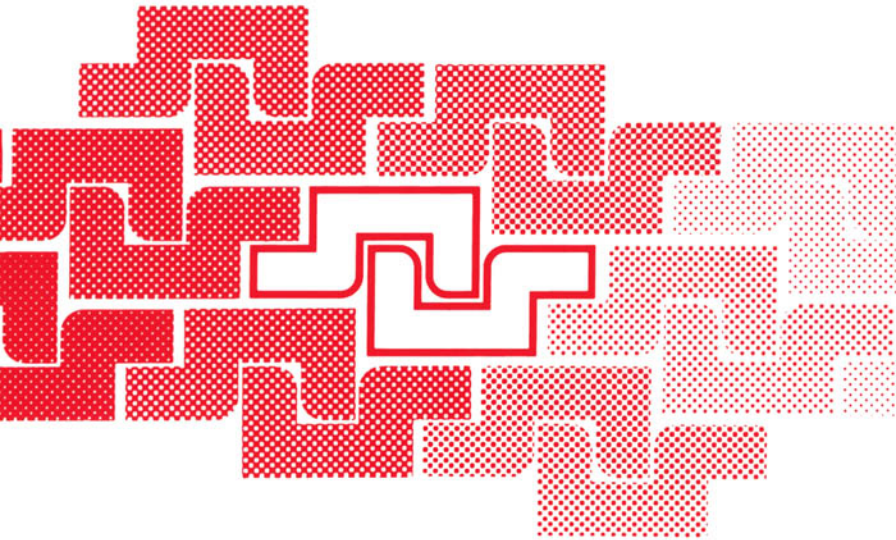

A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications

Edited by
Edwin L. Cooper
Alain Beschin
Martin Bilej

IOS Press
Ohmsha

NATO Science Series

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Welcome address by Blanka Rihova

Director of the Institute of Microbiology,
Academy of Sciences of the Czech Republic,
Prague, Czech Republic

It is a great pleasure to welcome the participants to this NATO Advanced Research Workshop. As one of the co-sponsors, we are pleased since the subject of the workshop fits most appropriately within the context of certain ongoing projects. In this context, it is instructive to offer some history and overview of our activities. The Institute of Microbiology, Czechoslovak Academy of Sciences was founded in 1962. In 1992, the Institute became part of the newly established Academy of Sciences of the Czech Republic. Recently, the following four topical groups of the research aims have been defined: 1. molecular and cellular microbiology, 2. biosynthesis of natural compounds and biotechnology, 3. ecology and bioremediation, 4. immunology and gnotobiology.

Since its foundation, activities of the Institute have been on a broad front, including research projects in microbiology, study of microbial products and their formation, research of biodegradation activities of microorganisms and symbiotic relationships of biological models including the development of new biotechnological procedures. The Institute has pursued research in the field of physiology and regulation of immune mechanisms, both natural and acquired, investigation of the ontogenetic development of these mechanisms in conventional and germ-free models, and the study of causes and possible treatment of malignant and autoimmune diseases. The number of employees varied between 377 plus 160 students in 2000 to 389 plus 190 students in 2001. The present structure of the Institute of Microbiology includes 23 laboratories that are associated into five scientific divisions: Division of Biogenesis and Biotechnology of Natural Compounds, Division of Cell and Molecular Microbiology, Division of Ecology, Division of Immunology and Gnotobiology (two of its five laboratories are located in Novy Hradek in East Bohemia) and Division of Autotrophic Microorganisms located in Trebon in South Bohemia.

The Division of Immunology and Gnotobiology has five laboratories: Laboratory of Specific Cellular Immunity, Laboratory of Humoral immunity, Laboratory of Natural Cell Immunity, Laboratory of Physiology, Immunity and Ontogenesis of Gnotobionts and Laboratory of Immune Regulations. Its main research interest is concentrated on the development of cellular immunity in phylogeny and ontogeny, development and immunopathology of mucosal immunity, molecular basis of humoral and cellular immunity, immune regulation, pathogenetic mechanisms of some immunologically based diseases, targeted anticancer therapy, immunomodulation and mucosal vaccines.

Models of germ-free animals, *e.g.*, germ-free mice, pigs and rats developed in gnotobiological laboratories are widely used for the study of the development of hemopoietic cells, membrane markers of T and B cells and of the development of the immunocompetency under germ-free conditions. Immunodeficient and immunocompetent gnotobiological models are used to study the role of microflora components in the development of intestinal inflammation and autoimmune diseases such as insulin-dependent diabetes and spondylarthrosis. Analysis of the pathogenetic mechanisms of celiac disease concentrates on the immunological effects of the food component, gluten on intestinal mucosa of patients and animal models. Identification of new autoantigens (calreticulin) is bringing new light into the pathogenetic mechanisms.

A very important line of study concerns molecular and cellular mechanisms of immune recognition and activation of cytotoxic cells as critical effectors in antitumor immunity. Work at the molecular level deals with the recombinant expression and structural studies of important lymphocyte receptors possessing an extracellular C-terminal

motif related to C-type animal lectins. The regulatory role of neurotransmitters in stress-mediated NK cell depression and effector-target interaction during antitumor immune response are studied in relation to the use of dopaminergic drugs. Studies in applied immunology concentrate on design, properties and effects of macromolecular therapeutics based on N-(2-hydroxypropyl) methacrylamide copolymer carrier non-targeted or targeted with monoclonal antibodies, carbohydrates and lectins. The studies are aimed at improving the efficacy and therapeutic index of immunoconjugates by optimizing their selectivity and potency. Mucosal vaccines with anti-infectious effects are developed and tested.

Here is the relevance of the Division to the NATO Advanced Research Workshop. We aim to better understand early stages in evolution of immune competence. To this end, we utilize a complex invertebrate, the earthworm. The immune system development allows identification and analysis of immunologically active invertebrate molecules. These molecules are synthesized and secreted by earthworm leukocytes into the coelomic fluid of our model, *Eisenia foetida*. The results are important for analyzing the structure-function relationship of these antimicrobial molecules and for their potential therapeutical use.

We trust that your stay in Castle Trest will be both helpful and beneficial to the science of earthworm immunobiology and to immunology in general.

Prof. Blanka Rihova, PhD, DSc
Director

Welcome address by Ivo Hana

President of the Czech Immunological Society

I feel greatly honored by the invitation of the organizers to open on behalf of the Czech Immunological Society this noted NATO Advanced Research Workshop. Our Society welcomes and tries to strengthen any kind of scientific exchange of experience and knowledge, and therefore there was no space for any hesitation when the opportunity appeared to organize a NATO Workshop in our country. We have chosen this memorable castle as the site of the Workshop since it offers a quiet and isolated setting in a beautiful and less known countryside far from the busy centers of our age. We hope that you will enjoy both the Workshop and the environment, and who is eager to learn more about our country will without any doubt find some time to visit Prague or other remarkable cities as soon as the Workshop is adjourned.

Our Immunological Society started its activities already more that 30 years ago and in 1970 the first issue of our national immunological periodical "Imunologicky zpravodaj" (*Immunological Bulletin*) appeared. Since that time this bulletin has covered already 30 volumes bringing to our members Society's announcements, scientific reviews, original papers, methodological articles and summaries of national and international seminars and congresses. Nowadays, we have more than 400 members and are active in organizing both national and international meetings and publishing various printed materials including books and proceedings. After a period in the seventies and eighties when our activities were heavily suppressed by bans of the communist regime from traveling abroad and communicating with our colleagues in the Western countries (due to the overt opposition by our scientific community to the Soviet-led military invasion of Czechoslovakia in 1968), we enjoy now the freedom of contact and are happy to welcome you here.

Allow me now a short and maybe amusing personal remark. As I was able to discern, your studies heavily concentrate on immunity processes in earthworms. Those beings have been far from my field of interest in immunology. Up to now I considered earthworms to be a lure only for fishes and therefore of interest for people who fish. Today I have to admit that earthworms – to my surprise – are also a fundamental lure for immunologists and may substantially help in elucidating fascinating questions in immunological research. So sorry for my lack of familiarity with earthworms!

I wish you a nice stay in Czechia, a lot of new knowledge and fruitful discussions!

Prof. Ivo Hana, MD, PhD
President of the Czech Immunological Society

Participants

Castle Třešt

Preface

From the total number of extant animal species probably surpassing 2 million 95 % are included in the invertebrate taxa, encompassing a diversity of organisms ranging from unicellular protozoans to the complex protochordates. Invertebrates have evolved for hundreds of millions of years often surviving in very hostile environments. All invertebrate species have evolved a variety of active immunodefense pathways efficiently recognizing and responding to nonself components despite the absence of an adaptive immune system based on antibodies or lymphocytes. The success of invertebrate taxa in evolution buttresses arguments about the potential value of gaining deep insights in their diverse immune recognition/effector defense mechanisms. In this respect, knowledge of the less complex invertebrate immune defense strategies may contribute to our understanding of the seemingly more sophisticated vertebrate immune system, as well as lead to the identification of new molecules with possible biomedical and therapeutic applications.

Since the pioneering works of Elie Metchnikoff at the end of the 19th century, invertebrate immunology has become a credible subject of significant interest and analysis. However, it is only in the last two decades that detailed analyses of invertebrate immune reactions and their molecular basis have begun to emerge. Moreover, the available data so far are still somewhat scarce and restricted to a few animal models, in particular arthropods, mollusks, and now annelids (earthworms, marine worms and leeches). Earthworms belonging to oligochaete annelids became a model for comparative immunologists in the early sixties with the publication of results from transplantation experiments. American (Edwin L. Cooper) and French (Pierre Valembois) scientists reported that *autogeneic* transplants (self to self) of earthworm body wall were accepted but not transplants even between individuals of the same (non-self *allogeneic*) or another (nonself *xenogeneic*) earthworm species. These transplantation experiments proved the existence of self/nonself recognition in earthworms, paving the way for extensive studies on analyzing other earthworm immune mechanisms that evolved to prevent possible destruction by pathogens. The choice of earthworms for comparative immunology studies becomes significantly pertinent since they represent an inexpensive, noncontroversial, and socially acceptable experimental model. Moreover, earthworms are essential for maintenance of soil and are now considered as a source of biologically active molecules with potential biomedical use. For example, earthworm powder has been used in traditional medicine as a drug for treatment of various diseases in the Far East since a few thousands years ago. The therapeutic effect of earthworm active factors is being re-evaluated by a modern scientific approach. A potent fibrinolytic enzyme extracted from *Lumbricus rubellus* has been reported to be in a clinical trial as a possible antithrombotic drug.

Within the field of *comparative immunology*, particularly *invertebrate immunology*, data concerning antimicrobial lytic factors is considered worthy of continued analysis for several reasons. First, there are the potential biomedical applications. Second, there is a need to clarify and standardize their classification. The aim of this NATO Advanced Research Workshop was to shed a new light on the nomenclature of earthworm lytic molecules in one major invertebrate group, the annelid worms. Within this context, our workshop sought to (1) unravel the mechanism of annelid lytic and antimicrobial activities; (2) identify their target molecules; (3) discuss and plan long range and perhaps immediate potential application of these molecules; (4) place our findings within the context of

homologous and analogous data concerning similar molecules from other invertebrate models species.

Planning and execution of the workshop: It was the suggestion and insistence of Edwin Cooper that the ARW be held in an elegant, secluded location, conducive to intense analysis and discussion. Away from the beautiful city of Prague and receiving the full sanction of Dr. Walter Kaffenberger, Castle Třešt, which is actually associated with the Czech Academy of Science, was our superb venue. The relative isolation of this location from other "attractions" (approximately 150 km from Prague) was advantageous for intense discussion.

We have succeeded, as organizers, to get together an extraordinary group of comparative immunologists. In addition to active workers using the earthworm as a model to focus on the objectives of the ARW, we decided on several approaches. First, we wanted all active earthworm workers to participate. Second, we wanted to use the NATO ARW as an opportunity to be inclusive, *i.e.* by inviting active workers involved in the use of other annelids (notably the leeches and marine annelids). Third, we wanted to have student participation, since after all, they represent the lifeblood of any discipline – they will perpetuate this field of study. Strong worker was Dr. Kenneth Söderhäll from Sweden who is the authority on crustacean immunodefense molecules. Dr. Philippe Roch was considered as an absolutely essential participant since he along with Dr. Pierre Valembois, were the first to discover antimicrobial molecules in earthworms. Moreover, Dr. Ph. Roch is now in charge of a marine laboratory that examines the antimicrobial molecules isolated from edible crustaceans and mollusks. The molecular perspectives of Dr. K. Söderhäll and Dr. Ph. Roch were of inestimable value in providing suggestions of new technological advances. In addition Dr. Werner Mohrig was invited as a pioneer in earthworm immunology. Dr. Matthias Leippe was also an essential contributor having worked on earthworm lytic molecules and on those derived from protozoans. Finally, the point of view of Dr. Patrick De Baetselier, an authority in parasitology and cancer immunology was of critical help.

Edwin L. Cooper
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The earthworm: a new model with biomedical applications

Edwin L. Cooper

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Abstract. From the 1960s there were varied investigations concerning the immune system of the earthworm, mostly on transplantation of integument. The results yielded valuable information that reinvigorated investigations of invertebrate immune systems. Roughly sixty years earlier, Metchnikoff did his classic experiments on phagocytosis in the water flea, *Daphnia*, heralding invertebrates as potent models for demonstrating elements of what is recognized as innate immunity. During the 1990s there was a surge of interest in the humoral immune system of the earthworm with an emphasis on the molecules that cause lysis of various types of experimental targets, including erythrocytes, bacteria, tumor cells. There is hardly any work at all on the role of lytic factors in the destruction of parasites and viruses. This does not mean that the earthworm's potent lytic system is not capable of destroying these sorts of foreign components. In any case, the earthworm immune system especially the lytic components may prove to be of tremendous value with respect to clinical problems that require the use of chemicals that are not toxic but yet will dissolve clots in human subjects. Although reportedly used in Asian countries for this precise reason, these approaches have not been tried in Europe and the US.

1. What can invertebrates tell us about innate immunity?

There are at least three strong reasons for analyzing innate invertebrate immunity. First, we can learn more from invertebrates concerning the expansive, evolutionary development of innate immunity, which protects millions of metazoans, a vastly greater number than the handful of vertebrate model. Second, because the humoral products derived from these organisms are usually potent antimicrobial molecules, we may understand better the mechanisms of natural protection against disease. Extant species have probably relied heavily on such immune competence for survival. This work has the potential to reveal new and significant modes of therapy. Understanding the immune system of invertebrates is essential to understanding their survival strategies and our ecological relationships with them. Pathogenic threats to any of these three invertebrate contributors to human detriment or well being (disease vectors, sources of food, ecological balancers) could pose immediate problems. Biospheric viability rests partially on the capacity of organisms (including plants) to be protected from the ravages of disease. Thus at every level of animal evolution, immune components help to maintain and perpetuate life (Tables 1, 2). The focus here will concern the immunodefense system of earthworms that play an essential role in the biosphere. Earthworms are really not new to biologists in modern times since Darwin was one of the firsts to publish about them in a most animated fashion [1]. Third, our species is part of an intricate web of ecological relationships. Some invertebrates are parasites, pests, and purveyors (vectors) of infectious diseases. Many are dependable and important sources of food (*e.g.* crustaceans, mollusks). Earthworms and others provide some ecological balance

and biospheric maintenance. This NATO ARW will focus on earthworm immunity in order to 1) identify certain molecules that sequester experimental antigens (bacteria, erythrocytes, cancer cells); 2) present evidence of their possible therapeutic applications; some are already employed in certain countries.

Table 1: Some elements of innate immune components in invertebrates

Phagocytic cells
Encapsulation (granuloma/nodule formation see ERP)
Transplant rejection (evidence of accelerated second-set rejection)
Proteases and protease inhibitors
Lysozyme
Lysins
Agglutinins
C-Type lectins [Ca^{++}-dependent lectins]
S-Type lectins (Galectins)
Prophenoloxidase system (proPO)
Metal-binding proteins (cysteine-rich metallothioneins)
Cytokine-like (functional analogues; no sequence data)
Transferrin and lactoferrin
Bacterial Permeability Increasing Protein (BPI)

Table 2: Some innate immune components in particular invertebrates

Component/Characteristic	Metazoan model
Encapsulating-Relating Proteins (ERPs)	Insect (*Tenebrio molitor*)
Eicosanoids	Insects
Inducible inflammatory system (Toll, NFκB)	Insects/arthropods, *Caenorhabditis elegans*
Acute phase proteins: Pentraxins, Ig superfamily, complement	Insects/arthropods
α_2-macroblobulin family (protease inhibitor)	Nematodes, crustaceans, mollusks, echinoderms, tunicates; horseshoe crab (*Limulus polyphemus*)
Lipoprotein-receptor related protein (LRP/α_2 M-R)	*C. elegans*
Mannose Binding Lectin (MBL)	*C. elegans*, tunicates
Antimicrobial peptides (Defensins):	
Cecropins, Drosomycin, Drosocin, Attacin	
Metchnikowin	*Drosophila*
Mytilins; Myticins	*Mytilus galloprovincialis*
Lytic and Antimicrobial Components:	Earthworm
Lysins (fetidins, lysenin, eiseniapore)	
Antimicrobial peptide: non-lytic lumbricin I	
TNF analogue	
Pattern recognition molecule (CCF)	
Reactive oxygen species (ROS)	Mollusks
Fibrinogen Related Proteins (FREPS)	Mollusks (planorbid snails)
NK-like cells associated with CD markers	Sipunculids, annelids, mollusks
Tachylectins	Horseshoe crab
Acute Phase Proteins:	
C-reactive protein, LPS-binding protein (LBP)	Crustaceans, horseshoe crab
C3 homologue	Echinoderms; tunicates
Collectin (collagenous, carbohydrate binding protein)	Tunicates, horseshoe crab
Lectin-mediated complement pathway	Tunicates, horseshoe crab
Mannose Binding Protein (MBP)	Tunicates
Mannose Associated Serine Protease (MASP)	Tunicates
Antimicrobial peptides: Clavanins, styelins	Tunicates

2. Cells and molecules of the earthworm immune system

Earthworms are as numerous as there is habitable soil and they range from small familiar species the gargantuan *Megascolides australis*, an Australian species that reaches lengths of over 3 meters! Innate immunity in earthworms is a system that has received less attention than that of *Drosophila* but which has much interest from the dual perspective of both immune system function and of evolution. In fact, the capacity to destroy bacteria and other microbes apparently exists in parallel with the capacity to lyse foreign, eukaryotic cells, two responses found in many invertebrates. The earthworm's body cavity contains coelomic fluid and leukocytes that are as varied as they are in other equally complex invertebrates and they resemble certain vertebrate leukocytes with respect to morphology, cytochemistry and function [2]. Both the leukocytes and the fluid that they synthesize and secrete, effect immunobiological responses [3-10]. They do so by various routes: 1) opsonization; 2) inflammation and phagocytosis; 3) agglutination; 4) mitogenesis; 5) lysis; 6) destruction of experimentally introduced allogeneic, xenogeneic but not autogeneic transplants *in vivo* and various target cell types *in vitro*. With respect to leukocytes, Roch [11] proposed that cellular activities are based mainly upon phagocytosis and leukocyte cell-to cell recognition. The latter leads to cytotoxicity, mixed lymphocyte stimulation like-reactions and cellular cooperation [12-14]. Humoral activities include lysozyme, synthesis and secretion of agglutinins, a phenoloxidase/peroxidase system, and synthesis and expression of the first to be discovered lytic components, the fetidins [15-19]. We will focus on earthworm immunity by: 1) identifying certain molecules that sequester experimental antigens (bacteria, erythrocytes, cancer cells: the lysins [primarily fetidins lysenin, eiseniapore] and agglutinins [lumbricin I]); 2) presenting evidence of their possible therapeutic applications that may be already employed in certain countries.

3. Earthworm effectors can bind to and lyse K562 targets

We were among the first invertebrate immunobiologists to demonstrate cytotoxicity using leukocyte effectors cocultured with a variety of targets including allogeneic cells [20, 21]. Results of more rigorous analyses follow, especially the all-important question concerning the nature of viability in reactions involving autogeneic cells alone or in mixtures as allogeneic cells [22]. Information would then give us other relevant, newer parameters against which to clarify possible mechanisms of cytotoxicity, especially after contact, *i.e.* initial binding between effectors and targets. In the following experiments, we defined autogeneic (self) cells as leukocytes derived from a single earthworm and those that are allogeneic (nonself) as derived from at least two different earthworms of the same genus and species. In the remaining descriptions, two main leukocytes will be highlighted: small coelomocytes (SC) and large coelomocytes (LC), revealing for the first time their different behavior when cocultured together or with tumor cell targets (Fig. 1).

After cultivation, we observed a higher incorporation of [^{3}H]-thymidine in autogeneic cultures accompanied by a significantly greater number of cells in S, G2, or M phases than in allogeneic cultures. The disparity and apparent preferential killing resulting in varied efficacy of cytotoxicity has been interpreted in several ways. First, [^{3}H]-thymidine incorporation results suggest that autogeneic coelomocytes are in a better overall state of cellular health than allogeneic populations. Second, when viewed with respect to histocompatibility, autogeneic coelomocytes "*face to face*" are not confronted by potentially deleterious *nonself* alloantigens as when allogeneic cells are "*face to face*". Putative allogeneic effectors are vulnerable to reactions against themselves even if the alloantigens are weak or the effectors may lack

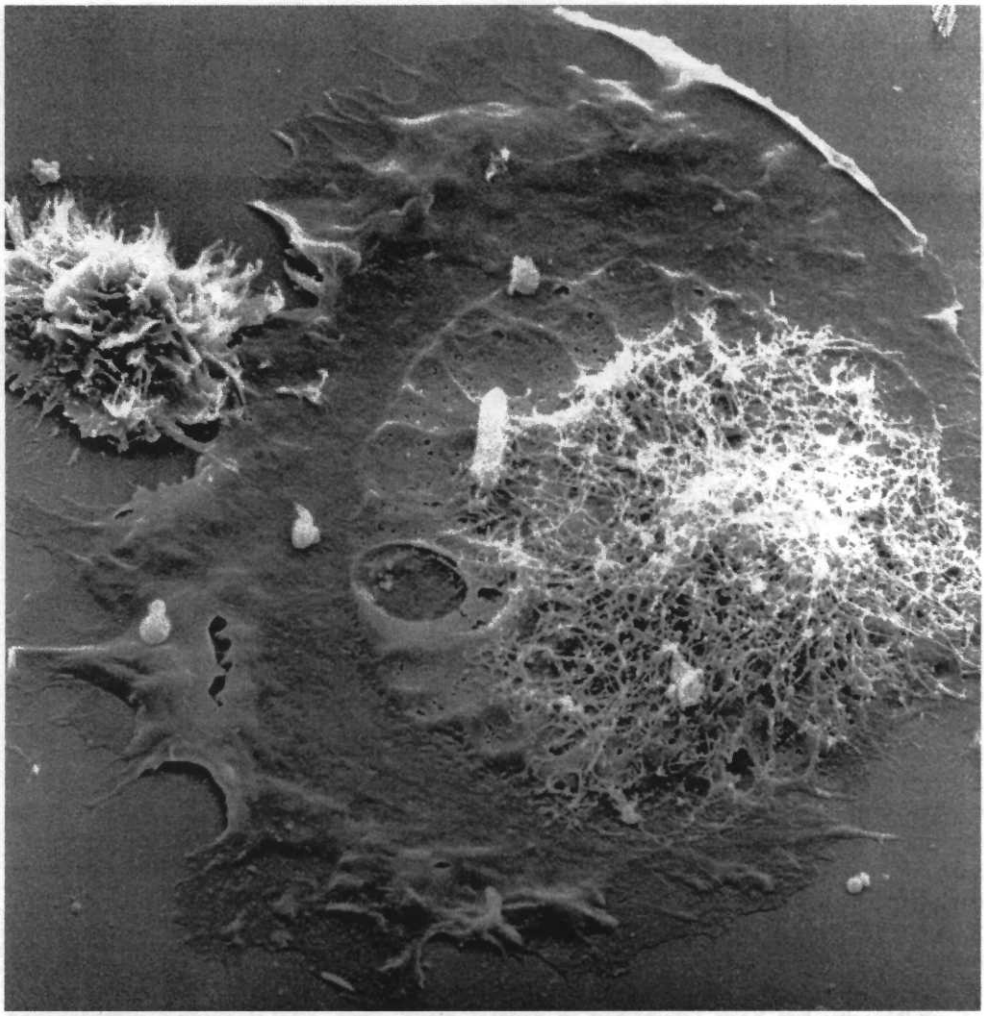

Fig. 1: Scanning electron micrograph showing small (SC) coelomocyte at left and large (LC) coelomocyte that dominates the field. Note the attempt to phagocytose bacteria and other foreign material. Numerous cytoplasmic extensions provide wide coverage for any nearby foreign material (x 4000).

receptors that can recognize minor, nonself, and histocompatibility differences. One controversial explanation suggests a primitive "allogeneic inhibition" as has been observed in mammalian bone marrow transplantation systems [22]. We suggest two other more plausible interpretations: 1) recognition of/binding to and lysing of foreign cells in a NK cell-like reaction may reflect innate immunity; 2) cytotoxicity is a response that is more advanced than phagocytosis [23].

Small coelomocyte lyse classical NK-sensitive cells like K562, and NK-resistant targets recalling the nonspecific, innate character of the response [24]. Cell contact is essential as revealed by the intricacies of lysis after binding of effectors to target cell membranes and an actual tearing away of target membranes by voracious effectors. We have observed intimate contacts and holes (pores), reminiscent of those effected by eiseniapore, between effectors and targets by scanning electron microscopy (Fig. 2). By contrast to this apparently exclusive cell mediated mechanism, lysis of targets will also occur in cell free coelomic fluid and this suggests humoral mechanisms as the more immediate mediators of lysis. Lysis is only one component of immunodefense and lysins are dependent upon one or more of the coelomocyte types, as mentioned earlier, for their synthesis and secretion.

The complexity of the earthworm cellular armamentarium as revealed by light and electron microscopy as well as by flow cytometry is significantly diverse. For critical future research, it will be essential to determine the molecular mechanisms of what appears to be a pathway involving sphingomyelin in the lytic process of eukaryotic cells such as tumors as revealed in the following results. Mediators of cytotoxic reactions would then suggest effectors of primordial NK cell activity [21]. Invertebrate lytic systems spontaneously kill various targets without evidence that they possess clonal-specific immunocytes (the adaptive system) such as cytotoxic lymphocytes (CTLs), suggesting that a NK-like activity is present [22]. To support this view, dissociation of phagocytosis from NK-like killing has been defined in earthworms wherein cellular and humoral inflammatory responses and transplant rejection have been consistently observed (Fig. 3).

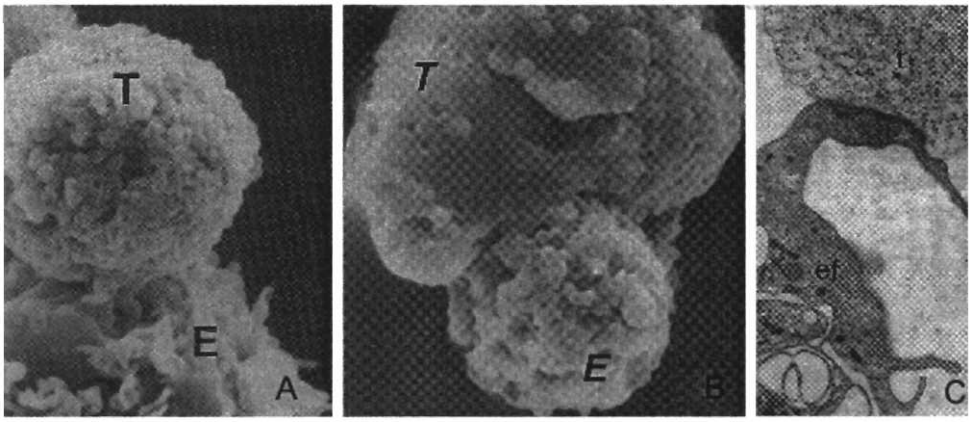

Fig. 2: Interaction between an effector (E) and a target (T) after 5 min. Contact has been established, but lytic activity has not commenced (A). Interaction between an effector (E) and a target (T) after 15 min. The effector is embedding itself into the target (B) (x 6000)[from M.M. Suzuki and E.L. Cooper, Spontaneous cytotoxic earthworm leukocytes kill K562 tumor cells, *Zool. Sci.* **12** (1995) 443-451]. Small effector coelomocytes in contact with K562 human targets. Magnification higher that 4000 x showing tight binding (C)[from A. Cossarizza *et al.*, Earthworm leukocytes that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines, Exp. Cell Res. **224** (1996) 174-182][from E.L. Cooper *et al.*, Digging for innate immunity since Darwin and Metchnikoff, *Bioessays* 2002, in press].

4. Small coelomocytes recognize and lyse before phagocytosis by large coelomocytes

As previously defined, there are two primary leukocytes involved in lytic responses: the killer cells (small coelomocyte, SC) and the phagocytic cells (large coelomocyte, LC) [24]. Two cell types accompanied by specificity and weak memory were first discovered *in vivo* during the rejection of allo- and xenografts [25, 26]. Small electron dense (as revealed by transmission electron microscopy) coelomocytes bind to targets, are not exclusively phagocytic and are CD11a-, CD45RA-, CD45RO-, CDw49B-, CD54-, Thy-1- (CD90-) and $\beta_2$microglobulin-positive; this confirms previous observations [27, 28]. Large coelomocytes C do not bind to K562 but are mostly phagocytic and negative for the above markers. Both SC and LC are negative for other CD and MHC class I and class II markers. Although the positive CD markers are associated with human lymphocytes, monocytes and macrophages, these cells are interpreted as putative (convergent?) evolutionary descendants of leukocytes like those of earthworms that also effects the two functional activities of cytotoxicity and phagocytosis. Dissociation of two, perhaps independent functions, phagocytosis and cytotoxicity, mediated by two different cell types, suggests that leukocytes are polyfunctional and not exclusively phagocytic. After lysis, what is the fate of K562 targets *in vitro*?

After determining that target cells are killed *in vitro* as it occurs *in vivo*, it was necessary to determine if after the killing process certain events take place that lead to disposal of resulting dead material. After all, once destruction occurs there must be a mechanism that relieves the system of remaining waste. Under *in vivo* conditions, disposal occurs by means of phagocytosis and when material is too large, encapsulation occurs. Often referred to as brown body formation in earthworms, this event is crucial in the riddance of foreign material [17]. When cocultured with K562, SC become agitated, extending numerous pseudopodia that bind to and spontaneously kill tumor cells. Then LC, now the most active, aggregate around lysed material, thus "encapsulating" it, according to invertebrate immunologists or forming "granulomas", in the jargon of mammalian immunologists [29] (Fig. 3).

These new results, *i.e.* dissociating cytotoxicity from phagocytosis, suggest several future paths worthy of exploration. First, we must relate the cytomorphological events to humoral mediators that may play a role such as the inflammatory cytokines IL-1 and TNF whose presence has been tentatively identified in certain invertebrates [for review 30]. Second, since there has been obvious tumoricidal activity that is interpreted to result from direct cytotoxicity, other events require analysis, *e.g.* the production of H_2O_2 and mediating factors (proteases have already been identified). Third, it would not be surprising if the granulomas that form *in vitro* actually represent previously described brown bodies that form *in vivo* and that are connected to the prophenoloxidase system.

5. Lysins from Eisenia foetida earthworms: fetidins, lysenin, eiseniapore, and coelomic cytolytic factor

5.1. Fetidins

In *Eisenia foetida* earthworms, fetidins are polymorphic and multifunctional, including responses that involve cytolysis, those that are antibacterial and certain clotting capacity [31-34]. The clotting effect probably evolved and was deployed to eliminate nonpathogenic bacteria, and is mediated by a serine protease/serine protease inhibitor equilibrium. Several bits of information suggest that clotting of the fetidins is a normal response occurring at low but constant rates on the outer surface of earthworms [35]. Mixed with mucus, fetidins cover the body and they constitute an external nonspecific antimicrobial barrier (Fig. 4).

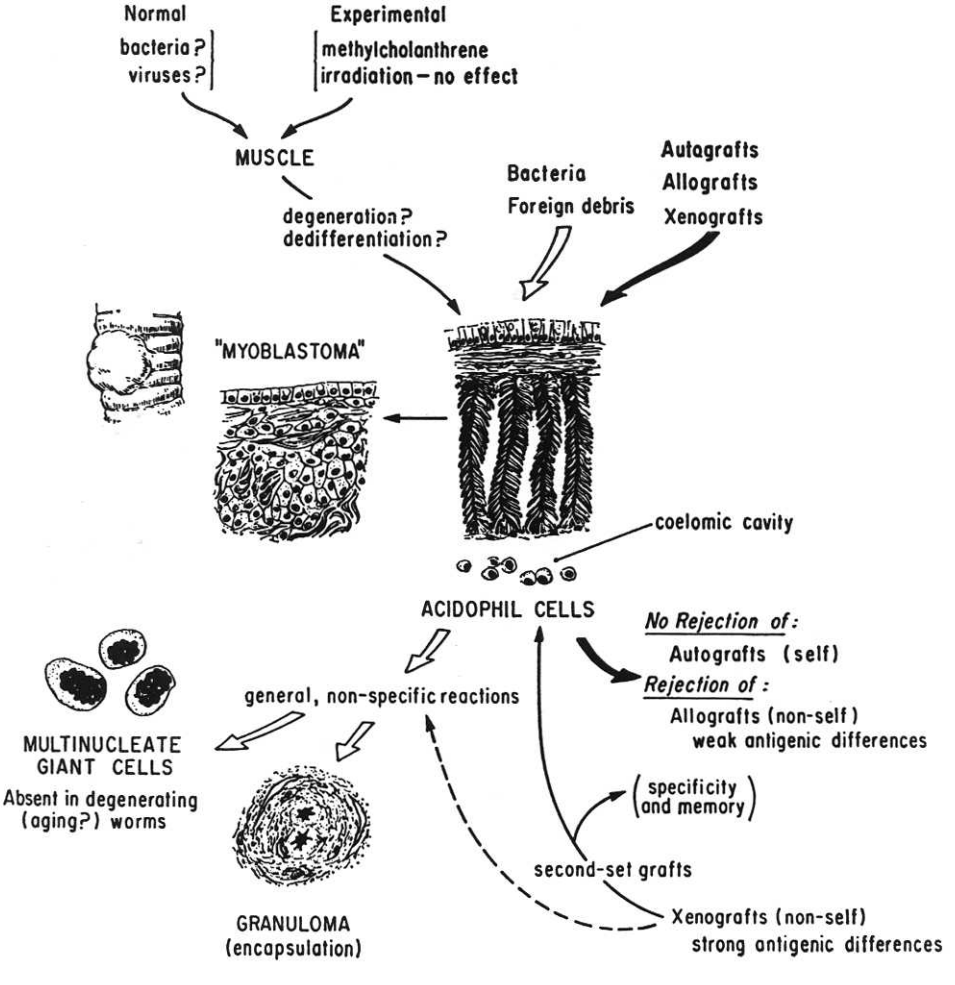

Fig. 3: Hypothetical interrelationships of inflammatory processes in earthworms [from E.L. Cooper, Neoplasia and transplantation immunity in annelids. *J. Natl. Cancer Inst.* **31** (1969) 655-669].

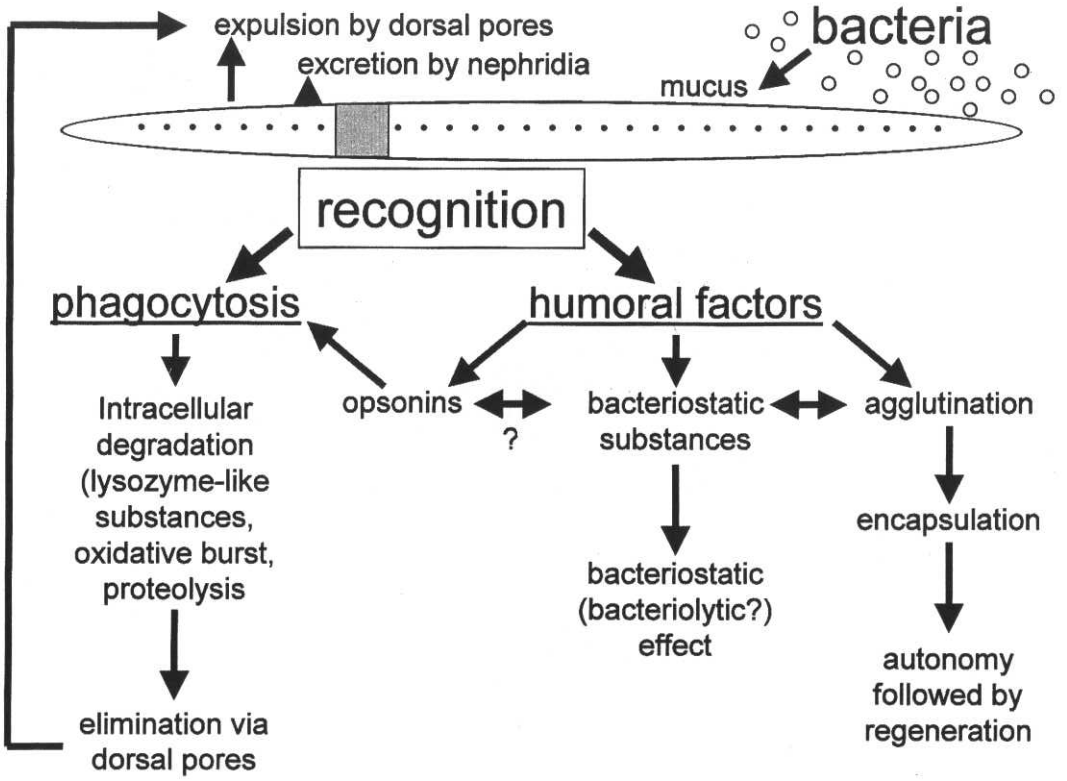

Fig. 4: General armamentarium of natural resistance of the earthworm. The first protective barrier is represented by secreted mucus containing agglutinins. Bacteria invading the coelomic cavity are directly expelled via dorsal pores and excreted by nephridia or can be engulfed by coelomocytes. Moreover, humoral factors are involved in their elimination: agglutinated bacteria can be encapsulated, antibacterial substances prevent bacteria from multiplying, and opsonins facilitate phagocytosis. All humoral factors involved in antibacterial defense seem to be connected in complex protective system [from L. Tuckova and M. Bilej, Mechanisms of antigen processing in invertebrates: Are there receptors? *Adv. Comp. Env. Physiol.* **23** (1996) 41-72][from E.L. Cooper *et al.* Digging for innate immunity since Darwin and Metchnikoff *Bioessays* 2002, in press].

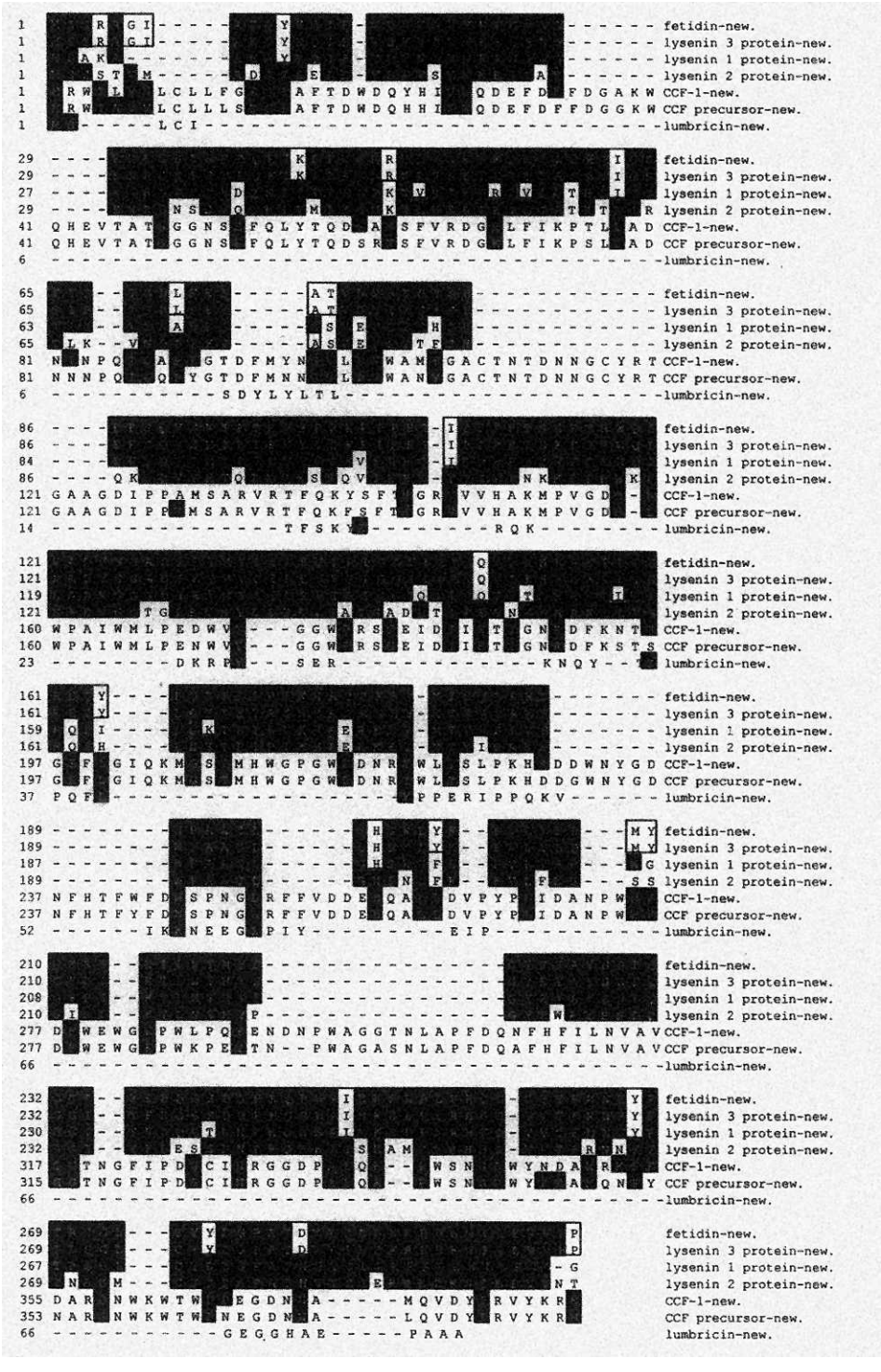
fetidin-new.
lysenin 3 protein-new.
lysenin 1 protein-new.
lysenin 2 protein-new.
CCF-1-new.
CCF precursor-new.
lumbricin-new.

Fig. 5A

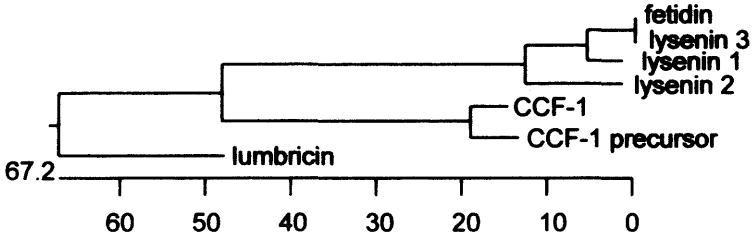

Fig. 5: Molecular characteristics of the lysins from *Eisenia foetida*: fetidin, CCF-1, lysenin and the peptide lumbricin I from *Lumbricus rubellus* (See also Table 3). **A.** Clustal alignment MegAlign (DNA STAR Inc). Fetidin and lysenin 3 show 100 % amino acid identity and 99 % nucleotide homology, suggesting the same gene. **B.** Dendogram depicts the relationships among lytic molecules. The length of each pair of branches represents the distance between sequence pairs. The scale beneath measures the distance between sequences. Units may represent the number of substitution events or percent difference (Courtesy of Duane E. Keith, Jr.)[from E.L. Cooper *et al.*, Digging for innate immunity since Darwin and Metchnikoff, *Bioessays* 2002, in press].

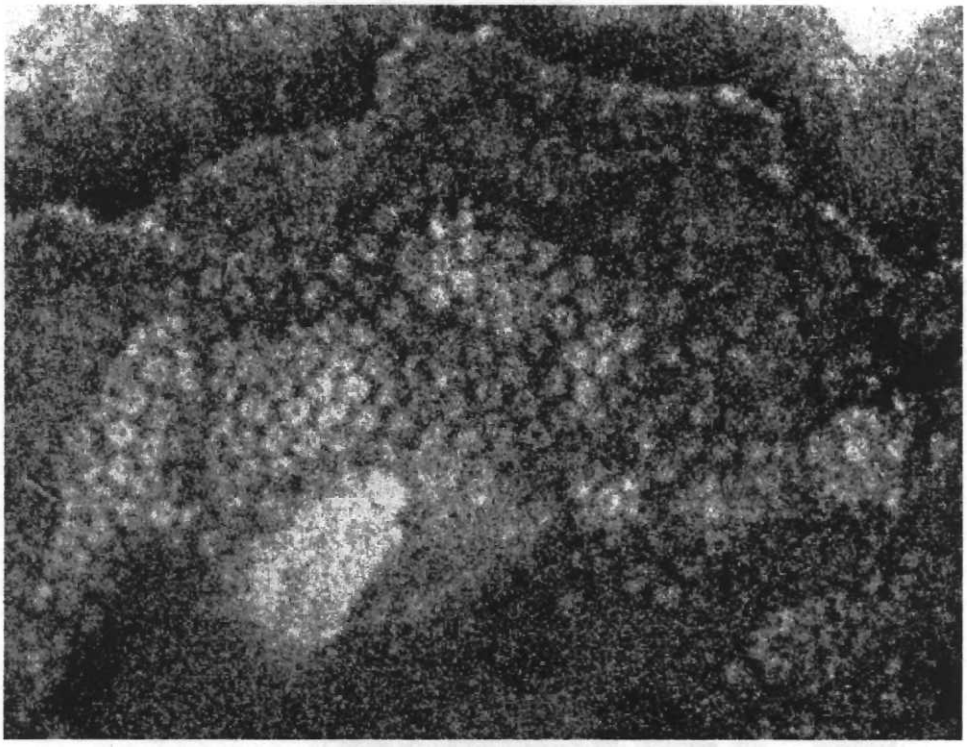

Fig. 6: Negative staining transmission electron microscopy of eiseniapore-treated sheep erythrocyte membranes and untreated membranes. The images clearly show many pore-like structures with an outer diameter of 10 nm. Note that each of the pores contains a dark inner-channel of 3 nm. The image has a honeycomb appearance [from S. Lange *et al.*, Biochemical characteristics of Eiseniapore, a pore-forming protein in the coelomic fluid of earthworms, *Eur. J. Biochem.* **262** (1999) 547-556][from E.L. Cooper *et al.*, Digging for innate immunity since Darwin and Metchnikoff, *Bioessays* 2002, in press].

