fetida,* namely epidermis, body wall muscles, ventral nerve chord, sperm cells and mesenchymal lining. Considering its chaperoning function, calreticulin can be a useful tool for identification and affinity purification of novel cytolytic proteins.

The coelomic fluid of *E. fetida* earthworms contains hemolytic molecules showing bactericidal and/or bacteriostatic activity (Valembois et al., 1986; Roch et al., 1991). These molecules represent a heterogenous set of proteins (Roch, 1984; Lange et al., 1997; Lassegues et al., 1997; Sekizawa et al., 1997; Koenig et al., 2003). Even though their nomenclature and terminology differ, their characterization indicates that they are the same or similar molecules. In our study two *E. fetida* hemolytic proteins - fetidin and lysenin - molecules displaying a high amino acid sequence homology, were described to be encoded by two separate genes on the basis of the combination of the specific primers together with stringent temperature. Further, the levels of expression of these molecules were evaluated by quantitative real-time PCR. We found out that while the expression of fetidin is similar in all individuals, the expression of lysenin strongly varies. Moreover, these two hemolytic proteins seem to be unique for *E. fetida* earthworms, which could reflect their living conditions. *E. fetida* earthworms living in the compost could have developed distinct defense mechanisms to better tolerate a wide range of environmental conditions and their fluctuations.

The prophenoloxidase cascade is an important defense process of invertebrates resulting in production of melanin and high reactive and toxic quinone intermediates (Ashida and Yamazaki, 1990; Söderhäll et al., 1994; Cerenius

and Söderhäll, 2004). We proved the presence of phenoloxidase and its inhibitors in the coelomic fluid of *E. fetida* earthworms, but the level of phenoloxidase activity is lower as compared to other invertebrate species. Moreover, the activation of prophenoloxidase cascade is slower. These data suggest that this cascade does not represent the main defense system in earthworms.

6. ORIGINAL PUBLICATIONS

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