Mystery surrounding lytic components of coelomic fluid from *Eisenia* has been extended and partially clarified (Tables 3, 4). Fetidins appear to be controlled by two independent genes: one expresses and codes for a pI 6.1 hemolysin; the other is composed of four alleles that define ten genetic families. Significant similarities are characterized by large quantities of aspartic acid, glutamic acid, and glycine; polyclonal and monoclonal antibodies do not discriminate between purified hemolysin [31]. Lysis usually depends upon the presence of glycoprotein molecules and when erythrocytes are used as targets, lysis is mediated by two monomeric lipoproteins of 40 and 45 kDa termed fetidins (from the earthworms, *Eisenia foetida,* so called because of their fetid odor if they are disturbed), two related polymorphic immunodefense factors [31]. A cDNA containing an insert of 1.44-kb encoding a 34-kDa protein that corresponds to the size of deglycosylated fetidins has been cloned [9]. The cDNA sequence contains a peroxidase signature that confirms peroxidase activity of fetidins. The recombinant protein is antibacterial since it inhibits growth of *Bacillus megaterium.* Fetidin possesses varying degrees of significant homology with lysenins 1, 2, 3; there is no significant homology with CCF nor lumbricin I (Fig. 5).

Table 3: *Eisenia foetida* proteins exerting cytolytic functions (except antimicrobial lumbricin I from *Lumbricus rubellus*)

Name	Accession number	Homology	Presence	Function
Lysenin, 42 kDa *EfL1* (L1) *EfL2* (L2) *EfL3* (L3) [36]	 D85846 D85847 D85848	 U02710 (Fetidin 1)	Coelomic fluid, chloragocytes, coelomocytes	Contracts rat smooth muscle
Fetidin 1, 40 kDa Four isoforms Fetidin 2, 45 kDa monomorphic [9]	U02710	D85848 (Lysenin)	Coelomic fluid, chloragocytes, inducible, increases after injecting pathogenic bacteria	Hemolysis, bacteriolysis, agglutination, clotting, opsonization, heme-binding enzymes, peroxidase
Coelomic Cytolytic Factor, 42 kDa [44]	AF030028	AF395805 (*L. terrestris* CCF)	Coelomic fluid, coelomocytes	Opsonization, Not hemolytic, pattern recognition
Lumbricin I, 7.2 kDa [80]	AF060552		Whole worm, not inducible, constitutive	Antimicrobial, not hemolytic

Table 4: Lytic factors found in *Eisenia foetida*

Name	Assay System	Mol. mass	References
Lytic Factor	NK-cell assay Targets: NK-dependent K562 NK-independent U937, BSM, CEM	ND	[22, 24, 29]
Fetidin	Binding to sphingomyelin on RBC membranes, bactericidal activity	40, 45 kDa	[9, 18, 19, 31, 32, 34]
Lysenin	Binding to sphingomyelin on RBC membranes, liposome	41kDa	[37]
Eiseniapore	Relief of fluorescence quenching from liposomes	38 kDa	[41, 42]
Hemolysin (H_1, H_2, H_3)	Isolated by preparative PAGE	46, 43, 40 kDa	[81]

5.2. Lysenin

Lysenin, isolated from *E. foetida*, is another protein of similar character, *i.e.* sharing biological roles and biochemical properties with fetidins, and eiseniapore, but it has been shown to effect different and perhaps more important functional properties [36, 37](Tables 3, 4). Lysenin was isolated as a 41-kDa protein, causes contractions of smooth muscle from the rat-isolated aorta. Although recombinant and native lysenin had similar contractile activities when tested on rat aorta, the amino acid sequence of lysenin revealed no significant homology to previously characterized vasoactive substances. Like the other lytic components, especially eiseniapore, lysenin induces hemolysis and binds specifically to sphingomyelin among various phospholipids in cellular membranes, including those found in various spermatozoa. However, lysenin differs in amino acid sequence and certain biological activities from the other lytic proteins isolated from coelomic fluid of *E. foetida*. There was no binding of lysenin to other phospholipids including sphingomyelin analogues such as sphingosine, ceramide, and sphingosylphosphorylcholine, suggesting recognition of the precise molecular structure of sphingomyelin. Further analyses revealed that if cholesterol incorporation changes the topological distribution of membrane, sphingomyelin accessibility to lysenin increases. Northern blot analysis of the RNA from earthworm tissues indicated that lysenin is produced by coelomocytes [36, 38]. In particular, immunoreactive lysenin was detected in LC and in the free large chloragocytes present in the lumen of the typhlosole, a depression in the dorsal wall of the intestine. These coelomocytes and chloragocytes seemed to be mature and separate from the chloragogen tissue that lined the typhlosole. The free large chloragocytes in the typhlosole contained numerous vacuoles. The nuclei were small and irregular in shape, and glycogen granules and mitochondria were occasionally found between vacuoles. The chloragocytes of the chloragogen tissue that surrounded the coelomic side of the intestine and the dorsal blood vessel did not react with the lysenin antiserum and no expression of lysenin mRNA was detected in these cells. Furthermore, no evidence of the protein or of the mRNA was found in the cells of the pharyngeal gland. Their findings suggest that lysenin is produced in the free large chloragocytes in the lumen of the typhlosole.

Kobayashi *et al.* [39, 40] found that the coelomic fluid of *E. foetida* was not toxic to 42 species, belonging to seven invertebrate phyla, almost all in aquatic adults and larvae. Eleven teleostean species tested died in 0.2-1 % coelomic fluid mostly between 10 and 120 min and the effects were dose-dependent. Tadpoles of the toad *Bufo japonicus formosus* died in 0.4-2 % coelomic fluid between 80 and 225 min depending upon size, with larger tadpoles

surviving longer. Before dying, all experimental tadpoles developed curled and shrunken tails. The Okinawa tree lizard, soft-shelled turtle, Japanese quail, mouse and rat all died after i.v. injection of coelomic fluid (above 20 μl/kg). Thus, coelomic fluid was not toxic to invertebrates, but toxic to vertebrates. After heating, coelomic fluid lost its toxicity to fish, tadpoles and mice. Coelomic fluid incubated with sphingomyelin-liposomes was no longer toxic, suggesting the involvement of sphingomyelin in the toxicity. Since lysenin, which is a constituent of the coelomic fluid and known to bind specifically to sphingomyelin, exhibited toxicity similar to that of coelomic fluid, it was probably responsible for the toxic effects of coelomic fluid in vertebrate tissues. Accordingly, lysenin exerted lethal effects on spermatozoa of 5 of 33 species of invertebrates tested, and on spermatozoa of 30 of 39 species of vertebrates. It was postulated that plasma membranes of the spermatozoa of most invertebrates might not contain sphingomyelin whereas those of most vertebrate species might contain sphingomyelin. These possibilities were supported by the failure to detect sphingomyelin chemically in the testes of three species of invertebrates, none of which spermatozoa responded to lysenin. In contrast, sphingomyelin was detected in the testes of all 25 vertebrate species examined, irrespective of a negative or positive response of spermatozoa to lysenin. None of the six species of *Protista* examined was affected by lysenin. This work suggests that, in general, the spermatozoa of animals can be grouped into two categories, invertebrate and vertebrate, depending on the absence or presence of sphingomyelin in their plasma membrane. The incorporation of sphingomyelin into spermatozoa seems first to have occurred in protochordates during the course of evolution.

5.3. Eiseniapore

To approach the mechanism of synthesis and secretion of lytic components in *E. foetida*, a novel new system has been defined and lytic activity ascribed to eiseniapore, a protein of 38-kDa [41](see also Kauschke *et al.* in this book)(Tables 3, 4). Lipid vesicles of various compositions were used to determine whether specific lipids might serve as receptors to eiseniapore. The lysins bind to and disturb the lipid bilayer only when distinct sphingolipids consisting of a hydrophilic head group as phosphorylcholine or galactosyl and the ceramide backbone, (*e.g.* sphingomyelin), are present. Cholesterol enhances eiseniapore lytic activity toward sphingomyelin-containing vesicles probably due to interaction with sphingomyelin. Leakage of vesicles was most efficient when the lipid composition resembled that of the outer leaflet of human erythrocytes.

Further analysis revealed that the secondary structure of eiseniapore did not change upon binding to lipid membranes and lytic activity of eiseniapore was completely abolished after its denaturation or after preincubation with polyclonal antibodies. Absence of lysis suggests two mechanisms: 1) presence of specific sphingolipids is sufficient for eiseniapore mediated lytic activity; 2) membrane glycoproteins, as receptors for eiseniapore, are not required as long as specific sphingolipids are present. Still further analyses by electron microscopy revealed interesting characteristics concerning eiseniapore and erythrocyte membranes [42]. Several ring shaped structures (pores) were found to be composed of a central channel that contained outer (10 nm) and inner (3 nm) diameters. This channel complex consists of six monomers as deduced from the molecular mass of 228 kDa. Functional evidence of pore formation by eiseniapore was revealed since protection of lysis by carbohydrates occurred at an effective diameter above 3 nm (Fig. 6). Channel formation complex suggest two conclusions: 1) the existence of a channel or a "barrel stave model" (closed beta-pleated sheet) for vesicle and cell interaction of eiseniapore; 2) a plausible explanation for the mechanism by which components of the earthworm humoral immune system directly destroys nonself cells.

5.4. Coelomic cytolytic factor

Bilej *et al.* in experiments aimed at identifying cytolytic molecules from the coelomic fluid of *E. foetida* purified a 42-kDa protein that was named coelomic cytolytic factor (CCF). This protein is able to lyse TNF-sensitive tumor cell line in a protease-independent way but is not hemolytic. CCF displays high homology with invertebrate pattern recognition molecules, and participates in *Eisenia* defense mechanisms triggering the activation of the prophenoloxidase cascade upon binding to conserved microbial polysaccharides. The lytic activity of CCF observed on mammalian tumor cells may not be relevant for the immune defense of earthworms. It was shown that CCF displays functional analogies with the mammalian cytokine TNF, despite a lack of gene or amino acid sequence homologies [30, 43-50]. Beschin *et al.* and De Baetselier *et al.* in this book summarize the biological activities of CCF.

5.5. Serine proteases may play a role in the regulation of the lytic activity of earthworm molecules

Serine proteases are important enzymes involved in regulating many physiologic events, including extrinsic pathways of blood coagulation and immune processes. There are several types of serine proteases, *e.g.* trypsin, thrombin, granzyme B, subtilisin/kexin-related endopeptidases and other neuropeptide endopeptidases. Serine proteases have also been implicated in the processing of peptide precursors into active immune signaling molecules (*e.g.* methionine enkephalin), which may stimulate proinflammatory events. As such they are of interest in the earthworm immune response capacities.

Three serine proteases have been isolated from coelomocyte lysates of *E. foetida andrei*, one of which was a trypsin-type (Table 4)[10, 51]. Serine protease activity has been reported in *Lumbricus terrestris* [52, 53]. According to interactions observed principally in insects, extracellular serine protease inhibitors regulate intracellular proteolytic enzymes [54]. Purified to homogeneity by affinity chromatography on trypsin, the serine protease inhibitor from *E. foetida* is a monomer of 14 kDa. Its partial N-terminal amino acid sequence revealed a basic hydrophobic fragment that shared 68-75 % homologies and 47-60 % identities with several plant serine protease inhibitors. The cytotoxic activity of fetidins was stimulated *in vitro* by several serine proteases while incubation with soybean trypsin inhibitor variant a (STIa) resulted in less cytotoxicity [10]. The inhibitory effect occurred only when STIa was added before cell lysis. According to one interpretation, cytotoxicity involves the release of intracellular cytotoxic proteins, intracellular trypsin-like activator and extracellular serine protease inhibitor. One can imagine that this proposed chain of events provides a regulatory mechanism for both cellular/humoral immune responses of earthworms, linking them.

6. Clinical applications of earthworm lytic molecules?

Earthworm lytic molecules are antimicrobial and may prove useful as antibacterial agents and prophylactic molecules, an idea that is not farfetched since the discovery of antibiotics was serendipitous. Lysenin and eiseniapore depend to some extent on intracellular lipid trafficking mechanisms. In fact, trafficking dysfunctions lead to disease development, such as Tangier disease and Niemann-Pick disease type C, or contribute to the pathogenesis of diseases such as Alzheimer disease and atherosclerosis. Lysenin reacts specifically with fibroblast membranes from patients with Niemann-Pick disease, a rather curious finding but one that may have some clinical relevance [37](Table 4). Thus, specific binding of lysenin to sphingomyelin on cellular membranes may prove to be a useful tool to probe the molecular

motion and function of sphingomyelin in biological membranes especially in an effort to explain the mechanism of lysis, at least in earthworms. These results stress the need for concerted analyses of various lytic pathways that may be mediated by the earthworm immunodefense system.

Two points deserve consideration with respect to lysis induced by earthworm factors: 1) source of targets *i.e.* intrafamilial allogeneic or xenogeneic targets; those derived from mammals, *e.g.* K562 and erythrocytes; 2) nature of cell death (Fig. 7, 8). Regardless of the target, in either instance, cell death may be due to necrosis or apoptosis [55-57]. This clarification would strengthen proposals concerning evolutionary views of apoptosis as have been deciphered for *Caenorhabditis elegans* and *Drosophila.* We may even reveal more informed clues concerning the absence of proven cancer in earthworms and most other invertebrates (with the possible exception of *Drosophila*)(Fig. 3). Assuming that cancer poses a natural threat despite the presence of an efficient innate immune system, we can question whether natural cell killing capacity of earthworms is so efficient that any potentially malignant cells never escape its surveillance. Products of the earthworm innate immune system could be tested in clinical trials against tumors as has been reported for the didemnins, a family of seven amino acid, cyclic depsipeptides isolated from deuterostome marine invertebrates (tunicates, *Didemnidae*)[58, 59]. Although not clear, didemnin effects may be mediated by high-abundance of a low-affinity cytosolic receptor similar to that for cyclosporine, FK-506, and rapamycin, cytosolic proteins named the immunophilins [59].

7. Earthworm lytic molecules that require more definition in relation to clinical application

In this portion of the review, the work of investigators representing a perhaps-different approach to the problem of earthworm immunity will be presented in some details. The previous paragraphs were more concerned with the possible applications of lytic components from the earthworm as vital to the immune response armamentarium. This next series of works from Japan, Korea, China and Croatia present a different picture, one aimed at finding some more clinical relevance. This activity from the decade of the 1990s provides possible clinical approaches. From earthworms, one factor (lombricine) inhibits growth of spontaneous tumors in SHN mice [60] whereas another, the "killer" glycolipoprotein extract called G-90 retards murine tumor growth *in vivo*. However, all other biological activities associated with coelomic fluid are retained. These include hemolysis, agglutination, mitogenicity, bacteriostatic activity and cytotoxicity *in vitro* [61]. There are even claims that earthworms are used by the pharmaceutical industry in China [62] and elsewhere [63, 64] to prepare certain medicinals (lumbrokinase) for treatment and prevention of thrombus and embolic diseases.

7.1. Japanese discover fibrinolytic activity in Lumbricus rubellus

Earthworm derivatives have been used as drugs against various diseases in China and the Far East for a few thousand years. However, modern scientific pharmacological studies have not yet been performed. Experiments involved designing trials for *in vivo* experiments on human volunteers, where 120 mg of lyophilized earthworm powder was orally administered to 7 healthy volunteers (28-52 years old) three times a day after meals for 17 days. Blood was withdrawn once a day before and at 1, 2, 3, 8, 11 and 17 days after the administration. The fibrin degradation products value, tissue plasminogen activator (t-PA) antigen level and t-PA activities were measured in the blood. After administration, the t-PA antigen level gradually increased until the seventeenth day. The fibrin degradation products level increased on the

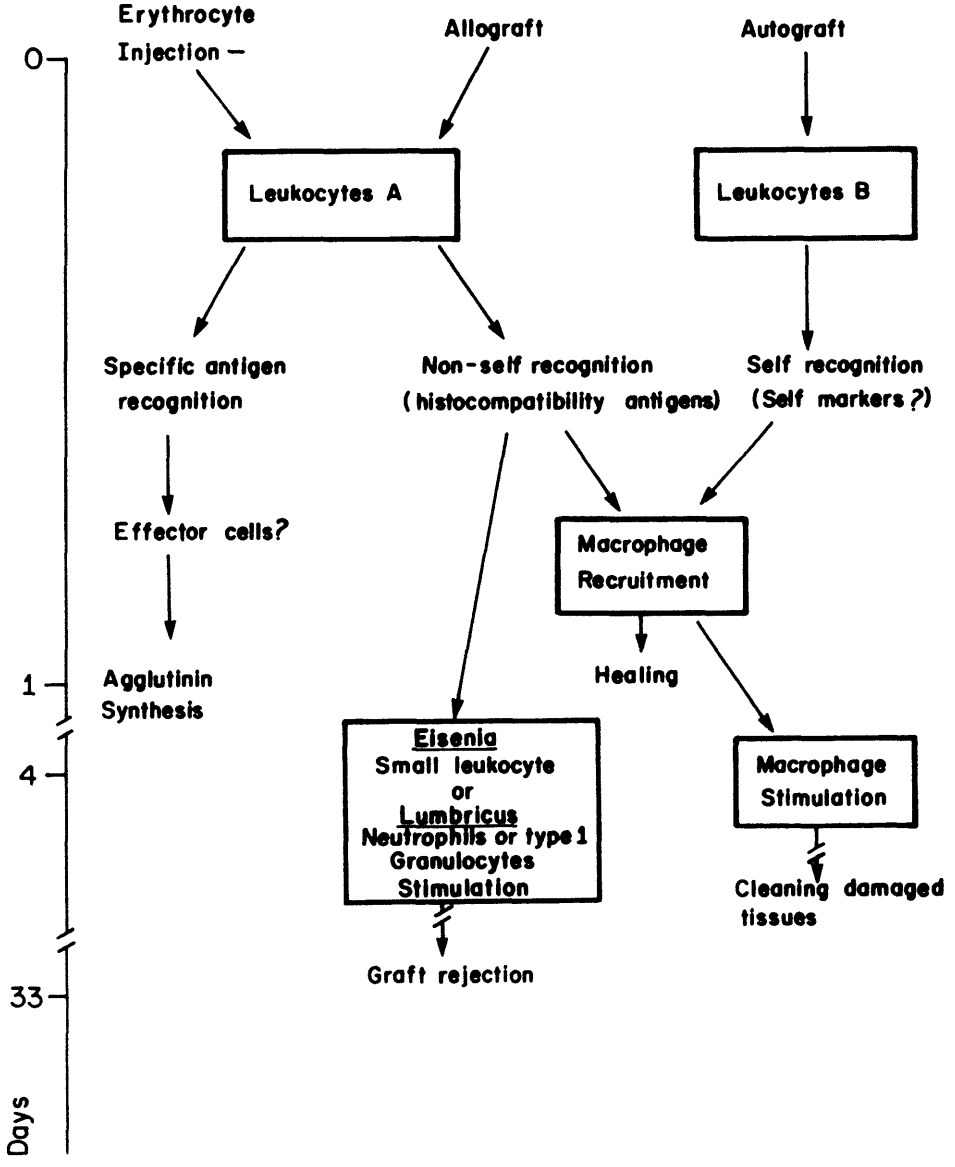

Fig. 7: Hypothetical scheme of earthworm leukocyte interactions occurring in grafting and agglutinin synthesis following erythrocytic injection, allografting, or autografting. Note that graft rejection occurred at 33 days, whereas cleaning damaged tissues after day 33, not before [from E.L. Cooper, Evolution of histoincompatibility. In M. Brehelin (Ed.), Immunity in Invertebrates (Cells, Molecules and Defense Reactions), Springer Verlag, Berlin, 1986, pp. 139-150].

first and second days but decreased and normalized by day 17. Because fibrinolytic activities increased, these results suggest that earthworm powder represents a possible oral thrombolytic agent. This earthworm enzyme may be applicable for treating patients with thalassemia.

As an attempt to discover possible therapeutic effects previously ascribed to historically recorded traditions, the groups of Mihara *et al.* and Nakajima *et al.* [65-68] have obtained strong fibrinolytic enzymes in saline extracts of the earthworm, *Lumbricus rubellus*. These enzymes hydrolyze both plasminogen-rich and free plates, are heat-stable (up to 60 °C) and display a broad optimal pH range (1-11). Three partially purified fractions of enzymes have been further subdivided. The first fraction (F-I) is divided into three fractions (F-I-0, F-I-1, and F-I-2), that exhibit similar biochemical characteristics, but the second fraction (F-II) is not subdivided. The third fraction (F-III) is divided into two more fractions (F-III-1 and F-III-2). Based on enzymatic activities against various substrates, fraction I enzymes are chymotrypsin-like and fraction III enzymes are trypsin-like. Fraction II is neither a trypsin- nor an elastase or chymotrypsin-like enzyme. Amino acid compositions of the six enzymes have been estimated and found to contain abundant asparagine or aspartic acid, sparse amounts of proline or lysine, and do not contain sugar components. These enzymes are therefore regarded as novel fibrinolytic enzymes, and referred to collectively as *lumbrokinase* from the earthworm generic name. Analyses of substrate specificity and inhibition indicated that these enzymes were trypsin-like serine proteases. The N-terminal amino acid sequences of the enzymes revealed similarities to those of trypsin-like enzymes such as elastase and coagulation factor IX. These six enzyme proteins were suggested to derive as isozyme(s) from at least four different genes.

The strongest fibrinolytic protease of the six enzyme proteins purified from *Lumbricus rubellus* (F-III-2) have been modified chemically with fragmented human serum albumin (10-30 kDa)[68]. This modified enzyme lost antigenicity characterized by the native enzyme and reacted with antisera against human serum albumin as well as their fragments, and a conjugate with the native enzyme to form precipitation lines that fused with each other. The conjugate was increasingly resistant to inactivation by protease inhibitors in rat plasma. The enzyme was a nonhemorrhagic protein and induced no platelet aggregation. It also retained potent proteolytic activity for fibrin and fibrinogen as that of human plasmin. This protein easily solubilized actual fibrin clots (thrombi) of whole blood induced by thrombin in a rat vena cava. The continuous fibrinolysis for fibrin suspension in an enzyme reactor system that uses the modified enzyme immobilized to oxirane-activated acrylic beads had been achieved without any inactivation of the activity at least for more than 1 month.

7.2. Korea: Lumbrokinase is discovered as a fibrinolytic enzyme

Lumbrokinase is a potent fibrinolytic enzyme purified from the earthworm, *Lumbricus rubellus* that was stable and showed greater antithrombotic activity than other fibrinolytic proteins [69, 70]. A lumbrokinase fraction showing the most potent fibrinolytic activity was immobilized onto a polyurethane surface to investigate its enzymatic and antithrombotic activity. The stability of immobilized lumbrokinase was determined by caseinolytic activity assay and the specificity of immobilized lumbrokinase on fibrinogen/fibrin was observed by SDS-PAGE. Immobilized lumbrokinase retained about 34 % of its activity, when compared to its soluble state and it also demonstrated stability against thermal inactivation and degradation and within a pH range. The optimal pH of immobilized lumbrokinase shifted 1.0 pH unit upward when compared with the soluble enzyme. Immobilized lumbrokinase demonstrated stable proteolytic activity during various incubation periods in addition to proteolyzing fibrinogen and fibrin almost specifically, while hardly hydrolyzing other plasma proteins including plasminogen and albumin. Upon exposure to the human whole blood, less amounts

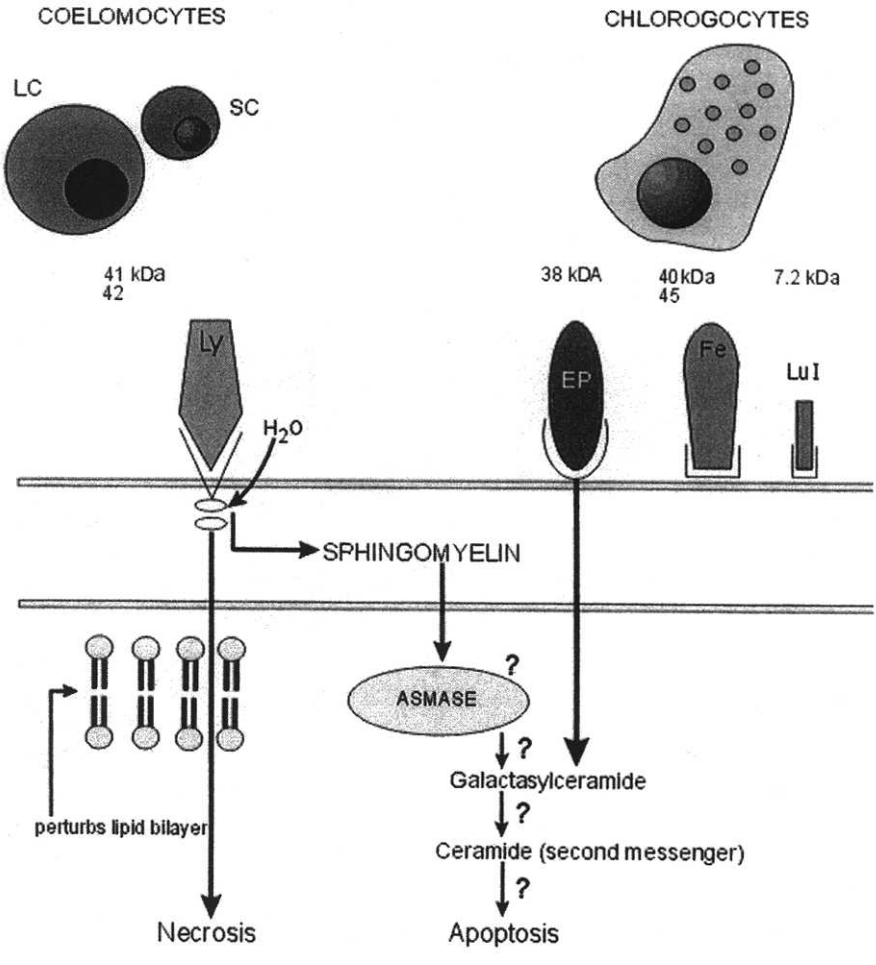

Fig. 8: Hypothetical scheme showing the relationship between the various lytic components, lysenin (Ly), fetidin (Fe), eiseniapore (EP) and the antimicrobial nonlytic peptide lumbricin I (Lu I) and the target cell membrane (sphingomyelin). Targets for lysis have included various erythrocytes, and tumor cells. Defects in acidic sphingomyelinase leads to Niemann Pick disease and lysenin binds specifically to these fibroblasts. Lysenin also causes contraction of rat smooth muscle. The interaction is suggested to occur primarily through a perturbation of the outer leaflet of the membrane sphingomyelin reaching the lipid bilayer. A series of changes that result in cell death are set into motion [modified from E.L. Cooper *et al.*, Digging for innate immunity since Darwin and Metchnikoff, *Bioessays* 2002, in press].

of ^{125}I-fibrinogen were adsorbed to the lumbrokinase-immobilized surface than to the polyurethane control surface. The lumbrokinase-immobilized surface showed less platelet adhesion than did the MAMEC-grafted surface. Initially, the number of adhered platelets increased on the lumbrokinase-immobilized surface with time; yet, the platelet number drastically decreased on the lumbrokinase-immobilized surface after 80-min incubation. This suggests that lumbrokinase-immobilized polyurethane digested the adsorbed fibrinogen and inhibited platelet adhesion on the surface, probably by making fibrinogen adsorption be highly antithrombogenic. In the *ex vivo* A-A shunt experiment, the lumbrokinase-immobilized surface significantly prolonged occlusion time over control surfaces, which is primarily due to the high thrombolytic activity of immobilized lumbrokinase. Consequently, a highly efficient surface modification method on the polyurethane surface was developed, and this lumbrokinase immobilization technique could be useful in improving blood compatibility of blood-contacting devices. Moreover, these results lead to the suggestion that clinical applications of this material to artificial organs should be developed in the near future.

7. 3. China: The Eisenia foetida enzyme

In Beijing, Yang and Ru [71] have purified a SDS-activated fibrinolytic enzyme from *E. foetida,* the *E. foetida* enzyme. This 45 kDa protein was composed of two subunits (26 and 18 kDa) held together by hydrophobic interactions. The enzyme displayed four activities on fibrin plates used to detect proteolytic activity. These activities were designated as CFPg (complete fibrinolysis in the plasminogen-rich plate), uCFPg (uncompleted fibrinolysis in the plasminogen-rich plate), CF (complete fibrinolysis in the plasminogen-free plate), and uCF (uncompleted fibrinolysis in the plasminogen-free plate). SDS-activated CFPg and rendered the enzyme more sensitive to some inhibitors. For example, leupeptin, chymostatin, pepstatin, aprotinin, phenylmethylsulfonyl fluoride, and dithiothreitol had no effect on uCF. Pepstatin stimulated CFPg and uCFPg, while E-64, a thiol inhibitor, activated uCFPg and uCF. The large subunit alone had catalytic activity, while the small subunit did not. Using plasminogen as the substrate for defining peptide-bond specificity, the *E. foetida* enzyme was observed to cleave the carboxyl side of basic amino acids, small neutral amino acids, and methionine residue.

7. 4. Croatia: a G-90 is a biologically active glycolipoprotein

Hrzenjak *et al.* [61] isolated a biologically active glycolipoprotein extract from whole earthworm tissue homogenates called G-90. G-90 forms precipitation arcs in gel with different animal and human sera. It alters murine cell growth rate *in vitro* in serum in a dose-dependent manner and slows murine tumor growth *in vivo*. G-90 does not contain mutagens or carcinogens. Later this same group separated G-90 into seven fractions by gel-filtration [72]. Radioimmunoassays revealed that each of the fractions, except the lightest one, is crossreactive with porcine anti-insulin antibodies. Also, all fractions, except the heaviest and the lightest one, stimulate mammalian normal and transformed cell proliferation in serum-free conditions *in vitro*. The intensity of stimulation depends on cell type. Stimulation is completely abolished if the medium is supplemented with fetal calf serum.

The glycolipoprotein mixture G-90 was shown to contain two serine proteases (P I, P II) with fibrinolytic and anticoagulative activities [63, 64]. The fibrinolytic activity of G-90, P I and P II was tested in an *in vitro* euglobulinic test applied to fibrin clot from blood plasma of patients suffering from malignant tumors. G-90 and proteases showed euglobulinic time proportionally with the concentrations of added substances. The influence of G-90 on the fibrinolysis rate does not depend only on its concentration, but depends as well on histological

tissue type (organ) where malignant tumors are located. Both P I and P II exhibit fibrinolytic and anticoagulative activities, but the activity of P I is much higher. P I in concentration of 10^5 ng/ml of plasma shortened the physiological time of fibrin clot lysis by 54 % and completely inhibited blood clotting at a concentration of 10^3 ng/ml of venous blood.

8. What have we learned from the earthworm as a model invertebrate?

Most of our knowledge of defense mechanisms is mainly based on data obtained in vertebrates, particularly mice and men, while about 95 % of all two million animal species on the earth belong to invertebrate phyla. Moreover, invertebrates have evolved for hundreds of millions of years often surviving in hostile environments. Their survival strategies surely involve short life span combined with numerous offspring. In addition, despite the absence of an adaptive immune system, invertebrates have evolved a variety of active innate defense mechanisms based on "pattern recognition receptors" that in most cases do not discriminate between individual pathogens. These invertebrate nonadaptive defense mechanisms include both unique pathways unknown in vertebrates and more universal mechanism present throughout the whole animal kingdom. In this respect, knowledge of the less complex invertebrate defense strategies may contribute to understand the sophisticated vertebrate immune system, as well as lead to the identification of new factors with possible therapeutic use. Such insights may also contribute to understand the evolution of immunity.

Coelomic fluid of earthworms exerts not only enzymatic activities, but it has also potent cytotoxic and cytolytic function [for reviews 23, 73, 74]. Kauschke and Mohrig [75] described the toxic effect of *E. foetida* coelomic fluid on different cell types, such as chicken fibroblasts, guinea-pig polymorphonuclear leukocytes, and insect hemocytes, while the viability of coelomocytes of other lumbricids as well as cells of some mollusks, nematodes, and protozoans was not affected. The toxic effect seems to be correlated with the hemolytic/antibacterial activity, since three out of seven hemocytic antibacterial fractions exerted cytotoxic activity. Compounds with antitumor activities are of particular interest especially when they exert pleiotropic functions (tumoristatic, mitogenic, fibrinolytic)

Earthworms, belonging to oligochaete annelids, became a model for comparative immunologists in the early sixties with the publication of results from transplantation experiments. These results proved the existence of self/nonself recognition in earthworms, paving the way for more extensive studies on the earthworm immune mechanisms that evolved to prevent the invasion of pathogens. Within the last 35 years numerous papers on proteolytic, hemolytic, antibacterial, and cytolytic properties of earthworm coelomic fluids were published [for reviews 46, 73]. Moreover, annelids represent one of the animal groups traditionally studied by biologists to seek new molecules with potentially therapeutic use. Though the earthworm has been used as a drug for various diseases in China and the Far East since a few thousand of years ago, more detailed scientific studies have been performed only recently [65, 66]. Lumbrokinase, a fibrinolytic enzyme isolated from the earthworm *Lumbricus rubellus*, has been suggested for therapeutic use as a potent thrombolytic agent and recently is commercially available as a novel orally administered fibrinolytic agent for the prevention and treatment of cardiac and cerebrovascular diseases [76].

Invertebrates, like vertebrates, possess factors regulating responses to infection or wounding. In fact, as mentioned earlier, vertebrate innate immunity resembles a mosaic of invertebrate immune responses [77]. One mechanism in earthworms has identified and focused on lytic molecules. At least three of them have come into sharper focus with respect to sequence and possible interrelations. We now know the sequences of molecules derived from *Eisenia foetida*: fetidin, lysenin and CCF, but this must be deciphered definitively for

eiseniapore, H_1, H_2, and H_3. Clearer explanations are needed concerning lumbricin I, a small peptide isolated from *Lumbricus rubellus*. Evidently, within the oligochaete annelid group, there are vast differences that suggest families of peptides. With respect to lysis, clearly the picture may now emerge as even more complicated.

Recently, Kauschke *et al.* [78] and Komiyama *et al.* [79 and this issue] have presented interesting evidence concerning the existence of yet another lytic component that is "perforin-like". The function appears similar to lysins but the molecular structure is different. In the face of this new evidence, one possible explanation suggests that perforin perforates the holes in target cell membranes thus facilitating the entry of either or all of the family of lysins. Although not yet investigated in depth, the lysins appear to be derived from chlorogogue tissue whereas perforin is associated with SC but less so with the LC. Coordinated regulation of lytic molecules may occur via earthworm serine proteases, but this must be more clearly defined.

Well known extant species have probably relied heavily on immune competence as a highly successful survival strategy. We could exploit this strategy and with enlightened comprehension discover modes of therapy beneficial to humans. In good conscience and at the same time, we should preserve and not severely exploit any species that have inhabited the earth for millions of years. Earthworms possess advantages other than bait for those who fish. Earthworms are not parasites nor pests, and in some cultures (including certain advocates in the west) they have been proposed as another source of nutritious food. Moreover, their presence in and importance to soil maintenance is undisputed where they constitute a sizeable biomass.

The last and most important question to ask is what do these earthworm defense molecules have to do with clinical or biomedical application. On the one hand with respect to molecular data we do know something about the lysins fetidins and lysenin as well as CCF. We also know their relationships to each other. However, as the latter portion of this paper has shown there is a wide degree of variability of technical approaches to determine their activity in biomedical applications. How to translate and to apply these earthworm molecules to humans remains seemingly problematic outside of certain countries in Asia.

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References

[1] C. Darwin, Formation of vegetable mould through the action of worms (With a foreword by Stephen Jay Gould) *The University of Chicago Press,* 1881, pp. 326.

[2] V. Vetvicka *et al.* 1994. Immunology of Annelids. CRC Press, Boca Raton, 1994 p. 300.

[3] E. Kauschke and W. Mohrig, Cytotoxic activity in the coelomic fluid of the annelid *Eisenia foetida* Sav, *J. Comp. Physiol.* B **157** (1987) 77-83.

[4] E. A. Stein and E.L. Cooper, Carbohydrates and glycoprotein inhibitors of naturally occurring and induced agglutinins from the earthworm, *Lumbricus terrestris, Comp. Biochem. Physiol.* **76B** (1983) 197-206.

[5] E.A. Stein and E.L. Cooper, *In vitro* agglutinin production by earthworm leukocytes, *Dev. Comp. Immunol.* **6** (1988) 613-624.

[6] F. Hirigoyenberry *et al.*, Antibacterial activity of *Eisenia fetida* andrei coelomic fluid: transcription and translation regulation of lysozyme and proteins evidenced after bacterial infestation, *Comp. Biochem. Physiol.* **95B** (1990) 71-75.
[7] A. Wojdani *et al.*, Mitogenic effect of earthworm (*Lumbricus terrestris*) coelomic fluid on mouse and human lymphocytes, *Immunobiology* **166** (1984) 157-167.
[8] R. Hanusova *et al.*, Identification of a coelomic mitogenic factor in *Eisenia foetida* earthworm, *Immunol. Lett.* **65** (1990) 203-311.
[9] M. Lassègues *et al.*, Sequence and expression of an *Eisenia foetida* derived cDNA clone that encodes the 40 kDa fetidin antibacterial protein, *Eur. J. Biochem.* **246** (1997) 756-762.
[10] Ph. Roch *et al.*, Characterization of a 14 kDa plant-related serine protease inhibitor and regulation of cytotoxic activity in earthworm coelomic fluid, *Dev. Comp. Immunol.* **22** (1998) 1-12.
[11] Ph. Roch, Invertebrate immune responses: cells and molecular products, *Adv. Comp. Env. Physiol.* **23** (1996) 116-145.
[12] P. Valembois *et al.*, Natural and induced cytotoxicities in sipunculids and annelids. In M.J. Manning (Ed.), Phylogeny of Immunological Memory, Elsevier Biomedical Press, North-Holland, 1980, pp. 47-55.
[13] P. Valembois *et al.*, Evidence of an MLR-like reaction in an invertebrate, the earthworm *Eisenia fetida*. In J. B. Solomon (Ed.), Aspects of developmental and comparative immunology, Pergamon Press, Oxford, 1980, pp. 23-30
[14] E.L. Cooper and Ph. Roch, Earthworm leukocyte interactions during early stages of graft rejection, *J. Exp. Zool.* **232** (1984) 67-72.
[15] F. Lassalle *et al.*, Protein analysis of earthworm coelomic fluid. IV. Evidence, activity, induction and purification of *Eisenia fetida andrei* lysozyme, *Comp. Biochem. Physiol.* **91** (1988) 187-192.
[16] E.A. Stein *et al.*, Separation and partial purification of agglutinins from coelomic fluid of the earthworm, *Lumbricus terrestris, Comp. Biochem. Physiol.* **97** (1990) 701-705.
[17] P. Valembois *et al.*, Evidence and cellular localization of an oxidative activity in the coelomic fluid of the earthworm *Eisenia fetida andrei, J. Invert. Pathol.* **57** (1991) 177-183.
[18] Ph. Roch *et al.*, Protein analysis of earthworm coelomic fluid. II. Isolation and biochemical characterization of the *Eisenia fetida andrei* factor (EFAF), *Comp. Biochem. Physiol.* **69** (1981) 829-836.
[19] A. Milochau *et al.*, Purification, characterization and activities of two hemolytic and antibacterial proteins from coelomic fluid of the annelid *Eisenia fetida andrei, Biochim. Biophys. Acta* **1337** (1997)123-132.
[20] E.L. Cooper, Phylogeny of cytotoxicity. *Endeavor* **4** (1981) 160-165.
[21] E.L. Cooper, Comparative immunology: the value of annelids In V. Vetvicka *et al.* (Eds.), Immunology of Annelids, CRC Press, Boca Raton, 1994, pp. 1-12.
[22] E.L. Cooper *et al.*, Autogeneic but not allogeneic earthworm effector coelomocytes kill the mammalian tumor target K562, *Cell. Immunol.* **166** (1995) 113-122.
[23] E.L. Cooper, Earthworm immunity. In B. Rinkevich and W.E.G. Müller (Eds.), Invertebrate Immunology, Springer-Verlag, Heidelberg, 1996, pp. 10-45.
[24] A. Cossarizza *et al.*, Earthworm leukocytes that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines, *Exp. Cell Res.* **224** (1996) 174-182.
[25] E.L. Cooper, Specific tissue graft rejection in earthworms, *Science* **166** (1969) 1414-1415.
[26] E.L. Cooper and Ph. Roch, Second-set allograft responses in the earthworm *Lumbricus terrestris*: Kinetics and characteristics, *Transplantation* **4** (1986) 514-520.
[27] E.L. Cooper and M.H. Mansour, Distribution of Thy-1 in invertebrates and ectothermic vertebrates. In A.E. Reif and M. Schlesinger (Eds.), Cell Surface Antigen Thy-1. Immunology, Neurology, and Therapeutic Applications, Marcel Dekker, 1989, pp. 197-219.
[28] Ph. Roch *et al.*, Serological evidences for a membrane structure related to human β_2 microglobulin expressed by certain earthworm leukocytes, *Eur. J. Immunol.* **13** (1983) 1037-1042.
[29] D. Quaglino *et al.*, Earthworm coelomocytes *in vitro*: cellular features and "granuloma" formation during cytotoxic activity against the mammalian tumor cell target K562, *Eur. J. Cell Biol.* **70** (1996) 278-288.
[30] A. Beschin *et al.*, On the existence of cytokines in invertebrates, *Cell. Mol. Life Sci.* **58** (2001) 801-814.
[31] Ph. Roch, Protein analysis of earthworm coelomic fluid. I. Polymorphic system of the natural hemolysin of *Eisenia fetida andrei, Dev. Comp. Immunol.* **3** (1979) 599-608.
[32] Ph. Roch *et al.*, Interactions between earthworm hemolysins and sheep red blood cell membranes, *Biochim. Biophys. Acta* **983** (1989) 193-198.
[33] P. Valembois *et al.*, Bacteriostatic activity of a chloragogen cell secretion, *Pedobiologia* **24** (1982) 191-195.
[34] P. Valembois *et al.*, Evidence of plasma clotting system in earthworms, *J. Invert. Pathol.* **15** (1988) 221-228.
[35] P. Valembois *et al.*, Scanning electron-microscopic study of the involvement of coelomic cells in earthworm antibacterial defense, *Cell Tissue Res.* **240** (1985) 479-484.

[36] Y. Sekizawa *et al.*, Molecular cloning of cDNA for lysenin, a novel protein in the earthworm, *Eisenia foetida* that causes contraction of rat vascular smooth muscle, *Gene* **191** (1997) 97-102.
[37] A. Yamaji *et al.*, Lysenin, a novel sphingomyelin-specific binding protein, *J. Biol. Chem.* **273** (1998) 300-5306.
[38] N. Ohta *et al.*, Sites of expression of mRNA for lysenin, a protein isolated from the coelomic fluid of the earthworm *Eisenia foetida, Cell Tissue Res.* **302** (2000) 263-270.
[39] H. Kobayashi *et al.*, Lethal and non-lethal responses of spermatozoa from a wide variety of vertebrates and invertebrates to lysenin, a protein from the coelomic fluid of the earthworm *Eisenia foetida, J. Exp. Zool.* **286** (2000) 538-549.
[40] H. Kobayashi *et al.*, Toxicity of coelomic fluid of the earthworm *Eisenia foetida* to vertebrates but not invertebrates: probable role of sphingomyelin, *Comp. Biochem. Physiol.* **128C** (2001) 401-411.
[41] S. Lange *et al.*, Interaction of earthworm hemolysin with lipid membranes requires sphingolipids, *J. Biol. Chem.* **272** (1997) 20884-20892.
[42] S. Lange *et al.*, Biochemical characteristics of eiseniapore, a pore forming protein in the coelomic fluid of earthworms, *Eur. J. Biochem.* **263** (1999) 1-11.
[43] M. Bilej *et al.*, Identification of a cytolytic protein in the coelomic fluid of *Eisenia foetida* earthworms, *Immunol. Lett.* **45** (1995) 123-128.
[44] A. Beschin *et al.*, Identification and cloning of a glucan- and lipopolysaccharide-binding protein from *Eisenia foetida* earthworm involved in the activation of prophenoloxidase cascade, *J. Biol. Chem.* **273** (1998) 24948-24954.
[45] M. Bilej *et al.*, Cellular expression of the cytolytic factor in earthworms *Eisenia foetida*, *Immunol. Lett.* **60** (1998) 23-29.
[46] M. Bilej *et al.*, Antimicrobial defense of earthworm, *Folia Microbiol.* **45** (2000) 283-300.
[47] M. Bilej *et al.*, Distinct carbohydrate recognition domains of an earthworm defense molecule recognize Gram negative and Gram positive bacteria, *J. Biol. Chem.* **276** (2001) 45840-45847.
[48] A. Beschin *et al.*, Convergent evolution of cytokines, *Nature* **400** (1999) 627-628.
[49] E. Olivares Fontt *et al.*, *Trypanosoma cruzi* is lysed by coelomic cytolytic factor-1, an invertebrate analogue of TNF, and induces phenoloxidase activity in the coelomic fluid of *Eisenia foetida foetida*, *Dev. Comp. Immunol.* **26** (2002) 27-34.
[50] A. Bloc *et al.*, An invertebrate defense molecule activates membrane conductance in mammalian cells by means of its lectin-like domain, *Dev. Comp. Immunol.* **26** (2002) 35-43.
[51] Ph. Roch *et al.*, Purification of three serine proteases from the coelomic cell of earthworms (*Eisenia fetida*), *Comp. Biochem. Physiol.* **98** (1991) 597-602.
[52] C. Leipner *et al.*, Serine proteases in coelomic fluid of annelids *Eisenia fetida* and *Lumbricus terrestris, Comp. Biochem. Physiol.* **105** (1993) 637-641.
[53] E. Kauschke *et al.*, Characterization of proteolytic activity in coelomic fluid of *Lumbricus terrestris, Comp. Biochem. Physiol.* **116** (1997) 235-242.
[54] Polanowski and T. Wilusz, Serine proteinase inhibitors from insect hemolymph. *Acta Biochim. Polinica* **43** (1996) 445-454.
[55] E.W. Skowronski *et al.*, Fas-mediated apoptosis and sphingomyelinase signal transduction: the role of ceramide as a second messenger for apoptosis, *Death Different.* **3** (1996) 171-176.
[56] S. Rowan and D.E. Fisher, Mechanisms of apoptotic cell death, *Leukemia* **11** (1997) 457-465.
[57] S. Bourteele *et al.*, Tumor necrosis factor induces ceramide oscillations and negativity controls sphingolipid synthases by caspases in apoptotic Kym-1 cells, *J. Biol. Chem.* **273** (1998) 31245-31251.
[58] K.L. Rinehart *et al.*, Antiviral and antitumor compounds from tunicates, *Fed. Proc.* **42** (1983) 87-90.
[59] D.W. Montgomery *et al.*, Immunomodulation by didemnins. Invertebrate marine natural products, *Ann. N.Y. Acad. Sci.* **712** (1994) 301-314.
[60] H. Nagasawa *et al.*, Inhibition by lombricine from earthworms (*Lumbricus terrestris*) of the growth of spontaneous mammary tumours in SHN mice, *Anticancer Res.* **11** (1991) 1061-1064.
[61] T. Hrzenjak *et al.*, A new source of biologically active compounds earthworm tissue (*Eisenia foetida, Lumbricus rubellus*), *Comp. Biochem. Physiol.* **102A** (1992) 441-447.
[62] R. Fan, The new medicine "lumbrokinase capsules" for treatment and prevention of thrombus and imbolic diseases, *Pub: Nat. Lab. Mol. Virol. Gen. Eng. 100 Ying Xin Jie, Xuan Wu Qu, Beijing,* 100052, China. (1996) 1-7.
[63] T.M. Hrzenjak *et al.*, Fibrinolytic activity of earthworms extract (G-90) on lysis of fibrin clots originated from the venous blood of patients with malignant tumors, *Pathol. Oncol. Res.* **4** (1998) 206-211.
[64] T. Hrzenjak *et al.*, Fibrinolytic and anticoagulative activities from the earthworm *Eisenia foetida, Comp. Biochem. Physiol.* **119B** (1998) 825-832.
[65] H. Mihara *et al.*, A novel fibrinolytic enzyme extracted from the earthworm, *Lumbricus rubellus, Jpn. J. Physiol.* **41** (1991) 461-472.

[66] H. Mihara *et al.*, Novel thrombolytic therapy discovered from traditional oriental medicine using the earthworm, *Southeast Asia J. Trop. Med. Public Health* **23** (1992) 131-140.
[67] N. Nakajima *et al.*, Characterization of potent fibrinolytic enzymes in earthworm, *Lumbricus rubellus*, *Biosci. Biotechnol. Biochem.* **57** (1993) 1726-1730.
[68] N. Nakajima *et al.*, Chemical modification of earthworm fibrinolytic enzyme with human serum albumin fragment and characterization of the protease as a therapeutic enzyme, *Biosci. Biotechnol. Biochem.* **60** (1996) 293-300.
[69] G.H. Ryu *et al.*, Antithrombogenicity of lumbrokinase-immobilized polyurethane, *J. Biomed. Mater. Res.* **28** (1994) 1069-1077.
[70] G.H. Ryu *et al.*, Surface characteristic and properties of lumbrokinase-immobilized polyurethane, *J. Biomed. Mater. Res.* **29** (1995) 403-409.
[71] J.S. Yang *et al.*, Purification and characterization of an SDS-activated fibrinolytic enzyme from *Eisenia fetida*, *Comp. Biochem. Physiol.* **118B** (1997) 623-631.
[72] M. Hrzenjak *et al.*, Mitogenicity of the earthworm's (*Eisenia foetida*) insulin-like proteins, *Comp. Biochem. Physiol.* **104B** (1993) 723-729.
[73] M. Bilej, Cellular defense mechanisms. In V. Vetvicka *et al.* (Eds.), Immunology of annelids, CRC Press, Boca Raton, 1994, pp. 167-200.
[74] E.L. Cooper *et al.*, Digging for innate immunity since Darwin and Metchnikoff, *Bioessays* (2002) in press.
[75] E. Kauschke and W. Mohrig, Comparative analysis of hemolytic and hemagglutinating activities in the coelomic fluid of *Eisenia foetida* and *Lumbricus terrestris* (*Annelida, Lumbricidae*), *Dev. Comp. Immunol.* **11** (1987) 331-341.
[76] Daedo Pharmaceutical Co. Ltd., Yongshim Capsule, 1990, pp. 1-2.
[77] M. Salzet, Vertebrate innate immunity resembles a mosaic of invertebrate immune responses, *Trends Immunol.* **22** (2001) 285-288.
[78] E. Kauschke *et al.*, Evidence for perforin-like activity associated with earthworm leukocytes, *Zoology* **32** (2001) 13-24.
[79] K. Komiyama *et al.*, Identification of perforin gene and its protein in the earthworm coelomocytes, *Dev. Comp. Immunol.* **21** (1997) 115.
[80] J.H. Cho *et al.*, Lumbricin I a novel proline-rich antimicrobial peptide from the earthworm: purification, cDNA cloning and molecular characterization, *Biochim. Biophys. Acta* **1408** (1998) 67-76.
[81] I. Eue I *et al.*, Isolation and characterization of earthworm hemolysins and agglutinins, *Dev. Comp. Immunol.* **22** (1998) 13-25.

Coelomocytes (Leukocytes)

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Cytochemical properties of earthworm coelomocytes enriched by Percoll

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Abstract. Coelomocytes of *Eisenia fetida* were separated by a Percoll gradient and based on cytomorphology and cytochemistry, classified into four major categories: acidophils, basophils, neutrophils and chloragocytes cells. Basophils exhibited heterogeneity with respect to staining properties of granules. The enzyme acid phosphatase was present in all coelomocytes, but was especially abundant in basophils and neutrophils. Alkaline phosphatase was detected in basophils and acidophils, and α-esterase was found in all types except neutrophils. Basophils and neutrophils were more active in killing the tumor target, K562, which partially reflects their role in the earthworm's immune system.

1. Introduction

Earthworm coelomic fluid contains cells, which are generally differentiated in acidophilic, basophilic and neutrophilic amoebocytes (or coelomocytes) and chloragocytes. The majority of chloragocytes forms the chloragogen, which covers tissue like intestinal tract (gut) from outside, but still a great number of chloragocytes circulates in the coelomic fluid and their yellow pigmentation is a striking characteristic of that cell type. Functionally, chloragocytes have often been described as trophocytes, which are taking up nutritive components like proteins, lipids and glycogen into storage chloragosomes and transport them via circulation into the coelomic fluid as well as to different cells and organs. However, as early as 1942, Liebmann [1] proposed that chloragocytes also may release activating substances and be involved in wound healing and regeneration processes.

Coelomocytes are the most numerous cell population in the coelomic fluid and heterogeneous regarding size, cytochemical properties and immune functions. They float as single cell in the coelomic fluid-filled coelomic cavity. They are derived from the mesenchymal lining and involved in important immune functions as phagocytosis, encapsulation, NK-like cytotoxicity, inflammation, graft rejection and granuloma formation. Moreover, they synthesize and secrete humoral immune components like lysins and agglutinins into the coelomic fluid [2-14].

Coelomocyte classification remained a subject of investigations for more than a century [12, 15-21]. Common characteristics for differentiation are cell and nucleus size, content and chemical nature of granules as well as adherence and chemotaxis. The use of regular blood cell stain (Wright stain) provides a differentiation in acidophilic, basophilic and neutrophilic

coemolocytes but FACS analysis showed that coelomocytes also differ regarding surface markers [22-25].

Wounding as well as intracoelomic presence of foreign material provokes an increase in number of coelomocytes within 24 hours. That might be due to a cell release out of storage nodes since proliferation rates of any coelomocyte types have always been shown low or absent.

As long as long-term culture of coelomocytes remains a problem, attempts to separate coelomocyte subpopulations are seen as useful tools for analyzing cell functions, interactions and differentiation. Many reports have been provided about morphology, function and cytochemistry of coelomocytes in *Lumbricus terrestris* [2, 3, 7, 10, 19, 20, 26-29]. Our data here focus upon coelomocytes of *Eisenia fetida,* separated by using a Percoll gradient, and their cytochemical characteristics and immune function.

2. Cytochemical characteristics of four cell types in the coelomic fluid of Eisenia fetida

In *E. fetida*, four major cell types are characterized as: basophils, acidophils, neutrophils and chloragocytes based on light microscopy and Wright staining after cytospin preparation (Fig. 1). Kits to detect cytoplasmic enzymes in vertebrate peripheral leukocytes, bone marrow or tissue touch preparations (acid phosphatase, alkaline phosphatase, specific and nonspecific esterases) were used for enzyme detection in coelomocytes

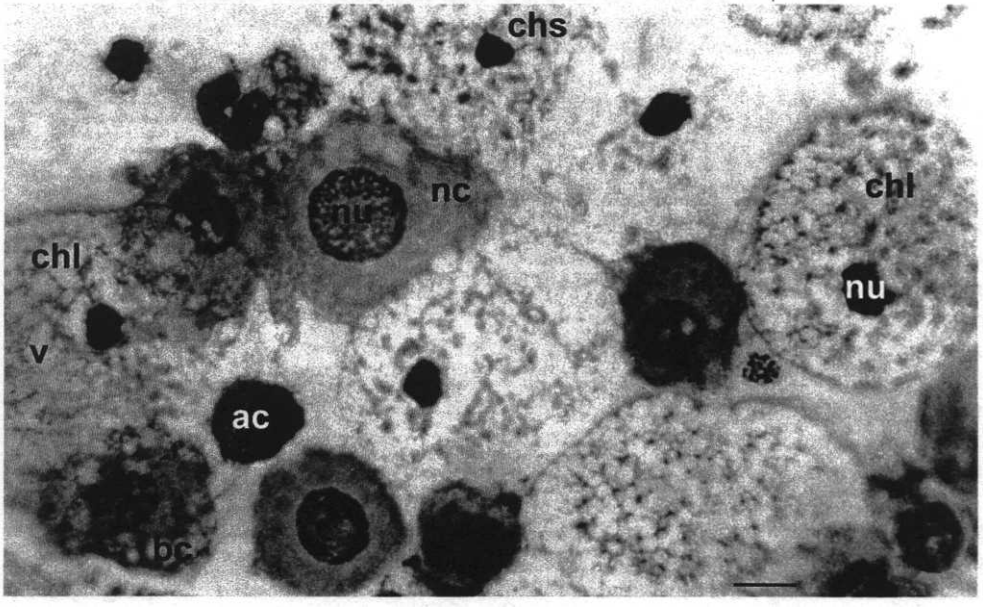

Fig. 1: Cell types of *Eisenia fetida* coelomic fluid after cytospin glass adherence and Wright stain.
About 35 % of the whole cell population in the coelomic fluid of *E. fetida* are chloragocyte (ch) were seen to be different in sizes, small (chs) and large (chl). They have a significantly smaller nucleus (nu) than coelomocytes and their cytoplasm contains numerous vacuoles (v) and lipid inclusions. The most numerous coelomocytes in the coelomic fluid are basophilic cells (bc), their nucleus is always eccentric and the cytoplasm contains many granules. Neutrophils (nc) have a relatively large nucleus, which is often centrally located. Acidophils (ac) appear in small numbers and size. Their cytoplasm contains many red to orange granules.

2.1. Basophils

Basophils were the most numerous coelomocyte types, stained strongly basophilic with occasional small, dark blue granules. They possess abundant cytoplasm colored lighter blue and clear vacuoles. Basophils vary in size sometimes from as small as 12 μm to as large as 21 μm, whereas smaller basophils display a strong tendency to aggregate. The nuclei are compact, about 6 μm in diameter, and either centrally or peripherally located. Chromatin is condensed, stained dark blue violet and the nucleoli are not visible (Fig. 1). Three enzymes are demonstrated in the cytoplasm of basophils: acid phosphatase, which is present in large amounts, alkaline phosphatase as deep blue granules of different sizes, but to lesser extent (Fig. 2B) and α-esterase mainly as brown granules (Fig. 2C, Table 1).

Basophils also contain significant amounts of the enzyme acid phosphatase in discrete granules or vesicles that are presumed to be lysosomes. Using electron micrographs, large (4-6 μm) acid phosphatase-positive vacuoles were found in basophils and neutrophils of *L. terrestris* and have been thought to be phagosomes [19]. Acid phosphatase is not as abundant in acidophils as in basophils and neutrophils [2].

2.2. Neutrophils

Neutrophils are granular cells with distinct outlines, which usually are classified into two types based upon granules and cell size. In type I (large cells 20 ± 2.3 μm), although there are few granules, they still fill the cells completely (Fig. 1). The nuclei are always in eccentric positions and their sizes are about 6.7 ± 0.6 μm. The cytoplasm of smaller type II cells (13.9 ± 1.8 μm) sometimes appeared homogeneous and have no granulation. The nuclei (7 ± 0.8 μm) are located either centrally or peripherally, and appeared flattened. The nucleoli are not visible. Two enzymes were detected in neutrophil cells: acid phosphatase in low to moderate frequency, distributed diffusely in discrete granules throughout the cytoplasm and alkaline phosphatase moderately as deep blue cytoplasmic granules (Fig. 2A). No α-esterase activity was shown for neutrophils (Table 1).

Neutrophils contain large nuclei and less heterochromatin than those of other coelomocytes. These characteristics are usually associated with relatively undifferentiated cells. Neutrophils are of particular interest to invertebrate immunologists. In addition to being highly phagocytic, they are responsible for making initial contact, invasion and early destruction of foreign tissue grafts [14]. Basophils have been found at the graft site, but they appear to play a secondary or 'scavenger' role and seem not to be the primary cell responsible for graft rejection. Experiments involving chemotaxis toward foreign tissue or bacteria [30] revealed that neutrophils, comprise 92-94 % of the responding cells, although they comprise an average of only 18 % of the total coelomocyte population [2].

2.3. Acidophils

A most prominent characteristic of acidophils, easily observed is the profusion of granules scattered throughout the cytoplasms (Fig. 1). Acidophils appear to be relatively small cells (11.3 ± 2 μm) and contain numerous granules of red to orange color. The nuclei are 6.1 ± 0.8 μm, stained medium to dark purple and display condensed chromatin. Although indications of acid phosphatase are significant, they are stained less intensely than basophils (Fig. 2A). The percent of alkaline phosphatase in acidophils is moderate and that of α-esterase is low (Table 1).

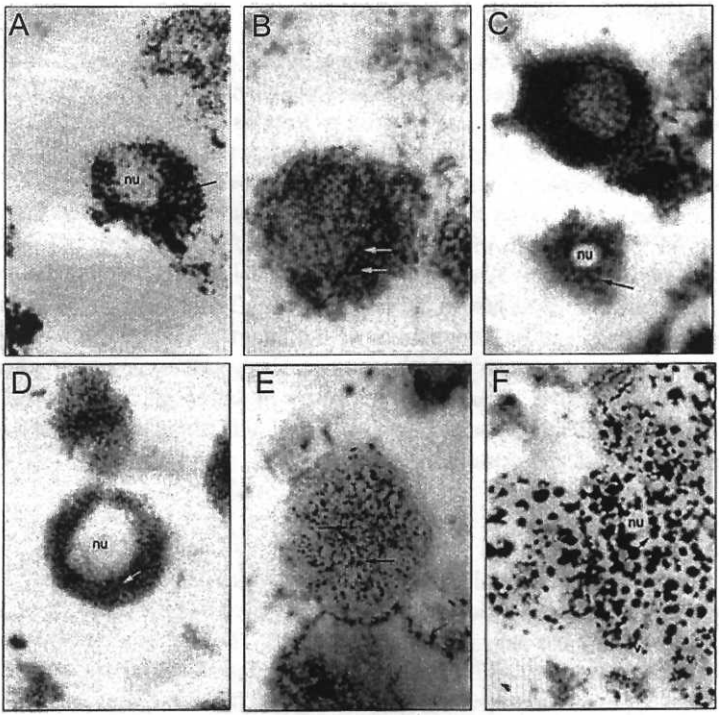

Fig. 2: Cytochemical properties of *Eisenia fetida* coelomocytes and chloragocytes.
A: acid phosphatase (black arrow) in neutrophilic coelomocyte; B: alkaline phosphatase (white arrow) in basophilic coelomocyte; C: esterase in basophilic coelomocyte; D: alkaline phosphatase in acidophilic coelomocyte; E: acid phosphatase (black arrow) in chloragocyte; F: esterase on chloragocyte; (nu) nucleus.

Table 1: Acid phosphatase, alkaline phosphatase, specific and nonspecific esterase activities of the population of coelomocytes.

Enzyme	Type of cell			
	Basophil	Neutrophil	Acidophil	Chloragocyte
Acid phosphatase	Intense	Low to moderate	Moderate	Low
Alkaline phosphatase	Moderate	Negative	Moderate	Negative
Specific esterase	Negative	Negative	Negative	Negative
Nonspecific esterase	Low to moderate	Negative	Low to moderate	Moderate

The acid phosphatase showed the highest intensity in basophils while the alkaline phosphatase was moderate in basophils and acidophils and the α-esterase was ranged from low to moderate in all types except neutrophils.

Wright's-stained acidophils in *L. terrestris* contain strongly acidophilic materials, but lack observable granules [2]. This condition probably reflects a particular developmental stage in which the materials have been synthesized but not yet 'packaged' into discrete granules. In cytochemical preparations, acidophils have been observed and appear to have unknown secretory activity. In other invertebrates, substances secreted by granular cells range from clotting factors in *Limulus* and several species of crustaceans to lysosomal enzymes in the mollusk, *Mercenaria mercenaria* [31]. Although the coelomic fluid of the earthworm *Lumbricus* is known to contain agglutinins secreted *in vitro* in response to stimulation by rabbit erythrocytes [32, 33], the specific cell(s) responsible for synthesizing and secreting them has not been identified.

2.4. Chloragocytes

Chloragocytes occur in two forms of different cell size and are sometimes arranged in clusters of four to six cells. Both types are larger than acidophils, basophils, and neutrophils (Fig. 1). Large chloragocytes are oblong with a cell size of 32 ± 1.8 μm; the nuclei are 4.5 ± 1.7 μm. Cytoplasmic granules are spheroid and stained bright blue with Wright-stain. Small chloragocytes (23.4 ± 3.4 μm) have circular nuclei that measured 4.1 ± 0.7 μm in diameter. The amount of acid phosphatase in chloragocytes was less than in basophils and neutrophils (Fig. 2E). There was no indication of alkaline phosphatase, but nonspecific α-esterase was moderately frequent in the cytoplasm as brown granules (Fig. 2F; Tab. 1). Chloragogen cells have been compared to the liver of vertebrates [29] and postulated to have a trophic function containing as well two types of phospholipids.

3. Coelomocyte separation by Percoll

Percoll is a density gradient medium, non toxic to cells that maintains physiological conditions during centrifugation experiments. It allows the separation of *E. fetida* coelomocytes and chloragocytes on a six step gradient (55-45-35-25-15-5 %). Coelomocyte suspensions transferred onto the Percoll gradient yield four coelomocyte populations at concentrations of 10, 25, 35 and 45 % (Fig. 3). Neutrophilic cells are enriched in the 10 % Percoll fraction. The 25 % fraction comprises mainly basophils and few chloragocytes, which are enriched in the 35 % Percoll fraction. The 45 % fraction contains acidophils and neutrophils as well as basophils, which display an enriched appearance.

Coelomocytes without Percoll separation reveal varying percentages of cell and nuclear sizes for each coelomocyte type. The percentage of coelomocytes and cell size of each type after Percoll separation also revealed variation, but the chosen setup allows the best enrichment for neutrophils and basophils. Further centrifugation in Percoll media could be necessary for a more complete isolation of all coelomocyte subpopulations. Nevertheless, all cells are highly viable after Percoll separation and therefore most suitable for further functional analysis as illustrated in the next paragraph.

4. Coelomocyte cytotoxicity using Percoll-separated coelomocytes

We measured the cytotoxic activity of coelomocyte effectors obtained from Percoll gradients against the erythromyeloid human tumor cell line K562 tumor cell targets in a

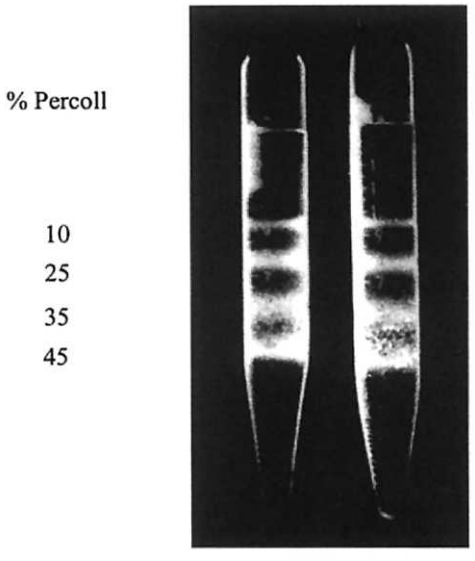

Fig. 3: Separation of *Eisenia fetida* coelomic fluid cells by using a Percoll gradient (55 - 45 - 35 -25 - 15 – 5 %). Four separated cell bands became visible at 10, 25, 35 and 45 %Percoll concentrations. The cell band at 10 % mainly consists of neutrophils; the second at 25 % mainly of basophils and some chloragocytes. Chloragocytes were enriched at 35 % Percoll. The fourth band (45 % Percoll) consisted of acidophils, basophils, and neutrophils. Basophils appeared enriched in the 45 % band.

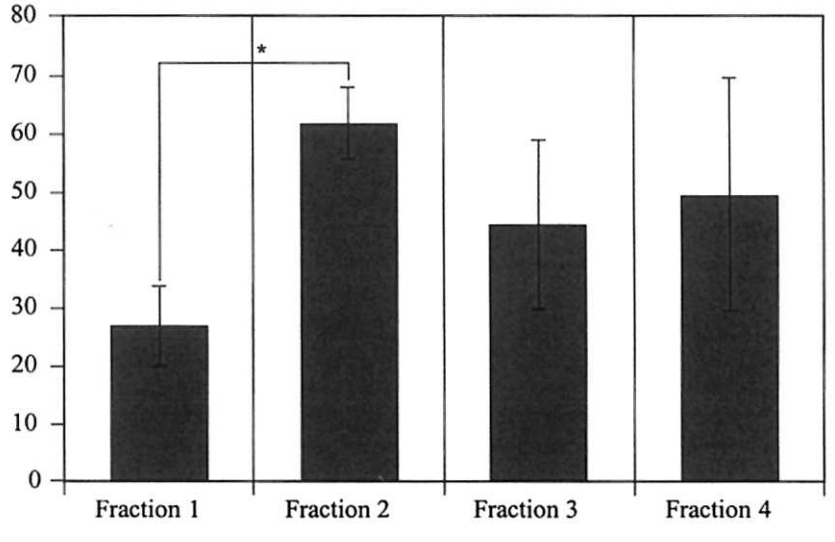

Fig. 4: Cytotoxicity caused by *Eisenia fetida* coelomic fluid cells (effectors) upon K562 targets.
Coelomocytes separated into 4 fractions by using a Percoll gradient (Fig. 3) were incubated with ^{51}Cr-labeled K562 targets (4 h, 37 °C). The ^{51}Cr release was measured with a gamma counter and cytotoxicity (%) calculated. Percoll fraction 2 and 4, which were either enriched with basophils or neutrophils, displayed a high cytotoxicity.
* Significance level at $p<0.02$.

classical 4 h ^{51}Cr-release assay according to previous methods [12-13, 25]. Results showed a cytotoxic effect of all Percoll fractions of effector cells, and more than 50 % cytotoxicity was found in fractions 25 and 45 % (Fig. 4), which were enriched with basophils and neutrophils. Fraction 35 % enriched with the majority of chloragocytes, which are known to secrete lytic proteins, expressed a lower cytotoxicity than cells from fraction 25 and 45 %. These results indicates that two types of coelomocytes at certain stages of differentiation are actively involved in the killing of K562 tumor target cells.

Newer findings in relation to coelomocyte multiplication have been observed during cytotoxic activity against K562 *in vitro*. Two cell types (i.e. small and large coelomocytes) retained their morphological features, their DNA content was significantly less than that of human K562, and significant percentages of coelomocytes were found to be in S or G2/M phases of the cell cycle. When cultivated alone for up to 3 h, coelomocytes formed no aggregates. However, upon mixing with K562, coelomocytes spontaneously killed tumor cells and cytotoxic reactivity was accompanied by the formation of multiple aggregates similar to granulomas. These results were described as nonspecific ''inflammatory'' responses of earthworms *in vitro* against tumor cells [13]. In this *in vitro* system, as analyzed by FACS, the small basophil (SC) seems to be responsible for killing whereas the larger neutrophilic phagocytic coelomocytes (LC) are responsible for cleaning up debris [25].

5. Conclusion

Percoll gradient preparation is valuable and rapid for separating coelomocyte populations. Percoll is inexpensive and non-toxic not affecting immunocyte function, and small cell numbers with good yields have made emphasizing it as a routine laboratory tool. Our study revealed that fraction 25 and 45 % Percoll gradient showing the highest cytotoxic activity were enriched with small and large cells. These data implicate basophils as the cell population responsible for target cell killing, analogous to mammalian NK activity as has been shown recently [25]. ^{51}Cr-release data from the Percoll fractions, in conjunction with percent cell population (Fig. 4) and cell size also suggest that the origin of NK-like activity in earthworms is from a unique stage of basophil development/differentiation corresponding with basophils of up to 16 μm. (fraction 25 %). We propose stem or embryonic cells that may differentiate into putative "basoblasts". As cells grow into basocytes and reach the stage of development where cells are 16 μm in size, they then develop the capacity for NK activity. Once past this stage as cells grow into larger basocytes (large basophils), they loses their NK activity. Therefore, two basophil subpopulations exist. Further investigations are needed to pinpoint the exact subpopulations with NK activity, which may be associated with both. We suggest a step involving the use of gold-conjugated antibodies in electron microscopic assays to detect the precise location of effector molecules within the effector cells responsible for killing. At the moment this may be partially due to perforin-like molecules [22].

Since these are the first cytochemical and ^{51}Cr-release observations using Percoll separation for earthworm coelomocytes, two specific points remain unresolved: 1) the nature and the enzymatic changes that occur in both small and large cells during phagocytosis and killing; 2) exact stage at which coelomocytes become involved in NK- or perforin-like activities. The cytochemical information reported here should serve as a foundation for further studies coupled with more intense assays related to immune responses.

Acknowledgments

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References

[1] E. Liebmann The coelomocytes of *Lumbricidae, J. Morphol.* **71** (1942) 221-249.
[2] E.A. Stein *et al.*, The coelomocytes of the earthworm *Lumbricus terrestris*: morphology and phagocytic properties, *J. Morphol.* **153** (1977) 415-425.
[3] A. Laulan *et al.*, The phagocytic activity of *Lumbricus terrestris* leukocytes is enhanced by the vertebrate opsonins: IgG and complement C3b fragment, *Dev. Comp. Immunol.* **12** (1988) 269-277.
[4] M. Bilej *et al.*, Phagocytosis of synthetic particles in earthworms: absence of oxidative burst and possible role of lytic enzymes, *Folia Biol.* **36** (1991) 227-231.
[5] M. Bilej *et al.*, Phagocytosis of synthetic particles in earthworms: effect of antigenic stimulation and opsonization, *Folia Biol.* **36** (1991) 273-280.
[6] V. Vetvicka *et al.*,, Immunology of Annelids, CRC Press Boca Raton Florida (1994) pp. 300.
[7] E.L. Cooper, Transplantation immunity in annelids. I. Rejection of xenografts exchanged between *Lumbricus terrestris* and *Eisenia foetida, Transplantation* **6** (1968) 322-337.
[8] E.L. Cooper, Specific tissue graft rejection in earthworms, *Science* **166** (1969) 1414-1415.
[9] R.K. Hostetter and E.L. Cooper, Coelomocytes as effector cells in earthworm immunity, *Immunol. Commun.* **1** (1972) 155-183.
[10] E.L. Cooper and P. Roch, Second-set allograft responses in the earthworm *Lumbricus terrestris*. Kinetics and characteristics, *Transplantation* **41** (1986) 514-520.
[11] M.M. Suzuki and E.L. Cooper, Spontaneous cytotoxic earthworm leukocytes kill K 562 tumor cells, *Zool. Sci.* **12** (1995) 443-451.
[12] E.L. Cooper *et al.*, Autogenic but not allogenic earthworm effector coelomocytes kill the mammalian tumor target K562, *Cell. Immunol.* **166** (1995) 113-122.
[13] D. Quanglino *et al.*, Earthworm coelomocytes *in vitro*: cellular features and "granuloma" formation during cytotoxic activity against the mammalian tumor target K562, *Eur. J. Cell Biol.* **70** (1996) 278-288.
[14] D.S. Linthicum *et al.*, Graft rejection in earthworms: An electron-microscopic study. *Eur. J. Immunol.* **7** (1977) 871-876.
[15] D. Rosa, Les lymphocytes des Oligochètes, *Arch. Ital. Biol.* **25** (1896) 455-458.
[16] M. Kollman, Recherches sur les leucocytes et le tissu lymphoïde des invertébrés, *Ann. Sci. Nat. (Zool)* T. **8** (1908) 269-289.
[17] A. Dehorne, Sur les expansions petaloïdes des leucocytes des Chetopodes, *C. R. Acad. Sci.* Paris **180** (1925) 333-335.
[18] E. Fuare-Fermiete, Les amibocytes des invertébrés a l'état quiescent et a l'état actif, *Arch. Anat. Microsc.* Paris **23** (1927) 99-173.
[19] D.S. Linthicum *et al.*, Electron microscopic observations of normal coelomocytes from the earthworm *Lumbricus terrestris, Cell Tissue Res.* **185** (1977) 315-330.
[20] M. Ehlers, Resistenzmechanismen bei *Lumbricus terrestris* L. Dissertation (Thesis) Ernst-Moritz-Arndt-Universität Greifswald (1986) pp. 113.
[21] M. Bilej *et al.*, The flow cytometric analysis of *in vitro* phagocytic activity of earthworm coelomocytes (*Eisenia foetida*; *Annelida*), *Cell. Biol. Int. Rep.* **14** (1990) 831-837.
[22] E. Kauschke *et al.*, Evidence for perforin-like activity associated with earthworm leukocytes, *Zoology* **1:104** (2001) 13-24.
[23] P. Roch *et al.*, Serological evidences for a membrane structure related to human β_2-microglobulin expressed by certain earthworm leukocytes, *Eur. J. Immunol.* **13** (1983) 1037-1042.
[24] A.H. Saad and E.L. Cooper, Evidence for a Thy-1-like molecule expressed on earthworm leukocytes, *Zool. Sci.* **7** (1990) 217-222.
[25] A. Cossarizza *et al.*, Earthworm leukocytes that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines, *Exp. Cell Res.* **224** (1996) 174-182.
[26] W. Mohrig *et al.*, Rosette formation of the coelomocytes of the earthworm *Lumbricus terrestris* L. with sheep erythrocytes, *Dev. Comp. Immunol.* **8** (1984) 471-476.

[27] E.A. Stein and E.L. Cooper, Cytochemical observation of colomocytes from the earthworm, *Lumbricus terrestris, Histochem. J.* **10** (1978) 657-678.

[28] E.A. Stein and E.L. Cooper, Oligochaetes. In R.A. Ratcliffe and A.F. Rowley (Eds.), Invertebrate blood cells. Academic Press, San Diego (1981) pp.75-140.

[29] E.L. Cooper and E.A. Stein, Leucocytes of Annelids: The *Oligochaeta*. In R.A. Ratcliffe and A.F. Rowley (Eds.), Invertebrate Blood Cells, Academic Press, New York (1981) 75-140.

[30] D.H. Marks *et al.*, Chemotactic attraction of *Lumbricus terrestris* coelomocytes to foreign tissue, *Dev. Comp. Immunol.* **3:2** (1979) 277-85.

[31] T.C. Cheng, Functional morphology and biochemistry of molluscan phagocytes, *Ann. NY Acad. Sci.* **266** (1975) 343-79.

[32] E.L. Cooper *et al.*, Agglutinins and cellular immunity in earthworms, *Ann. NY Acad. Sci.* **234** (1974) 45-50.

[33] E.A. Stein and E.L. Cooper, *In vitro* agglutinin production by earthworm leukocytes, *Dev. Comp. Immunol.* **12** (1988) 531-547.

A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
E.L. Cooper et al. (Eds.)
IOS Press, 2002

Earthworm coelomocytes: convenient model for basic and applied sciences

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Abstract. Earthworm coelomocytes were extruded via dorsal pores after an electric shock (5 V) or 5 % ethanol treatment. Both methods were equally efficient in respect of the number and viability of coelomocytes retrieved and did not affect the animal survival. Coelomocyte populations differ between *Dendrobaena veneta*, *Eisenia foetida*, *Allolobophora chlorotica* and *Lumbricus terrestris*. Cell survival was high in L-15 medium and in balanced HANKS solution at the neutral pH and at the osmolarity characteristic for terrestrial animals (285-320 mOsm). *In vitro*, cell viability and activity were dependent on the temperature of animal adaptation and on the temperature of cell incubation. Heavy metals *in vitro* caused the decrease of cell viability and activity. Soil pollution strongly affected earthworm viability, reproduction and the number of coelomocytes.

1. Introduction

Heterogeneous earthworm coelomocytes deriving from coelomic cavity play various functions, including the recognition and elimination of foreign materials (primarily by phagocytosis and encapsulation), participation in clotting, wound healing, and some aspects of nutrition and excretion. Coelomocytes contribute to a non-specific immune defense against microbial invaders. Some coelomocytes take part in detoxification of earthworm tissues [1-7].

Earthworms are often exposed to a wide range of anthropogenic compounds released to the soil and may be affected by the toxicity of some of them. For this reason, earthworms have been used extensively in ecotoxicological studies. Temperature is one of the most important environmental factors standardized in lethal and sub-lethal toxicity tests [8].

2. Coelomocyte retrieval: defining the optimal in vitro *culture conditions*

Earthworm coelomocytes are very convenient to work with as they may be retrieved from the coelomic cavity without killing the animals. They are liberated via the dorsal pores of animals immersed in extrusion fluid and stimulated for 1 min either by 5 % ethanol [9] or by 5 V electric shock [10]. The comparison of these methods showed that both of them are equally efficient in respect of the number and viability of coelomocytes retrieved (Fig. 1) and does not affect the animal survival. However, the general vigor of animals is much better after electric stimulation than after ethanol treatment [11].

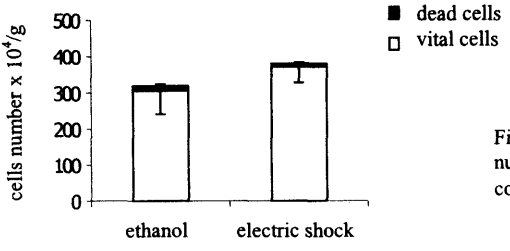

Fig. 1: Effects of retrieval method on the number and the viability of the extruded coelomocytes of *Eisenia foetida*; means ± SD.

Coelomocytes consist of three apparent cell populations: eleocytes, granular amoebocytes, and hyaline amoebocytes [5, 6]. Eleocytes are rare in *Lumbricus terrestris*, but represent a large portion of total coelomocytes retrieved from *Eisenia foetida*, *Dendrobaena veneta* and *Allolobophora chlorotica* (Fig. 2)[11]. In the four investigated earthworm species, eleocytes are always the largest, while granular and hyaline amoebocytes vary considerably in size (Fig. 3)[12].

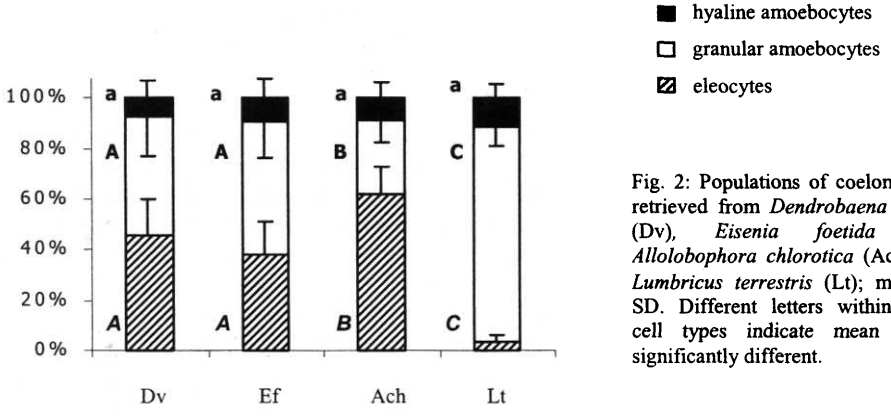

Fig. 2: Populations of coelomocytes retrieved from *Dendrobaena veneta* (Dv), *Eisenia foetida* (Ef), *Allolobophora chlorotica* (Ach) and *Lumbricus terrestris* (Lt); means ± SD. Different letters within given cell types indicate mean values significantly different.

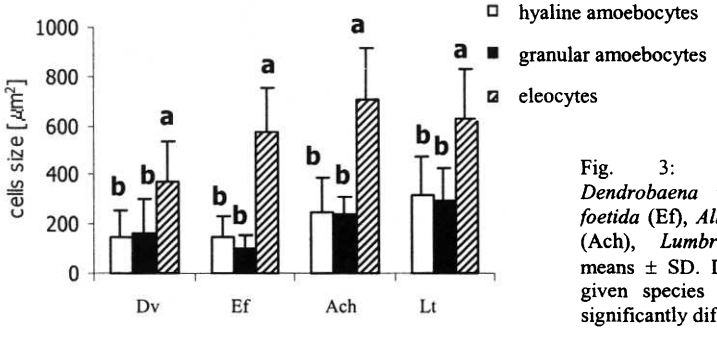

Fig. 3: Coelomocyte size: *Dendrobaena veneta* (Dv), *Eisenia foetida* (Ef), *Allolobophora chlorotica* (Ach), *Lumbricus terrestris* (Lt); means ± SD. Different letters within given species indicate mean values significantly different.

For a short lasting *in vitro* incubation (7 h), highest viability of coelomocytes is achieved in L-15 medium (Sigma Chemical Co.), similar in balanced HANKS solution (320 mOsm) [9, 11], and lowest in buffered physiological saline, PBS (Fig. 4). In all instances, their preferred osmolarity is 285-320 mOsm (characteristic for terrestrial animals) at neutral pH (Fig. 5, Fig. 6)[11].

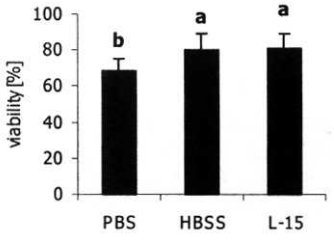

Fig. 4: *In vitro* viability of *Eisenia foetida* coelomocytes after 7 h incubation in PBS, HBSS or L-15; means ± SD. Different letters indicate mean values significantly different.

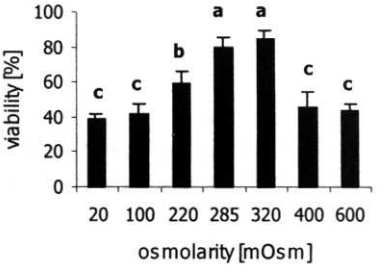

Fig. 5: *In vitro* viability of *Eisenia foetida* coelomocytes after 2 h incubation in PBS of various osmolarities; means ± SD. Different letters indicate mean values significantly different.

Under optimal composition of the incubation fluid, coelomocytes of *E. foetida* are vital at wide range of temperatures (0-22 °C), while their viability is significantly diminished at 37 °C [11]. The *in vitro* viability is the best at the temperature close to that used for animal maintenance, e.g. coelomocytes retrieved from animals kept at the room temperature are more vital at 22 °C than at 10 °C, while coelomocytes retrieved from earthworms kept at 10 °C are more vital in the cold (10 °C) than in the warmth (22 °C)(Fig. 7)[11]. The above mentioned thermal adaptation of coelomocytes to the cold or to the warmth is achieved only after relatively long-lasting (about 2 weeks) *in vivo* adaptation (Fig. 8)[11].

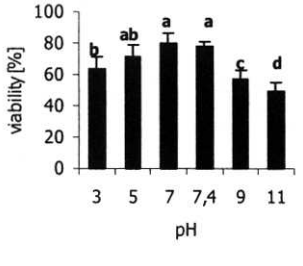

Fig. 6: *In vitro* viability of *Eisenia foetida* coelomocytes after 2 h incubation in PBS of various pH; means ± SD. Different letters indicate mean values significantly different.

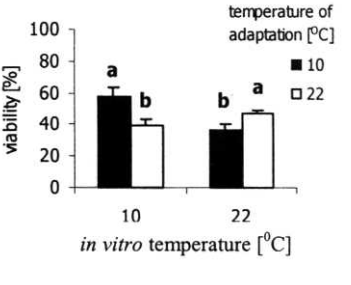

Fig. 7: *In vitro* viability of *Eisenia foetida* coelomocytes after 7 h incubation in PBS at 10 or 22 °C following *in vivo* animal adaptation to 10 or 22 °C; means ± SD. Different letters within given temperature indicate mean values significantly different.

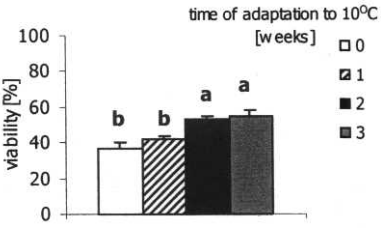

Fig. 8: *In vitro* viability of *Eisenia foetida* coelomocytes after 7 h incubation in PBS at 0 °C following *in vivo* animal adaptation to the cold for 0 to 3 weeks; means ± SD. Different letters indicate mean values significantly different.

3. *Assessment of* in vitro *coelomocyte activity*

Besides checking of *in vitro* coelomocyte viability, several simple tests may be used for an assessment of *in vitro* coelomocyte activity: pinocytosis of neutral red, plastic adherence, MTT reduction by mitochondrial dehydrogenases [13]. The results of all them are considerably temperature-dependent (Fig. 9a-c).

The ability of thermal adaptation is a great importance for the animals inhabiting a temperate climatic zone. The results of tests performed at different *in vitro* temperatures on coelomocytes retrieved from animals collected in the field in January (cold-adapted) or June (warm-adapted) are different. Pinocytosis, adherence and dehydrogenase activity are most efficient at low temperatures (0-10 °C) in January, but at 22 °C in June [14, 15]. Fig. 10 shows annual changes of dehydrogenase activity in coelomocytes collected in the filed in the course of the year.

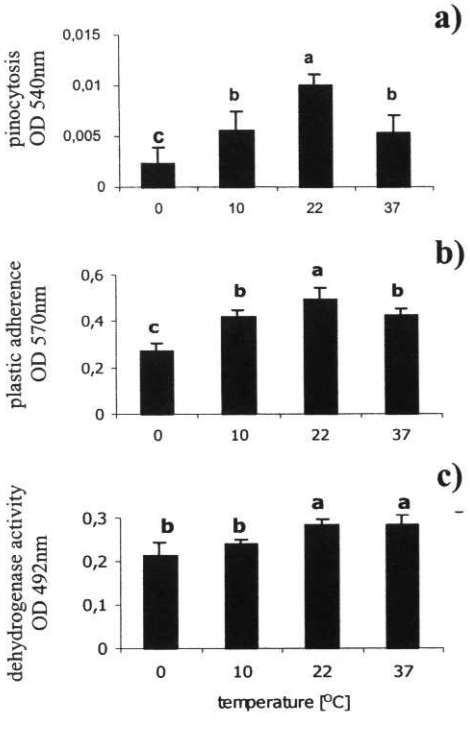

Fig. 9: Effects of *in vitro* temperature on *Eisenia foetida* coelomocyte activity: pinocytosis of neutral red (a); plastic adherence measured by crystal violet stain (b); mitochondrial dehydrogenase activity measured by MTT reduction to formasan (c); means ± SD; OD - optical density. Different letters in given assay indicate mean values significantly different.

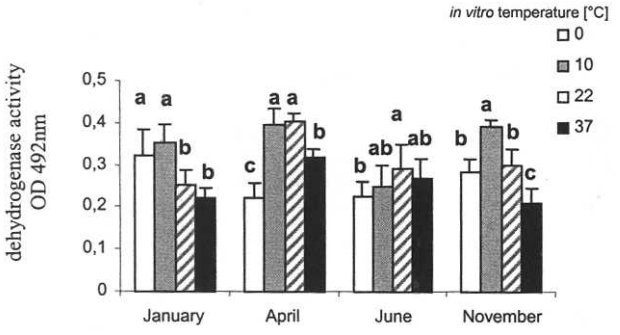

Fig. 10: The annual cycle of dehydrogenase activity of *Allolobophora chlorotica* coelomocytes at different *in vitro* temperatures after 2 h incubation; means ± SD. Different letters within given season indicate mean values significantly different.

Phylogenetically conservative heat shock proteins HSP70 and HSP72 may be detected in earthworm coelomocytes by immunocytochemistry using murine anti-human monoclonal antibodies. In *E. foetida*, the HSP70 is expressed constitutively in cells from animals adapted to the room temperature (22 °C) but its expression is significantly stronger in thermally shocked cells. In contrast, the HSP72 is almost undetectable in coelomocytes from animals adapted to the room temperature (22 °C) but is expressed in cells subjected to the thermal shock. In conclusion, both constitutive HSP70 and inducible HSP72 proteins are involved in the protection of *E. foetida* coelomocytes against thermal shock during the sudden shift of temperature (Table 1, Fig. 11)[16].

Table 1: Expression of HSP70 and HSP72 proteins in coelomocytes of *Eisenia foetida* kept at the constant temperature 22 °C or thermally shocked by transferring from 22 to 37 °C. Intensity of dark products of immunoenzymatic reaction after *in vitro* exposure to various temperatures was quantified.

Thermal conditions	HSP72	HSP70
22 °C constant	-/+	+++
22 °C → 37 °C	+++	+++++

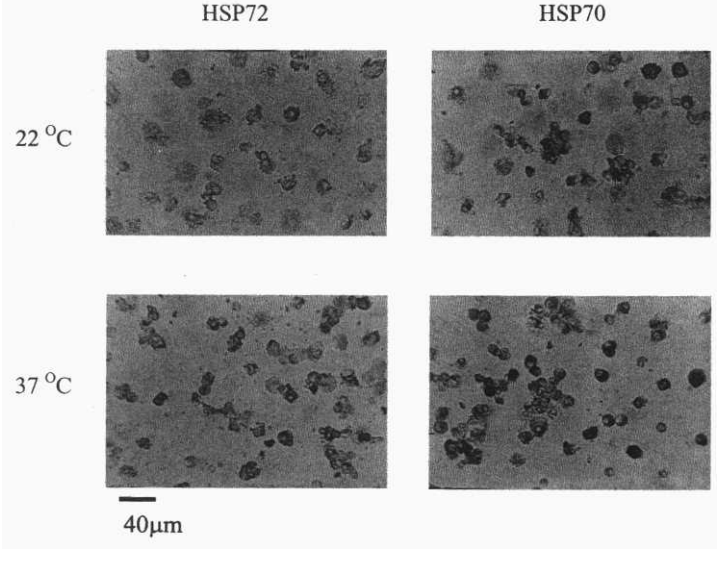

Fig. 11: Expression of HSP70 and HSP72 proteins in coelomocytes of *Eisenia foetida* determined by immunoenzymatic reaction after *in vitro* exposure to various temperatures.

4. Coelomocytes as bioindicators to investigate the toxicity of heavy metals

Coelomocytes of *L. terrestris* were incubated *in vitro* for 3 h in medium supplemented with various concentrations of Zn, Cd, Pb or Cu ions at various concentrations: 0, 11, 22, or 44 mg/l. Cell viability was assessed by Trypan blue exclusion test (Fig. 12a). Coelomocyte activity was estimated by the neutral red pinocytosis assay (Fig. 12b), plastic adherence (Fig. 12c), and MTT reduction by mitochondrial dehydrogenases (Fig. 12d). It turned out that the cell viability and activity was not affected at any Zn ion concentrations. In contrast, other ions reduced coelomocyte viability and activities in dose-dependent manners in order Cd < Pb < Cu (Fig. 12a-d)[17].

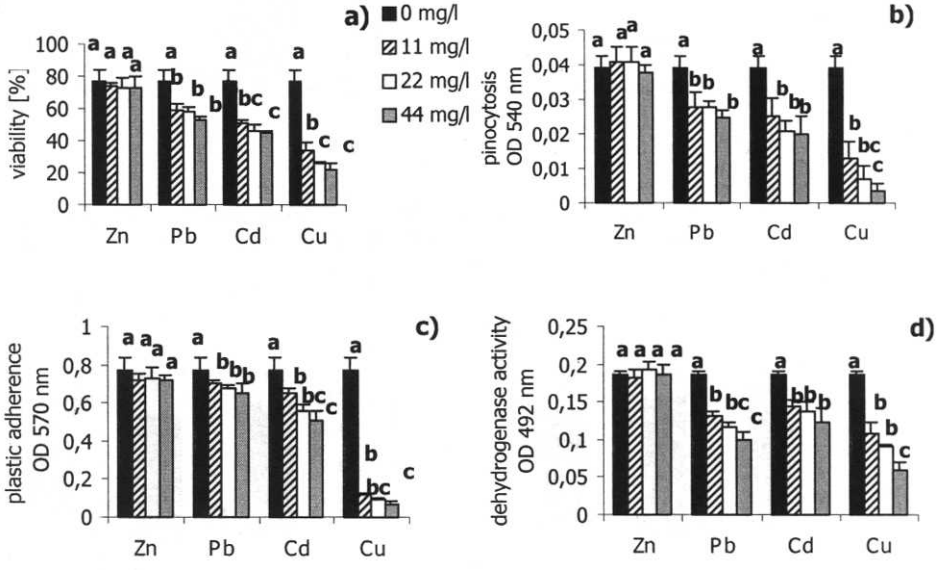

Fig. 12: Effects of heavy metals on viability (a), pinocytosis of neutral red (b), plastic adherence measured by crystal violet stain (c) and mitochondrial dehydrogenase activity measured by MTT reduction to formasan (d) of *Lumbricus terrestris* coelomocytes; means ± SD; OD - optical density. Different letters within given heavy metals ions indicate mean values significantly different.

The soil samples were collected from three localities (S, K, and B) with low (S), moderate (K) and heavy (B) pollution with Zn, Pb, Cd, and Cu (Fig. 13). The adult individuals of *A. chlorotica* were field-collected from the K population. They were divided in three groups, 24 individual each, and kept for 4 weeks in S, K, or B soil samples. Animals were fully vital and reproduced (as evidenced by the presence of cocoons and juveniles) in S and K soil samples. In contrast high mortality, no reproduction and decreased coelomocyte number per body weight were recorded in animals from heavily polluted B soil samples (Fig. 14a-d)[18].

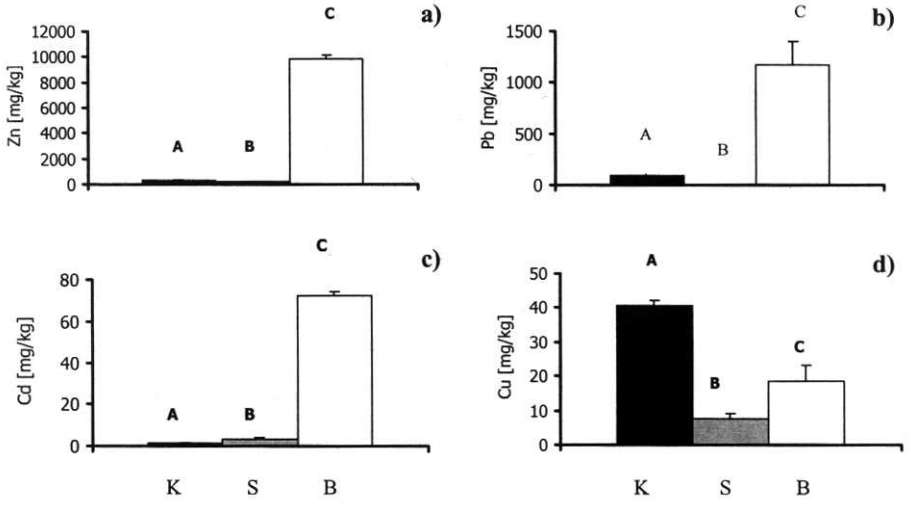

Fig. 13: Soil pollution in three localities: Sierbowice (S), Kraków (K) and Bukowno (B); means ± SD. Different letters within given heavy metal ions: Zn (a), Pb (b), Cd (c) and Cu (d) indicate mean values significantly different.

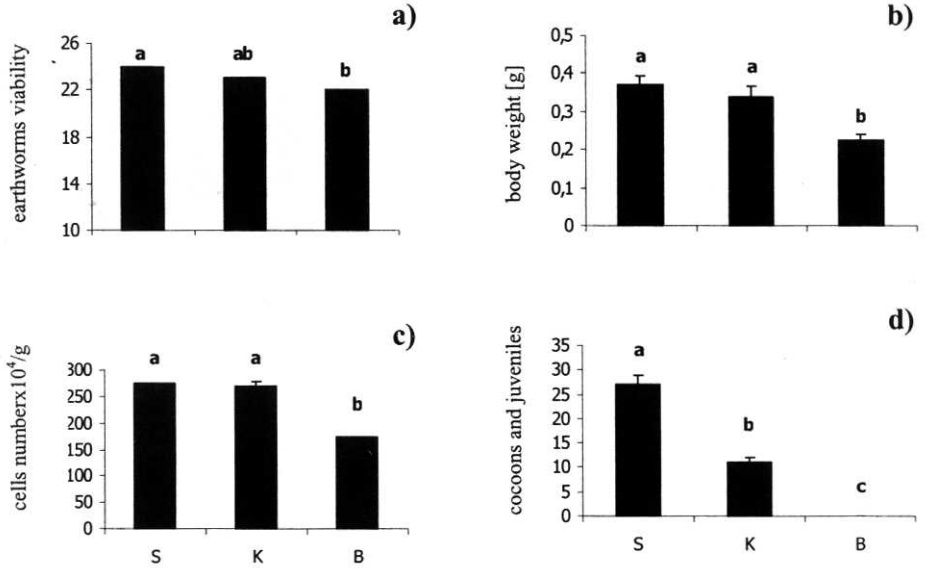

Fig. 14: Effects of soil pollution on *Allolobophora chlorotica* earthworms: viability (a), body weight (b), numbers of cells per body weight (c), and numbers of cocoons and juveniles (d); means ± SD. Different letters within given group indicate mean values significantly different.

5. Conclusions

The earthworms are able to adapt to ambient temperature. Such thermal adaptation is a gradual and rather slow process. The obvious effects of the ambient temperature on coelomocyte viability correspond with several functions of these cells connected with their participation in nonspecific immunity [14, 15]. Immunocompetent cells from other ectothermic animals (e.g. fish, amphibians) also show ability of thermal adaptation [19]. Such abilities may be responsible for an effective defense of ectothermic animals inhabiting the moderate climatic zone during both cold winters and warm summers.

Numerous invertebrate species have been used to evaluate risks, usually as whole organisms exposed *in vivo* to toxicants in the form of neat chemicals, chemical mixtures, solid matrices or their elutriates [20]. Earthworms are model soil organisms for the terrestrial ecotoxicology thus they are frequently used for the monitoring of heavy metals in the environment [7, 21-23]. Toxicity rating using earthworm species gives approximately the same results as that obtained using vertebrate species [23].

Acknowledgements

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References

[1] M. Bilej *et al.*, Phagocytosis of synthetic particles in earthworms: effect of antigenic stimulation and opsonization, *Folia Biol.* **36** (1990) 273-280.
[2] M. Bilej *et al.*, Antimicrobial defense of the earthworm, *Folia Microbiol.* **45** (2000) 283-300.
[3] S.W. Burch *et al.*, *In vitro* earthworm *Lumbricus terrestris* coelomocyte assay for use in terrestrial toxicity identification evaluation, *Bull. Environ. Contam. Toxicol.* **62** (1999) 547-554.
[4] E.L. Cooper and A. Stein, Oligochaetes. In: N.A. Ratcliffe, A.F. Rowley (Eds), Invertebrate blood cells. Academic Press, New York, 1981, pp. 75-140.
[5] E.L. Cooper, General immunology. Pergamon Press. Oxford, New York, Toronto, Paris, Frankfurt, 1982, pp. 287- 289.
[6] E.L. Cooper, Invertebrate Immunology. Springer-Verlag, Berlin-Heidelberg, 1996.
[7] L.C. Fitzpatrick *et al.*, Comparative toxicity in the earthworms *Eisenia fetida* and *Lumbricus terrestris* exposed to cadmium nitrate using artificial soil and filter paper protocols, *Bull. Environ. Contam. Toxicol.* **57** (1996) 63-68.
[8] J.J. Scott-Fordsmand and J.M. Weeks, Biomarkers in earthworms, *Rev. Environ. Contam. Toxicol.* **165** (2000) 117-159.
[9] A. Cossarizza, Earthworm leukocytes that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines, *Exp. Cell. Res.* **224** (1996) 174-182.
[10] P. Roch, Protein analysis of earthworm coelomic fluid: polymorphic system of the natural hemolysin of *Eisenia fetida andrei*, *Dev.Comp. Immunol.* **3** (1979) 599-608.
[11] A. Stankiewicz and B. Plytycz, Effects of *in vitro* conditions and *in vivo* thermal adaptation on viability of the earthworm (*Eisenia fetida*) coelomocytes, *Folia Biol.* **46** (1998) 183-188.
[12] A. Kurek *et al.*, Earthworm coelomocytes: inter-species differences in activity and sensitivity to heavy metals. Proceeding of the *European Federation of Immunological Societies – EFIS 2000* (2001) 323-328.
[13] A. Stankiewicz and B. Plytycz, Optimization of *in vitro* assays for activity of earthworm (*Eisenia fetida*) coelomocytes, *Scientific Papers of Agricultural Academy in Krakow* **334** (1998) 183-190.
[14] A. Kurek and B. Plytycz, The annual cycle of earthworm coelomocyte activity. *Scientific Papers of Agricultural Academy in Krakow* **372** (2001) 125-131.
[15] A. Kurek and B. Plytycz, Annual changes in coelomocytes of four earthworm species, submitted.
[16] A. Kurek *et al.*, Thermal shock induced expression of HSP70 and HSP72 proteins in coelomocytes of the earthworm *Eisenia fetida*, *Centr. Eur. J. Immunol.* **26** (2001) 88-92.

[17] J. Homa and B. Plytycz, Effects of heavy metals on *Lumbricus terrestris* coelomocytes. *Scientific Papers of Agricultural Academy in Krakow* **372** (2001) 183-192.
[18] J. Homa *et al.*, Effect of heavy metals on the earthworm *Allolobophora chlorotica*, submitted.
[19] B. Plytycz and A. Józkowicz, Differential effects of temperature on macrophages of ectotermic vertebrates, *J. Leuk. Biol.* **56** (1994) 729-731.
[20] A.J. Goven and J. Kenedy, Environmental pollution and toxicity in invertebrates: an earthworm model for immunotoxicology, In: E.L. Cooper (Ed), Invertebrate immune responses: cells and molecular products, Thomson Press, Berlin, 1996, pp. 169-206.
[21] K.E. Bunn *et al.*, Effects of agrochemicals on the immune systems of earthworm, *Bull. Environ. Contam. Toxicol.* **57** (1996) 632-639.
[22] N. Fugére *et al.*, Heavy metal-specific inhibition of phagocytosis and different *in vitro* sensitivity of heterogenous coelomocytes from *Lumbricus terrestris* (*Oligochaeta*), *Toxicology* **109** (1996) 157–166.
[23] L. Lagadic and T. Caquet, Invertebrates in testing of environmental chemicals: are they alternatives? *Environ. Health Perspect.* **106** (1998) 593-611.

A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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Use of fluorescent probes to analyze the killing, encapsulation and chemotaxis functions of coelomocytes of the earthworm, *Lumbricus terrestris*

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Abstract. Coelomocytes play a central role in the earthworm immune system. Coelomocyte function is often assessed using microscopy techniques. Microscopic analysis of the immune cell functions of phagocytosis, encapsulation and chemotaxis are often limited by the kind of stain used to label the cells and targets. Fluorescent probes can be used to greatly enhance the ability of the observer to view these immune cell functions using microscopy. The fluorescent probe FUN-1 enhanced the analysis of phagocytosis and gave a reliable report of the "killing" of FUN-1-labeled yeast cells. FUN-1 appeared to remain stable in the cytoplasm of cells in long-term killing assays. The fluorescent probe Sytox green allowed the observer to view cells which were part of globular clusters of cells which tend to form capsules as part of the encapsulation process. Furthermore, Sytox green also aided in the detection of cells migrating through porous filters in a modified Boyden chamber chemotaxis assay.

1. Introduction

The immune function of earthworm coelomocytes can be assessed by measuring, phagocytosis, killing of microorganisms, chemotaxis, and encapsulation of foreign substances and pathogens [1-4]. Phagocytosis has been studied extensively and is often used as a standard test of coelomocyte function. In contrast, only a few studies have investigated the killing function of coelomocytes [5]. For instance, yeast cells are often used to analyze coelomocyte phagocytosis but the killing of yeast targets is seldom included in studies which assess coelomocyte function [2]. Furthermore, studies of killing function typically employ an indirect measurement of the killing event which often involves assessing microbial growth after killing has occurred [5]. Coelomocytes also eliminate pathogens by encapsulation [1, 6]. During this process coelomocytes create a multicell structure that surrounds and encapsulates the foreign particle. The entire capsule can then be eliminated by the host organism. Examination of the morphology of individual cells in the multicell capsule can be problematic because the coelomocytes differentiate into a tissue like cell morphology. Chemotaxis can also be used as a measurement of coelomocyte function [3]. Chemotaxis is often assessed by observing cells migrating through filters. However, identifying individual cells migrating through filters can be difficult to assess by microscopy. In this paper we demonstrate how fluorescent probes can be used to enhance the measurement of microbial killing, encapsulation and chemotaxis in earthworm coelomocytes.

2. Assessment of phagocytosis and killing using fluorescently labeled yeast cells

Phagocytosis and killing of pathogens are a common innate immune functions present in all metazoan species. Phagocytes kill intracellular pathogens by a variety of antimicrobial mechanisms. A general survey of the published scientific literature involving the invertebrate immune system shows that phagocytosis, is perhaps, the most commonly studied immune cell function [1]. However, surprisingly few studies have focused on the fate of internalized pathogens. This may be due to the fact that, in general, immune cell functions occur at a slower rate in invertebrate systems compared to mammalian systems making experiments more cumbersome to perform. For instance, the killing of bacterial pathogens by coelomocytes may require several hours and consequently viable bacteria may also have time to reproduce, complicating how one can determine the number of pathogens killed. We have used the fluorescence probe FUN-1 to determine the fate of intracellular yeast targets. FUN-1 is a halogenated cyanine compound, which is virtually non-fluorescent in aqueous solution [7]. The fluorescence of FUN-1 is increased in the cytoplasmic environment of cells. In viable yeast cells FUN-1 fluorescence is primarily confined to cylindrical refractile bodies. This produces a localized bright-red punctate fluorescence in the cytoplasm. Upon cell death the yeast cytoplasm displays a diffusive green fluorescence.

The yeast, *Saccharomyces cerevisiae,* were maintained in Yeast extract Peptone Dextrose media (Difco, Detroit MI, USA). Yeast in log phase growth were stained with the FUN-1 fluorescent probe (10 μM) for 30 min at 30 °C. The cells were washed by centrifugation at 2000 x g and resuspended in 1 ml of *Lumbricus* balanced salt solution (LBSS) [2] and incubated for an additional 30 min at 30 °C. Yeast were greater than 85 % viable as assessed by this method. Fluorescently stained yeast were added to glass-adherent coelomocytes at approximately a 25:1 yeast to coelomocyte ratio. The adherent coelomocyte population was derived from coelomic fluid harvested from earthworms using a 21-gauge butterfly needle. LBSS was mixed with the coelomic fluid on a glass microscope slide. Coelomocytes were allowed to adhere for 30 min to the glass slides and the nonadherent coelomocyte cell fraction and debris was removed by gentle rinsing with LBSS. After rinsing, 100 – 300 μl of fresh LBSS was added to the adherent cells. All subsequent incubations were maintained in portable humidified chamber at 22 °C. The yeast were incubated with the adherent cells in humidified chambers at 22 °C for up to 21 h. Before analysis by fluorescence microscopy the glass-adherent cells were gently rinsed with LBSS to remove non-cell associated yeast. Killing of yeast cells was determined by counting the number of green and red yeast cells associated with coelomocytes using fluorescence microscopy.

Fig. 1 shows yeast cells stained with the FUN-1 fluorescent probe. The viable yeast cells display a bright red punctate compartmentalized fluorescence (Fig. 1b). The stain is bright enough to be recorded by a standard video camera with nominal light sensitivity. Dead yeast cells also stained stain brightly and are easily distinguished from viable cells because they display a diffusive green fluorescence (Fig. 1d). A small percentage of the yeast population in all experiments exhibited both red and green fluorescence. This double-labeling is hypothesized to be due to a yeast cell that is dying. Fluorescent yeast cells internalized by coelomocytes or bound to them can be readily detected (Fig. 1c, d). The killing of coelomocyte associated yeast peaks at roughly 16 h after yeast are added to the glass adherent coelomocytes (Fig. 2). Yeast not exposed to coelomocytes remain 90 % viable under similar incubation conditions (Fig. 2). The potential for cytolytic affects due coelomic fluid factors is minimized by rinsing the adherent coelomocytes with LBSS prior to addition of FUN-1 labeled yeast. Furthermore, coelomic fluid does not cause a significant decrease in the viability of FUN-1 yeast when incubated with yeast for 2 h.

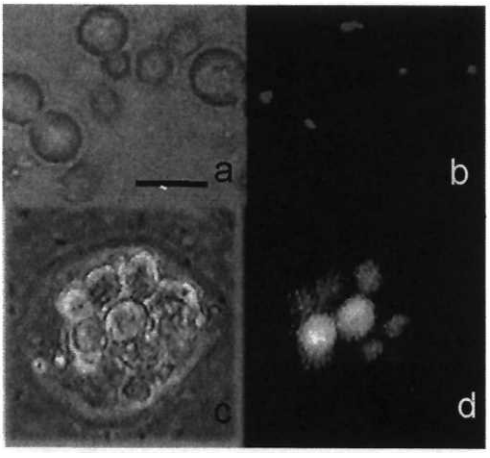

Fig. 1: FUN-labeled yeast targets in the presence and absence of earthworm coelomocytes. FUN-1 is an intracellular metabolic stain that shifts from a red to green fluorescence upon cell death. FUN-1-labeled yeast cells killed in the presence of earthworm coelomocytes were detected by fluorescence video-microscopy. Live yeast cells display a red fluorescence located in discrete sequestered areas in the cytoplasm (a, b). Yeast cells were killed in the presence of earthworm coelomocytes after incubation for several hours. Upon cell death the fluorescence shifts to green and becomes more diffuse throughout the yeast cytoplasm (c, d). A microscope equipped with a halogen light source, excitation and emission filters and a video camera (Javelin Smartcam, Javelin electronics, Torrance CA, USA) was used to obtain bright field and fluorescence microscopy images. Digital images were captured directly from the video output or from video tape using a Digital Still Recorder (Sony, New York, NY, USA).

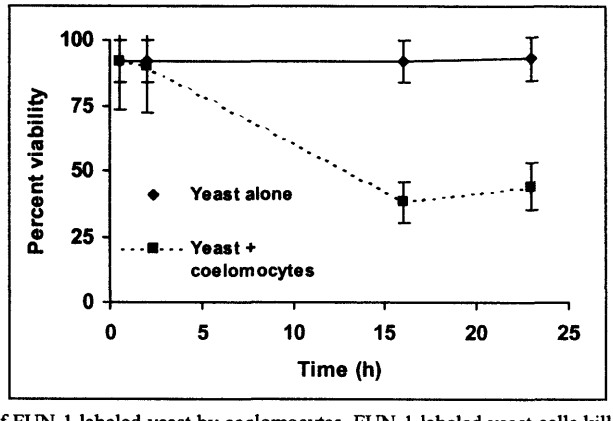

Fig. 2: Killing of FUN-1 labeled yeast by coelomocytes. FUN-1 labeled yeast cells killed in the presence of earthworm coelomocytes were detected by fluorescence video-microscopy. For each experimental trial, several fields were observed and recorded by video microscopy. A killing-index was determined by enumerating the live and dead yeast per 10 coelomocytes. Typically, a minimum of 50 coelomocytes were observed for each experimental trial. The experiments were repeated 4 times for each of the four time points; 0.5, 2, 16 and 23 h. Fresh earthworm coelomocytes were used for each trial. The data in the graph shows the average killing index expressed as percent viable yeast remaining after exposure to coelomocytes. Greater than 50 % of the yeast were killed in the presence of coelomocytes (noted by a decrease in yeast viability) at 16 and 23 h ($P< 0.01$ and $P< 0.05$ respectively) as compared to controls.

Perhaps one of the greatest advantages of the FUN-1 probe is its stability. The probe remains fluorescent during killing assays, which involve 21 h of incubation. Furthermore, the probe does not noticeably affect yeast viability. In addition, we predicted that the probe would have a tendency to leak from yeast cells during long incubation periods and cause unintended background fluorescence. However the probe remains confined to the yeast cytoplasm during long incubation periods.

3. Use of the Sytox green viability stain to assess encapsulation

Sytox green can be used to enhance the analysis of encapsulation by fluorescence microscopy. Encapsulation is a process used by invertebrates to eliminate large foreign particles [1, 6]. During this process coelomocytes congregate and adhere to the foreign object and each other. A dense capsule composed of flattened cells is formed within several days. The capsule is walled off from the body and eventually eliminated by the organism. It is difficult to study the cell morphology of cells, which make up the capsule using conventional stains and light microscopy. We found that the Sytox green stain greatly assisted in determining the location of the cells in the capsule. In Fig.3a and Fig. 3b, a large aggregate of cells can be seen. Large aggregates of coelomocytes are often observed in the coelomic fluid extracts and are consistent with the formation of capsules. When examined by light microscopy it is difficult to determine how many cells are part of this aggregate. However examination of Sytox green labeled nuclei allows the investigator to determine the number of cells involved and also determine the approximate location of each cell in the aggregate. In addition, some cells show thin flattened nuclei, which correspond to a similar cell morphology. Some areas of the capsule structure were not stained by Sytox green indicating an area of nuclear exclusion or perhaps the location of the encapsulated foreign particle.

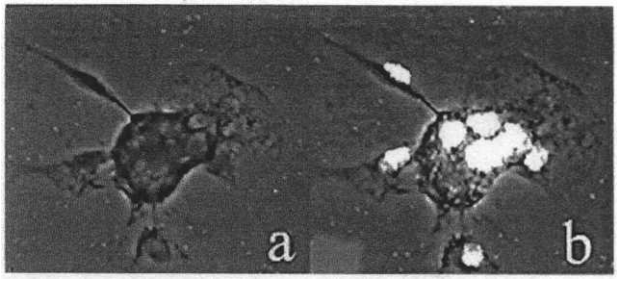

Fig. 3: Detection of individual cell nuclei in large clusters of earthworm coelomocytes using Sytox green. Large clusters of cells consistent with the phenomenon of encapsulation are often observed in coelomocytes extracted from earthworms. Individual cells within the cluster are difficult to distinguish by light microscopy. However, individual nuclei are readily distinguished after paraformaldehyde fixation and staining with Sytox green. Some cells displayed a thin-flattened structure which is postulated to occur in cells which participate in the encapsulation process. The fluorescence in this image (b) was enhanced by stacking several images together.

4. Use of the Sytox green viability stain to assess chemotaxis

Sytox green can also be used to analyze coelomocytes undergoing chemotaxis through filter substrates. Conventional methods for detecting cells on filters have involved using stains like hematoxylin-eosin [8]. These staining methods use multiple steps and often do not allow one to distinguish the individual cells in a cell cluster. Fluorescent nuclear stains offer several advantages over these conventional stains including one step staining methods and labeling that is clear and unambiguous. DAPI and Sytox green are stains which have been used to stain the nuclei of cells [8-10]. DAPI has also been used to enhance the detection of migrating cells in chemotaxis assays [8]. Sytox green staining of migrating cells offers many of the same advantages that the DAPI staining method provides. In addition, Singer and Jones have documented that the Sytox green probe has a higher quantum yield compared to propidium iodide stains leading to a higher signal to background ratio [10].

To assess chemotaxis, a boyden chamber assay is used. In this assay polycarbonate filters with 8 micron pores were used in the chambers (Neuroprobe, Gaitherburg MD, USA). Filters were pretreated by boiling in a 1 % EDTA solution for 10 min, followed by boiling 20 min in distilled water. Chemoattractant or buffer (200 µl) was placed in the lower chamber and two filters were placed above the lower chamber. Coelomocytes (2 x 10^7 cells/ml) were placed in the upper chamber over the filters. The chambers were stored in a humidified environment at room temperature for 21 h. The chemoattractant is allowed to diffuse into the filters from a lower cavity in the chamber and coelomocytes which are placed above the filters, migrate towards the chemoattractant. At the end of the incubation period filters were separated and the bottom filter was fixed in 1 % paraformaldehyde for 25 min. The paraformaldehyde was removed and the filters were exposed to Sytox green (6 µM solution) in LBBS for 30 min at 22 °C. Filters were then washed with several changes of LBBS and mounted on a microscope slide. Cells trapped in the lower filter are enumerated. The Sytox green stain used in our chemotaxis studies displayed very bright stained nuclei which could be easily detected and enumerated on the chemotaxis filters. The Sytox green stain did not appear to stain the cytoplasm and it sharply delineates the nucleus within the cell. Many stained nuclei correspond with a filter pore opening, suggesting that the coelomocyte was fixed while in transit within the filter pore (Fig. 4). The nuclei of the adherent coelomocytes displayed a very uniform morphology and size (Fig. 4). Only cells which had nuclei of similar morphology and size to the coelomocyte preparations were considered to be migrating cells in the chemotaxis assay. In some cases cells with large fluorescent nuclei appear on the chemotaxis filters. These cells were considered to be contaminants since they had nuclei which did not match the morphology or size of cells in a typical coelomocyte preparation. It is possible that fungal spores or other contaminating cells could collect on the filters since the chemotaxis assay required long periods of incubation.

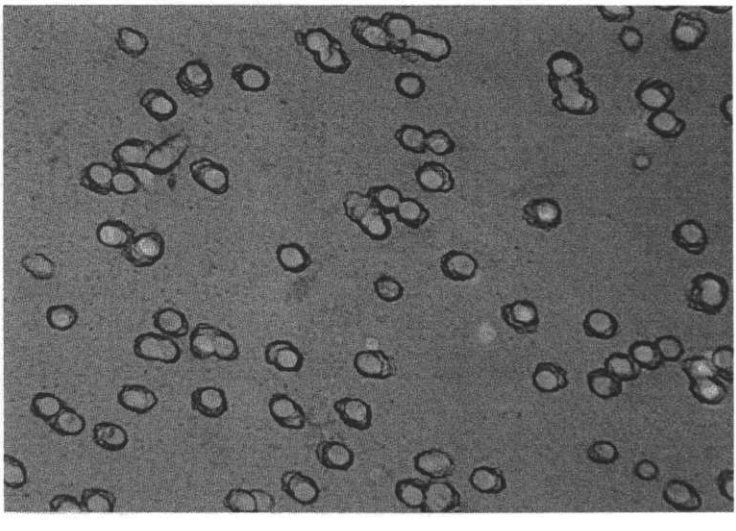

Fig. 4: Sytox green-labeled coelomocytes migrating through polycarbonate filters. Coelomocytes were allowed to migrate through filters in the modified Boyden chamber assay. This digital image was constructed from the merger of the bright-field and fluorescence digital images of the same field. The filters with trapped and adhered coelomocytes were fixed in paraformaldehyde and stained with Sytox green. The cell nuclei can be observed on the filter and within the filter pores. Sytox green stained cells are enumerated on several randomly chosen fields to determine the extent of chemotaxis. Digital images of coelomocytes migrating through the filters were captured using a Spot camera and Spot software (Diagnostics Instruments Inc., Sterling Heights, MI USA).

5. Conclusion

In this paper we have highlighted how fluorescent probes can enhance the study of coelomocyte immune cell function. These probes and others like them will aid our understanding of how the coelomocyte functions as an immune cell.

References

[1] V. Vetvicka *et al.*, Immunology of Annelids, CRC Press, Boca Raton, Florida, 1994.
[2] E.A. Stein and E.L. Cooper, The role of opsonins in phagocytosis by coelomocytes of the earthworm, *Lumbricus terrestris*, *Dev. Comp. Immunol* **5** (1981) 415-425.
[3] D.H. Marks *et al.*, Chemotactic attraction of *Lumbricus terrestris* coelomocytes to foreign tissue, *Dev. Comp. Immunol.* **3** (1979) 277-285.
[4] P. Valembois *et al.*, Immunity in Invertebrates, Springer-Verlag, Berlin, 1986.
[5] R.P. Dales and Y. Kalac,. Phagocytic defence by the earthworm *Eisenia foetida* against certain pathogenic bacteria, *Comp. Biochem. Physiol.* **101A** (1992) 487-490.
[6] P. Valembois *et al.*, Formation of brown bodies in the coelomic cavity of the earthworm *Eisenia fetida andrei* and attendant changes in shape and adhesive capacity of constitutive cells, *Dev. Comp. Immunol.* **16** (1992) 95-101.
[7] P.J. Millard *et al.*, Development of the FUN-1 family of fluorescent probes for vacuole labeling and viability testing of yeast, *Appl. Env. Microbiol.* **63** (1997) 2897-2905.
[8] G. Boguslawski *et al.*, Use of DAPI staining for quantitation of cell chemotaxis, *BioTechniques* **30** (2001) 42-48.
[9] B.L. Roth *et al.*, Bacterial viability and antibiotic susceptibility testing with Sytox green nucleic acid stain, *Appl. Env. Microbiol.* **63** (1997) 2421-2431.
[10] L.J. Jones and V.L. Singer, Fluorescence microplate-based assay for tumor necrosis factor activity using Sytox green stain, *Anal. Biochem.* 293 (2001) 8-15.

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Comparative analysis of earthworm immune system using cell surface and intracellular markers

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Abstract. Coelomocytes located in the coelomic cavity of the earthworms *Eisenia foetida* are involved in the immune protection of these animals. Three coelomocyte subpopulations were identified by flow cytometry. The three subpopulations were different in size and granularity and showed different reactivity with monoclonal antibodies against mammalian cell surface markers, CD markers and intracellular antigens. We found positivity with anti-Thy-1, CD24, TNF-α and TSH antibodies in two cell subpopulations while the third population was found to be negative. These results were confirmed by confocal microscopic detection both on the cell surface and in the intracellular compartment.

1. Introduction

In the 1960s and early 1970s there were only a few useful techniques for the identification of the cell surface molecules of eukaryotic cells, and important data obtained from erythrocyte and other model systems did not result in a general method for analyzing cell membrane. Considerable progress was possible with the solubilization of membrane proteins by detergent and affinity chromatography. Mouse H2 antigens were discovered by agglutination assays on the red blood cells combined with the use of highly specific monoclonal antibodies against well-defined cell surface markers [1, 2]. The basic method was to produce antibodies that would selectively recognize the different subpopulations of leukocytes. This was done by raising "alloantibodies", by immunizing mice with immunocytes of other strains. Such techniques successfully led to the development of antibodies that reacted with mouse T lymphocytes (anti-Thy-1 antibodies) and even against functionally different subsets of T lymphocytes. Biochemical analyses of cell surface proteins recognized by a monoclonal antibody demonstrated that in many instances these antibodies specifically link to the identical molecular structures on different cells, tissues or species. In the beginning some confusion occurred because these surface markers were initially named according to the clonal name of antibodies reacting with them. In order to solve this, a uniform nomenclature system initially used for human leukocytes was adopted. According to this system, a surface marker that identifies a particular lineage or differentiation stage, that has a defined structure and that is recognized by a group (cluster) of monoclonal antibodies with identical specificity is defined as a member of Cluster of Differentiation (CD). Although this nomenclature was originally used for human leukocyte antigens, it is now common practice to refer to crossreactive markers in other cellular components of different individuals or species and on

cells other than leukocytes by the same CD designation. The two most frequent functions attributed to various CD antigens are to promote cell-cell interactions and adhesion or to transduce signals that lead to cell activation [3].

2. Presence for defense molecules in invertebrate species

The ancient form of nonself recognition/defense function is conserved through phylogenesis. Cell adhesion molecules play role in cell-cell cross talk in physiological conditions and in the encapsulation of foreign invaders. Invertebrates do not express specific receptors for the recognition of antigens (immunoglobulins, B- and T-cell receptors) but they can produce other recognition molecules e.g. lectins. These proteins are capable to bind to carbohydrate components. Lectins are involved directly in host defense mechanisms as opsonins, LPS-binding molecules and toxins and indirectly as cell adhesion molecules. They can also induce cell activation as mitogens. Receptor molecules are localized in the plasma or on the surface of immunologically active cells of invertebrate animals. Different pathways are responsible for the recognition of foreign molecules:

1. Direct binding of carbohydrate determinants (glycoconjugates) of the foreign particles to membrane-bound lectins (named as agglutinins).
2. Binding of humoral agglutinin to the carbohydrate determinants of the foreign cell and the opsonin receptors of the immune cells via different binding sites [4].
3. Binding of surface lectins of foreign particles to carbohydrate determinants on the surface of the immune cells.

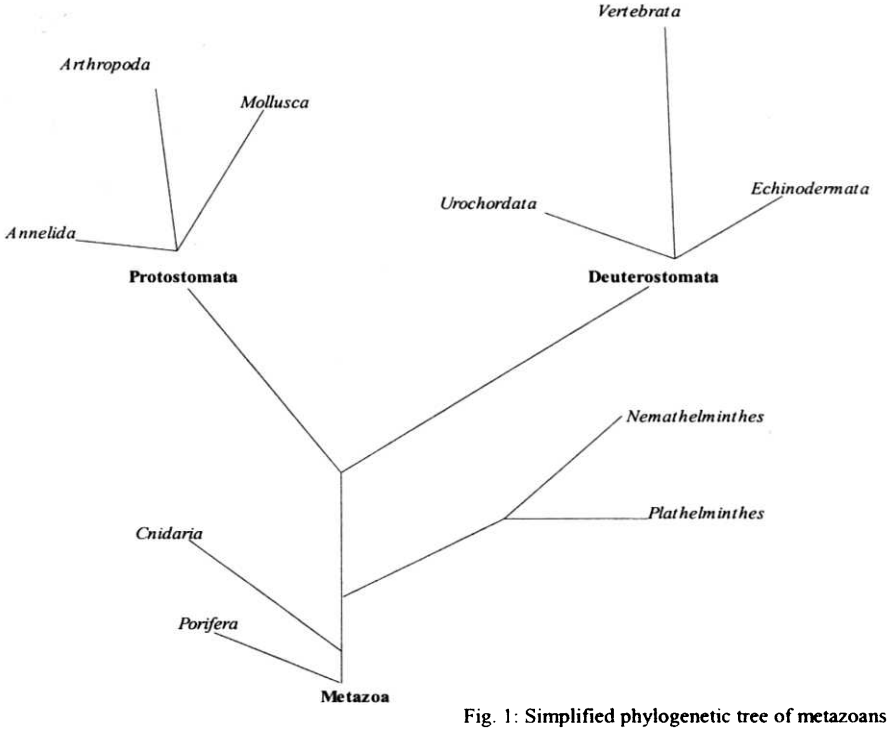

Fig. 1: Simplified phylogenetic tree of metazoans.

Cell adhesion molecules playing a role in cell aggregation are present since the most ancient phyla like sponges (Fig. 1). Other molecules typically participate in the self/nonself discrimination. Two groups of molecules were found in *Geodia cydonium* that contain an immunoglobulin (Ig)-like motif, a receptor tyrosine kinase and the sponge adhesion molecules (SAM). During the last few years studies on autografting in sponges, which demonstrated self-tolerance were supported by the identification of molecules involved in this recognition process. In autografts integrin receptors are strongly upregulated in the zones between the grafts suggesting their functional commitment [5]. It was noted that the SAM structures from *G. cydonium* are similar to the T cell receptor [6, 7, 8]. In the nematode *Caenorhabditis elegans* and in the fruitfly *Drosophila melanogaster* cadherin-like cell adhesion proteins were described. Examination of the structure and the function of these molecules revealed similarities. The classic cytoplasmic domain is present in these two model systems as well as in humans, but the extracellular domains share a different arrangement [9].

A further important recognition system, the prophenoloxidase cascade in arthropods, utilizes nonself recognition molecules. These molecules, known as "pattern recognition molecules", can recognize for instance the β-1,3 glucan on the surfaces of fungi (yeast) and the LPS in Gram-negative bacteria, initiating the cascade. In crayfish *Astacus*, 10^{-10} g/ml LPS or 10^{-9} g/ml glucan increases protease activity leading to prophenoloxidase cascade activation. In crayfish a cell adhesion molecule with peroxidase activity was found (peroxinectin). The peroxidase activity is not necessary for cell adhesion but peroxinectin can rather act as an opsonin. Recently myeloperoxidase was showed to act as an adhesion molecule in mammals [10]. The conserved residue of the peroxinectin molecule was described in *Drosophila*.

Recently a variety of cell surface molecules was detected in annelids, by flow cytometry analysis and immunocytochemistry using monoclonal antibodies against mammalian CD markers. Different research groups described two populations of coelomocytes in earthworms reacting with monoclonal antibodies specific for mammalian cell surface molecules [11]. A small electron dense cell population gives signal with anti-CD11a, CD45RA, CD45RO, CDw49b, CD54, β_2 microglobulin, and anti-Thy-1 monoclonal antibodies. The large cell population is are negative for these markers [11, 12 13]. Three cell populations (R1, R2, R3) were defined by flow cytometry in our experiments differing in size and granularity. In harmony with the literature data, we found positivity with anti-Thy 1 antibodies. However, the detection of CD24- and TNF-cross-reactive epitopes on the surface of coelomocytes seems to be new results of our comparative studies. These CD24- and TNF-like molecules are localized in two coelomocyte populations (R1, R2). The R3 population contains cells relatively smaller and highly granulated as compared with the two others populations. In leeches, another annelid class, CD markers were also found, allowing to divide immunologically active cells into three different types. The macrophage like cells are positive for anti-CD25, CD14, CD11b, CD11c antibodies; NK-like cells are positive for anti-CD68, CD61, CD56, CD57 antibodies; and granulocyte-like cells are stained with anti-CD11b, CD11c antibodies [14, 15, and de Eguileor in this issue].

3. *Cytokines and neuropeptides mediate cross talk between the neuroendocrine and immune system*

The cross talk between the nervous and the immune system has a common ancient origin. These interactions are mediated by different molecules, including adrenocorticotropin hormone (ACTH), monoamines, opioid peptides and cytokine-like factors (IL-1, IL-6, TNF). These molecules are produced by macrophages in vertebrates and by macrophage-like immunocytes in invertebrates [16]. Some neuropeptides have important role in defense

mechanisms. For instance, in mollusks, ACTH is able to induce modification of the cytoskeleton of macrophages and can play a role in cell migration and motility. Moreover, β-endorphins and other pro-opiomelanocortin (POMC)-derived peptides (encephalin) mediate stress response and inflammation during bacterial infection. The presence of glucocorticoids and cortisol was also reported. All these molecules participate in the vertebrate stress response and may have a similar role in invertebrate species [17].

In recent years the presence of cytokine-like molecules in invertebrate animals was suggested. These cytokine-like peptides are functionally similar to the vertebrate cytokines, but nucleotide sequence homology is not given yet. Some groups tried to determine in invertebrates the presence of membrane molecules similar to vertebrate cytokine receptors [18-20]. By polymerase chain reaction (PCR), a cDNA sequence from insect (*Manduca sexta*) shows 35 % homology to sheep, rat, rabbit, mouse, human IL-1α, IL-1β and IL 1 receptor antagonist [19]. In *Drosophila*, a membrane receptor called Toll was described [21]. Toll is involved in defense mechanisms and this led to the definition of the IL-1R/Toll-like receptor [22]. Another example is that the activation of mollusk immunocytes by IL-1 partly upregulates the synthesis of TNF-like molecules [23]. These studies suggest the presence of cytokine analogues in invertebrates. There are cytokine-like factors in earthworms. In our experiments, we found TNF-like activity in earthworms. Strong positivity was detected with anti-TNF and anti-TGF-α monoclonal antibodies in the supernatant of coelomocyte lysates. TNF positivity was found in two coelomocyte populations (R1, R2) by flow cytometry.

Thyroid-stimulating hormone (TSH) was detected in the R1 and the R2 populations of coelomocytes by immunochemistry and by flow cytometry in our recent study. This new observation together with the presence of TSH supports some papers suggesting the expression of hormone-like molecules in invertebrate leukocytes [16].

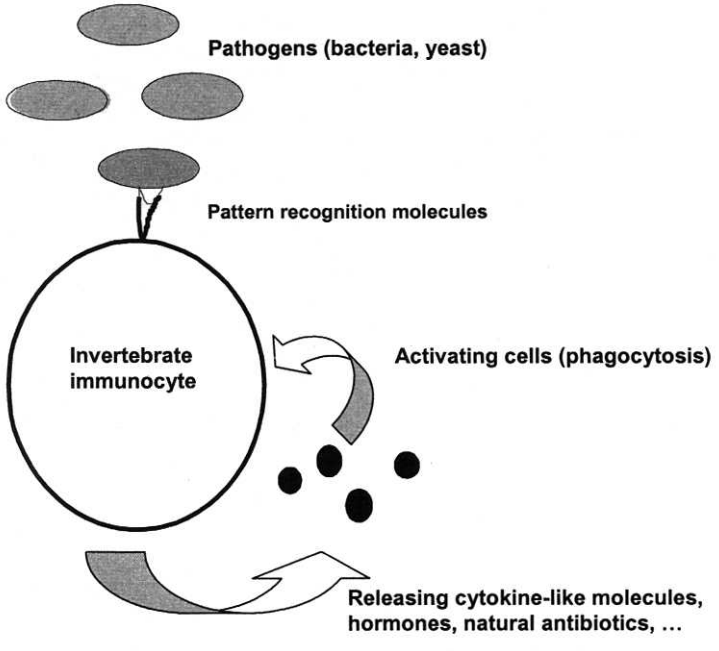

Fig. 2: Invertebrate immunocytes participate in the innate immune response with humoral and cellular reactions.

4. Conclusion

In this review, we discussed some aspects of cell surface markers such as lectins, cell adhesion molecules, cytokine-like factors, neuropeptides and steroid hormones (Fig. 2). In some cases, proteins and peptides of critical importance remained unchanged or underwent only minor modifications during evolution from invertebrates to vertebrates. In some others we can detect the presence of some well-conserved determinants, but we have no evidence of the function of the expressed markers yet.

References

[1] N. Barclay *et al.*, The Leucocyte Antigen Factsbook, Academic Press 1993.

[2] G. Köhler and C. Milstein, Continuous cultures of fused cells secreting antibody of predefined specificity, *Nature* **256** (1975) 495-497.

[3] A. K. Abbas *et al.*, Cellular and molecular immunology W.B. Saunders Company 2000.

[4] N. A. Ratcliffe *et al.*, Invertebrate immunity: basic concept and recent advances, *Int. Rev. Cytol.* **97** (1985) 183-350.

[5] W. Wimmer *et al*, Origin of the integrin mediated signal transduction. Functional studies with cell cultures from the sponge *Suberites domuncula, Eur. J. Biochem.* **260** (1999) 156-165.

[6] X. Fernandez-Busquets and M. M. Burger, Cell adhesion and histocompatibility in sponges, *Micr. Res. Tech.* **44** (1999) 204-218.

[7] W. E. G. Müller *et al.*, Aggregation of sponge cells: stage dependent, distinct adhesion mechanisms in *Cliona celata, Eur. J. Cell Biol.* **28** (1982) 243-250.

[8] W. E. G. Müller *et al.*, Evolution of the innate and adaptive immune systems: Relationships between potential immune molecules in the lowest metazoan phylum (*porifera*) and those in vertebrates, *Transplantation* **68** (1999) 1215-1227.

[9] E. Hill *et al.*, Cadherin superfamily proteins in *Caenorhabditis elegans* and *Drosophila melanogaster, J. Mol. Biol.* **305** (2001) 1011-1024.

[10] Johansson *et al.*, Peroxinectin, a novel cell adhesion protein from crayfish blood, *Biochem. Biophysic. Res. Commun.* **216** (1995) 1079-1087.

[11] A. Cossarizza *et al.*, Earthworm leukocytes that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines, *Exp. Cell Res.* **224** (1996) 174-182.

[12] E. L. Cooper *et al.*, Cell adhesion and the immune system: a case study using earthworms, *Micr. Res. Tech.* **44** (1999) 237-253.

[13] E. L. Cooper, Earthworm immunity, *Prog. Mol. Subcell. Biol.* 16 (1996) 10-45.

[14] P. Roch *et al.*, Serological evidences for a membrane structure related to human β_2-microglobulin expressed by certain earthworm leukocytes, *Eur. J. Immunol.* **13** (1983) 1037-1042.

[15] M. de Eguileor *et al*, Lipopolysaccharide-dependent induction of leech leukocytes that cross-react with vertebrate cellular differentiation markers, *Tissue Cell* **32** (2000) 437-45.

[16] E. Ottaviani and C. Franceschi, The invertebrate phagocytic immunocyte: clues to a common evolution of immune and neuroendocrine systems, *Immunol. Today* **18** (1997) 169-174.

[17] E. Ottaviani *et al.*, Pro-opiomelanocortin-derived peptides, cytokines, and nitric oxide in immune responses and stress: an evolutionary approach, *Int. Rev. Cytol.* **170** (1997) 79-140.

[18] A. Beschin *et al.*, On the existence of cytokines in invertebrates, *Cell. Mol. Life Sci.* **58** (2001) 801-814.

[19] G. Beck, Macrokines: invertebrate cytokine-like molecules, *Front. Biosci.* **3** (1998) 559-569.

[20] G. Beck and G. S. Habicht, Primitive cytokines: Harbingers of vertebrate defense, *Immunol. Today* **12** (1991) 180-183.

[21] J. Maxton-Kuchenmeister *et al.*, Toll homologue expression in the beetle *Trilobium* suggests a different mode of dorsoventral patterning than in *Drosophila* embryos, *Mech. Dev.* **83** (1999) 107-114.

[22] A. Bowie *et al.*, The interleukin 1 receptor/Toll like receptor superfamily: signal generators for proinflammatory interleukins and microbial products, *J. Leuk. Biol.* **67** (2000) 508-514.

[23] T. K. Hughes *et al.*, Interaction of immunoactive monokines (interleukin 1 and tumor necrosis factor) in the bivalve mollusc *Mytilus edulis, Proc. Natl. Acad. Sci.* USA **87** (1990) 4426-4429.

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Antimicrobial Activity and Cytotoxicity

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A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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Coelomic fluid from *Lumbricus terrestris* and *Eisenia foetida* inhibits *Escherichia coli*

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Abstract. This study shows preliminary evidence of antimicrobial molecules and their dose-dependent activities in earthworm coelomic fluid. Coelomic fluids from *Lumbricus terrestris* and *Eisenia foetida*, when compared to controls: 1) suppressed bacterial growth of *Esherichia coli* K-12, as measured in colony forming units (cfu); 2) increased the area inhibition zones as measured in centimeter square. Antimicrobial activities from both species were dose-dependent. Bacterial growth was measured in two ways using agar plates: 1) counting of bacterial colonies in the drop zone; 2) measuring of the area of the zone of inhibition. As the amount of coelomic fluid was increased, the amount of bacterial colonies decreased, and the size of the zone of inhibition was increased.

1. Introduction

Antibiotics kill microorganisms without damaging the body tissues. Most antibiotics work in one of two ways. They can block a metabolic pathway that yields nutrients necessary for bacterial life or they can inhibit a specific enzyme required for the construction of bacterial cell walls thereby leading to death [1]. Over the past twenty years, bacteria have acquired resistances to many common antibiotics. In fact, many bacterial pathogens found in hospitals have multiple antibiotic resistance. Bacteria have become resistant by circumventing the specific pathways that antibiotics are designed to inhibit [2]. Scientists then began to find new antibiotics that inhibited other pathways, however, it was only a matter of time before bacteria became resistant to the newer antibiotics. The scientific community has been puzzled by the question of finding an antibiotic that works completely different from the conventional ones, that is, one that bacteria cannot become resistant to.

Therefore, the search for new antibiotics that have antimicrobial actions different from the common antibiotics is increasingly timely and important. Hence, the proposed experiments, to use the coelomic fluid from earthworms so as to seek a new antibiotic are particularly relevant to the today's scientific dilemma on antibiotics. According to one hypothesis coelomic fluid can be used as an antibiotic because it possesses certain lytic compounds [3]. These lytic compounds are believed to be antibiotic peptides that inhibit bacterial growth through pathways different from conventional antibiotics [4].

When experiments studying effects of coelomic fluid on common vertebrate or human bacteria revealed no positive results [5], Valembois *et al.* [6] focused their interest on bacteria habitually associated with the earthworm biotype. They isolated 23 strains of telluric bacteria from manure and exposed them to the coelomic fluid of *Eisenia foetida andrei*. Only six bacterial strains, both Gram-positive and Gram-negative, manifesting high pathogenicity when inoculated into the coelomic cavity, were sensitive to the activity of the coelomic fluid. All sensitive bacteria expressed at least one surface antigen that was common to vertebrate erythrocytes but distinct from the Forssman antigen [7]. These results suggested that at least one of the bacteriostatic substances belonged to the hemolytic system [6]. Gel filtration of coelomic fluid revealed that there were eleven fractions, and significant antibacterial

molecules characterized seven of which. At least four of the isolated fractions were involved in hemolytic or hemagglutinating activities (175-kDa pentamer of 35-kDa hemagglutinating subunits [8]; 20-, 40-, and 45-kDa molecules possessed either hemolytic or hemagglutinating activities [9]). From a viewpoint of relatedness of antibiotic and hemolytic substances, the 40- and 45-kDa molecules seem to be of particular interest. These proteins are encoded by two distinct genes one of which possesses four different alleles [10].

These proteins cause lysis of bacteria forming pores on their cell walls. Holes expose bacterial cytoplasms directly to the environment, which disturbs normal cellular functions and eventually causes cell death. This killing activity is more effective even to resistant strains of bacteria and makes it difficult for them to acquire resistance [1]. These proteins with their strong lytic activity found in the coelomic fluid of *Lumbricus terrestris* may open possibilities for applications as new-wave antibiotics. In this context, this study demonstrated preliminary evidence of dose-dependent antimicrobial activities of coelomic fluid from *L. terrestris* and *E. foetida*.

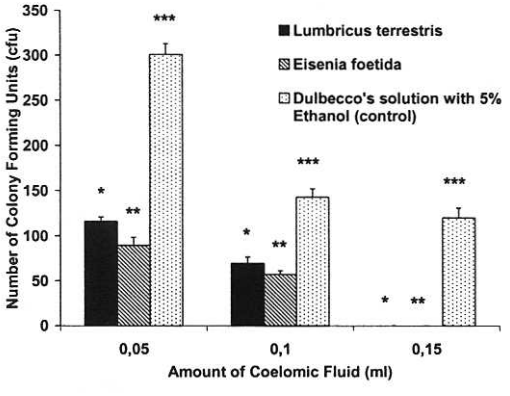

Fig. 1: The effects of *Lumbricus terrestris* and *Eisenia foetida* coelomic fluid on the number of *Escherichia coli* bacteria in the drop zone. Note that the controls remain higher than the experimental, and that *E. foetida* had the strongest effect against bacteria. Mean ± S.D.; n = 4; Student *t*-test: $p < 0.05$

2. Evidence of antimicrobial molecules in earthworm coelomic fluid

Coelomic fluids were obtained from two different worm species (*L. terrestris* and *E. foetida*), and controls were also set up. Worms were placed into Dulbecco's cell culture medium with 5 % ethanol. The ethanol in the medium stimulates earthworms to expel their coelomic fluids and they contain lytic molecules that we suspect will work against bacteria. As controls, the medium (95 % Dulbecco's medium with 5 % ethanol without coelomic fluids) was used. After removing cell debris by centrifugation, coelomic fluids and controls were inoculated onto agar plates in specified drop zones. Then, suspensions of *Escherichia coli* were placed on agar plates and cultured. Counting the number of colonies inside of the drop zones assessed bacterial growth, and the area of the zones of inhibition were measured. These procedures were performed with different amount of coelomic fluids and controls.

Results showed that as the amount of coelomic fluid was increased, the bacteria growth decreased, and the area of inhibition zones increased. In addition, *E. foetida* had strongest bacterial inhibition at each amount of solution, closely followed by the *L. terrestris* (Fig. 1).

The different effectiveness of coelomic fluids and controls suggested that the coelomic fluids were truly the factor of bacterial inhibition, while the ethanol in the Dulbecco's solution was not. The measurement of inhibition zones revealed that *L. terrestris* and *E. foetida* were significantly more effective to kill bacteria than controls (Fig. 2). Controls had relatively less potent activities in inhibiting bacterial growth, while the coelomic fluid actually killed more bacteria than controls (Fig. 1).

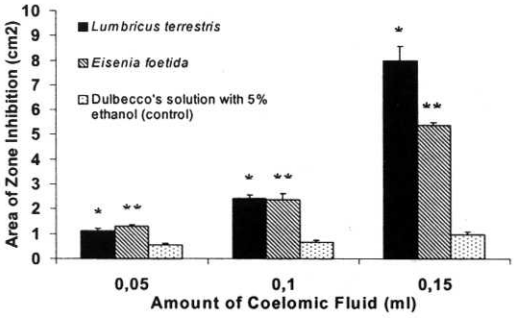

Fig. 2: The effects of *Lumbricus terrestris* and *Eisenia foetida* coelomic fluid on the area of the zone of inhibition. Note that the control had the smallest area of inhibition, whereas the cultures with the coelomic fluids had a much more potent effect on the area of bacteria. Mean ± S.D.; n = 4; Student *t*-test: $p < 0.05$

3. Conclusion

The efficacy of coelomic fluids to inhibit bacterial growth is most likely to derive from the effect of the lytic molecules present in coelomic fluids. The lytic substances, produced by immune cells in coelomic fluids of these two earthworms are synthesized as a reaction to antigen or a life-threatening predicament [1]. These lytic molecules, presumably peptides, kill bacteria by inserting themselves beneath bacteria cell walls, and forming pores [2]. These peptides are likely to be responsible for the large zones of inhibition and for decrease of bacteria in the drop zones.

Further experiments must be implemented in order to determine: 1) exact amount of coelomic fluid released by a specific organism; 2) the amount of bacteria in specific drops. This preliminary study demonstrated, however, significant differences of antimicrobial activities between earthworm coelomic fluids and controls.

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References

[1] K. Isselbacher (Ed.), *Harrison's Principles of Internal Medicine*. New York: McGraw Hill. 1994.
[2] G. McCallister, Quantitative phagocytosis, *The American Biology Teacher* **58** (1996) 6.

[3] A. Cotuk and R.P. Dales, The effect of coelomic fluid of the earthworm *Eisenia foetida* Sav. on certain bacteria and the role of the coelomocytes in internal defense, *J. Exp. Zool.* **232** (1984) 67.

[4] R.S. Anderson, Hemolysins and hemagglutinins in the coelomic fluid of a polychaete annelid, *Glycera dibranchiata*, *Bio. Bull.* **159** (1980) 259.

[5] E.L. Cooper *et al.*, Lack of bactericidal response in the earthworm *Lumbricus terrestris* after immunization with bacterial antigens, *J. Invert. Pathol.* **14** (1969) 402-406.

[6] P. Valembois *et al.*, Antibacterial activity of the hemolytic system from the earthworm *Eisenia fetida andrei*, *J. Invert. Pathol.* **40** (1982) 21-27.

[7] P. Roch *et al.*, Protein analysis of earthworm coelomic fluid. II. Isolation and biochemical characterization of the *Eisenia fetida andrei* factor (EFAF), *Comp. Biochem. Physiol.* **69B** (1981) 829-836.

[8] P. Roch *et al.*, Isolation of agglutinins from lysins in earthworm coelomic fluid by gel filtration followed by chromatofocusing, *J. Chromatogr.* **290** (1984) 213-235.

[9] J. Vaillier *et al.*, Protein analysis of earthworm coelomic fluid. III. Isolation and characterization of several bacteriostatic molecules from *Eisenia foetida andrei*, *Dev. Comp. Immunol.* **9** (1985) 11-20.

[10] P. Roch *et al.*, Antibacterial activity of *Eisenia fetida andrei* coelomic fluid. II. Relationship within the polymorphic hemolysins, *Dev. Comp. Immunol.* **15** (1991) 27-32.

A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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Earthworm humoral immune system: interaction of hemolysins with lipid membranes requires sphingolipids

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Abstract. Earthworms possess a number of membrane active compounds, able to agglutinate or lyse foreign material. Eiseniapore is one molecule of the well-investigated lytic system in *Eisenia fetida*. It is activated by thiol-groups and affected by anions ($SO_4^{2-} > PO_4^{3-}$) and cations ($Au^{3+} > Fe^{3+} > Fe^{2+} > Zn^{2+}$). Ions of higher oxidation number significantly inhibited the lytic action of eiseniapore stronger, which suggests that this effect is due to oxidative damage. Eiseniapore-induced pore formation differentiates two steps: (1) the Ca^{2+}- and temperature-independent binding to target membranes; (2) membrane lysis. Incubation of eiseniapore with erythrocyte membranes or liposomes results in pore-like structures with an outer diameter of 10 nm and an inner diameter of 3 nm as well as the disintegration of these membranes. Eiseniapore-induced pore formation is due to oligomerization of at least six eiseniapore monomers to a macromolecular complex of 268 kDa. The presence of sphingomyelin is an essential prerequisite for an eiseniapore-induced leakage of liposomes. Because of its sphingomyelin specificity, eiseniapore may potentially be applicable as a useful tool in membrane biology, especially in analyzing and modulating membrane events.

1. Introduction to the earthworm immune system

Earthworms are relatively long-lived organisms, which belong together with arthropods, mollusks and echinoderms to the well-investigated species in the field of comparative immunology. Even though they lack strong mechanical barriers like the exoskeleton of arthropods, earthworms successfully survive in a hostile, soil environment, contaminated with bacteria, fungi, protozoa and other parasites. Moreover, they are substrate feeders with a relatively high intestinal uptake of soil living pathogens. However, their effective innate immune mechanisms allow survival. The segmentally divided coelomic cavity, filled with coelomic fluid, a protein-rich physiological medium that contains circulating coelomocytes and chloragocytes, is essential for earthworm immune functions. Coelomocytes participate in numerous cellular immune mechanisms like phagocytosis, granuloma and brown body formation, graft rejection as well as cytotoxicity [1-12].

Many coelomic fluid proteins e.g. agglutinins/lectins, antibacterial molecules, proteases and cytolysins are involved in earthworm innate immunity. These proteins often act as pattern recognition receptors for pathogens and other foreign material, contribute to the elimination of nonself and mediate cellular immunity. Moreover, they can be stimulated by wounding or intracoelomic injection of foreign material [13-16].

2. *Why to study hemolysins in* Eisenia fetida?

The natural lytic system of *Eisenia fetida* characterizes a high titer of activity, which acts upon erythrocytes as well as different cell types, including tumor cells [17, 18]. Since the lytic system is active against erythrocytes, activity can easily be analyzed by using a very simple test model, the hemoglobin release. Moreover *E. fetida* is a low cost and non-controversial animal model with no problems of husbandry. These conditions allow *E. fetida* to be an efficient source of bioactive molecules, most suitable to study effector-target interactions that cause membrane disruption and may have potential importance for biomedical applications. The lytic activity of *E. fetida* has been characterized as polymorphic and multifunctional system, including responses that involve cytolysis, antibacterial action and clotting capacities [19-28]. Therefore molecules involved in the lytic activity are considered as factors of major importance in earthworm immunity. Today, a number of different lytic molecules are described so far in *E. fetida*.

1. *Eisenia fetida andrei* Factor (EFAF) recently characterized as fetidins, are hemolytic and antibacterial glycoproteins (40 and 45 kDa) occurring in chloragocytes and coelomic fluid [21, 22, 26, 29].
2. H_1, H_2 and H_3 are cytolytic coelomic fluid proteins of 46, 43 and 40 kDa [30].
3. Lysenin is a 41-kDa protein, derived from coelomocytes and contracts smooth vascular muscles [31, 32].
4. Lysenin related protein is a 42-kDa coelomic fluid protein with sphingomyelin-binding specificity.
5. Eiseniapore is a pore-forming coelomic fluid protein of 38 kDa with sphingomyelin-binding specificity [33, 34].
6. Coelomic Cytolytic Factor (CCF-1) is a 42-kDa coelomic fluid protein, which lyses tumor cells and trypanosomes, but is not hemolytic [35, 36].

3. *Hemolytic molecules H_1, H_2 and H_3*

In contrast to other authors [20, 26, 37, 38] we did not show only two but three lytic molecules for the coelomic fluid of *E. fetida*, which we called H_1, H_2 and H_3 [30]. We identified these lytic molecules after electrophoretic separation in a native PAGE followed by placing buffer equilibrated sample gel stripes onto an erythrocyte containing agarose gel. After localization, the proteins were cut from the gel, eluted and analyzed. H_1 and H_2 share many characteristics, they are cytotoxic/hemolytic, heat-sensitive, and monomeric molecules of 46 and 43 kDa. H_3 is a dimeric molecule of 40 kDa, which splits under reducing conditions into subunits of 18 and 21 kDa. It is bifunctional because it is lytic and cytotoxic like H_1 and H_2 but it also agglutinates erythrocytes. The activity of all three lytic proteins is independent of divalent cations. Glycoproteins like fetuin, α1-acid glycoprotein and thyroglobulin, but also lipoproteins (HDL) inhibit the lytic action of these molecules. Today, we know that lytic molecules, present in coelomic fluid, originate from chloragocytes as well as large

coelomocytes [39-41]. H_1 and H_2 are secreted into the coelomic fluid. H_3 could neither been shown neither for cell lysate nor in the coelomic fluid 24 hours after challenging the immune system by injecting foreign material. Secretion of H_1 and H_2 into the coelomic fluid is a process that results in glycosylation of these molecules and causes an increase in molecular mass [29].

4. Eiseniapore

The hemolytic molecule, called eiseniapore [33] has been isolated from the coelomic fluid by preparative PAGE. Preparative PAGE allows a pure preparation of proteins that show a high affinity for separation media commonly used in column chromatography that may lead to aggregation and peak overlapping. The estimated molecular mass of eiseniapore is about 38 kDa. Eiseniapore is one of the lytic molecules in *E. fetida*, which has been analyzed revealing biochemical and biophysical characteristics as well as mechanisms of membrane disruption [33].

4.1. Biochemical characteristic of Eiseniapore

Eiseniapore has been characterized as a thiol-activated hemolysin [34]. It can be activated by thiol-groups but in contrast to thiol-activated bacterial lytic toxin, eiseniapore lytic properties are not irreversibly lost in the presence of cholesterol [33]. Eiseniapore activity is temperature-dependent and declines upon prolonged incubation at higher temperatures. Its hemolytic activity is affected by the anions ($SO_4^{2-} > PO_4^{3-}$) and cations ($Au^{3+} > Fe^{3+} > Fe^{2+} > Zn^{2+}$). Metal ions of higher oxidation number inhibited significantly the lytic action of eiseniapore that may indicate oxidative damage of eiseniapore by these chemicals.

The eiseniapore-induced pore formation process can be differentiated into two steps, a Ca^{2+}- and temperature-independent binding step and the membrane lysis step. Target cells (sheep erythrocytes) incubated with eiseniapore at 0 °C showed no lysis, but hemolysis occurred after centrifugation, intensive washing and a temperature increase up to 37 °C for 30 min. Binding of eiseniapore to erythrocytes does not necessarily lead to lysis. It has been shown that copper ions enhance the binding capacity of eiseniapore to erythrocytes, but inhibit its lytic activity. Ca^{2+}, Mg^{2+}, EDTA and EGTA exert no effect on binding and lytic activity of eiseniapore. Vitronectin, heparin and lysophophatidylcholine suppressed eiseniapore binding to membranes, which may be due to masking the binding site [34]. These results suggest that different structures/characteristics of eiseniapore are responsible either for binding or for lysis [34].

4.2. Interaction of eiseniapore with artificial membranes

In order to identify the potential target receptor of eiseniapore on membranes, defined liposomes of various compositions as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, cholesterol, sphingomyelin and galatosylceramide were used in leakage experiments. In order to measure eiseniapore-induced leakage, liposomes were labeled with a fluorescence marker (ANTS/DPX) that is released upon liposome disruption. It has been shown that the presence of sphingomyelin is an essential prerequisite for eiseniapore-induced leakage [33]. In the absence of sphingomyelin, no leakage occurred neither in phosphatidylcholine, phosphatidylethanolamine nor phosphatidylserine liposomes (Fig. 1A). In the presence of sphingomyelin leakage occurred with a maximum at neutral pH and 37 °C [34].

A significant leakage increase of phosphatidylcholine/sphingomyelin (PC/SM) vesicles was evident in the presence of cholesterol, whereas cholesterol alone did not promote the lytic activity of eiseniapore. The exchange of sphingomyelin by galactosylcerebroside did not increase the leakage. Addition of cholesterol to PC/SM vesicles increased the leakage, implicating an interaction of cholesterol with sphingomyelin and suggesting the SM/cholesterol complex formation or microdomains, as currently discussed by some authors [42-44]. Preincubation of eiseniapore with liposomes of different lipid composition without sphingomyelin did not change leakage intensity. In contrast no leakage occurred in PC/Cholesterol/SM liposomes when sphingomyelin was present during preincubation. Binding to lipid membranes without sphingomyelin did not occur. Therefore, it can be excluded that other lipids like cholesterol known as receptors for thiol-activated toxins from bacteria may act as receptor for eiseniapore. These detailed analyses revealed that two characteristics of sphingolipids are required for an eiseniapore-induced membrane leakage. These are, first, a hydrophilic head group as phosphorylcholine or galactosyl, and second, the double chain skeleton of sphingolipids, the ceramide (Fig. 1B).

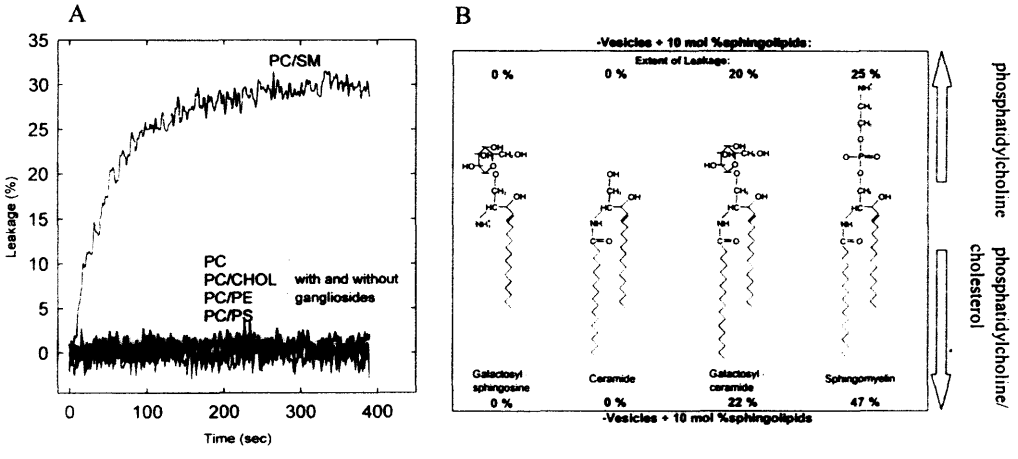

Fig. 1: Eiseniapore induced leakage of small unilamilar vesicles (SUVs) of different lipid composition.

A. Eiseniapore induced leakage of SUVs at pH 7.4 and 25 °C for vesicles of different lipid composition (PC- phosphatidylcholine, CHOL- cholesterol, PE- phosphatidylethanolamine and PS- phosphatidylserine).

B. Eiseniapore induced leakage of liposomes with different sphingolipid composition. Leakage was measured after 5 min incubation with eiseniapore. Values above the component indicate leakage in percent for vesicles without cholesterol; values below the component indicate leakage in percent for vesicles with cholesterol.

Dilution of ANTS/DPX complexes caused by their release from vesicles leads to complex dissociation and thereby to the relief of fluorescence quenching of ANTS. Thus, leakage is accompanied by an increase of ATNS fluorescence intensity. To determine 100% leakage Triton X-100 (0.1% v/v) was added, which led to a maximal dilution and dissociation of the ANTS/DPX complex.

4.3. Pore formation by eiseniapore

Negatively stained TEM images of erythrocytes incubated with the purified hemolysin reveal pore-like structures in the target membrane [33, 40]. The ultrastructural lesions in the target membrane were of an outer diameter of 10 nm. An inner diameter of about 3 nm was concluded from lysis protection by carbohydrates as dextran, inulin and polyethyleneglycol [34]. It has been assumed that the pore-like structure was formed by oligomerization of eiseniapore monomers, because of the similarity to channel formation caused by membrane attack complex of the vertebrate complement or the *Staphylococcus* α-toxin [45-47].

4.4. Pore formation by oligomerization

By TEM analysis of liposomes which were incubated with eiseniapore it could be shown pore-like structures on the surface with an inner diameter of 2-3 nm and an outer diameter of 10 nm (Fig. 2A-D), [33, 34]. Moreover pore formation is due to an oligomerization as concluded from the molecular mass of a pore-forming complex obtained after membrane treatment with TritonX-100. The complex was identified in SDS-PAGE with a molecular mass of about 268 kDa, which seems to consist of at least six eiseniapore monomers (Fig. 2E-G).

The pore structure/complex itself is very stable since it requires 30 min of boiling in the presence of reducing agents to obtain monomers. Its stability in the presence of reducing substances as well as pore size let assume that eiseniapore integrates into the target membrane as a so called "protein walled channel". This is a model favored by Bhakdi and Tranum-Jansen during a debate with Esser about complement pores [48, 49]. The formation of multimeric complexes that are responsible for leakage is also supported by leakage half-time, depending to a first approximation linearly on the inverse eiseniapore concentration. These results suggested that the protein acts at least at some stages as a dimer. The change from a soluble hydrophilic to a membrane-associated lipophilic conformation by dimerization is also known for human defensins, cytolytic toxins of bacteria and enzymes such as HIC-1 protease [50-53].

5. Conclusion: Perspectives on the future of eiseniapore

Many participants of this NATO workshop made major contributions to our current knowledge of earthworm hemolysins, their molecular structure and mechanisms, as recently reviewed [19]. Looking for a potential natural target of eiseniapore, we would assume that bacteria should be excellent candidates. However, as our results demonstrated, eiseniapore requires sphingomyelin to interact with the target membranes but sphingomyelin is not present on the surface of bacteria. Therefore, we would like to propose that eiseniapore might be preferably targeting membranes of eukaryotic organisms including invading parasites of annelids, for example protozoans or even nematodes.

At least two explanations seem plausible with respect to lysis of bacteria. First, eiseniapore may not interact with the surface of bacteria and therefore molecules of the lytic system other than eiseniapore should cause the known antibacterial effects. Second, we cannot exclude that eiseniapore may act as an antibacterial molecule via another receptor. Promising candidates in this sense are carbohydrates since the lytic activity of *Eisenia* hemolysins can be inhibited by α-1-acid glycoprotein, fetuin, blood group substance A and thyroglobulin [15]. More detailed analyses are needed to support this hypothesis. So far, there

is only one other hemolytic lectin (CEL III) acting by hexameric oligomerization and channel formation [54].

The strong binding specificity of eiseniapore to sphingomyelin may indicate that it is a potential marker protein for sphingomyelin detection in cytological, cytochemical and histological analysis. This might potentially be of medical relevance, since under certain pathological conditions, for example the Niemann-Pick syndrome type A and B in humans, sphingomyelin is accumulated in cells and tissues. The sphingomyelin accumulation is due to a genetically determined sphingomyelinase deficiency, which leads to changes in cell function and impairs organs such as liver, spleen, lungs as well as the central nervous system. Moreover eiseniapore might be a useful tool for analyzing and modulating membrane mechanisms, since hydrolysis of sphingomyelin is involved in signal transduction, which leads to apoptosis and other cellular processes.

Oligomerization of homologous molecules is phylogenetically an old mechanism evolved to the sophisticated complement system in vertebrates. Although, cross-reactivity between complement C9 and *Eisenia* hemolysin has been shown [55], no sequence similarities have been demonstrated yet. Gene expression, as revealed by molecular as well as ligand analysis may provide new results regarding molecular structure of channel/pore forming proteins and the phylogenetic establishment of this very efficient immune mechanism.

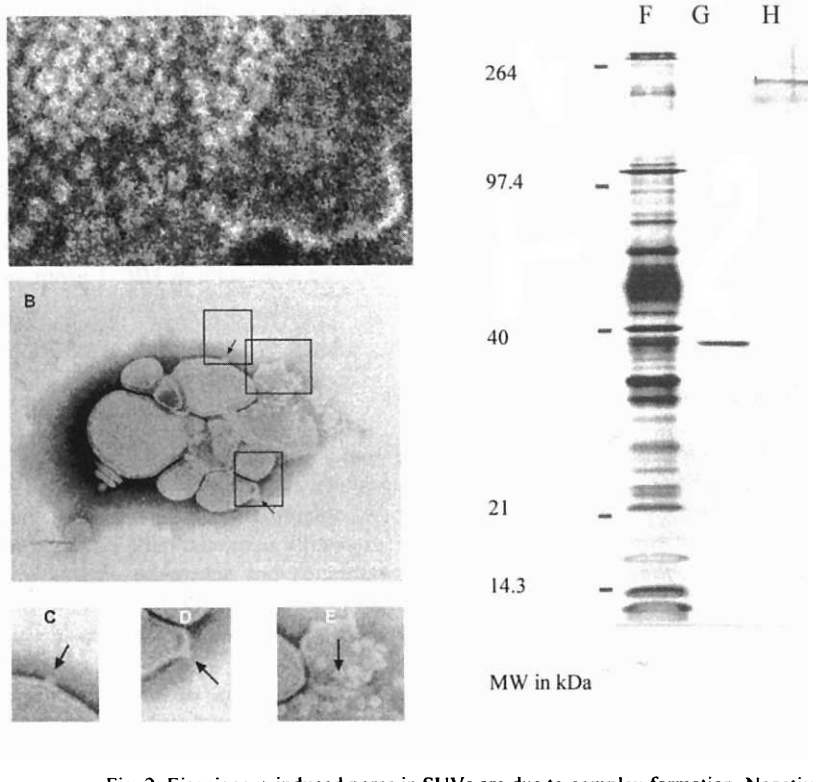

Fig. 2: Eiseniapore-induced pores in SUVs are due to complex formation. Negatively stained TEM images of A: eiseniapore-treated sheep erythrocyte membranes, B-E: egg-PC/Chol/SM liposomes in the presence of eiseniapore; F-H analytical SDS-PAGE (5-10 %) silver stained of F: *E. fetida* coelomic fluid (25 µg), G: eiseniapore (2.1 µg), H: eiseniapore after preincubation in the presence of egg-PC/Chol/SM liposomes (1.4 µg). Originally published in [33, 34].

References

[1] E.A. Stein *et al.*, The coelomocytes of the earthworm *Lumbricus terrestris*: morphology and phagocytic properties, *J. Morphol.* **153** (1977) 415-425.

[2] A. Laulan *et al.*, The phagocytic activity of *Lumbricus terrestris* leukocytes is enhanced by the vertebrate opsonins: IgG and complement C3b fragment, *Dev. Comp. Immunol.* **12** (1988) 269-277.

[3] M. Bilej *et al.*, Phagocytosis of synthetic particles in earthworms: absence of oxidative burst and possible role of lytic enzymes, *Folia Biol.* **36** (1991) 227-231.

[4] M. Bilej *et al.*, Phagocytosis of synthetic particles in earthworms: effect of antigenic stimulation and opsonization, *Folia Biol.* **36** (1991) 273-280.

[5] V. Vetvicka *et al.*, Immunology of Annelids, CRC Press Boca Raton, FL (1994) pp. 300.

[6] E.L. Cooper, Transplantation immunity in annelids. I. Rejection of xenografts exchanged between *Lumbricus terrestris* and *Eisenia foetida*, *Transplantation* **6** (1968) 322-337.

[7] E.L. Cooper, Specific tissue graft rejection in earthworms, *Science* **166** (1969) 1414-1415.

[8] R.K. Hostetter and E.L. Cooper, Coelomocytes as effector cells in earthworm immunity, *Immunol. Commun.* **1** (1972) 155-183.

[9] E.L. Cooper and P. Roch, Second-set allograft responses in the earthworm *Lumbricus terrestris*. Kinetics and characteristics, *Transplantation* **41** (1986) 514-520.

[10] M.M. Suzuki and E.L. Cooper, Spontaneous cytotoxic earthworm leukocytes kill K 562 tumor cells, *Zool. Sci.* **12** (1995) 443-451.

[11] E.L. Cooper *et al.*, Autogeneic but not allogeneic earthworm effector coelomocytes kill the mammalian tumor target K562, *Cell. Immunol.* **166** (1995) 113-122.

[12] D. Quanglino *et al.*, Earthworm coelomocytes *in vitro*: cellular features and "granuloma" formation during cytotoxic activity against the mammalian tumor target K562, *Eur. J. Cell Biol.* **70** (1996) 278-288.

[13] E. Kauschke and W. Mohrig, The occurrence of bacterioagglutinating, hemagglutinating and hemolytic compounds in the coelomic fluid of different species of European earthworms (*Annelida, Lumbricidae*), *Zool. Jb. Physiol.* **91** (1987a) 467-477.

[14] E. Kauschke *et al.*, Characterization of the proteolytic activity in the coelomic fluid of L. *terrestris* L. (*Annelida, Lumbricidae*), *Comp. Biochem. Physiol.* **116** (1997) 235-242.

[15] W. Mohrig *et al.*, Cross reactivity of hemolytic and hemagglutinating proteins in the coelomic fluid of earthworms (*Annelida*), *Comp. Biochem. Physiol.* **115** (1996) 19-30.

[16] E.L. Cooper *et al.*, Recognition receptors in annelids. In E. Cohen (Ed.) Recognition proteins, receptors and probes: Invertebrates, A.R. Liss, Inc. New York, 1984, pp.43-55.

[17] A. Cossarizza *et al.*, Earthworm leukocytes that are not phagocytic and cross-react with several human epitops can kill tumor cell lines, *Exp. Cell Res.* **224** (1996) 174- 182.

[18] E. Kauschke and W. Mohrig, Cytotoxic activity in the coelomic fluid of the annelid *Eisenia foetida* Sav, *J. Comp. Physiol.* **157** (1987b) 77-83.

[19] M. Bilej *et al.*, Antimicrobial defense of the earthworm, *Folia Microbiol.* **45** (2000) 283-300.

[20] L. Du Pasquier and P. Duprat, Aspects humoraux et cellulaires d'une immunité naturelle non spécifique chez l'oligochète *Eisenia fetida, C.R. Acad. Sci. Paris Serie D* **266** (1969) 538-54.

[21] P. Roch, Protein analysis of the earthworm coelomic fluid: I. polymorphic system of the natural hemolysin of *Eisenia fetida andrei, Dev. Comp. Immunol.* **3** (1979) 599-608.

[22] P. Roch *et al.*, Genetic and biochemical polymorphism of earthworm humoral defenses, *Dev. Comp. Immunol.* **11** (1987) 91-102.

[23] P. Roch *et al.*, Antibacterial activity of *Eisenia foetida andrei* coelomic fluid: III Relationship within the polymorphic haemolysins, *Dev. Comp. Immunol.* **15** (1991) 27-32.

[24] E. Kauschke and W. Mohrig, Comparative analysis of hemolytic proteins of the coelomic fluid of *Eisenia foetida* and *Lumbricus terrestris* (*Annelida, Lumbricidae*), *Dev. Comp. Immunol.* **11** (1987) 331-341.

[25] P. Roch *et al.*, Interactions between earthworm hemolysins and sheep red blood cell membranes, *Biochim. Biophys. Acta* **983** (1989) 193-198.

[26] A. Milochau *et al.*, Purification, characterization and activities of two hemolytic and antibacterial proteins from coelomic fluid of the annelid *Eisenia fedida andrei, Biochim. Biophys. Acta* **1337** (1997) 123-132.

[27] L. Tuckova *et al.*, Lytic activities in the coelomic fluid of *Eisenia foetida* and *Lumbricus terrestris, Dev. Comp. Immunol.* **10** (1986) 181-189.

[28] P. Valembois *et al.*, Antibacterial activity of the hemolytic system from the earthworm *Eisenia fetida andrei, J. Invert. Pathol.* **40** (1982a) 21-27.

[29] M. Lassegues *et al.*, Sequence and expression of an *Eisenia-fetida*-derived cDNA clone that encodes the 40kDa fetidin antibacterial protein, *Eur. J. Biochem.* **246** (1997) 756-762.

[30] I. Eue *et al.*, Isolation and characterization of earthworm hemolysins and agglutinins, *Dev. Comp. Immunol.* **22** (1998) 13-26.

[31] Y. Sekizawa *et al.*, Molecular cloning of cDNA for lysenin, a novel protein in the earthworm, *Eisenia foetida* that causes contraction of rat vascular smooth muscle, *Gene* **191** (1997) 97-102.
[32] A. Yamaji *et al.*, Lysenin a novel sphingomyelin-specific binding protein, *J. Biol. Chem.* **273** (1998) 5300-5306.
[33] S. Lange *et al.*, Interaction of earthworm hemolysin with lipid membranes requires sphingolipids, *J. Biol. Chem.* **272** (1997) 20884-20892.
[34] S. Lange *et al.*, Biochemical characteristics of eiseniapore, a pore-forming protein in the coelomic fluid of earthworms, *Eur. J. Biochem.* **262** (1999) 1-11.
[35] M. Bilej *et al.*, Identification of a cytolytic protein in the coelomic fluid of *Eisenia foetida* earthworms, *Immunol. Lett.* **45** (1995) 123-128.
[36] A. Beschin. *et al.*, Convergent evolution of cytokines, *Nature* **400** (1999) 627-628.
[37] P. Roch *et al.*, Protein analysis of the coelomic fluid. II: Isolation and biochemical characterization of the *Eisenia fetida andrei* Factor (EFAF), *Comp. Biochem. Physiol.* **69** (1981) 829-836.
[38] P. Roch *et al.*, Isolation of agglutinins from lysins in the earthworm coelomic fluid by gel filtration followed by chromatofocusing, *J. Chromat.* **290** (1984) 231-235.
[39] E. Kauschke *et al.*, Evidence for perforin-like activity associated with earthworm leukocytes, *Zoology* **104** (2001) 13-24.
[40] P. Valembois *et al.*, Scanning electron-microscopy study of the involvement of coelomic cells in earthworm antibacterial defense, *Cell Tissue Res.* **240** (1985) 479-484.
[41] P. Valembois *et al.*, Bacteriostatic activity of chloragogen cell secretion, *Pedobiologia* **24** (1982) 191-195.
[42] L. Groenberg *et al.*, Interaction of cholesterol with synthetic sphingomyelin derivates in mixed monolayers. *Biochem.* **30** (1991) 10746-10754.
[43] J.P. Slotte, Enzyme-catalyzed oxidation of cholesterol in mixed phospholipid monolayers reveals the stoichiometry at which free cholesterol cluster disappears, *Biochemistry* **31** (1992) 5472-5477.
[44] K. Simons and E. Ikonen, Functional rafts in cell membranes, *Nature* **387** (1997) 569-572.
[45] M.C. Peitsch and J. Tschopp, Assembly of macromolecular pores by immune defense systems, *Curr. Opin. Cell Biol.* **3** (1991) 710-716.
[46] J.P. Arbuthnott *et al.*, Lipid-induced polymerization of staphylococcal α-toxin, *J. Gen. Microbiol.* **75** (1973) 309-319.
[47] S. Bhakdi and J. Tranum-Jensen, Alpha-toxin of *Staphylococcus aureus*, *Microbiol. Rev.* **55** (1991) 733-751.
[48] S. Bhakdi and J. Tranum-Jensen, Complement lysis: a hole is a hole, *Immunol. Today* **12** (1991) 318-320.
[49] A.F. Esser, Big MAC attack: complement proteins cause leaky patches, *Immunol. Today* **12** (1991) 316-318.
[50] K. Hristova *et al.*, Interactions of monomeric rabbit neutrophil defensin with bilayers: comparison with dimeric human defensin HNP-2, *Biochemistry* **35** (1996) 11888-11894.
[51] F.G. Van Der Goot *et al.*, Dimerization stabilizes the pore-forming toxin aerolysin in solution, *J. Biol. Chem.* **268** (1993) 18272-18279.
[52] H.D. Robertson and M.B. Mathews, The regulation of protein kinase PKR by RNA, *Biochemie* **78** (1996) 909-914.
[53] S.K. Grant *et al.*, Use of protein unfolding studies to determine the conformational and dimeric stabilities of HIV-1 and SIV proteases, *Biochemistry* **31** (1992) 9491-9501.
[54] T. Hatakeyama *et al.*, Interaction of the hemolytic lectin CEL-III from the marine invertebrate *Cucumaria echinata* with the erythrocyte membrane, *J. Biol. Chem.* **270** (1995) 3560-3564.
[55] S. Lange, Eiseniapore - ein dem komplementprotein C9 analoges porenformendes hämolysin aus Anneliden: Charakterisierung der membranwechselwirkung und identifizierung des molekularen targets, Dissertation an der Math.-Nat.-Fakultät der HU Berlin, 1996, pp. 161.

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Evidence for a perforin-like cell-mediated cytolysis by earthworm coelomocytes

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Abstract. Small earthworm leukocytes express lytic activity against K562 target cells, as identified by standard 4-h ^{51}Cr release assay. Lytic capacity appears associated with a molecule that resembles human and mouse perforin (70 kDa). The presence of putative perforin in earthworm suggests several mechanisms: a) a conserved immune defense mechanisms across phylogenetic lines; b) rapid evolution of the earthworm immune system; c) functional analogy of lytic mechanism during evolution. Earthworm perforin may belong to a family of lytic molecules with unique immunodefense properties.

1. *Introduction*

Perforin, a pore-forming protein, has been isolated from human, mouse, and rat cytolytic cells [1-5]. This is not surprising since perforin is believed to have evolved to meet the complex immune requirements of higher animals. However, with respect to ancient primitive species, our results confirm previous works on earthworm cytotoxic responses. Cossarizza *et al.* [6] verified that earthworm leukocytes (coelomocytes) effected cytotoxicity against the human tumor cell line K562. There are two effector cells: 1) small (8-11 μm) electron dense cells (SC) stained by monoclonal antibodies against human cell adhesion molecules (CD11a, CD45RA, CD45RO, CDw49b, CD54) and against β_2-microglobulin and Thy-1 (CD90); 2) large (12-15 μm) electronlucent cells (LC) negative for these markers. By serologic methods, β_2-microglobulin [7] and Thy-1 (CD90) [8] had been previously demonstrated. Both cell types were negative for other CD and MHC class I and class II markers.

Electron microscopy of earthworm effector-target cell interaction suggests perforin-mediated activity [9-14]. Kauschke *et al.* [15] confirmed the expression of a lytic factor by immunocytochemistry using light and transmission electron microscopy. Earthworm leukocytes were labeled (nearly 20 %) by a rat anti-mouse-perforin monoclonal antibody. Transmission electron microscopy using immunogold showed homogeneous antibody binding in the cytoplasm. The highest number of immunogold particles was evidenced in coelomocytes possessing cytoplasmic granules. No antibody binding was observed in chloragocytes, another coelomocyte type, either by light or electron microscopy, suggesting no role for these cells concerning perforin production.

By MALDI analysis we also found evidence for calreticulin that is known to associate with perforin in mammalian NK cells and CTL [15]. Taken together, we suggest that lytic activity is associated with the presence of putative perforin in earthworms. This in turn supports the

hypothesis that perforin is a conserved component of immune defense mechanisms across phylogenetic lines.

2. Evidence for perforin-like activity in earthworm

2.1. Earthworm leukocytes (coelomocytes)

Coelomocytes, suspended in coelomic fluid, are a heterogeneous population (basophils, acidophils, and neutrophils), that differs in size, cytomorphological and cytochemical properties. Immediately after harvesting, two coelomocyte types can be distinguished. There are small dark basophilic cells 8-11 μm and large light neutrophilic cells 12-15 μm. (Fig. 1A, C). FACS analysis confirms two major cell types (Fig. 1B). Chloragocytes another coelomocyte type is granular, often oblong and ranges in size from 10-25 μm wide by 30-60 μm long. They are frequently found in coelomic fluid in partial disintegration.

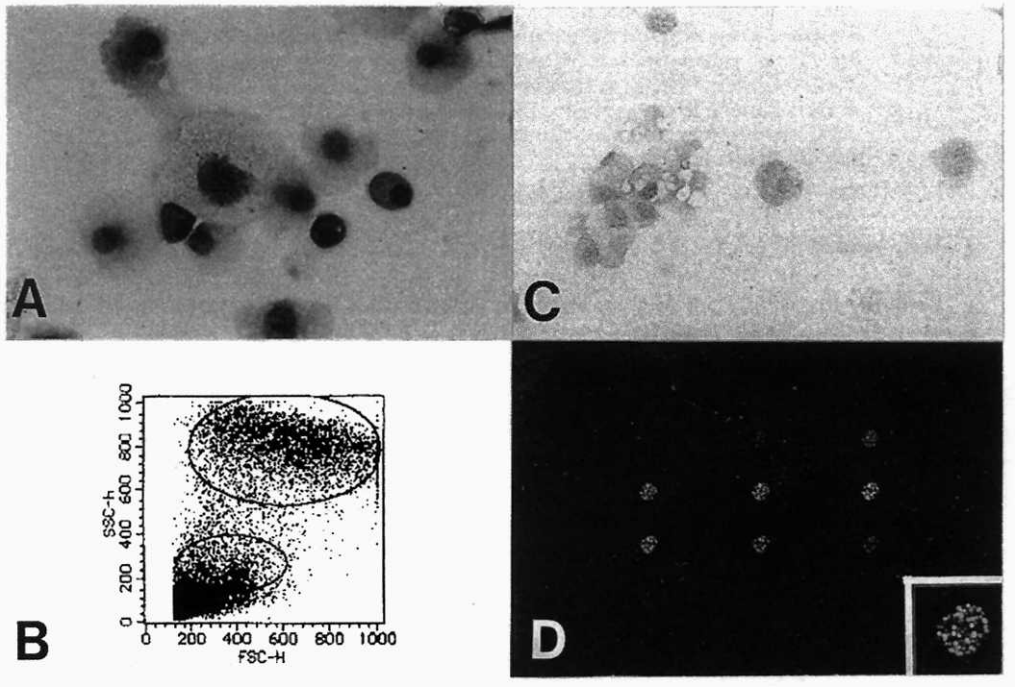

Fig. 1: Coelomocytes (leukocytes). Coelomocyte types are (i) small, dark cells 8-11 μm and (ii) large, light cells 12-15 μm. Small cells have numerous cytoplasmic protrusions and may also possess vacuoles. Chloragocytes show numerous vacuoles (A). Note the distribution of small and large cells as revealed by FACS (B). In immunocytochemistry, the immune reaction was detected by avidin-biotin complex method and hematoxylin for counterstaining the nucleus. Large coelomocytes contain few granules. About 20-25 % of pooled coelomocytes was labeled with antibodies as small basophil, perforin-positive cells (C). Immunostaining was positive in cytoplasm, negative in the nucleus. Chloragocytes were perforin-negative. Presence of perforin-positive granules in coelomocytes shown by confocal laser microscopy (D – window). Fine granules positive for perforin antibody show uniform cytoplasmic spreading. After cutting sections at 0.5 μm intervals by laser, perforin positive granules of varying density were identified in each sections (D).

In immunocytochemistry, 10-20 % of pooled coelomocytes were labeled with anti-perforin antibodies (Fig. 1C). Perforin-containing coelomocytes are relatively small, basophilic, immunostained exclusively in cytoplasm but not in nuclei. By contrast, all chloragocytes were negative. We confirmed the presence of perforin-positive granules in coelomocytes by confocal laser microscopy (Fig. 1D). After cutting sections, fine perforin positive granules of varying intensity were spread evenly in the cytoplasm.

2.2. *FACS analysis of coelomocytes revealed positive responses to mouse lymphocyte markers*

By FACS analysis, coelomocytes were divided into two major populations: large and small (Fig. 1B). We found that coelomocytes were positively stained with anti-CD90, CD5, CD8, CD45RA, CD45RO and anti-perforin antibody, while they were negative for Ia, CD4 and CD11c. In general, only small, and to a lesser extent, large coelomocytes reacted with these antibodies (Fig. 2a and Table 1). FACS analysis showed similar values for CD90- and CD5-positive cells (approximately 20 %). The percentage of CD8- and perforin-positive cells was significantly lower (approximately 10 % of total coelomocyte number). CD90-positive cells were detected in the small cell population (45 %), while the large cells were mostly negative. For perforin, about 20 % of small cells were positive whereas the large cells were negative, (Fig. 2b).

Table 1: Summary of immunocytochemical analysis of coelomocytes

Positive	Negative
CD90 (Thy-1) [8]	HLA-DQ [8], HLA-DR
CD5 (Lyt-1), CD8 (Lyt-2)	Ia
CD45RA, CD45RO [6]	CD4 (L3T4)
CD11a [6], CD54 [6]	CD11c
β_2-microglobulin [7]	
Perforin	

2.3. *Western blotting*

Coelomocyte lysates were used to determine the molecular mass of earthworm perforin-like molecule. Samples were run on the SuperDex 200 column that yielded 19 fractions (Fig. 3A), which were then analyzed in SDS-PAGE (Fig. 3B). They were then transferred to nitrocellulose membranes and probed with anti-perforin antibody. Three protein bands with a molecular mass of about 70 kDa were identified (Fig. 3C).

2.4. *Evidence for the role of calcium in binding of coelomocyte effector against K562 and cytotoxicity*

We used the tumor cell target K562 to assay killing capacity of coelomocytes. We found that coelomocytes affected cytotoxic activity against K562 cells significantly higher than IL-2-stimulated human peripheral blood lymphocytes even at low effector-target ratios (Fig. 4a). The released soluble proteins were polymerized forming pores in target cell membranes. To assay for the role of Ca^{2+}, we added the calcium ionophore to the NK assay. Human peripheral blood lymphocytes showed reduced cytotoxic activity in the presence of

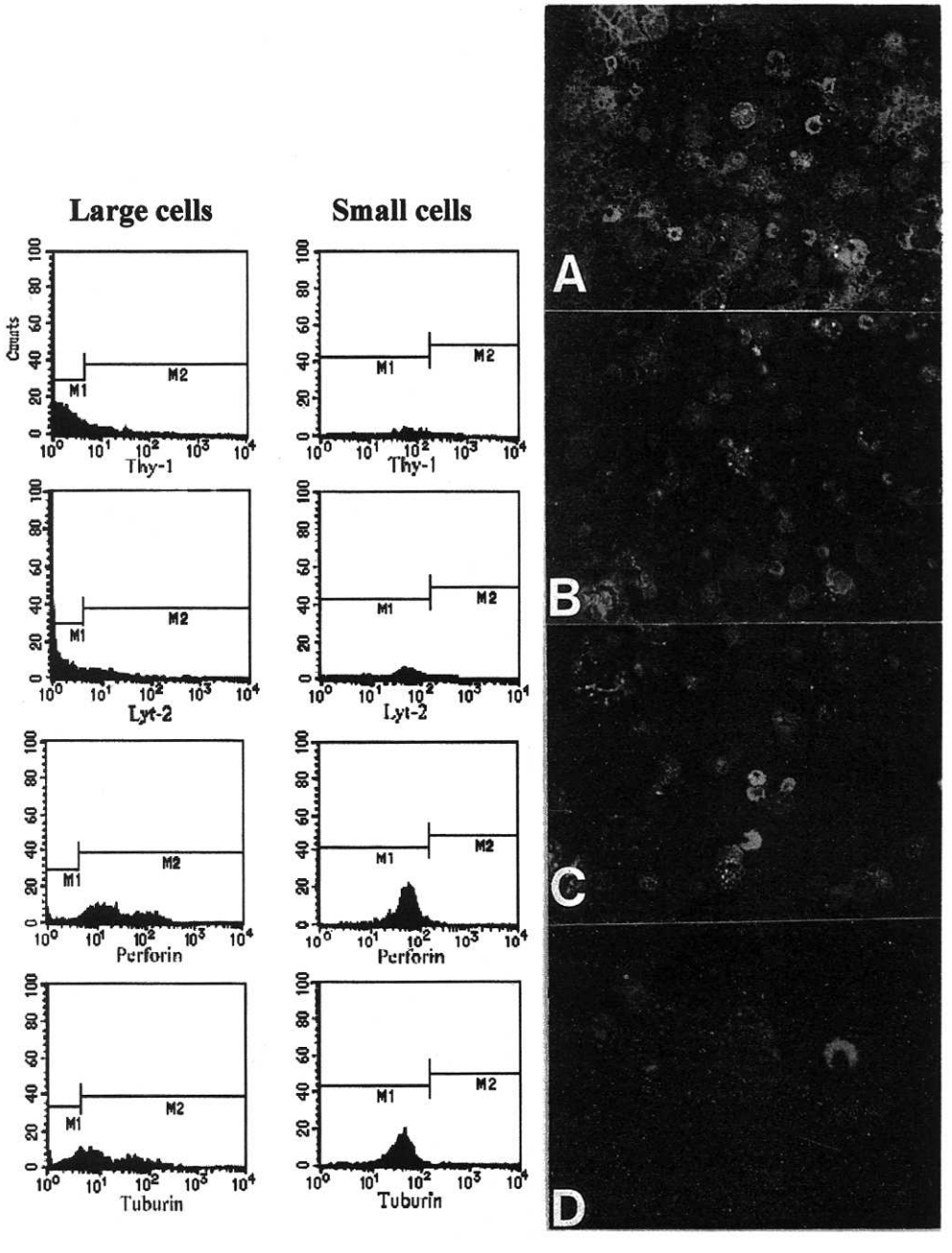

Fig. 2: Immunohistochemistry and FACS analysis. (a) Coelomocytes stained with CD90 (Thy-1), CD5 (Lyt-1), CD8 (Lyt-2) and CD4 (L3T4), rat anti-mouse monoclonal antibody (Table 1 for comparison). Only small coelomocytes reacted with all antibodies except for CD4. CD90, CD5 (A, C) showed similar values of approximately 20 %. Note no staining of cells for CD4 (B) and cytoplasmic granules positive for perforin (D). (b) By FACS analysis, coelomocytes were divided into two major populations: large and small. Only small leukocytes reacted with anti-CD90 (45 %) and anti-CD5 whereas large cells were negative. For perforin, small cells (~20 %) were positive whereas large cells were negative.

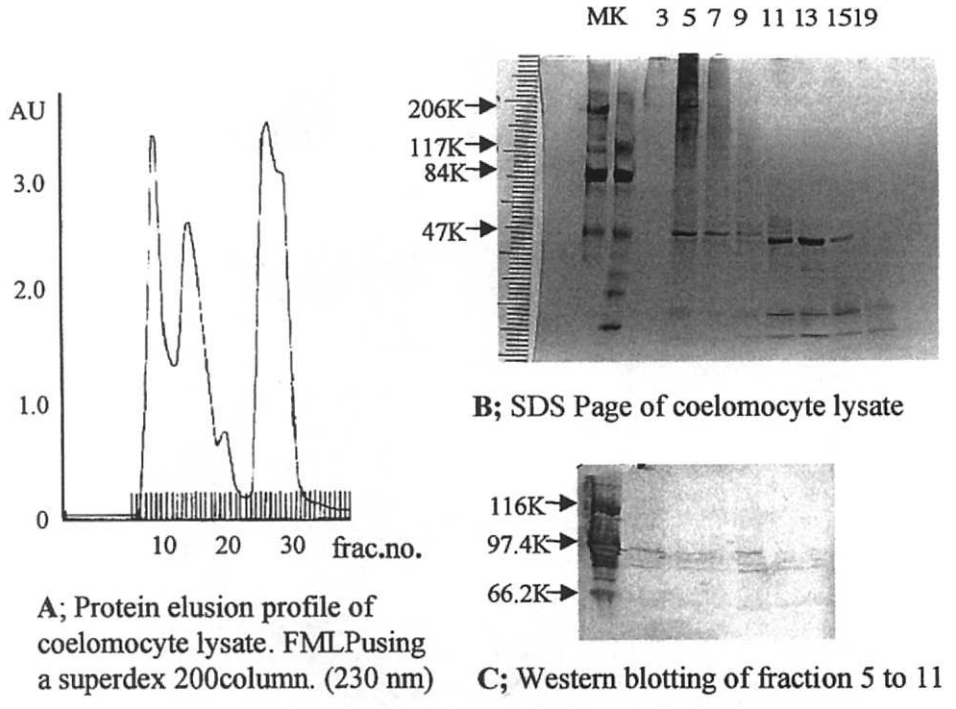

Fig. 3: Protein purification, SDS-PAGE and immunoblotting. (A) Protein elution profiles of coelomocyte lysates run on a SuperDex 200 column; (B) SDS-PAGE of a number of coelomocyte lysate fractions; (C) By western blot three protein bands were identified with the anti-perforin antibody. Molecular mass of these proteins was determined as of about 68, 70 and 72 kDa.

ionomycin. However, cytotoxic activity of coelomocytes was less affected by the presence of ionomycin as compared with human peripheral blood lymphocytes (Fig. 4a). By means of transmission (TEM) and scanning (SEM) electron microscopy, we found interesting arrangement of coelomocyte against the targets. There were intimate associations between the membranes of coelomocyte against K562 tumor cell (Fig. 4b).

3. Perspectives of cell killing by earthworm immune components

We initiated this study after observing that earthworm leukocytes (coelomocytes) make intimate contact with K562 targets [9-14] as has been found when mammalian CTL and NK cells are cocultured with targets. Although the cytotoxic action of leukocytes has been shown for several invertebrates, there is no information concerning the mechanism of cytolysis [12, 13]. In this paper, we showed for the first time that small leukocytes known for their cytolytic activity, expressed a lytic component that is crossreactive with mouse anti-perforin antibody.

The data suggests a perforin-like molecule in earthworm leukocytes, supporting the hypothesis that perforin appeared early in evolution. In agreement with shared molecular

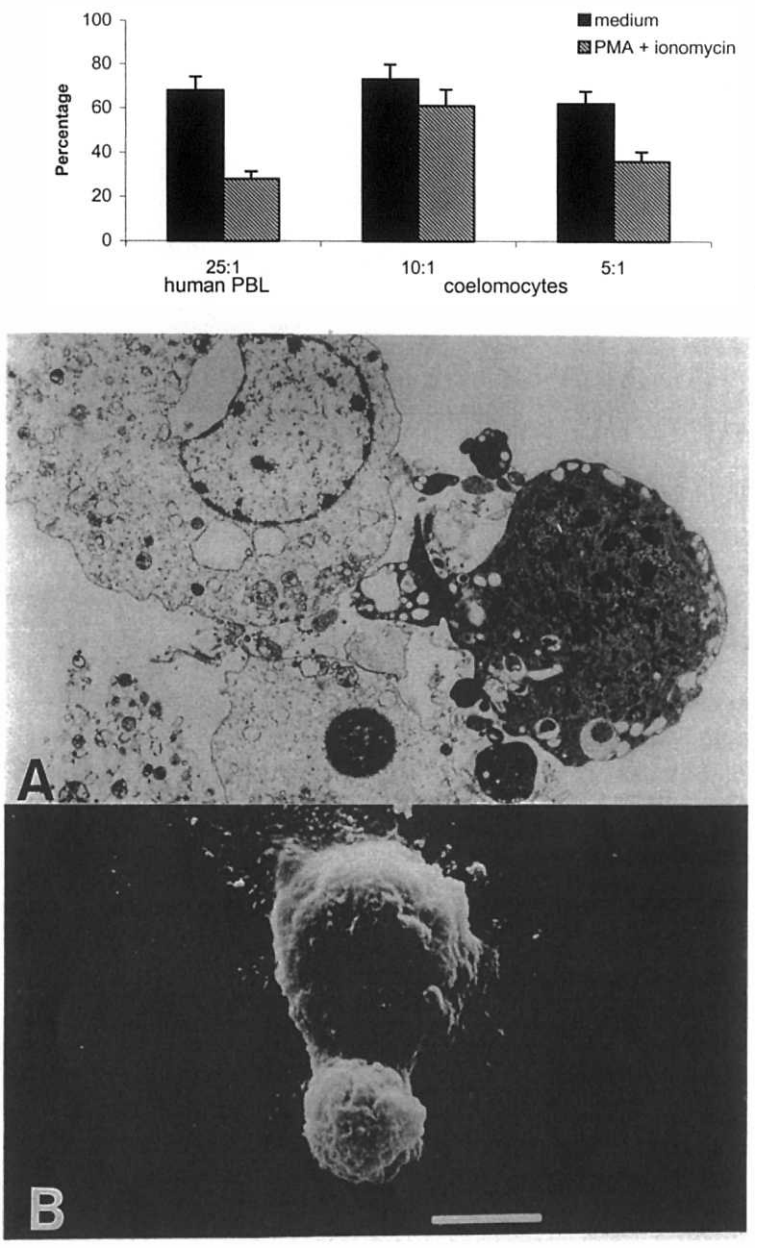

Fig. 4: Evidence for the role of calcium in binding of coelomocyte effectors against K562 and cytotoxicity. (a) Assay for the role of calcium by addition of calcium ionophore, ionomycin to the NK cell assay. Controls of human peripheral blood lymphocytes stimulated with IL-2 showed normal cytotoxicity responses (E/T ratio at 25:1). Coelomocytes showed higher cytotoxic capacity for killing than human peripheral blood lymphocytes (E/T ratio at 10:1 and 5:1). Control human peripheral blood lymphocytes showed reduced activity whereas coelomocytes affected significantly less killing in the presence of ionomycin. (b) TEM of direct tight contact of coelomocyte effectors (small dark cells) against K562 targets (A). SEM revealed similar tight and intimate association between the membranes of effectors against targets (B).

sequences across phylogenetic barriers and divergent species, there is evidence for sequence homology of a plant toxin, purothionin that supports this hypothesis. There is significant homology of amino acid sequence between purothionin and mammalian pore-forming proteins, such as complement components or perforin [16]. This similarity is apparently due to an EGF-like cysteine-rich repeated motif. Studies now in progress have been initiated (1) to extend biochemical evidence confirming the specificity of anti-perforin antibodies, (2) to use the perforin-specific antibody to purify the putative earthworm perforin homologue and to sequence it, and (3) to isolate of a complete cDNA for the putative earthworm perforin.

Our preliminary data revealed the following information. Three protein bands with a molecular mass of about 68, 70 and 72 kDa crossreacting with anti-perforin antibodies were identified in western blot. Future deglycosylation experiments may also reveal that the three bands are due to different N-glycosylation sites of one protein that possesses a molecular mass closer to 65 or 70 kDa, the mass assigned to vertebrate perforin [2, 17, 18]. Furthermore, we detected a perforin mRNA expression in earthworm coelomocytes and human CTL by RT-PCR and Southern blot analysis. The size of PCR product in coelomocytes was similar to that of CTL [Komiyama *et al.*, unpublished].

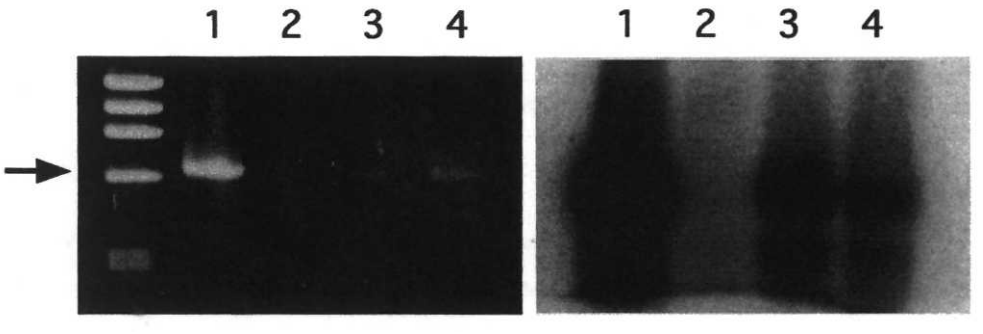

Fig. 5: RT-PCR. Detection of perforin mRNA expression in earthworm coelomocytes and human CTL by RT-PCR and Southern blot analysis. The size of PCR product in coelomocytes (552 bp, arrow) is similar to that of CTL. Negative controls, HT-29 cells show no amplified product.

Chloragocytes (another coelomocyte types) were not labeled with the anti-perforin monoclonal antibody. This was not surprising since chloragocytes have never been involved in either tumor target attachment or granuloma formation, but they have been assumed to be the source of other lytic components. The perforin-like component, evident in small effector coelomocytes, is thus not identical with the hemolysins/cytolysins in the coelomic fluid of *Eisenia fetida* (assumed to be chloragogen-derived) [13].

The cytotoxic function of coelomocyte lysates against the tumor K562 target was demonstrated earlier using the MTT assay by Kauschke *et al.* [15] and confirmed in this investigation using the ^{51}Cr release assay. A cell lysate diluted 1:8 significantly decreased the viability of K562 cells about 50 %. Thus cytosol proteins are apparently involved in coelomocyte-mediated cytotoxicity against K562 cells.

4. Relationship of perforin-like activity to other lytic molecules

The expression of a lytic component behaving like perforin expands the existing repertoire of the earthworm's immune system and suggests relatively advanced and probable functional analogy to the immune system of higher animals. Concerning leukocytes and lytic molecules that they synthesize and secrete, other molecules may mediate earthworm effector activity against experimental targets such as cancer cells [6, 9, 11, 12, 19, 20]. Coelomic fluid causes lysis by means of a protein that has been referred to as eiseniapore [21, 22]. Other investigators have isolated and characterized similar molecular products involved in lysis [23-26]. Some of these lytic molecules might reveal regulation by serine proteases [27-29], possible relationships to other humoral mediators, *e.g.* agglutinins (H_1, H_2, H_3) [30] and to antimicrobial peptides that are not hemolytic [31].

These research groups propose several names for earthworm lytic molecules: fetidin, lysenin, eiseniapore, and now perhaps perforin. Cloning cDNA that encodes some cytolytic proteins has revealed shared characteristics. One component, the 40 kDa fetidin, has at least four isoforms with different isoelectric points whereas the other, lysenin, represents two isoforms of molecular masses of 41 and 42 kDa. Fetidins and lysenin, as well as eiseniapore, 38-kDa protein, effects lysis in relation to sphingomyelin. Thus coelomic fluid of *E. fetida* contains several sphingomyelin-binding cytolytic proteins with molecular masses around 40 kDa. Viewed together, there is the need for reaching a consensus among these several laboratories for further characterization of earthworm lytic molecules using identical methods. Expected results should clarify differences that could help to codify lytic components of earthworms and to distinguish them functionally from humoral agglutinins.

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References

[1] L.S. Zalman *et al.*, Mechanism of cytotoxicity of human large granular lymphocytes: relationship of the cytotoxic lymphocyte protein to the ninth component (C9) of human complement, *Proc. Natl. Acad. Sci.* USA **83** (1986) 5262-5266.

[2] D. Masson and J. Tschopp, Isolation of a lytic, pore-forming protein (perforin) from cytolytic T-lymphocytes, *J. Biol. Chem.* **260** (1985) 9069-9072.

[3] G. Konjevic *et al.*, Analysis of perforin expression in human peripheral blood lymphocytes, CD56+ natural killer cell subsets and its induction by interleukin-2, *Immunol. Invest.* **24** (1995) 499-507.

[4] P.J. Millard *et al.*, Purification and properties of cytoplasmic granules from cytotoxic rat tumors, *J. Immunol.* **132** (1984) 3197-3204.

[5] G. Berke, Unlocking the secrets of CTL and NK cells, *Immunol. Today.* **16** (1995) 343-346.

[6] A. Cossarizza *et al.*, Earthworm leukocytes that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines, *Exp. Cell Res.* **224** (1996) 174-182.

[7] P. Roch *et al.*, Serological evidences for a membrane structure related to human β_2-microglobulin expressed by certain earthworm leukocytes, *Eur. J. Immunol.* **13** (1983) 1037-1042.

[8] A.H. Saad and E.L. Cooper, Evidence for a Thy-1-like molecule expressed on earthworm leukocytes, *Zool. Sci.* **7** (1990) 217-222.

[9] D. Quaglino *et al.*, Earthworm coelomocytes *in vitro*: cellular features and "granuloma" formation during cytotoxic activity against the mammalian tumor cell target K562, *Eur. J. Cell Biol.* **70** (1996) 278-288.

[10] E.L. Cooper, Earthworm immunity, In B. Rinkevich and W.E.G. Müller (Eds.), Invertebrate Immunology, Springer Verlag, Heidelberg, 1996, pp. 10-45.

[11] E.L. Cooper *et al.*, Autogeneic but not allogeneic earthworm effector coelomocytes kill the mammalian tumor target K562, *Cell. Immunol.* **166** (1995) 113-122.
[12] E.L. Cooper *et al.*, Cytotoxic reactions in invertebrates: The earthworm model. In K. Söderhäll *et al.* (Eds.), New Directions in Invertebrate Immunology, SOS Publications, Fair Haven, NJ, 1996, pp. 23-42.
[13] E.L. Cooper *et al.*, Cell adhesion and the immune system: a case study using earthworms, *Micr. Res. Tech.* **44** (1999) 237-253.
[14] K. Komiyama *et al.*, Identification of perforin gene and its protein in the earthworm coelomocytes, *Dev. Comp. Immunol.* **21** (1997) 115.
[15] E. Kauschke *et al.*, Evidence for perforin-like activity associated with earthworm leukocytes, *Zoology* **104** (2001) 13-24.
[16] T. Oka *et al.*, Similarity, in molecular structure and function, between the plant toxin purothionin and the mammalian pore-forming protein, *Mol. Biol. Evol.* **9** (1992) 707-715.
[17] J.D. Young *et al.*, The ninth component of complement and the pore-forming protein (perforin 1) from cytotoxic T cells: structural, immunological, and functional similarities, *Science* **233** (1986) 184-190.
[18] N. Rochel and J. Cowan, Negative cooperativity exhibited by the lytic amino-terminal domain of human perforin: implications for perforin-mediated cell lysis, *Chem. Biol.* **3** (1996) 31-36.
[19] M. Bilej *et al.*, Identification of a cytolytic protein in the coelomic fluid of *Eisenia foetida* earthworms. *Immunol. Lett.* **45** (1995) 123-128.
[20] E. Kauschke *et al.*, Characterization of proteolytic activity in coelomic fluid of *Lumbricus terrestris, Comp. Biochem. Physiol.* **116B** (1997) 235-242.
[21] S. Lange *et al.*, Interactions of earthworm hemolysin with lipid membranes requires sphingolipids, *J. Biol. Chem.* **272** (1997) 20884-20892.
[22] S. Lange *et al.*, Biochemical characteristics of eiseniapore, a pore forming protein in the coelomic fluid of earthworms, *Eur. J. Biochem.* **262** (1999) 547-556.
[23] M. Lassègues *et al.*, Sequence and expression of an *Eisenia fetida* derived cDNA clone that encodes the 40 kDa fetidin antibacterial protein, *Eur. J. Biochem.* **246** (1997) 756-762.
[24] Milochau *et al.*, Purification, characterization and activities of two hemolytic and antibacterial proteins from coelomic fluid of the annelid *Eisenia foetida andrei, Biochim. Biophys. Acta* **1337** (1997) 123-132.
[25] Y. Sekizawa *et al.*, Molecular cloning of cDNA for lysenin, a novel protein in the earthworm *Eisenia foetida* that causes contraction of rat vascular smooth muscle, *Gene* **191** (1997) 97-102.
[26] A. Yamaji *et al.*, Lysenin, a novel sphingomyelin-specific binding protein, *J. Biol. Chem.* **273** (1998) 5300-5306.
[27] M. Leipner *et al.*, Serine proteases in coelomic fluid of annelids *Eisenia foetida* and *Lumbricus terrestris, Comp. Biochem. Physiol.* **105B** (1993) 637-641.
[28] P. Roch *et al.*, Purification of three serine proteases from the coelomic cells of earthworms (*Eisenia foetida*), *Comp. Biochim. Physiol.* **98B** (1991) 597-602.
[29] P. Roch *et al.*, Characterization of a 14 kDa plant-related serine protease inhibitor and regulation of cytotoxic activity in earthworm coelomic fluid, *Dev. Comp. Immunol.* **22** (1998) 1-12.
[30] I. Eue *et al.*, Isolation and characterization of earthworm hemolysins and agglutinins, *Dev. Comp. Immunol.* **22** (1998) 13-25.
[31] J.H. Cho *et al.*, Lumbricin I, a novel proline-rich antimicrobial peptide from the earthworm: purification, cDNA cloning and molecular characterization, *Biochim Biophys Acta* **1408** (1998) 67-76.

A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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The evolution of cell killing: when a target cell became "invited" to choose how to die

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Abstract. During evolution, in the world of cytotoxic reactions a new mechanism has emerged that was based upon the direct "responsibility" of targets in their death. In other words, effector cells thought targets to use their own genetic material to trigger their suicide. To investigate this hypothesis, we have used an experimental model represented by *Eisenia foetida* coelomocytes that kill human cells, and asked whether coelomocytes can trigger apoptosis, or are able to provoke their death *via* necrosis, or even both. Using a strategy based upon a technique we have developed, that evaluates the expression of different forms of Fas (CD95/APO-1, *i.e.* the pro-apoptotic membrane form and the anti-apoptotic soluble form) mRNA in target cells, we tested the capacity of either coelomocytes from *E. foetida* or supernatant from *E. foetida* coelomocyte cultures to kill human cell lines of different origin. When target cells were incubated with coelomocytes, an upregulation of the membrane form of Fas occurred, along with an increase of the total form. On the contrary, cells treated with supernatant (containing cytotoxic molecules) had a significant reduction of the production of both forms of Fas mRNA, suggesting that mechanisms devoted to triggering of apoptosis were downregulated. Our data suggest that the production of soluble mediators, that could be considered the ancestors of humoral immunity, causes death of the foreign cell because of an aspecific activity of lytic molecules, that likely bind target membrane for physicochemical reasons, and determine necrosis. The development of cellular mechanisms to kill targets, *i.e.* likely representing the onset of cellular immunity, could act by modulating the expression of genes involved in apoptosis, determining an increase of total Fas expression.

Metazoans have to protect themselves from invasion of microorganisms, parasites, viruses and even from cells of individuals from the same species. In all phyla, different strategies of molecular recognition allow to discriminate between self and nonself, so avoiding the danger of contamination or infection. Recognition of allopolymorphisms and of foreign substances is indeed universal and, in that sense, all metazoans have developed an "immune system". However, this does not mean that the recognition events and the resulting effector reactions are mediated by homologous systems across metazoans. Some features may be conserved throughout the species, while some others are specific to one phylum or even to one single class within a phylum.

The vertebrate immune system is formed by two main arms, one rapid and incomplete, and the other slower and definitive: innate immunity and adaptive immunity, respectively. Several mechanisms mediating innate immunity are conserved from protostomian to vertebrates. In invertebrates, however, the presence of phyla that have an immunity characterized by specific

memory, clonal expansion or by somatic generation of something similar to a B- or a T-cell repertoire of receptors is still matter of debate.

In invertebrates, local or systemic immune responses apparently do not involve a clonal amplification of the cells producing a given effector molecule (antibody-like, cytokine-like or chemokine-like), and all cells belonging to a class are identical. The production of a given antimicrobial agent is amplified by the regulation of its transcription and, strictly speaking, this phenomenon does not confer specific memory for a given antigen. The receptor used by invertebrate innate immunity is fixed in the genome and can recognize only molecular patterns (LPS, mannans, glycans) which are likely conserved within different pathogen species. The discrimination between self and nonself typical of invertebrate responses is considered "perfect" because selected over time by the evolution. However, several molecules bearing immunoglobulins or T-cell receptor motifs were present in invertebrates even before the arrival of somatic rearrangement [1].

The "core" of vertebrate immunity system is clonal amplification, and all cells belonging to a given class (*i.e.*, B or T lymphocytes) are distinct in their capacity to react with different antigens. This amplification is mediated by unique receptors encoded in gene segments that needs somatic rearrangement and can recognize a large variety of different epitopes presented by specialized antigen-presenting cells. As a consequence, this receptor able to recognize the cellular structures in details is considered "imperfect" because the selection for the above-mentioned discrimination is at single cell level. Although the mechanism of diversity generation offers an immense repertoire for the recognition of antigens, it can also determine the presence of autoreactive clones that can be responsible for the onset of autoimmune disease. This can be considered as a sort of price to pay to the increased complexity and sophistication of the immune responses. Therefore, behind somatic rearrangement, there are other mechanisms that can select and eliminate dangerous cells. Once created with its T-cell receptors, immunoglobulins and major histocompatibility complex, the system has little possibilities to evolve significantly. Then, at the level of lymphoid organs, the "complexification" that occurs seems to result, in mammals, in a better way of exploiting the mechanisms generating diversity: on the one side, somatic mutations improve the possibility to see antigens, and on the other side, immunoglobulin isotype switch allows an improvement of the response with time after immunization.

All the mechanisms conserved between invertebrates and vertebrates are related to innate immunity, suggesting they are indeed the main line of defense against pathogens. For instance, phagocytosis is conserved throughout metazoans. The complement, a group of soluble proteins that complex foreign cell surface components, presents three pathways of activation: the classical, the alternative, and the lectin pathway. The alternative and the lectin pathways that do not involve the binding of complement components to an antigen-antibody complex may exist in invertebrates. The echinoderm, or prochordate complement molecules that have been characterized so far, C3 and factor B, are homologous to their mammalian counterpart [2].

Innate and adaptive immunity concur to eliminate stress conditions determined by physical, biochemical or biological injury. The depletion of substances needed for survival determines different responses apt to balance the stress conditions. If an insult is minimal, the cell can restore its own function; otherwise cell death occurs and, if the organism is unicellular, loss of life is unavoidable. The passage from unicellular to multicellular organisms is characterized by a major cellular specialization, which requires another step of pivotal importance for the evolution: from some specialized groups of cells to the presence of tissues and organs in which the homeostasis maintenance is more complex. In multicellular organisms, cell death does not necessarily mean death of the whole organism, and has become an important response utilized by the immune system.

During the evolution, a separation has occurred between necrosis, a type of cell death not controlled neither controllable, and apoptosis or programmed cell death, a phenomenon largely used in physiological condition for homeostasis maintenance. Apoptosis is conserved from protozoan to humans and, if deregulated, can contribute to the pathogenesis of a large variety of diseases widely studied in the latter "animal model". The study of programmed cell death is important because this phenomenon is involved in several physiological mechanisms in different organisms: maintenance of normal cell turnover, elimination of "unwanted" cells during developmental processes in embryogenesis and metamorphosis, clonal selection of lymphocytes in thymus, immune-mediated cytotoxicity, clearance of antigens, rapid termination of an immune response without losing a large number of effector cells [5, 6]. Apoptosis needs energy production as well as the activation of several genes [3, 4]. It is a sort of asynchronous process that involves single cells without triggering inflammatory responses. In contrast, necrosis is exclusively pathological and involves simultaneously several cells, determining an inflammatory response. Morphologically, apoptosis shows a later loss of membrane integrity accompanied by cellular contraction and chromatine condensation while necrosis shows an early loss of membrane integrity and a swelling either of cells or of nucleus accompanied by chromatine disintegration.

The enormous interest around this apoptotic process resides in the fact that, in humans, it plays an important role not only in physiological but also in pathological conditions. In fact, a deregulation of apoptosis can be at the basis, or at least one of the main causes of several pathological processes such as viral infections, tumors and autoimmune diseases. From this point of view, the study of the immune system in various organisms is crucial. Interesting researches on invertebrates, and in particular on earthworms, did not reveal the presence of tumors in these organisms. Thus, innate immunity in earthworms is not only apparently able to eliminate foreign cells, but also does not allow the growth of neoplastic cells. Through the study of these mechanisms we can learn more from invertebrates concerning the expansive evolutionary development of innate immunity which protects millions of metazoans whose sheer numbers are infinitely more than the single human species. Indeed, the numerous humoral products from these invertebrate organisms are potent antimicrobial molecules, and we may better understand the mechanisms of natural protection against diseases.

Earthworms (*Eisenia foetida*) represent a meaningful model for the understanding of the innate immune system because of its complex effector activity, typical of either simple invertebrates with few leukocytes, or more complex organisms with more leukocytes [7]. The earthworm's body cavity contains coelomic fluid and coelomocytes (equivalent to mammalian leukocytes) that are as varied as in other equally complex invertebrates, and they resemble certain vertebrate leukocytes with respect to morphology, cytochemistry and function. Both the coelomocytes and the fluid which they synthesize and secrete exert immunobiological responses like opsonization, inflammation and phagocytosis, agglutination, lysis, and destruction of experimentally introduced allogeneic, xenogeneic but not autogeneic transplants *in vivo* and various target cell types *in vitro* [8].

Evolution of innate and adaptive immunity proceeds in parallel with the development of the mechanisms used for the elimination of possible noxious nonself. These mechanisms can be of two types and are represented in different ways along the evolutionary scale. The first is characterized by production of soluble molecules by specialized cells. The second uses effector cells. Multicellular invertebrates have an innate immunity based on either cells or their humoral product. Cells synthesize and secrete products that allow invertebrates to survive in hostile environment being in contact with potentially pathogenic microorganisms. The lytic capacity of these molecules has been investigated *in vitro* against eukaryotic cells, including tumor cells, along with their capacity to respond to soluble, modulating molecules such as vertebrate cytokines [9].

Lysis of foreign cells is a universal response that is common to several invertebrate species, and a cell component of immune system was observed in parallel with the capacity to kill foreign cells by humoral mechanisms. In the last years, we have investigated the capacity of coelomocytes to exert cytotoxicity against cancer cells. Invertebrate lytic system spontaneously kills various targets without any evidence for the presence of clonal/specific immunocytes such as CTLs, suggesting that an NK-like activity is present [10]. To support this view, we demonstrated the dissociation of phagocytosis from NK-like killing in earthworms where cellular and humoral aspects of inflammation, as well as rejection of allografts and xenografts but not of autografts, have been consistently observed.

In the coelomic fluid of the earthworm *E. foetida*, cytofluorimetric analysis revealed two major leukocyte-type cells: one kills, the other eats. Small dense coelomocytes (SC) bind to targets, are not exclusively phagocytic and are CD11a, CD45RA, CD45R0, CDw49B, CD54, Thy-1+ and β_2 microglobulin -positive, which confirms previous observations [11-13]. Large coelomocytes (LC) do not bind to K562 cells but are predominantly phagocytic and negative to the above markers. Both SC and LC are negative for other CD and MHC class I and class II markers. Although the positive CD markers are associated with human lymphocytes, monocytes and macrophages, they may serve as examples of putative evolutionary precursors of certain leukocyte lineages in earthworms associated but not exclusively, with those that exert cytolytic and phagocytic functions. This dissociation supports the view of polyfunctional and nonexclusively phagocytic roles of invertebrate leukocytes.

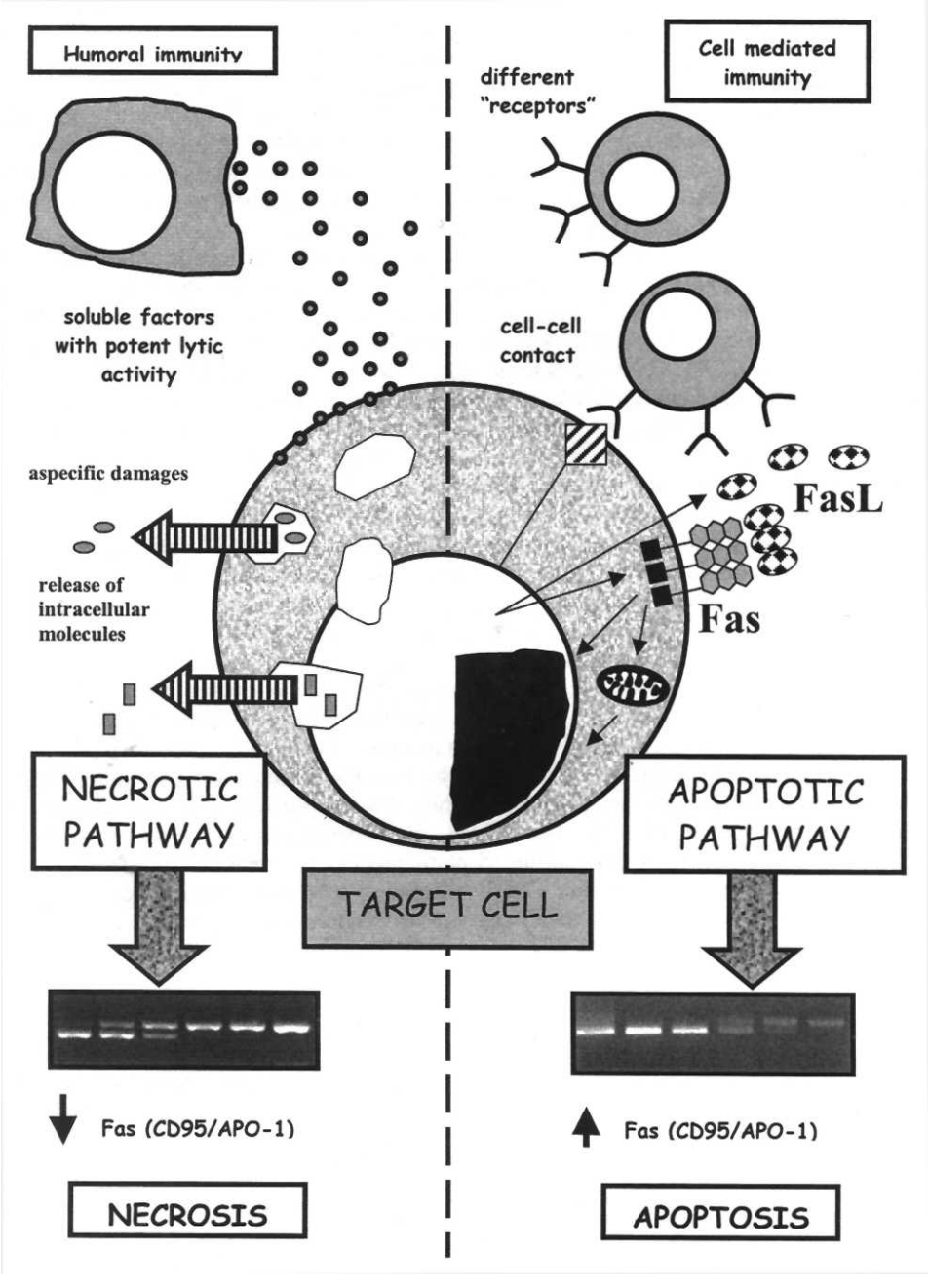
We then studied viability of effectors, and found that there was higher incorporation of [^{3}H]-thymidine in autogeneic (A⇔A self) and significantly greater numbers of cells in S, G2, or M cell cycle phases than in allogeneic (A⇔B non self) coelomocytes. When A⇔A or A⇔B were cultured, no significant cell killing occurred, as measured in a 4-h ^{51}Cr release assay. A⇔A but not A⇔B lysed K562 target cells and cytotoxicity was dependent upon membrane binding between SC to targets, and was enhanced by adding PHA [14]. The same effect was observed using the heat-labile supernatant from A⇔A but not from A⇔B, following cultivation for 10 min at 22 °C, but not immediately after washing. We suggest two interpretations: 1) recognition/binding to and lysing of foreign cells in an NK cell-like reaction may reflect innate immunity; 2) cytotoxicity is a response that is more advanced than phagocytosis [15]. SC lyses classical NK-sensitive cells like K562, but also NK-resistant targets [16]. Interestingly, different cell lines have different sensitivity to the action of coelomocyte supernatant, which seems to depend upon mitochondria functionality of targets (manuscript in preparation). We then analyzed the behavior of coelomocytes in culture, after killing of target cells [17]. When cultured alone for up to 3 h, coelomocytes remained confluent forming neither conjugates nor larger aggregates. When cocultured with K562 cells, coelomocytes spontaneously killed tumor cells and then formed multiple visible aggregates similar to granulomas. Almost simultaneously, once lysis is complete, coelomocytes then wall off the debris that results from lysis, thus "encapsulating" it according to invertebrate immunologists, or "forming granulomas" in the jargon of mammalian immunologists. It is assumed that the most active cell during the encapsulation process is the LC.

Recent experiments are lighting on further mechanisms of invertebrate immunity, paying attention to cytotoxic responses. In particular, it is of interest to search for a possible point at which a different mechanism than necrosis was created for triggering cell death. A big step was reached when target cells learned to die for apoptosis, such phenomenon occurring when a diversification of killing mechanisms took place. Evolutionarily speaking, the first way to kill a target cell was likely by producing soluble molecules: there is no necessity for specific receptors, and thus this method is highly aspecific yet efficient. Along with an increasing complexity of surface molecules and the development of sophisticated cell-cell recognition patterns, another mechanism has evolved, i.e. that of programmed cell death/apoptosis. Such

mechanism based on the development of a dedicated program in the target cell requires first a cell contact, then the activation of specific intracellular pathways. As depicted in Fig. 1, it can be hypothesized that the production of soluble effectors could kill target cells simply by provoking a direct damage to the plasma membrane and/or to other intracellular compartments. Subsequently, when effector cells became able to interact with their targets *via* cell-cell contact, a new mechanism has emerged that involved the direct "responsibility" of targets in their death. In a sense, effectors taught targets to use their own gene repertoire to trigger their suicide. Teaching how to die – and convincing targets to do so – has been a big evolutionary step!

To investigate this idea, we took advantage from the experimental model described above, *i.e.* coelomocytes that kill human target cells, and by the fact that analyses in human cells are facilitated by our knowledge of the effector cells. The main question was whether coelomocytes were able either to induce apoptosis in target cells (meaning that they possess a mechanism capable of triggering this type of death), or to provoke their death *via* necrosis, or both. We used a strategy based upon a recent technique we have developed that evaluates the expression of different forms of Fas (CD95/APO-1) mRNA in target cells. Fas is a type 1 transmembrane protein belonging to the TNF-receptor superfamily that can exist either as membrane molecule (mFas) or as a soluble form (sFas)[18]. Fas is involved in the regulation of several immune processes, including, among others, selection of T-cell repertoire, deletion of self-reactive cells and cytotoxicity against target cells or tissues [19-23]. Fas is present on the cell surface as a monomeric protein and can be bound by its natural ligand, called Fas ligand (FasL, CD178), another protein member of the TNF superfamily [24]. Crosslinking of Fas by FasL leads to trimerization of the so-called death domain (DD) of Fas present in the inner surface of plasma membrane; the trimeric Fas recruits then an adapter protein, the "Fas-associated DD" (FADD), by homotypic protein-protein interaction, forming a "death-inducing signaling complex" (DISC). FADD then recruits procaspase 8 to the DISC by homotypic interaction between their "death effector domains" (DED). Procaspase 8 is autolytically cleaved into active caspase 8, which activates caspase 3, and starts a complex signaling pathway that leads to apoptosis [25, 26]. Peripheral naive T cells express little or no mFas, whereas activated memory T cells express relatively high levels of mFas. This has been linked to the higher susceptibility of memory cells to undergo apoptosis, and underlines the role of Fas in the homeostasis of the immune system.

Fig. 1: Diversification of the mechanisms of cell death occurred when a new program of cell death, *i.e.* a new way to die has emerged. As depicted in the left part, the production of soluble molecules (similar to perforins or complement factors) by effector cell provokes irreversible, gross damages to several structures (plasma membrane, nucleus, etc.) of the target cell. As a result, such cell dies for necrosis, and downregulates – or does not produce at all – molecules involved in apoptosis. When more sophisticated cytotoxic mechanisms have emerged, that involved cell-cell contact and recognition, target cells learned to die in a different manner, using their own genetic material to trigger apoptosis. As shown in the right part of the figure, it can be hypothesized that the interaction between effector and target had provoked the creation of a signal through a phylogenetically newer receptor (here indicated as Fas). Activating this receptor provokes a signal that goes directly into the nucleus, and triggers the production of molecules (FasL) that are able to bind such a receptor. Then, the interaction between the receptor and its ligand causes a variety of phenomena that, involving organelles such as mitochondria and molecules such as caspases, ultimately lead cell to apoptosis. In this perspective, an increase in the production of Fas has to be expected for an active way of dying to occur.


Humoral immunity
Cell mediated immunity
different "receptors"
soluble factors with potent lytic activity
cell-cell contact
aspecific damages
FasL
Fas
release of intracellular molecules
NECROTIC PATHWAY
APOPTOTIC PATHWAY
TARGET CELL
Fas (CD95/APO-1)
Fas (CD95/APO-1)
NECROSIS
APOPTOSIS

Interestingly, besides the full-length mRNA that codifies for the membrane-bound form of Fas protein, a variety of cells are able to produce several mRNA variants by alternative splicing of the primary transcript. The variant called FasTMDel, which represents the large majority of soluble variants, produces a protein that can inhibit apoptosis by interacting with FasL. Even if the mechanism that regulate the production of different Fas mRNA variants are not known, such a phenomenon suggests that a target cell can decide (or is taught) what to do. By producing the membrane form of Fas, it can increase its propensity to undergo apoptosis; on the contrary, the upregulation of sFas has a defensive meaning.

We have developed an original method based upon quantitative-competitive (QC) RT-PCR assay that allowed quantifying the amounts of mRNA coding for the total (tFas) and membrane (mFas) forms of Fas in target cells after contact either with effector coelomocytes or with their soluble products [27]. We have used human leukemia cell lines of different origin (promyelocytic HL-60, erythroblastic K562, and promonocytic U937), and tested the capacity of either cells from *E. foetida* or supernatant from *E. foetida* coelomocyte cultures to kill such targets. The analysis of mRNA for different forms of Fas was performed after few hours of incubation at 37 °C, *i.e.* in optimal conditions for human targets. Interestingly, we have observed that when target cells were incubated with coelomocytes, an upregulation of the m Fas occurred, along with an increase of the tFas form. On the contrary, target cells treated with coelomocyte supernatant (containing cytotoxic molecules) significantly reduced the production of both forms of Fas mRNA, suggesting that mechanisms triggering apoptosis were downregulated. Thus, our data suggested that the production of soluble mediators by coelomocytes (the ancestor of humoral immunity?) causes death of the foreign cell because of an aspecific activity of lytic molecules, that likely bind target membrane for physicochemical reasons, and thus determine necrosis. The development of cellular mechanisms to kill targets, i.e. the onset of cellular immunity, seems to act modulating the expression of genes involved in the apoptosis regulation, and is reflected by an increase of total Fas expression.

During evolution, the basic mechanisms of the immune response became more complex with the increase of the organism complexity. Thus, the development of cellular mechanisms beside humoral mechanisms has determined the development of more sophisticated mechanisms that represent the basis of the apoptotic process. Necrosis can be considered pre-existing, and much more rudimental. The divergence of cytotoxic responses of humoral or cellular type might have provoked the divergence between the two above-mentioned way of death, *i.e.* necrosis and apoptosis.

Acknowledgements

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References

[1] C.A. Janeway Jr., How the immune system works to protect the host from infection: a personal view, *Proc. Natl. Acad. Sci.* USA **98** (2001) 7461-7468.

[2] L. Du Pasquier, The immune system of invertebrates and vertebrates, *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **129** (2001) 1-15.

[3] A. Cossarizza A. *et al.*, Mitochondrial modifications during rat thymocyte apoptosis: a study at the single cell level, *Exp. Cell Res.* **214** (1994) 323-330.

[4] C. Richter *et al.*, Hypothesis: control of apoptosis by the cellular ATP level, *FEBS Lett.* **378** (1996) 107-110.

[5] J.F.R. Kerr *et al.*, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, *Br. J. Cancer* **26** (1972) 239-257.

[6] S.V. Lennon *et al.*, Induction of apoptosis (programmed cell death) in tumour cell lines by widely diverging stimuli, *Biochem. Soc. Transact.* **18** (1990) 343-345.
[7] E.L. Cooper *et al.*, Digging for innate immunity since Darwin and Metchnikoff, *BioEssays* 2002 (in press).
[8] E.L. Cooper *et al.*, Annelid humoral immunity: cell lysis in earthworms. In: G. Beck *et al.* (Eds.), Phylogenetic perspectives on the vertebrate immune system, Kluwer Academic/Plenum Publishers, NY, USA., 2001 pp.169-184.
[9] C. Franceschi *et al.* Cytotoxicity and immunocyte markers in cells from the freshwater snail *Planorbarius corneus* (L.) (*Gastropoda, Pulmonata*): implications for the evolution of NK cells, *Eur. J. Immunol.* **21** (1991) 489-493.
[10] E.L. Cooper *et al.*, Cell adhesion and the immune system: a case study using earthworms. *Microsc. Res. Techn.* **44** (1999) 237-253.
[11] E.L. Cooper and M.H. Mansour, In: A.E. Reif and M. Schlesinger (Eds), Cell Surface Antigen Thy-1. Immunology, neurology, and therapeutic applications, Marcel Dekker Publisher, 1989 pp. 197-219.
[12] A.-H. Saad and E.L. Cooper, Evidence for a Thy-1 like molecule expressed on earthworm leukocytes, *Zool. Sci.* **7** (1990) 217-222.
[13] Ph. Roch *et al.*, Serological evidences for a membrane structure related to human β_2-microglobulin expressed by certain earthworm leukocytes, *Eur. J. Immunol.* **13** (1983) 1037-1042.
[14] E.L. Cooper *et al.*, Autogeneic but not allogeneic earthworm effector coelomocytes kill the mammalian tumor cell target K562, *Cell. Immunol.* **166** (1995) 113-122.
[15] E.L. Cooper, Phylogeny of cytotoxicity, *Endeavor* **4** (1981) 160-165.
[16] A. Cossarizza *et al.*, Earthworm leukocytes that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines, *Exp. Cell Res.* 224: 174-182, 1996.
[17] D. Quaglino *et al.*, Earthworm coelomocytes form granulomas in vitro during cytotoxic activity against the mammalian tumor cell target K562, *Eur. J. Cell Biol.* **70** (1996) 278-288.
[18] N. Itoh *et al.*, The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis, *Cell* **66** (1991) 233-243.
[19] S. Cory, Fascinating death factor, *Nature* **367** (1994) 317-318.
[20] S.T. Ju *et al.* Fas(CD95)/FasL interaction required for programmed cell death after T-cell activation, *Nature* **373** (1995) 444-448.
[21] S. Nagata and P. Golstein, The Fas death factor, *Science* **267** (1995) 1449-1456.
[22] D.H. Lynch *et al.*, Fas and FasL in the homeostatic regulation of immune responses, *Immunol. Today* **16** (1995) 569-574.
[23] M. Lenardo *et al.*, Mature T lymphocyte apoptosis - immune regulation in a dynamic and unpredictable antigenic environment, *Annu. Rev. Immunol.* **17** (1999) 221-253.
[24] S. Nagata, Fas ligand-induced apoptosis, *Annu. Rev. Genet.* **33** (1999) 29-55.
[25] V. Depraetere and P. Golstein, Fas and other cell death signaling pathways. *Semin. Immunol.* **9** (1997) 93-107.
[26] M.E. Peter and P.H. Krammer, Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis, *Curr. Opin. Immunol.* **10** (1998) 545-551.
[27] M. Pinti *et al.*, Quantitation of CD95 and CD95L mRNA expression in chronic and acute HIV infection by quantitative competitive PCR, *Ann. N.Y. Acad. Sci.* **926** (2000) 46-51.

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Other Annelid Models: Leeches

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A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
E.L. Cooper et al. (Eds.)
IOS Press, 2002

Leech immune responses: contributions and biomedical applications

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Abstract. Leeches can use a variety of selected defense systems against nonself. Different antigens and immunization conditions lead to responses that may vary both quantitatively and qualitatively; in fact some antigens can selectively stimulate proliferation and migration of immune cells, while others can induce phagocytosis and encapsulation. In addition *Hirudo medicinalis* can react to large and deep explantations through the induction of massive angiogenesis. Another interesting point about the variety of responses of the leech immune system regards first and second set graft rejection. Our data refer to all these topics and we speculate about the function of adopted defense systems in relation to various foreign antigens.

1. Inflammation and initiation of immune reactivity

The wound repair process in the class *Hirudinea* (*Annelida*) has received little attention and that of immune responses is even scarcer. This situation is probably referable to the absence of a true coelomic cavity in these organisms. Instead of a typical coelom as in other annelids, a peculiar botryoidal tissue occupies the inner part of the leech body and coelom is often involved in circulatory system formation [1]. Furthermore, *Hirudinea* are characterized by scarce regenerative phenomena. This, on one hand, gives rise to a more complex response following body injury and, on the other hand, the repair process of these animals is overshadowed by the great segmental regenerative ability of the other annelids the marine polychaetes and the terrestrial oligochaetes.

Studies of wound response in leeches are limited to the observations of repair of tissues injured during hypodermic insemination of the glossiphonid leech *Placobdella parasitica* [2], and to the studies of repair of body wall incision in *Piscicola geometra* [3] and regeneration following amputation of hind sucker in *Helobdella stagnalis* [4-6]. Despite these limitations in analyzing immune response in leeches, the study of wound healing in these animals is interesting for several reasons. First, it is fundamental to compare how two *taxa* phylogenetically distant, such as leeches and vertebrates, use their cellular repertoire to respond to wound. Second, it is important to understand which are the differences in wound healing when regenerative processes are reduced (leeches) or massive (oligochaetes). According to Sawyer [7] amoebocytes (called leukocytes or lymphocytes) carry out the defense response in wounded leeches. Few data are available about these immune cells: they might be involved in phagocytosis and are localized both in coelomic fluid and in connective tissue, ready to freely move and reach the injured area.

Le Gore and Sparks have described two different kinds of cells involved in the wound healing process – vasocentral cells and myofibroblasts – leading to the formation of a pseudoblastema [3]. Huguet and Molinas, in their studies on wound healing, have evidenced the combined action of two systems during the repair of injury [8, 9]. First, an important wound contraction leads to the approach of the wound margins to reduce the injury size and to prevent loss of body fluids: the two cell populations described by Le Gore and Sparks are massively involved in the formation of a pseudoblastema. Later, the adjacent epithelium closes in to cover the wound, even before the underlying tissue has fully recovered [10]. The data collected until the end of the 90's are incomplete and mainly lack any comparison between the immune system of leeches and that of vertebrates. Such analogy has been underlined in other annelids by Cossarizza *et al.* [11] and by Blanco *et al.* [12]. These authors have demonstrated that oligochaetes and related phyla (sipunculids) possess immune cells and mechanisms really similar to those found in vertebrates. Recently, these observations have found support by molecular studies showing the presence in invertebrates of the similar signaling molecules previously characterized in vertebrate immune system [13].

2. Leeches are capable of responding to different foreign antigens

In leeches, different antigens and immunization conditions lead to responses that may vary both quantitatively and qualitatively. As an example, different antigens can selectively stimulate both proliferation and migration of immune cells. When leeches are injected with LPS, immune cells migrate among grouped muscle fibers, in close contact in unlesioned leeches (Fig. 1.1), causing an enlargement of the spaces between two adjacent fields (Fig. 1.2). The cells involved in immune response in leeches can be classified as macrophage-like cells, NK-like cells or granulocytes not only by their morphological aspect but also by the expression of specific CD antigens [14]. Using mouse anti-human CD molecules typical of macrophage, NK cells and granulocytes, we found leech cells that are positive for CD25, CD14, CD61, CD68, CD11b, CD11c, CD56, CD57 and CD16. In addition, these CD-like molecules have comparable molecular mass with their mammalian counterparts. This is a suggesstive evidence even though it is not possible to speak of homology. As shown in Table 1, the morphological and histochemical identification of particular immune cell types in leeches confirms the detection of specific CD markers [15].

Table 1: Morphological and histochemical identification of leech immune cell types and their reactivity with CD markers

Cell type	Histochemistry positivity	Immunochemistry positivity
Macrophage-like	ATPase, COX, ORO, ALP, ACP, PAS, NADH/TR	CD25, CD61, CD68, CD14, CD11b, CD11c
NK-like	ATPase, COX, ORO, ALP, ACP, PAS-NADH/TR	CD25, CD16, CD56, CD57
Granulocytes	ALP, ACP	CD11b, CD11c

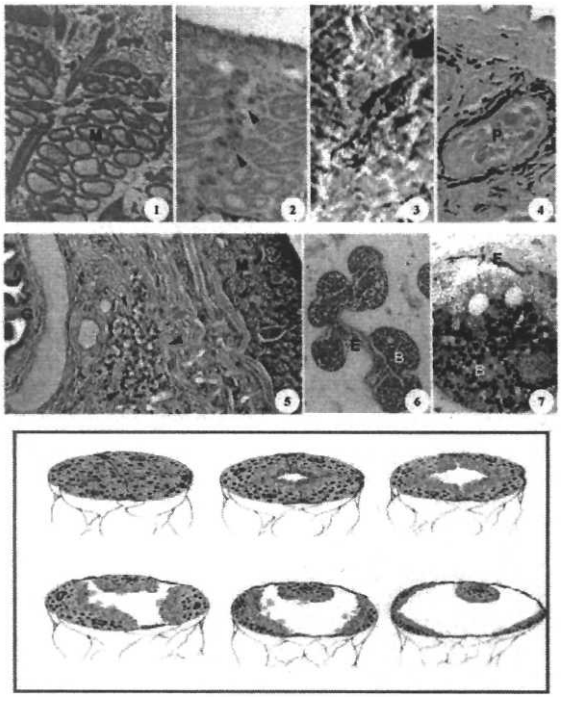

Fig. 1:
1. Semi-thin cross-section of unlesioned *Glossiphonia complanata.* Longitudinal muscular fields (M) are in close contact, separated only by dorsoventral fibers.
2. Semi-thin cross-section of *G. complanata* injected with LPS. The adjacent muscle fields are separated by a large number of migrating cells (arrowheads).
3. Cryo-section, superimposed confocal image. Fluorescent microspheres are phagocytozed by macrophage-like cells.
4. Semi-thin section of *G. complanata* with encapsulated parasite (P).
5. Longitudinal section of *Hirudo medicinalis.* The botryoidal tissue (arrowheads) is localized between the muscle layer (M) and the gut (G).
6, 7. Semi and thin -cross sections of botryoidal tissue. The tissue is composed by clustered botryoidal (B) and endothelial-like (E) cells. The botryoidal cells are easily recognizable by granule-filled cytoplasm (7).
8. Model of the angiogenic process in *H. medicinalis.* From solid cords of botryoidal tissue cells, a vascular lumen is shaped through a dehiscence process.

Defense responses can vary in relation to the dimension of the nonself [16]: leeches are able to phagocytoze small spheres (Fig. 1.3) or yeast, but can also encapsulate and subsequently melanize larger nonself, such as parasites (Fig. 1.4). These responses to foreign antigens are nonspecific events commonly used in invertebrates to free organisms from potentially pathogenic agents (phagocytosis) and/or to rapidly isolate large foreign antigens from the inner environment (encapsulation). All these responses towards the nonself show that leeches can use a variety of selected defense systems. In addition, *Hirudo medicinalis* can react to large and deep explantation through the induction of massive angiogenesis [17]. The formation of new vessels is essentially brought about by the remodeling of the botryoidal tissue (a peculiar tissue of *Hirudo*), which is located in the loose connective tissue between the body wall and the gut (Fig. 1.5). The botryoidal tissue, composed by clusters of botryoidal cells and endothelial-like cells (Fig. 1.6, 1.7), can change its shape from solid cords of cells to a tubular, prevascular structure through a dehiscence process (Fig. 1.8).

The ropes of clustered cells acquire a lumen, and the process occurs because botryoidal tissue cells undergo marked cell shape modification: thinning, flattening and tapering of these cells allow vessels to increase their diameter and length (Fig.2.9, 2.10). It is generally assumed that in adult vertebrates the outgrowth of a network of capillaries is achieved by endothelial cell sprouting, a well-described process in wound healing [18-20]. In our study

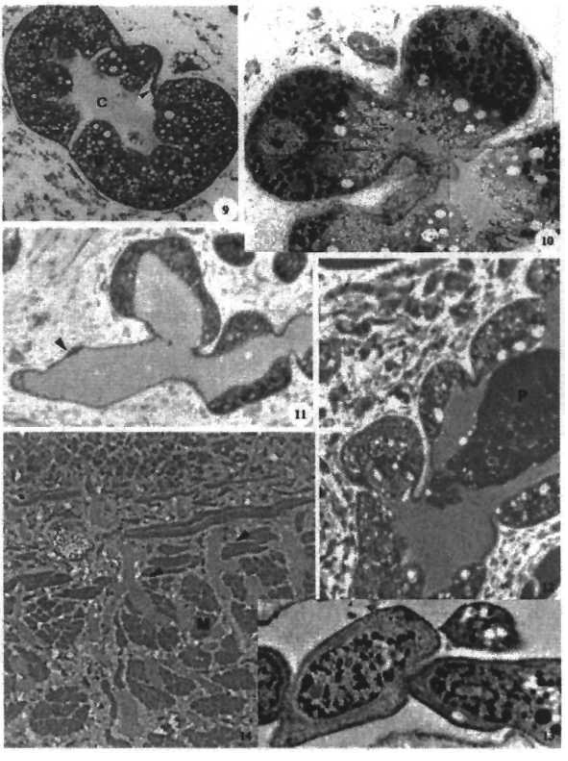

Fig. 2:
9-11. By vasculogenesis, the new vessel cavity (C) enlarges (9, 10), and by an angiogenic process, endothelial cells line the neovessel (arrowheads). B: Botryoidal cells.
12. During vessel development, precursors (P) of circulating cells are visible in the neolumen.
13. Detail of precursor cells in close contact.
14. Semi-thin cross-section of *Hirudo medicinalis*. New vessels (arrowheads) migrate from the center of the body towards the surface, through the grouped muscle fibers (M).

[21], we have evidenced that in adult leeches the growth of new vessels is characterized by a combination of two distinct events, i.e. an initial vasculogenic step followed by extensive angiogenesis. In fact, immediately after a surgical stimulus (deep explantation), we have observed the transformation of the botryoidal tissue into new immature vessels (vasculogenic step)(Fig. 2.9), from which new branches and capillaries can further outgrow following a "classic" angiogenic pattern (i.e. an extensive development of new vessels from pre-existing ones)(Fig. 2.11). In addition during this phase of vessel development, clusters of circulating precursors are evident (Fig. 2.12). These cells are lymphocyte-like [de Eguileor *et al.*, submitted], and are characterized by a large nucleus and the presence of centrioles and microfilament network underneath the plasmalemma (Fig. 2.13). As a tentative explanation of the occurrence of angiogenesis in districts of *Hirudo* which are virtually avascular in normal conditions, we speculate that new vessels can act as a piping system to rapidly transfer in the surgically lesioned area a large amount of cells involved in immune defense and wound healing (Fig. 2.14, 3.15, 3.16).

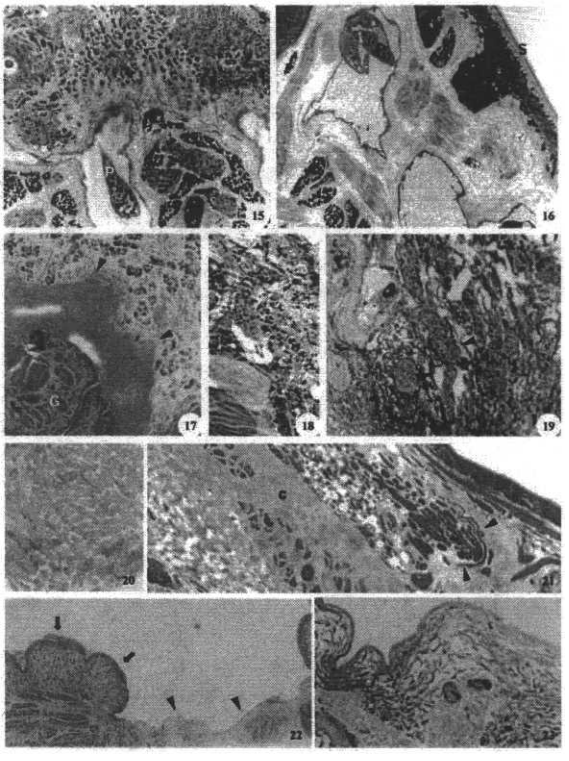

Fig. 3:
15, 16. Thin cross-sections. The neovessels piping the precursors (P) have reached the surface (S) of lesioned *Hirudo medicinalis* body wall.
17-19. Semi-thin and thin sections. The graft (G) is surrounded by migrating cells (arrowheads) that are initially disorderly disposed (18) and subsequently arranged in parallel rows (19, arrowhead).
20. Semi-thin section. The graft tissue organization (predominantly muscle fibers) shows a massive alteration.
21. Semi-thin section. The lesioned area is filled by migrating cells (arrowheads), fibroblasts and new collageneous matrix (c).
22, 23. Semi-thin sections. The new epithelium (arrowheads) made by flattened cells (23) is easily recognizable in respect to undamaged one (arrows).

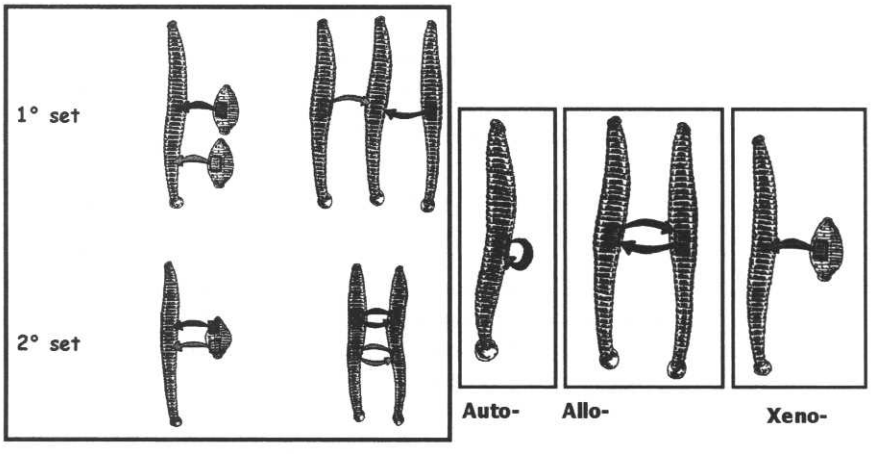

Fig. 4: First-set and second-set graft rejections, as well as auto-, allo- and xenografts.

Another interesting point about the variety of responses of the leech immune system regards the first-set graft rejection, but the especially second-set rejection [de Eguileor *et al.*, submitted]. In the first-set graft rejection, the second graft is transplanted in the same recipient from two different donors, while in the second-set graft rejection, the second graft is transplanted from the same donor to the same recipient (Fig. 4). The second-set results in an accelerated rejection of the second graft due to a secondary immune response. Our data refer to first and second -set rejection processes occurring at different time intervals (short and long time) between the implant of a first and a second graft, and observations are made at different times from the implant of the second graft (Fig. 5). Obviously, an autograft, which is a selftransplantation, leads only to modest inflammatory responses (Fig. 4). On the contrary, both allografts and xenografts show comparable vigorous responses.

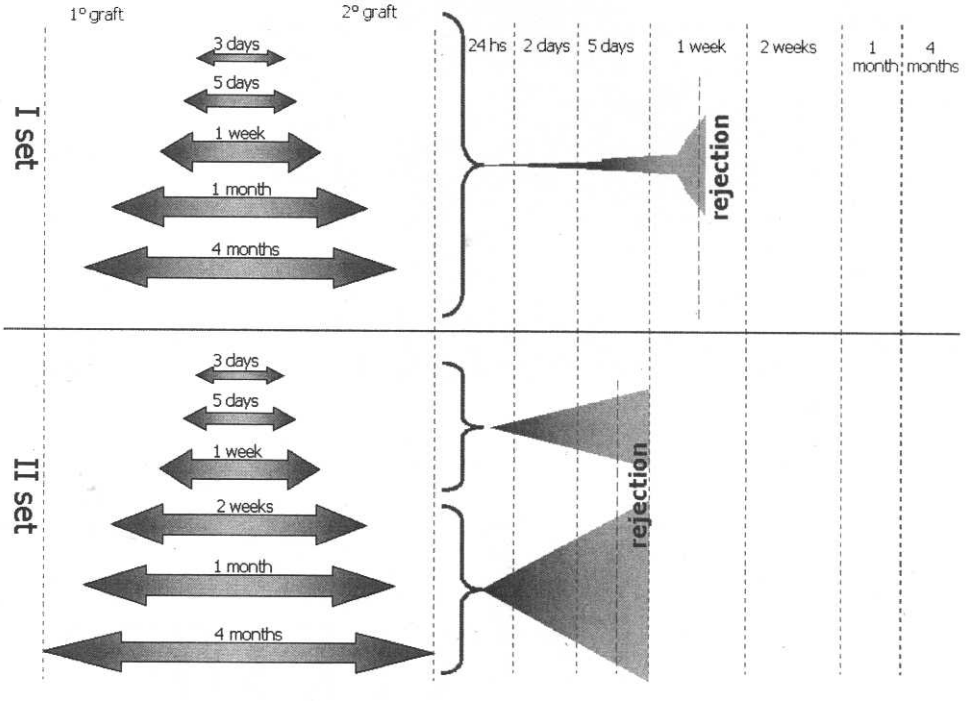

Fig. 5: Experimental schema for first- and second- set graft rejection

In both cases, we have comparable responses as far as host tissue modifications and graft tissue alteration are concerned. Host tissue modifications follow a sequence of steps: acute inflammatory reaction, rejection of graft, re-epithelialisation and reconstruction of lacking tissue. The first response to the nonself is mediated by migrating and proliferating cells. All immune cells move through the extra cellular matrix and are involved not only in the elimination of graft, of cell and matrix debris, but also in the production of cytokine-like molecules (as GM-CSF, VEGF, EGF). Migrating cells that have reached the graft area (Fig. 3.17) are initially distant and disorderly disposed (Fig. 3.18). Subsequently, all cells crowd

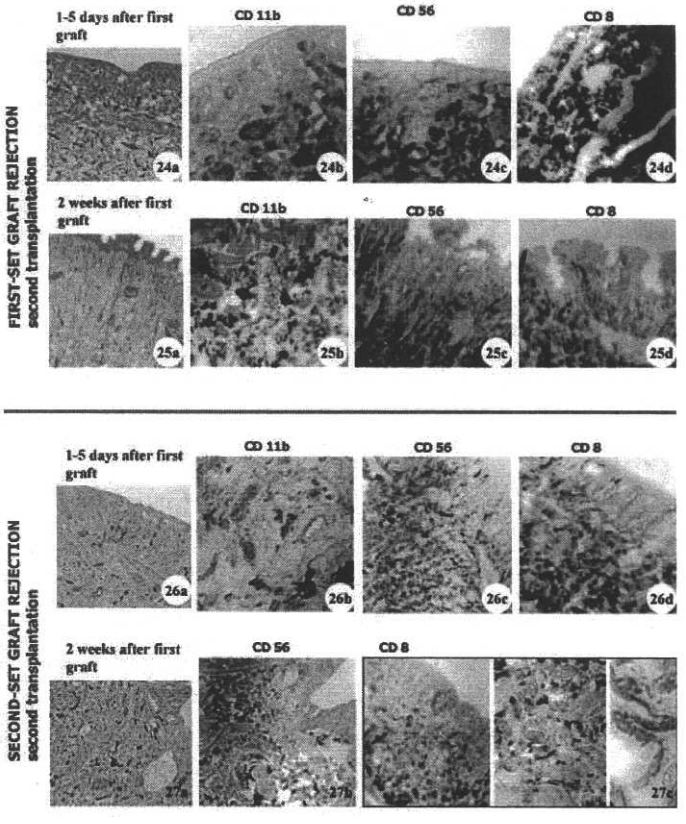

Fig. 6:
24, 25. First-set graft rejection. Immunofluorescence images of transplanted *Hirudo medicinalis*. At early time point many cells are positive for CD11b (24b, 25b) while the positivity for CD56 and CD8 is scarce (24c, 24 d). The signal for CD56 and CD8 decreases at two weeks after first graft (25c, 25d).
26, 27. Second-set graft rejection. Immunofluorescence images of transplanted *H. medicinalis*. Considering short time (from 1-5 days to 2 weeks) elapsed from the first graft, few CD11b-positive cells are present. CD56 and CD8 positive cells are largely represented.

and arrange themselves in parallel rows, around the graft thus completing the healing process (Fig. 3.19). At this time, the nonself tissue is completely restricted by host cells surrounding and coating the graft (Fig.3.17).

Two days after grafting, transplanted body wall fragments display massive structural alterations, and in the timespan of a week, the graft dissolves and disappears (Fig. 3.20). A dense population of macrophage-like cells and fibroblasts crowds the space previously occupied by the graft. Fibroblasts increase numerically, paralleling an increased production of collagen that can act as a scaffold to support cell growth and migration (Fig. 3.21). Superficially, it is possible to see a new epithelium, replacing the barrier between the inner and the outer part of the body, that is easily recognizable with respect to the undamaged one, because mostly consisting of flattened cells (Fig. 3.22, 3.23). The described situation concerns

not only the responses to first-set graft (where there are two different donors for the same recipient) but also to the second-set graft rejection (two grafts from the same donor to one recipient). Practically, the host responded to the second graft with a similar mechanism as if it was a first graft.

At early timepoints, 1-5 days after the first graft, many migrating cells are positive for CD11b (a granulocyte marker), few cells are positive for CD56 (a NK-like cell marker) and few cells are positive for CD8 (a NK and cytotoxic T cell marker) (Fig. 6.24, 6.25). After a long period of time from the first graft, only few cells are labeled by anti-CD56 antibody, and the antibody against CD8 doesn't detect any cell (Fig. 6.26). If we consider a second-set graft the response against the second graft is accelerated; the graft displays massive structural alteration and it is rejected more rapidly, usually in 3-4 days. Thus, in a very short time the graft dissolves and disappears. This quickness in the responsiveness may be related to the presence of immune cells remaining *in loco* after the first graft. If we consider the second graft implanted from 1-5 days to 2 weeks after a first graft, few CD11b-, CD56- and CD8 - positive cells are visible (Fig. 6.26, 6.27). At longer period of time between the two grafts (1-4 months), the rejection remains accelerated and many CD56- and CD8- positive cells are visible (Fig. 7.28-7.30). Considering a long time elapsed after the second graft, many cells located in the area previously occupied by the graft are labeled by anti-CD8 antibody. In contrast, few cells are positive for CD56 marker (Fig. 7.30).

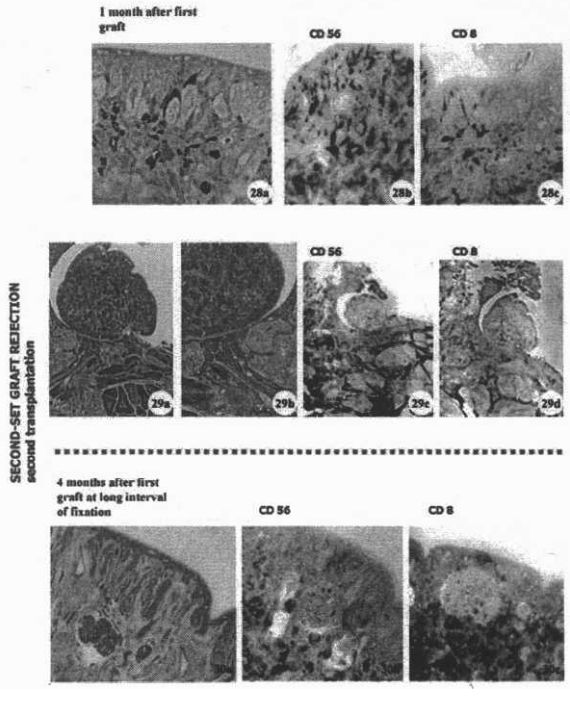

Fig. 7:
28, 29, 30. Second-set graft rejection. Immunofluorescence images of transplanted *Hirudo medicinalis*. Large population of CD56- and CD8-positive cells are largely distributed considering the second graft transplanted at long term. Comparing 28-29 with 30 underlines the different positivity for anti-CD56 and anti-CD8 antibodies in relation to the interval between the second graft and the specimen examination.

Morphological and immunocytochemical data in relation to first- and second-set graft rejection can be summarized as follows. Initially in both cases, the responses against the first graft are similar and a massive inflammatory reaction is observed. Afterwards, the responses against the second graft are different in relation to the first- or second-set. In the first-set, within 1-5 days after the second transplantation, macrophage-like cells, granulocytes and CD56-positive cells are present. In the second-set, we have two situations: the second graft transplanted at short term (1 day to 1 week interval between the two grafts) and the second graft transplanted at long term (2 weeks to 4 months interval between the two grafts). In both cases, a large population of CD56- and CD8-positive cells are distributed in a large section of the involved area. In addition, at longer period after the second graft, only CD8-positive cells are visible in the space previously occupied by the graft. The initial positivity for CD56 and CD8 could reflect the presence of a unique population of cells, possibly NK-like cells. The differential CD-staining at later timepoints might suggest the presence of two different types of cells that are NK-like cells and CD8-positive cells (generally classified as cytotoxic lymphocytes in vertebrates).

3. Perspectives on leeches and their immune system

These preliminary data raise a question: is it possible to propose the existence of a certain type of immune memory in leeches? We have tried to answer comparing some statements suitable for vertebrates with our data about leeches. Abbas *et al.* [22] in "Cellular and Molecular Immunology" define the immune memory as follow: "Exposure of immune system to a foreign antigen enhances its ability to respond again to that antigen. Thus, responses to second and subsequent exposures to the same antigen, called secondary immune responses, are usually more rapid, larger and often qualitatively different from the first, or primary, immune responses to that antigen. This property of specific immunity is called immunologic memory". Second-set *H. medicinalis* allo- and xenografts from the same donor to the same recipient undergo accelerated rejection both if the second graft is transplanted at short (1-5 days) and long (2 weeks-4 months) intervals. Thus, intense reactions at short interval between the two grafts could be justified by the presence of cells activated by first transplant. The reaction occurring at long intervals between two transplants can be justified assuming the presence of memory cells.

Always in Abbas *et al.* [22], it is possible to read about memory cells: "Lymphocytes proliferate when stimulated by antigens and each exposure to antigen expands the clone(s)..." and also: "Lymphocytes that have previously responded to antigenic stimulation, survive for prolonged periods even in the absence of the antigen. Thus, memory cells are programmed to respond rapidly to an antigenic challenge". In leeches, after stimulation as a graft, there is a massive angiogenesis and a correspondent massive production of lymphocyte-like cells deriving from one cluster that can circulate between the blood and the connective tissue. Even though the knowledge about protostome responses to nonself is incomplete, more and more evidences suggest the presence of conserved system in invertebrate and vertebrate immune mechanisms. This evidence could be considered striking but, in our opinion, if a defense system (i.e. immune system) is efficient, it can be maintained along the entire evolution tree and the differences among more or less evolved animals might be related to the complexity of the control of these basic mechanisms. Chervitz *et al.* [23] comparing *Saccharomyces cerevisiae* and *Caenorhabditis elegans* genomes, have shown that in this two phylogenetically distant species, the main part of their basic biological functions (i.e. intermediary metabolism, DNA and RNA metabolism, protein folding mechanism, intracellular trafficking systems) is

due to the presence of orthologue proteins (ie proteins whose origin is related to a common ancestor) that perform the same functions. There is nothing surprising about it if you think of an hypothetical ancestral animal, for which De Robertis and Sasai [24] proposed the name of Urbilateria (primitive bilateral animal) from which the arthropod and the chordate lineages diverged 600 million years ago.

References

[1] K.H. Mann, Leeches (*Hirudinea*): their structure, physiology, ecology and embryology, Pergamon Press, Oxford, 1962.

[2] R.J. Myers, Behavior and morphological changes in the leech, *Placobdella parasitica*, during hypodermic insemination, *J. Morphol.* **57** (1935) 617-648.

[3] R.S. LeGore and A.K. Sparks, Repair of body wall incision in the rhynchobdellid leech *Piscicola salmositica*, *J. Invert. Pathol.* **18** (1971) 40-45.

[4] J.P. Cornec, Régulation et régénération après amputation de la région postérieure de jeunes Hirudinées de l'espèce *Erpobdella octoculata*, *Arch. Zool. Exp. Gén.* **121** (1980) 173-181.

[5] J.P. Cornec, Modifications ultrastructurales après amputations dans le territoire de régénération postérieure de l'hirudinée Rhynchobdelle *Helobdella stagnalis*, *Arch. Anat. Micr.* **73** (1984) 269-289.

[6] J.P. Cornec, Etude ultrastructurale des bourgeons de régénération mis en place après amputation de la ventouse postérieure chez l'hirudinée rhynchobdelle adulte *Helobdella stagnalis*. *Can. J. Zool.* **68** (1990) 303-312.

[7] R.T. Sawyer, Leech Biology. Vol. I. Oxford Univ. Press, Oxford, 1986.

[8] G. Huguet and M. Molinas, The pseudoblastema in the wound healing process of the leech *Hirudo medicinalis* L. (*Hirudinea*): changes in cell junctions, *J. Exp. Zool.* **269** (1994) 23-36.

[9] G. Huguet and M. Molinas, Myofibroblast-like cells and wound contraction in leech wound healing, *J. Exp. Zool.* **275** (1996) 308-316.

[10] G. Huguet and M. Molinas, Changes in epithelial cells in *Hirudo medicinalis* during wound healing, *J. Invert. Pathol.* **59** (1992) 11-17.

[11] A. Cossarizza *et al.*, Earthworm leukocytes that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines, *Exp. Cell Res.* **224** (1996) 174-182.

[12] G.A Blanco *et al.*, LPS-induced stimulation of phagocytosis in the Sipunculan worm *Themiste petricola*: possible involvement of human CD14, CD11b and CD11c cross-reactive molecules, *Dev. Comp. Immunol,* **21** (1997) 349-362.

[13] A. Aderem and R.J. Ulevitch, Toll-like receptors in the induction of the innate immune response, *Nature* **406** (2000) 782-787.

[14] M. de Eguileor *et al.*, Lipopolysaccharide-dependent induction of leech leukocytes that cross-react with vertebrate cellular differentiation markers, *Tissue & Cell* **32** (2000) 437-445.

[15] M. De Eguileor *et al.*, Histopathological changes after injury in leeches, *J. Invert. Pathol.* **74** (1999) 14-28.

[16] M. De Eguileor *et al.*, Different types of response to foreign antigens by leech leukocytes, *Tissue Cell* **32** (2000) 40-48.

[17] M. De Eguileor *et al.*, Ultrastructure and functional versatility of hirudinean botryoidal tissue, *Tissue Cell* **33** (2001) 332-341.

[18] R.A.F. Clark, Regulation of fibroplasia in cutaneous wound repair, *Am. J. Med. Sci.* **306** (1993) 42-48.

[19] W.G. Jiang and S. Hiscox, Hepatocyte growth factor/scatter factor, a cytokine playing multiple and converse roles, *Histol. Histopathol.* **12** (1997) 537-555.

[20] L.K. Landeen *et al.*, Characterization of a human dermal replacement, *Wounds* **5** (1992) 167-175.

[21] M. de Eguileor *et al.*, *Hirudo medicinalis*: a new model for testing activators and inhibitors of angiogenesis, *Angiogenesis*, in press.

[22] A.K. Abbas *et al.*, Cellular and molecular immunology, Saunders, Philadelphia, USA, 1994.

[23] S.A. Chervitz *et al.*, Comparison of the complete protein sets of worm and yeast: orthology and divergence, *Science* **282** (1998) 2022-2028.

[24] E.M. De Robertis and Y. Sasai, A common plan for dorsoventral patterning in *Bilateria*, *Nature* **380** (1996) 37-40.

A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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New powerful cascade coagulant inhibitors isolated from the gut of the leech *Theromyzon tessulatum*

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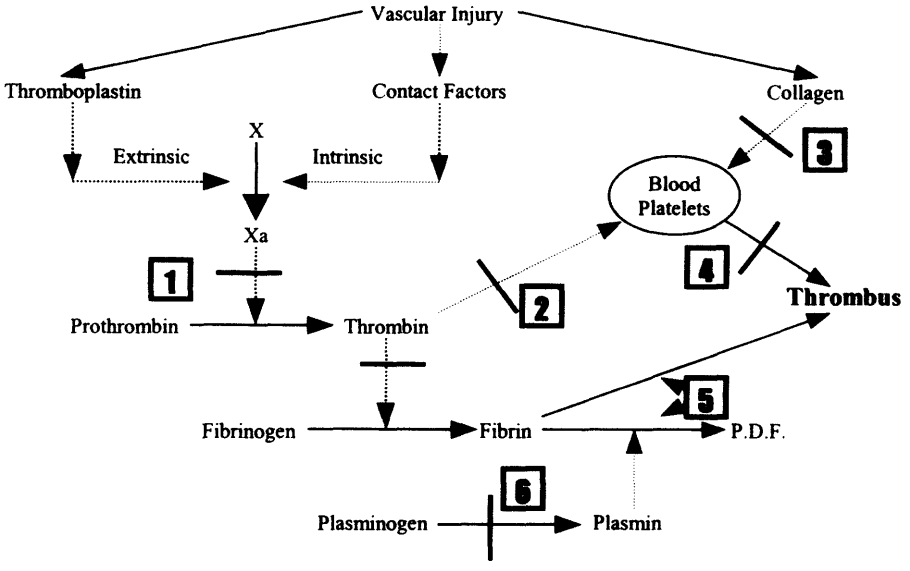
Abstract. Thrombin and factor Xa inhibitors, have already been isolated from jawed and gut leeches respectively. In this review, we report for the first time the isolation of both serine protease inhibitors in the same species, the gut leech *Theromyzon tessulatum,* and show that they are implicated in the modulation of coagulation and local inflammation. All the isolated protease inhibitors are highly active, around nmol, have a size < 10 kDa and are cysteine-rich. Interestingly, some of these molecules belong to a same family and are highly homologuous, leading the hypothesis of a gene duplication from an ancestral gene during evolution. Furthermore, all these molecules significantly diminish the level of human granulocyte and monocyte activation induced by LPS. The activity level of proteases inhibitors discovered in *T. tessulatum* is higher than that of aprotinin, another serine-protease inhibitor used biomedically. Therefore, this animal species is a real reservoir of new powerful drugs to treat ageing diseases like cardiovascular diseases.

1. Introduction

Animals depending on a diet of fresh blood have evolved mechanisms that interfere with the coagulation process of the blood donor. In this regard, a variety of coagulation inhibitors have been isolated from blood-sucking animals, *i.e.,* bat [1], tick [2, 3], leeches [4, 5] and hookworm [6]. Their salivas contain anticoagulant compounds that maintain the blood in a fluid state during intake and in their digestive structures [4] (Fig. 1).

In leeches, among the different anticoagulant molecules involved in the inhibition of the coagulation cascade, three substances have been investigated in great detail *e.g.* hirudin (thrombin inhibitor, [7]), antistasin (factor Xa inhibitor, [8]) and decorsin (antagonist of platelet membrane glycoprotein IIb-IIIa, [9]) (Fig. 1). Although these molecules are different in amino acid sequences and inhibitory activity, their three-dimensional structures share the same conformational motif with that of the Leech Antihemostatic Protein (LAP) [10]). Interestingly, their mechanisms of action and epitopes important for binding to their respective targets are distinct as well [7]. This may serve to demonstrate the significance of diverse mechanisms in inhibiting the coagulation process, as well as the evolvement of slightly different inhibitory processes. Most of these molecules have been studied in jawed leeches. Here, we report for the first time on the isolation and the characterization of highly specific anticoagulants isolated from the gut leech *Theromyzon tessulatum.*



1 Antistasin, Ghilanthen, Therostasin, Ankylostoma, TPA

2 Hirudin, Haemendin, Bufrudin, Theromin

3 Calin, LAP

4 Decorsin, Ornatin

5 Hementin, Destabilase

6 Hementerin, bat TPA

Fig. 1: Targets of leech anticoagulants on homeostasis

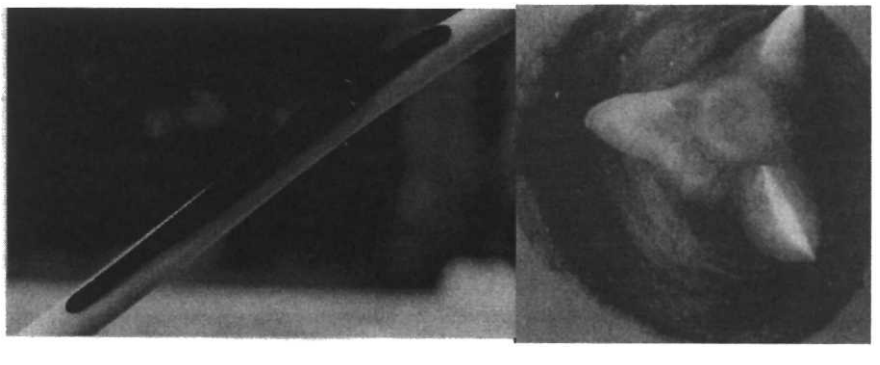


Fig. 2: Photographs of *Hirudo medicinalis* (left) and the jaws (right)

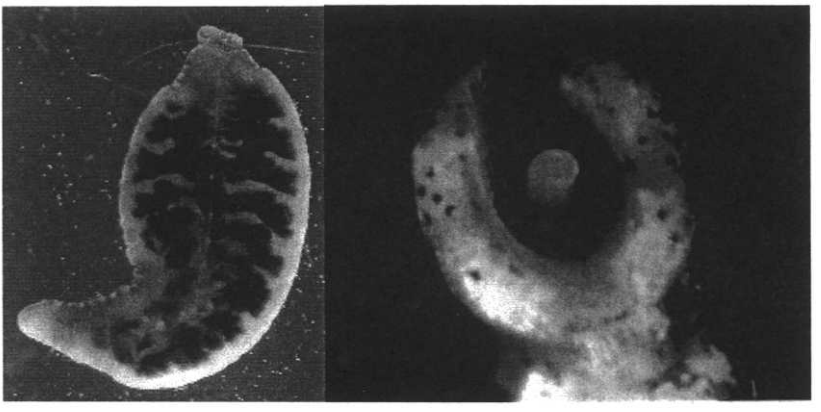


Fig. 3: Photographs of *Theromyzon tessulatum* (a) and the gut (b)

2. Coagulation inhibitors

Leeches belong from two families of family *i.e.* the jawed and the gut leeches. Moreover, in these two families, some are bloodsuckers and the others not. For example, in blood-sucking leech, *Hirudo medicinalis* is a jawed leech (Fig. 2) and the rhynchobdellid leech *T. tessulatum* is a gut one (Fig. 3).

2.1. Thrombin inhibitors

Most of the polypeptides belonging to the family of thrombin inhibitors have been isolated from jawed leeches, such as the amphibian parasite leeches *Hirudo medicinalis* [7] and *Hirudinaria nipponia* [11], the mammalian parasite *Hirudinaria manillensis* [12] or the land living *Haemadipsa sylvestris* [13]. We have for the first time isolated a specific tight-binding thrombin inhibitor, theromin, from the gut leech *T. tessulatum* [14]. In the same time, Hamberger and colleagues (US patent PCT/EP94/01404) have demonstrated the presence of such inhibitor in different species of *Theromyzon* (*T. binannulatum*, *T. cooperi*, *T. garjaewi*, *T. maculosum* and *T. sexoculatum*). They showed that this inhibitor is a 9-kDa peptide with a pI of 4.9, a clotting time in a fibrinogen test of >600 s/5 µl, and a specific activity at the final step of purification of 25 IU for thrombin inhibition and 0.2 IU for factor Xa inhibition. Clotting fibrinogen assays performed confirmed the presence of thrombin inhibitor(s) in these gut leeches. In *T. tessulatum*, we have demonstrated that theromin significantly reduces the level of immunocyte activation, probably by inhibiting a "secondary" processing, namely the release or freeing of immunocyte excitatory peptides. In this context, theromin also diminishes this action and acts in conjunction with other *Theromyzon* serine protease inhibitors [14]. In fact, we showed that LPS stimulate immunocytes in a process that exhibits a primary and secondary phase of stimulation [14-17]. The LPS-stimulated secondary phase appears to require enzyme-processed secretory products released from immunocytes. Aprotinin, a serine protease inhibitor used in clinical situations, reduces or prevents the adverse effects caused by serine proteases during surgery. It inhibits bleeding and reduces the need for blood transfusions. In addition to its antifibrinolytic activity, aprotinin also diminishes the diffuse inflammatory response associated with major surgery [18].

Table 1: Sequence alignment of theromin and antistasin-type protease inhibitors using PALIGN algorithm of PC/GENE program.

Name	Sequences	Homology
Theromin	CENTECPRACPGEYEFDE.DGCNTCVCKG.CDDA.QCRCS.SDANGCESFC.TCNTRCSAADEC.	
therostasin	.....CENTEPR.ACPGEYEFDE.DGCNTCLCKG.CNDA.QCRIY.CPLGFTTDAN.GCESFCTCNTR.	80 %
therin	...R.CN.PPCAFN QDCDTTLGKC FCNG.YCKMA CHTGFKRDKN GCEI.CEPN.. .	12 %
hirustasin	.....TQGN TCGGETCSAA QVCLK..GKC VCNEVHCRIR CKYGLKKDEN GCEYPCSCAK ASQ	17 %
guamerin I	VDENAEDTHG LCGEKTCSPA QVCLN..NEC ACTAIRCMIF CPNGFKVDEN GCEYPCTCA. ...	17 %
guamerin II	DDENA..VDH DCGGKTCSPA QICVN..NEC ACTAIRCMIF CPDGFKVDEN GCEYPCTCA. ...	17 %
antistasin	.....EGPFG PCEEAGCPEG GACNIITDRC TCSGVRCRVH CPHGFQRSRY GCEF.CKCRL ...	25 %
ghilanten	.....EPMKA TCDISECPEG MMCSRLTNKC DCKIDICRKT CPNGLKRDKL GCEY.CEC.. ...	16 %

Table 2: Comparison of putative active site sequences of Theromin and antistasin-type protease inhibitors.

	P4	P3	P2	P1	P1'	P2'	P3'	Inhibitors of
Antistasin I	V	R	C	R	I	H	C	Factor Xa, Trypsin
Antistasin II	I	N	C	R	K	T	C	Factor Xa, Trypsin
Ghilanten	V	R	C	R	V	Y	C	Factor Xa, trypsin
Guamerin I	I	R	C	M	I	F	C	Elastase
Guamerin II	I	R	C	M	I	F	C	Chymotrypsin
Hirustasin	V	H	C	R	I	R	C	Capthepsin G, Trypsin
Tessulin	C	L	C	K	E	P	C	Chymotrypsin, Trypsin
Therin	Y	L	C	K	M	A	C	Trypsin
Theromin	N	G	C	E	S	F	C	Thrombin
Therostasin	A	Q	C	R	I	Y	C	Factor Xa

In 1994, Merck Company deposited a foreign patent application regarding three thrombin inhibitors of mass 3 kDa, 9 kDa and 14 kDa. Interestingly, we also found, in *T. tessulatum,* two thrombin inhibitors named T1a and T1b, respectively of 18 and 14 kDa in SDS-PAGE. The 9 kDa inhibitor presents the N-terminal sequence EDDNPGPPRACPGE (US patent PCT/EP94/01404). This peptide possesses the same N-terminal sequence as theromin (T1b). Theromin is a homodimer of 67 amino acid residues, with 16 cysteines engaged in eight disulfide bridges. Compared with hirudin, a natural single-chain peptide of 65 residues with three intrachain disulfide bridges and a sulfated tyrosine residue, both peptides are anionic and rich in cysteine residue [19]. While the hirudin N-terminus is globular and very tight owing to the presence of the three-disulfide bridges, and its C-terminus is rather light, theromin N-terminal sequence is highly anionic and its C-terminal part very tight, owing to the ten cysteine residues present there. Sequence alignment between hirudin and theromin revealed a highly conserved core of amino acid residues (fragments 20-40 in the theromin sequence) that could explain the thrombin inhibitory activity of the two molecules. The hirudin N-terminal sequence is known to interact with the catalytic site of thrombin [21]. In this context, amino acid residues 46 to 48 (PKP) are extremely important for the link between hirudin and thrombin [20]. The same PKP sequence has also been found in heamadin [13] and *H. manillensis* antithrombin peptides [12]. These types of interactions explain why hirudin only binds to thrombin and not to other serine proteases. By contrast, theromin does not possess such a dramatic signature in its sequence.

Theromin has no sequence homology with any other animal thrombin inhibitors so far isolated. However, if theromin is aligned with potentially homologous inhibitors based on cysteine positions, these match best with antistasin-type protease inhibitors. In this case, it possesses a slight sequence homology (24 %) with the antistasin-type protease inhibitor family (Table 1). The P1 residue of the reactive site of these inhibitors generally determines their specificity [20]. In antistasin, only the N-terminal domain is inhibitory, and the P1 residue is Arg34 (21). This residue corresponds to Arg30 of hirustasin, and Met35 in guamerin (I and II) [22-24]. In theromin, we hypothesize that Glu45 could be the P1 residue (Table 2). However, considering the low level of general sequence identity between theromin and peptides of the antistasin-type family, it is difficult to consider theromin as a new member of this family. Additionally, these molecules differ in amino acid sequence and inhibitory activity, some of them in three-dimensional structures that share the same motif as that of LAP [10]. Interestingly, the mechanisms of action and epitopes important for binding to their respective targets are distinct [10]. However, theromin does not possess such a consensus motif. By contrast, sequence comparisons carried out for theromin with the five different protease inhibitors isolated from the leech *T. tessulatum* – therin, therostasin, cytin, antitrypsin A and B and tessulin [14-17, 25, 26] – revealed that, three of the five peptides (therostasin, theromin and tessulin) have a high degree of sequence similarity (> 80 %), except for the amino acid residues surrounding the putative active site (Table 2). These together probably constitute a new protease inhibitors family.

Taken as a whole, the present results demonstrate that gut leeches possess thrombin inhibitor that presents different biological action than the ones found in jawed leeches. In fact, under the same conditions as hirudin, theromin demonstrated an activity on the S-2238 chromogenic substrate with approximately 17500 IU/mg of protein and a K_i value of 12 ± 5 fM. This inhibitory value is higher than that obtained with hirudin (21 fM) [7] and heamadin (100 fM) [13]. Theromin is a strict tight-binding thrombin inhibitor. Reduced and s-β-pyridylethylated theromin had little thrombin-binding activity, suggesting that dimerization is necessary to give the protein an active folded configuration for complete binding to thrombin. Nevertheless, the reduced molecule does display antithrombin activity, suggesting that each monomer possesses an active site. This could explain the high value of 12 fM for

theromin (dimer) compared with 21 fM for hirudin (monomer). Each monomer thus acts in synergy to block thrombin. Moreover, hirudin, at concentrations from 0.1 to 2 μM, induces vasodilatation of PGF2α-precontracted ring segments of porcine pulmonary arteries with intact endothelium [14]. We sought to determine if theromin could release endothelium-derived nitric oxide (NO) as well [14]. However, theromin, regardless of concentration, did not stimulate NO release from human saphenous vein endothelial cells. The vein fragments were judged to be in good condition because 1 μM morphine released NO (33.6 ± 4.6 nM NO) as expected [14].

All these data reflect that theromin may well be used clinically to curtail pathological processes where seemed appropriate, such as the diffuse inflammatory responses associated with major surgery [16].

2.2. Factor Xa inhibitor

Although thrombin inhibitors were sough to be restricted to the jawed leeches, factor Xa inhibitors were considered as the ones for gut leeches. In fact, two molecules of 119 amino acid residues with a potent antimetastatic activity, antistasin and ghilianthen, have been isolated in glossiphonid leeches *i.e. Haementheria officinalis* [8, 9] and *H. ghilianii*, respectively [10]. More recently, in *Hydra*, a cDNA coding for an antistasin-like protein with 2-fold internal repeat has been cloned, suggesting that antistasin has evolved by gene duplication [11]. However, in the hookworm *Ancylostoma caninum*, a small molecule with a molecular mass of 8.7 kDa and a high intrinsic K_i (323.5 pM) has been isolated [6].

In the rhynchobdellid leech *T. tessulatum*, considered to be as the most primitive leech [4], we purified a 14-kDa peptide from SDS-PAGE. This peptide contains 82 amino acid residues and has a calculated molecular mass of 8985.6 Da which is in good agreement with the molecular mass measured by MALDI-TOF (8985.5 Da)[17]. Moreover, based on a PCR strategy, therostasin cDNA was cloned. Therostasin cDNA encodes a mRNA of 882 bp, sequence whose size is consistent with 1000-nt size in Northern blot analysis assuming the length of the poly(A)$^+$ as *ca.* 100 nt. Translation of the open reading frame gives a 101-amino acid residues sequence that matches with the sequence of the mature protein [17]. The N-terminal Asp residue of mature therostasin is preceded by a 19-amino acid pre-peptide beginning with a Met residue. The 19-amino acid sequence contains a hydrophobic core of 17 amino acid flanked by two charged residues typical of a signal peptide [17]. Using an antibody raised against the N-terminal part of synthetic therosatasin on serial cuts of *T. tessulatum* performed at stage 2, just after the blood meal, a specific immunolabeling was found in salivary glands. This confirmed its involvement in coagulation inhibition during the blood meal.

A comparison of the amino acid sequence of this new inhibitor with other inhibitors of serine proteases showed only 25 % sequence homology with the *Hydra* antistasin-like protein [17]. Despite its size close to the hookworm anti-factor Xa (8.7 kDa, [6]), no sequence homology was found between the two molecules. By contrast, therostasin presents an endothelin family signature (25-39:CLCKGCNDAQCRIYC) like the sarafotoxins and bibotoxin, potent vasoconstrictors isolated from venom of *Atractaspis* snakes family [28, 29]. The consensus pattern is the following: C-x-C-x(4)-D-x(2)-C-x(2)-[FY]-C. Moreover, although similar in size to Kunitz-type protease inhibitors, therostasin is an acidic protein (pI 4.3), whereas the Kunitz inhibitors are generally highly basic [30]. Therostasin appears to be highly specific for factor Xa (K_i: 34 pM, [17]), because it did not inhibit trypsin or any other serine proteases *e.g.* chymotrypsin, elastase, cathepsin G and thrombin (even in 100-fold molar excess of therostasin over each protease), in contrast to Kunitz inhibitors [30]. The putative active site of therostasin would be localized at position 34-35 in the molecule as in

antistasin. The putative active site sequence (P4-P4': AQCRIYCP) presents a high homology with the ones found in other inhibitors of factor Xa (Table 2).

Structural as well as kinetic evidence presented here suggests distinct dissimilarities between therostasin and the other known inhibitors of factor Xa. Therostasin represents a novel coagulation inhibitor whose effectiveness *in vitro* makes it attractive to test in various *in vivo* models of occlusive vascular disease. In this goal, we determined if therostasin was biologically active in diverse cell systems, we examined its ability to diminish LPS-induced excitation of human granulocytes and monocytes. These immunocytes were chosen for their ability to release numerous enzymes that may participate in a further enhancement of immunocyte activation [17]. As previously observed, once human immunocytes are separated and placed on a slide, they exhibit a low level of spontaneous activation as noted by an increase in the number of cells becoming ameoboid [14-17]. In the presence of a previously demonstrated effective dose of LPS (1 U/ml), the number of activated cells significantly increases [17]. Interestingly, this response to LPS continues after it is washed off (data not shown) suggesting that a cascading process has been initiated. Preincubation of immunocytes with LPS followed by the serine protease inhibitor aprotinin, significantly diminished the immunocyte excitation caused by LPS in a concentration-dependent manner. These results suggest that enzymes were secreted, which may process released signaling molecules that would lead to the higher level of activation in the absence of aprotinin. Furthermore, the level of activation that occurs in the presence of aprotinin (21.1 ± 3.7 for 10^{-6} M aprotinin) is higher than that found in non-LPS exposed cells (10.8 ± 3.4. This last result also supports the hypothesis that the higher level of cellular activation seen in the presence of aprotinin is due to a cascade of signaling molecules post-LPS stimulation. In this regard, addition of therostasin also resulted in a concentration-dependent inhibition of cell activation (20 % ± 3.5 at 10^{-6} M and 43.2 % ± 2.5 at 10^{-9} M *vs* 48 % ± 3.6 in control), which was similar to that induced by aprotinin in response to LPS stimulation [15, 16]. These results demonstrate that after a primary stimulus, the cells are capable of enhancing their own level of stimulation by generating signaling molecules that must be processed. Additionally, therostasin is as active as aprotinin in diminishing LPS-induced activation, demonstrating that the material is active in cells obtained from animals 500 million years divergent in evolution.

3. Conclusion

Taken together, the anticoagulants found in *T. tessulatum* demonstrate that leeches have developed panoply of molecules that interfere with coagulation cascade [14-20] and host-communication [20]). We speculate that during a bite, starved leeches inject substances able to block the host pain, inflammation and to induce vasodilatation. In fact, a nociceptive stress due to the injury will initiate an inflammatory response with a great amount of leukocytes. Leeches try to avoid this scenario, which will provoke in the intestine, during months used to digest the blood meal, a release of blood-degrading enzymes contained in leukocytes [19, 20] So, the challenge for leeches is to block the peripheral nociception immediately during the bite and the local inflammation. In this context, the production of endocannabinoids (opiates known to be antinociceptive), neurosignaling molecules (ACTH, MSH, angiotensin II) and serine protease inhibitors as well as cysteine protease inhibitors like cystatin (unpublished data) is for them a survival strategy to escape host-immune defense 31, 32]. Leeches are a real reservoir of new powerful molecules to treat inflammation, coagulation, thrombosis, all aging diseases.

References

[1] S.J. Gardell SJ *et al.*, Isolation, characterization, and cDNA cloning of a vampire bat salivary plasminogen activator, *J. Biol. Chem.* **264** (1989) 17947-17952.

[2] L. Waxman *et al.*, Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation factor Xa, *Science* **248** (1990) 593-596.

[3] S.P. Jordan *et al.*, Tick anticoagulant peptide: kinetic analysis of the recombinant inhibitor with blood coagulation factor Xa, *Biochemistry* **29** (1990) 11095-11100.

[4] R.T. Sawyer, Leech Biology and Behavior, Oxford Science Publications, Oxford, Vol II, 1986.

[5] R.T. Sawyer, Thrombolytics and anti-coagulants from leeches, *Biotechnology* **9** (1991) 513-518.

[6] M. Cappello *et al.*, *Ancylostoma caninum* anticoagulant peptide: a hookworm-derived inhibitor of human coagulation factor Xa, *Proc. Natl. Acad. Sci. USA* **92** (1995) 6152-6156.

[7] F. Markwardt, Isolation and chemical characterization of hirudin, *Hoppe Seyler Zschr.* **308** (1957) 147-156.

[8] G.P. Tuszynski *et al.*, Isolation and characterization of antistasin. An inhibitor of metastasis and coagulation, *J. Biol. Chem.* **262** (1987) 9718-9723.

[9] J.H. Han *et al.*, Cloning and expression of cDNA encoding antistasin, a leech-derived protein having anti-coagulant and anti-metastatic properties, *Gene* **75** (1989) 47-57.

[10] A.M. Krezel *et al.*, Structure of the RGD protein decorsin: conserved motif and distinct function in leech proteins that affect blood clotting, *Science* **264** (1994) 1944-1947.

[11] T.W. Holstein *et al.*, The primitive metazoan Hydra expresses antistasin, a serine protease inhibitor of vertebrate blood coagulation: cDNA cloning, cellular localisation and developmental regulation, *FEBS Lett.* **30** (1992) 288-292.

[12] F.S. Hong and WK Kang WK, Purification of granulin-like polypeptide from the blood-sucking leech, *Hirudo nipponia, Protein Expr. Purif.* **16** (1999) 340-346.

[13] A. Electricwala *et al.*, The complete amino acid sequence of a hirudin variant from the leech *Hirudinaria manillensis*, *J. Protein Chem.* **12** (1993) 365-370.

[14] K.-H. Strube *et al.*, Isolation, sequence analysis, and cloning of haemadin. An anticoagulant peptide from the Indian leech, *J. Biol. Chem.* **268** (1993) 8590-8595.

[15] M. Salzet *et al.*, Theromin, a novel leech thrombin inhibitor, *J. Biol. Chem.* **275** (2000) 30774-30780.

[16] V. Chopin *et al.*, Amino acid sequence determination and biological activity of therin, a naturally occuring specific trypsin inhibitor from the leech *Theromyzon tessulatum*, *Eur. J. Biochem.* 254 (1998) 565-570.

[17] V. Chopin *et al.*, Amino-acid-sequence determination and biological activity of tessulin, a naturally occurring trypsin-chymotrypsin inhibitor isolated from the leech *Theromyzon tessulatum*, *Eur. J. Biochem.* **256** (1998) 662-668.

[18] V. Chopin *et al.*, Therostasin, a novel clotting factor Xa inhibitor from the rhynchobdellid leech, *Theromyzon tessulatum*, *J. Biol. Chem.* **275** (2000) 32701-32707.

[19] D. Roston, Preventing the inflammatory response to open-heart surgery: the role of aprotinin and other protease inhibitors, *Int. J. Cardiol.* **53** (1996) suppl: S11-S37.

[20] M. Salzet, Anticoagulants and inhibitors of platelet aggregation derived from leeches, *FEBS Lett.* **492** (2001) 187-192.

[21] M. Salzet *et al.*, Serine protease inhibitors: an evolutionarily conserved survival strategy, *Immunol. Today* **20** (1999) 541-544.

[22] G.J. Gasic *et al.*, Leech salivary gland extract from *Haementeria officinalis*, a potent inhibitor of cyclophosphamide- and radiation-induced artificial metastasis enhancement, *Cancer Res.* **44** (1984) 5670-5676.

[23] C. Söllner *et al.*, Isolation and characterization of hirustasin, an antistasin-type serine-proteinase inhibitor from the medical leech *Hirudo medicinalis*, *Eur. J. Biochem.* **219** (1994) 937-943.

[24] H.I. Jung *et al.*, Isolation and characterization of guamerin, a new human leukocyte elastase inhibitor from *Hirudo nipponia*, *J. Biol. Chem.* **70** (1995) 13879-13884.

[25] D.R. Kim *et al.*, A cysteine-rich serine protease inhibitor (Guamerin II) from the non-blood sucking leech *Whitmania edentula*: biochemical characterization and amino acid sequence analysis, *J. Enzyme Inhib.* **10** (1996) 81-91.

[26] V. Chopin *et al.*, Amino-acid-sequence determination and biological activity of cytin, a naturally occurring specific chymotrypsin inhibitor from the leech *Theromyzon tessulatum*, *Eur. J. Biochem.* **249** (1997) 733-738.

[27] V. Chopin *et al.*, Biochemical evidence of specific trypsin-chymotrypsin inhibitors in the rhynchobdellid leech, *Theromyzon tessulatum*, *J. Enzyme Inhib.* **15** (2000) 367-379.

[28] Y. Kloog and M. Sokolosvsky, Similarities in mode and sites of action of sarafotoxins and endothelins, *Trends Pharmacol. Sci.* **10** (1989) 212-214.
[29] M. Sokolovsky, Endothelins and sarafotoxins: physiological regulation, receptor subtypes and transmembrane signaling, *Trends Biochem. Sci.* **16** (1991) 261-264.
[30] M. Laskowski and I. Kato, Protein inhibitors of proteinases, *Annu. Rev. Biochem.* 49 (1980) 593-626.
[31] M. Salzet, Molecular neuroimmune processes in invertebrates, *Brain Res. Rev.* **34** (2000) 69-79.
[32] M. Salzet *et al.*, Molecular cross-talk in host parasite relationships: schistosomes or leeches-host interactions, *Parasitol. Today* 12 (2000) 536-540.

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Other Invertebrate Models

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A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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The ancient principle of membrane permeabilization: structure and function of evolutionarily divergent cytolytic and antimicrobial polypeptides

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Abstract. As comparative immunobiology was not primarily concerned with unicellular organisms, important molecular mechanisms to fight against potential pathogens such as the use of antimicrobial and cytolytic peptides have neither been anticipated nor characterized below the phylogenetical level of metazoa. We summarize here our findings with membrane-permeabilizing polypeptides of amoeboid organisms and thereby demonstrate that the most ancient phylogenetic location of a gene-encoded antimicrobial and cytolytic armamentarium in eukaryotes has been found in protozoa. Accumulated evidence indicate that amoebapores, pore-forming polypeptides of *Entamoeba histolytica*, the causative agent of human amoebiasis, are essential elements of the cytolytic machinery of the pathogen. These factors diplay also activity against bacteria and this may reflect the primary function of amoebapores during the essential part of the life cycle within the human colon where the amoebae uses bacteria as a major nutrient source. We found similar proteins in free-living amoeboid protozoa which are potentially highly pathogenic for humans. Amoebapores reveal a substantial sequence similiarity with effector proteins of porcine and human cytotoxic lymphocytes, namely NK-lysin and granulysin. We have compared the structures of the amoebic and mammalian proteins and monitored their biological activities in parallel to extract the similarities and differences of effector molecules from organisms, the evolutionary paths of which diverged so early.

1. Introduction

Nowadays it is generally accepted that the ability of an organism to protect itself against potential pathogens and to combat infection is not a privilige of vertebrates. On the contrary, highly effective defense mechanisms and many potent effector molecules that kill prokaryotic and/or eukaryotic invaders have been found in invertebrates, the vast majority in arthropods. Recent overviews on the comparison of similar principles and molecules of arthropods and mammalia to combat infection are available [1, 2]. Despite the fact that for most people the search for mechanisms relevant in immunobiology starts with the defensive systems of metazoa, already the classical observations of Metchnikoff suggested that amoeboid cells are main players in cellular immunity of animals and that free-living amoebae may be the progenitors of professional phagocytes. As a consequence, the ancient principles of killing microbes by more or less specific proteins most likely has evolved before the appearance of metazoa and amoebae may be a rich source of such antimicrobial molecules. This article addresses the narrow but presumably to some extent representative topic of membrane-permeabilization of targets by similar polypeptides of amoebae and mammals.

2. *Amoebae – primitive phagocytes and 'unprofessional' killer cells*

Amoebae are a nonhomogeneous group of unicellular organism, which are widespread in nature and colonize various environments. Whereas the majority of them are free-living amoebae of soil and water, some are parasites of vertebrates. Modern techniques to evaluate the phylogenetic relationships between amoebic species made clear that the term amoebae suits rather to describe a form of lifestyle which is used to survive as a protozoon under various conditions than to a taxonomic definition. Amoebae may be viewed as insatiable phagocytic cells that use primarily bacteria or other protozoa as a nutrient source. The high capacity of amoebae to phagocyte these foreign cells implies that they possess a mechanism by which microbes can be continually engulfed and then efficiently killed and degraded.

Entamoeba histolytica is a particularly well known amoebic species because it is a human pathogen of substantial medical importance. As the name implies, lysis of cells and tissues is a prominent activity of the parasite. The extraordinary efficacy and rapidity of amoebae in killing host cells including immune effector cells in a contact-dependent and surface lectin-mediated reaction is highly impressive and accordingly *E. histolytica* has been viewed as an archaic cytotoxic cell that must possess an lytic principle which also kill metabolically active eukaryotic cells. We have hypothesized that the effective armamentarium used intracellularly against bacteria and extracellularly against host cells may overlap.

3. *Amoebapores and their relatives*

Lysosome-like granular vesicles of amoebae contain – beside several hydrolytic enzymes such as lysozymes, cysteine proteases and phospholipases – a family of small proteins that cause pore formation in artificial membranes that are termed *amoebapores*. As this amoebic species is a protozoan parasite and the causative agent of human amoebiasis, amoebapores have been studied rather as putative pathogenicity factors involved in the destruction of host tissues than as defensive molecules. Accumulated evidence suggests that these pore-forming proteins indeed represent an essential part of the cytolytic machinery of the pathogenic amoeba [3-5]. Amoebapores are cytolytic towards human nucleated cells *in vitro* in a dose-dependent manner and in a range comparable to the cytotoxic peptide of bee venom, melittin. Upon the contact-dependent cytolytic reaction, discharge of granules into the confined space of the contact zone between the amoeba and a host cell presumably mediates the deadly encounter of the cytotoxic amoebapores with target cells. As demonstrated recently, inhibition of amoebapore synthesis in amoebae by antisense technology resulted in drastically decreased cytolytic activity of the transformed trophozoites and impaired liver abscess formation in hamsters demonstrating that the pore-forming proteins are important amoebic virulence factors.

As pathogenic amoebae cannot proliferate in the tissues they invade, it has been suggested that coincidental selection for some other functions maintain genes encoding tissue-damaging proteins of *E. histolytica*. The observation that the related but non-pathogenic species *E. dispar* possesses homologous pore-forming proteins [6, 7] strengthened the notion that those gene products enabling the parasite *E. histolytica* to kill hosts cells so efficiently have other primary and more vital functions in the life of amoebae. Living on intestinal bacteria, the amoebae may first of all use these weapons to kill their prey. Indeed, the action of all these polypeptides against bacterial cytoplasmic membranes emphasizes that prevention of growth of engulfed bacteria within the amoebic phagosomes most likely is the primary functional role of these molecules. The amoebapores display potent antibacterial activity by perturbing

the integrity of bacterial cytoplasmic membranes. Immunofluorescence imaging by confocal laser microscopy revealed *in vivo* amoebapore-bacteria interactions in phagosomes. Taken together, the amoebapores most likely have primarily the function to combat growth of phagocytozed bacteria inside digestive vacuoles but appear to be instrumental in the extracellular killing of host cells by the protozoan parasite.

Among the several groups of antimicrobial and membrane-permeabilizing peptides classified so far, the one to that amoebapores belong is extraordinary. Its members are relatively large polypeptides and are characterized by a compact alpha-helical and disulfide-bonded fold. The primary structure of all three amoebapore isoforms was resolved by protein sequencing of the N-terminus and subsequent molecular cloning of the genes [8, 9]. The sequences each comprise a signal peptide of 19 to 24 amino acid residues and a mature protein of 77 residues. Analysis of the sequences reveals a membership to the family of saposin-like proteins (SAPLIP). This family is characterized by amphipathic sequence motifs with a conserved location of six cysteine residues and by their ability to interact with lipids regardless of the many different biological functions they fulfill [10]. Members of the family include saposins A-D, surfactant protein B, human acid sphingomyelinase and, in a circularly permutated form, some plant aspartic proteases called swaposins. Two functional analogs of amoepapores also bearing the cysteine motif were recognized in NK-cells and cytolytic T-cells of pig [11] and man [12] and were named NK-lysin and granulysin, respectively. Like amoebapores, these proteins also reside in intracellular granules, exhibit antibacterial and cytolytic activity and are not effective against red blood cells. As granulysin reveals a broad antimicrobial spectrum but does not permeabilize macrophages, a synergistic effect with the pore-forming protein of lymphocytes, perforin, has been hypothesized for killing of intracellular pathogens [13].

With regard to the sequence, granulysin is an exception within the SAPLIP family in that it contains only four cysteine residues involved in two disulfide bridges. The protein is N- and C-terminally processed. The truncation of 9 amino acid residues at the C-terminus implements the loss of one cysteine and the supposed partner for a disulfide bond at the N-terminus is exchanged to a tyrosine residue – presumably by evolution because there is no advantage of an unpaired cysteine residue for the stability of the protein. For NK-lysin, two slightly different sequences are reported which presumably represent isoforms. Several synthetic analogs of amoebapores, NK-lysin and granulysin, which are substantially reduced in size compared to the parent molecules, were tested as antimicrobial agents against clinical isolates of microbes [14-17]. As some of these peptides are highly active against Gram-positive and Gram-negative bacteria and the pathogenic yeast *Candida albicans* but are of low cytotoxicity towards human cell lines they may provide promising templates for the design of broad-spectrum peptide antibiotics.

We isolated and molecularly characterized amoebapore counterparts in related *Entamoeba* species. Moreover, we found most recently pore-forming proteins also in other species of amoeboid protozoa such as *Naegleria fowleri* and *Acanthamoeba culbertsoni*. These organisms are free-living in soil and water but are also known as potentially highly pathogenic species causing severe forms of encephalitis and abscesses of the cornea.

For several invertebrate species sequences containing this motif can be obtained from the database. Numerous putative gene products of unknown biological function with amoebapore-like sequence motifs are from the bacteria-feeding nematode *Caenorhabditis elegans* [18]. As other *C. elegans* gene products are quite similar to the amoeba lysozymes, which are substantially different from other animal lysozymes [19], one may speculate that a common bacteriolytic principle exists in such phylogenetically diverse organisms as amoebae and nematodes. However, without certainty about the natural existence of the encoded

proteins and characterization of their function, it is speculative to consider the nematode products as homologues of the amoebic proteins.

In view of the structural resemblance between amoebapores and the mammalian molecules NK-lysin and granulysin, it is tempting to propose that these proteins share a common ancestry and function by perforating various target cells [20]. In the following, we will outline the similarities and differences between the members of the "amoebapore group".

4. Structural implications and modes of membrane permeabilization

The three-dimensional structure of NK-lysin has been resolved by NMR [21]. This protein reveals a highly stable fold of five α-helices connected by three disulfide bonds. The amoebapores are also all α-helical proteins devoid of random regions or β-sheets as evidenced by circular dichroism studies with isoform A [8]. Due to the sequence homology in combination with the similarity in secondary structure, the conserved position of the cysteine residues and the shared function of membrane binding, all members of the SAPLIP family are postulated to adopt the same fold as shown for NK-lysin and the SAPLIP domain of phytepsin, an aspartic protease of barley [22]. Another strong indication for this hypothesis is the occurrence of gaps in the sequence alignment only in positions corresponding to loops in the NK-lysin structure. The putative three-dimensional structure of amoebapore A built by homology modeling on the template of NK-lysin [23] is shown in Fig. 1. The helices form two layers: the first comprises the hairpin of helix 2 and 3 and the second is represented by helix 1 packed against helix 5. The two layers are closed at one edge by helix 4 and the loop between helix 1 and 2. Between the two layers exclusively hydrophobic interactions are detectable. The disulfide bridges are each formed within a layer and contribute to the remarkable thermostability of the fold.

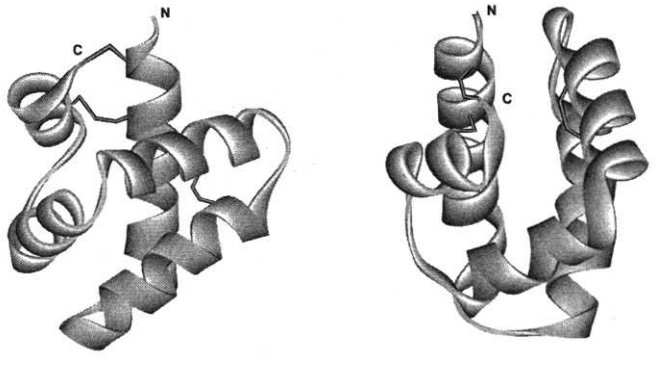

Fig. 1: Model of the three-dimensional structure of amoebapore A in two views rotated by 90°. The amoebapore molecule is modelled according to the tertiary structure of mammalian NK-lysin solved by NMR. The N- and C-termini are marked and the three disulfide bonds stabilizing the structure are shown as brackets.

Many studies aimed to understand the mode of action of membrane-active antimicrobial peptides have been reported in the past years, but the precise molecular mechanism of most of these molecules is still an unclear subject. For the class of linear α-helical peptides of up to 40

amino acids residues several possible mechanisms of membrane disruption have been described [for review 24]. In the classical view, a pore is formed by the barrel stave mechanism [25]: the peptides oligomerize and insert into the membrane resulting in a stable bundle of amphipathic helices in a transmembrane orientation facing their hydrophobic residues toward the lipids and the polar surface towards the center of the pore. An alternative mode of action postulated for the majority of such peptides is the carpet mechanism [26]: the peptides initially bind to the phospholipid headgroups by electrostatic interactions until a threshold concentration is reached. This enrichment enables the peptides to insert randomly into the membrane thereby disrupting it in a detergent-like manner. The step preceding the micellization is the formation of transient holes which differ from the pores created by the barrel-stave mechanism in that they are lined by phospholipid headgroups (toroidal model) [27, 28]. Any peptide that greatly changes the curvature of a stable membrane bilayer will promote pores or holes regardless whether this is caused by stable transmembrane oligomers or by membrane perturbation according to the carpet model.

For NK-lysin, membrane-permeating activity has been shown using large unilamellar vesicles [29]. By FTIR spectroscopy neither a transmembrane orientation of its helix bundles nor an arrangement of all the helices parallel to the membrane surface have been observed which suggests that the polypeptide perturbs the membrane without a gross conformational change and that a deep and permanent insertion of protein domains does not take place [29]. Considering the structural features, the charge distribution and moreover the electrostatic potential of NK-lysin supports this idea: the region with the most positive potential comprises helix 3 which presumably interacts with negatively charged phospholipid headgroups and would permit when orientated parallel to the membrane surface an adjacent hydrophobic patch to dip into the membrane [30]. The model of the three-dimensional structure of granulysin shows a strikingly positive charged surface virtually distributed over the entire molecule. These positive charges enable a strong interaction with negatively charged lipopolysaccharides and phospholipid headgroups of bacterial membranes resulting in an interference with the highly ordered lipid arrangement [31]. The plausible mode of action is again rather membrane perturbation than stable insertion into the phospholipid bilayer. Positively charged residues are significant for activity of amoebapores as well, particularly for binding to the membrane and oligomerization [32], but the amoebic polypeptides do not show any of the aforementioned characteristic charge distributions. On the contrary, they possess a virtually neutral net charge without a detectable bias of charged patches at the surface. Another feature that all amoebapores have in common is an unpolar region around the hairpin of helix 2 and 3 suitable for membrane insertion. Significantly, in contrast to NK-lysin [29], amoebapore A induces discrete current fluctuations in planar lipid bilayer experiments characteristic for an ion channel [33], also indicating a different mechanism of membrane interaction compared to NK-lysin and granulysin.

Concerning the size of the proteins, mechanisms proposed for small antimicrobial peptides may not suit a 77-residue entity and the insertion of the entire protein may be excluded. A remarkable feature of the amoebapores is large hydrophobic areas on the accessible surface, which contain in addition large caves appropriate for initial lipid binding. These caves reach into the hydrophobic core of the proteins indicating locally loose packing of the two layers and therefore reducing the energy barrier for structural rearrangement. These structural features of amoebapores suggest a mechanism of global reorganization and concomitant membrane insertion of the hairpin of helix 2 and 3 following membrane binding and oligomerization.

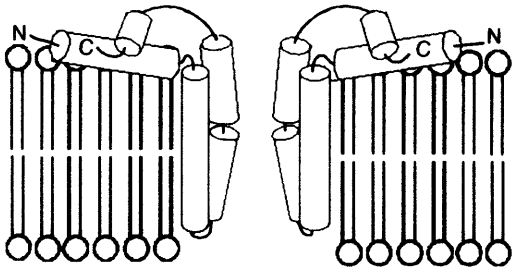

Fig 2: Model for the topology of amoebapores upon insertion into membrane phospholipid bilayers. Two molecules are shown. Whereas for the helices 1 and 5 an in-plane orientation is suggested, helices 2,3 and 4 are shown with a trans-membrane orientation. Although peptide oligomerization has been experimentally proven for amoebapores, the number of monomers needed to generate a transmembrane pore is unknown.

5. Conclusion

Amoebapores appear to fulfill the criteria of pore-forming proteins whereas the activities of NK-lysin and granulysin may be best described as membrane-perturbing. These differences in their mode of action are also reflected in their biological activities: only the amoebapores exhibit a pronounced pH-dependence of all their activities with virtually no activity at pH above 6. Whereas all proteins are potent against Gram-positive bacteria, only NK-lysin is highly efficient against Gram-negative bacteria. However, amoebapore A is about five times more active in pore formation than NK-lysin. Thus, structurally and functionally similar proteins achieve the aim of membrane destabilization by different mechanisms. This rises the interesting but unanswered question whether a common ancestor molecule had differentiated into several polypeptides with different modes of action. In other words: are amoebapores indeed ancient homologues of the mammalian effector molecules involved in innate immunity or has a structurally preferred protein fold asserted itself independently and took over comparable functions by convergent evolution? The identification and functional characterization of structural correlates from invertebrates that express antimicrobial and/or cytolytic activities and therefore may be viewed as belonging to the "amoebapore group" of the SAPLIPs will presumably shed some light on the evolution of these interesting effector polypeptides.

References

[1] T. Michel *et al.*, *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein, *Nature* **414** (2001) 756-759.

[2] M. Zasloff, Antimicrobial peptides of multicellular organisms, *Nature* **415** (2002) 389-395.

[3] M. Leippe *et al.*, Cytolytic and antibacterial activity of synthetic peptides derived from amoebapore, the pore-forming peptide of *Entamoeba histolytica*, *Proc. Natl. Acad. Sci.* USA **91** (1994) 2602-2606.

[4] O Berninghausen and M. Leippe, Necrosis versus apoptosis as the mechanism of target cell death induced by *Entamoeba histolytica*, *Infect. Immun.* **65** (1997) 3615-3621.

[5] R. Bracha *et al.*, Antisense inhibition of amoebapore expression in *Entamoeba histolytica* causes a decrease in amoebic virulence, *Mol. Microbiol.* **34** (1999) 463-472.

[6] M. Leippe *et al.*, Comparison of pore-forming peptides from pathogenic and nonpathogenic *Entamoeba histolytica*, *Mol. Biochem. Parasitol.* **59** (1993) 101-109.
[7] R. Nickel *et al.*, Pore-forming peptides of *Entamoeba dispar*. Similarity and divergence to amoebapores in structure, expression and activity, *Eur. J. Biochem.* **265** (1999) 1002-1007.
[8] M. Leippe *et al.*, Primary and secondary structure of the pore-forming peptide of pathogenic *Entamoeba histolytica*, *EMBO J.* **11** (1992) 3501-3506.
[9] M. Leippe *et al.*, Amoebapores, a family of membranolytic peptides from cytoplasmic granules of *Entamoeba histolytica*: isolation, primary structure, and pore formation in bacterial cytoplasmic membranes, *Mol. Microbiol.* **14** (1994) 895-904.
[10] R.S. Munford *et al.*, Saposin-like proteins (SAPLIP) carry out diverse functions on a common backbone structure, *J. Lipid Res.* **36** (1995) 1653-1663.
[11] M. Andersson *et al.*, NK-lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity, *EMBO J.* **14** (1995) 1615-1625.
[12] S.V. Pena *et al.*, Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins, *J. Immunol.* **158** (1997) 2680-2688.
[13] A.M. Krensky, Granulysin: a novel antimicrobial peptide of cytolytic T lymphocytes and natural killer cells, *Biochem. Pharmacol.* **59** (2000) 317-320.
[14] J. Andrä *et al.*, Shortened amoebapore analogs with enhanced antibacterial and cytolytic activity, *FEBS Lett.* **385** (1996) 96-100.
[15] J. Andrä and M. Leippe, Candidacidal activity of shortened synthetic analogs of amoebapores and NK-lysin, *Med. Microbiol. Immunol.* **188** (1999) 117-124.
[16] D. Andreu *et al.*, Identification of an anti-mycobacterial domain in NK-lysin and granulysin, *Biochem. J.* **344** (1999) 845-849.
[17] Z. Wang *et al.*, Bactericidal and tumoricidal activities of synthetic peptides derived from granulysin, *J. Immunol.* **165** (2000) 1486-1490.
[18] L. Banyai and L. Patthy, Amoebapore homologs of *Caenorhabditis elegans*, *Biochim. Biophys. Acta* **1429** (1998) 259-264.
[19] R. Nickel *et al.*, Molecular characterization of an exceptionally acidic lysozyme-like protein from the protozoon *Entamoeba histolytica*, *FEBS Lett.* **437** (1998) 153-157.
[20] M. Leippe, Ancient weapons: NK-lysin, is a mammalian homolog to pore-forming peptides of a protozoan parasite, *Cell* **83** (1995) 17-18.
[21] E. Liepinsh *et al.*, Saposin fold revealed by the NMR structure of NK-lysin, *Nat. Struct. Biol.* **4** (1997) 793-795.
[22] J. Kervinen *et al.*, Crystal structure of plant aspartic proteinase prophytepsin: inactivation and vacuolar targeting, *EMBO J.* **18** (1999) 3947-3955.
[23] H. Bruhn and M. Leippe, Comparative modeling of amoebapores and granulysin based on the NK-lysin structure-structural and functional implications, *Biol. Chem.* **380** (1999) 1001-1007.
[24] B. Bechinger, . The structure, dynamics and orientation of antimicrobial peptides in membranes by multidimensional solid-state NMR spectroscopy, *Biochim. Biophys. Acta* **1462** (1999) 157-83.
[25] G. Boheim, Statistical analysis of alamethicin channels in black lipid membranes, *J. Membr. Biol.* **19** (1974) 277-303.
[26] Y. Shai, Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides *Biochim. Biophys. Acta* **1462** (1999) 55-70.
[27] S.J. Ludtke *et al.*, Membrane pores induced by magainin, *Biochemistry* **35** (1996) 13723-13728.
[28] K. Matsuzaki, Magainins as paradigm for the mode of action of pore forming polypeptides, *Biochim. Biophys. Acta* **1376:** (1998) 391-400.
[29] J.M. Ruysschaert *et al.*, Lipid membrane binding of NK-lysin, *FEBS Lett.* **425** (1998) 341-344.
[30] M. Miteva *et al.*, Molecular electroporation: a unifying concept for the description of membrane pore formation by antibacterial peptides, exemplified with NK-lysin, *FEBS Lett.* **462** (1999) 155-158.
[31] W.A. Ernst *et al.*, Granulysin, a T cell product, kills bacteria by altering membrane permeability, *J. Immunol.* **165** (2000) 7102-7108.
[32] J. Andrä and M. Leippe, Pore-forming peptide of *Entamoeba histolytica*. Significance of positively charged amino acid residues for its mode of action, *FEBS Lett.* **354** (1994) 97-102.
[33] M. Leippe and H.J. Müller-Eberhard, The pore-forming peptide of *Entamoeba histolytica*, the protozoan parasite causing human amoebiasis, *Toxicology* **87** (1994) 5-18.

A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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From earthworm to mussel innate immunity

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Défense et Résistance chez les Invertébrés Marins (DRIM)
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Abstract. Cellular immune reactions were first studied in earthworms, both *in vivo* using graft rejection assays and *in vitro* involving various stimulations. Cytotoxic activity of the plasma was soon recognized as part of the innate immunity, and several large proteins were identified. Also in mussel, a cytolytic complex was isolated as well as number of small cationic proteins. Such peptides arranged in 4 families - defensins, mytilins, myticins and mytimycin - encompassed isoforms. Similarities between gene, cDNA and precursor organizations argue in favor of a common ancestor origin. The peptides are continuously synthesized, and stored as active molecules in specific granules. In the course of an anti-infectious response, the peptides are discharged into phagosome-containing bacteria as soon as they are phagocytozed, then are released into the plasma for a systemic response.

1. Introduction

Infections and injuries are of the major causes that threaten all the living creatures. Therefore, any mechanisms that limit their consequences have enormous survival value. Immunity is the first, as a function of being exempt or not affected by pathogens, healing referred to the second, restoring health. Since the pioneering works of Metchnikoff in the late 19th century, phagocytosis was not any longer seen as a simple nutrition process. The active free cells or macrophages "are among the most active of phagocytes" [1]. Inflammation referred to another process of segregation and elimination of relatively nontoxic elements and was reported in earthworms nearly a century ago [2]. Numerous works developed since on wound healing and graft rejection, with the conclusion on the existence of complex circulating cell populations continuously recruiting and interacting (Fig. 1; see [3-11]). Meanwhile, if histocompatibility in invertebrates was almost admit, both cellular antigens and the related genes are still missing.

Hemolytic capability of earthworm coelomic fluid was first reported in 1968 in *Eisenia fetida*, in an experiment designed to reveal complement-like activity [12]. Since that date, numerous reports concentrated on that activity (see other articles in this issue), including genetic basis [13], various biochemical characteristics [14–16], interaction with target cell membranes [17], cDNA cloning [18-19] and cellular expression [20]. Even if difficult to evidence, agglutination was also reported using fixed targets and active molecules partially purified [21-24]. From all this literature, it can be concluded that, in addition to free cells involved in immune recognition and antigen processing, several free molecules are also part of the earthworm immune system. Moreover, at least for the hemolytic activity, the large number of isoforms genetically coded [25] is questioning on the complexity-redundancy and/or specificity of the system.

Cytotoxic activity in mussel *Mytilus edulis* was first reported in 1984 [26] as lyzing erythrocytes, but few works were done since.

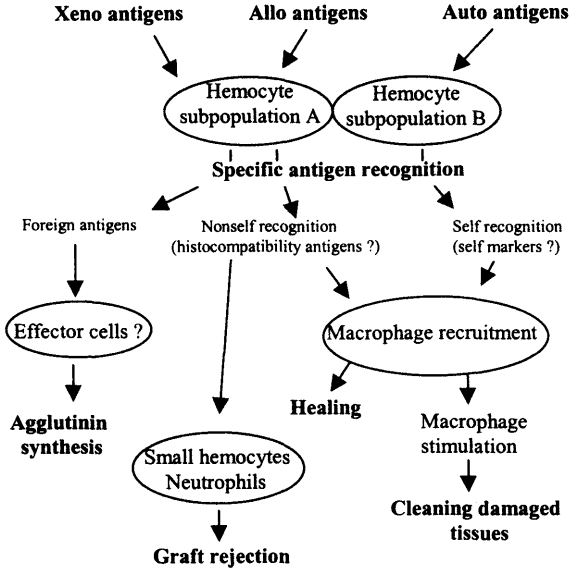

Fig. 1: Specific hemocyte activities and cell recruitment as deducted from numerous reports [3-10] and attempt to explain cell interactions [11].

Cecropin was the first antibacterial protein reported from diapausing pupae of the moth *Hyalophora cecropia* [27]. Its implication in immune defense was inferred from the fact that the activity was induced by a bacterial challenge. Since that date, more than two hundred antimicrobial small proteins, named peptides, have been isolated from insects [28, 29]. They are quite universal - as found either in plants, invertebrates and vertebrates, including mammals [reviewed in 30] - and they constitute the key elements of the innate immunity. One time, innate immunity was considered in vertebrates only to provide rapid but incomplete antimicrobial response, until the more specific and potent but slower acquired immunity develops. Nowadays, innate immune system is rather regarded to be essential to the function of adaptive immunity by determining which antigens will trigger the acquired immune system and what will be the nature of the response [31]. Acquired immunity does not exist in invertebrates. Meanwhile, similarities between pathogen recognition-signaling pathways, and effector mechanisms of innate immunity, both in mammals and in *Drosophila,* pointed to a common ancestry of these defenses [32].

No data is available on earthworm antibacterial peptides although antimicrobial defense due to larger molecules exists [33 and other articles of this issue]. On the opposite, the situation is well established in mussel, with the sequences of several peptides, the corresponding cDNAs and genes, and differential involvement in immune response described.

2. The mussel cytolytic complex

Up-to-date data on earthworm cytolytic molecules are reported in other articles of this issue. Concerning bivalves, a modification of the *in vitro* plaque assay has been employed to

demonstrate the presence of cytolytic molecules in plasma and their secretion by hemocytes [26, 34]. Also the plasma of the Mediterranean mussel, *Mytilus galloprovincialis,* contains cytotoxic activity against both vertebrate (erythrocytes and mouse tumor) and protozoan cells. The prokaryotic bacteria, *Escherichia coli* and *Vibrio alginolyticus,* were not sensitive to this cytotoxicity [35]. Injection of erythrocytes stimulated the cytotoxic activity of the plasma, suggesting that cytotoxic molecules be involved in immune defense [36]. Purification by anion exchange chromatography followed by gel filtration revealed a 320-kDa cytotoxic polymeric protein [37]. Composed of three different proteins, the complex acts through a heteropolymerisation process after binding onto target cell membranes as revealed by ultrastructure observation.

3. Investigating on antimicrobial peptides

A large variety of techniques have been used to purify antimicrobial peptides. In insects, the peptides are not present in the hemolymph of control animals, but are synthesized and released into the circulation following injury or injection. Consequently, the main source is hemolymph of challenged animals, mostly larvae, which will be submitted to different steps of purification (Fig. 2). In the case of the mussel *M. galloprovincialis*, both plasma and hemocytes, and particularly hemocyte granule fraction, were used.

As for insects, mussel antimicrobial peptides were purified assuming they are of small molecular mass and cationic [38]. Therefore, acidic treatment eliminated the large proteins and hydrophobic solid phase extraction allowed to recover only the positively charged molecules. Several other hydrophobic interactions were performed in HPLC, combined with high efficiency gel filtration. In each step, biological activities were located all along the fractions both against bacteria and fungi. Finally, purity of the peptides and molecular mass were estimated using mass spectrometry in electrospray ionisation. Primary amino acid sequences were established after reduction and alkylation of the possible cysteines.

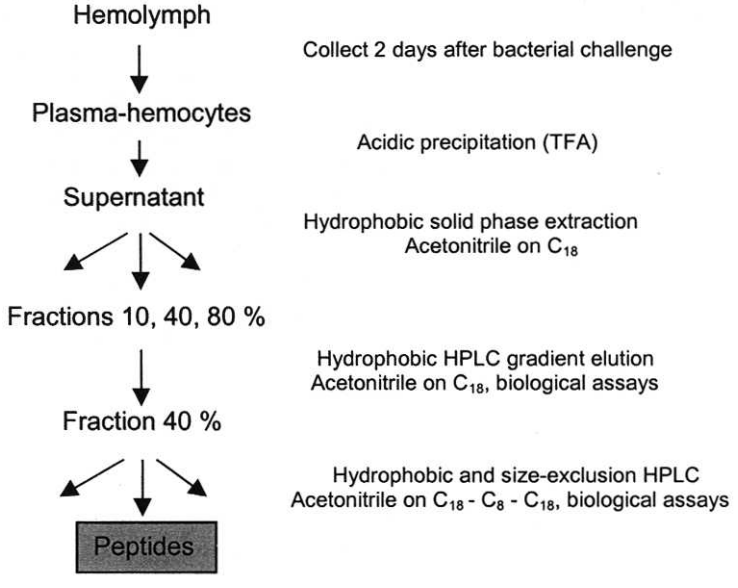

Fig. 2: General protocol to purify small molecular mass, cationic antimicrobial peptides.

4. The mussel antimicrobial peptides

Primarily, two arthropod defensin-like peptides were isolated from the plasma of bacteria-challenged *M. galloprovincialis* [39]. Hydroxylated tryptophan in position 28, non-essential for biological activities, represents a unique situation among the defensins isolated so far. Applying the same technology to hemocyte granules, two other peptide families, mytilin and myticin, were also identified [40-41]. All these families encompassed several slightly different molecules regarded as isoforms (Fig. 3). They all belong to the cysteine-rich antimicrobial peptides (Table 1), and are characterized by the presence of eight cysteines defining the typical patterns of the three families. Also in *M. edulis* collected in White Sea (North Karelia-Russia), several antimicrobial peptides were purified, two being antifungal and six being antibacterial [42]. The main difference is on the presence of only six cysteines instead of eight as a common feature for *M. galloprovincialis* peptides. In addition, a 6.2-kDa antifungal protein with probably twelve cysteines, mytimycin, and a 15-kDa non-sequenced antibacterial protein were also identified.

Even if sharing the term defensin, both the sequences and the 3D-structures of mussel and arthropod defensins are totally different from the ones of vertebrate defensins. Mytilins and myticins constituted original families without relationships with other known peptide structures. Admitting some gaps along the primary sequences revealed similarities, particularly in the C- and N-terms of defensins and myticins (Fig. 3).

Solution structure of defensin was established in ^{1}H NMR using synthetic MGD1. Mussel defensin mainly consists in CS$\alpha\beta$ motif made up of a α-helix (residues 7-16) and of a slightly twisted β-sheet made up of two strands [43]. Strand I (residues 20-25) is roughly antiparallel to the helix, and strand II (residues 33-37) is roughly parallel to the helix. The various elements of secondary structure, including N-term loop of six residues, turn I of three residues, turn II of seven residues and C-term loop of three residues, are tightly crosslinked together by four disulfide bonds regularly distributed along the sequence.

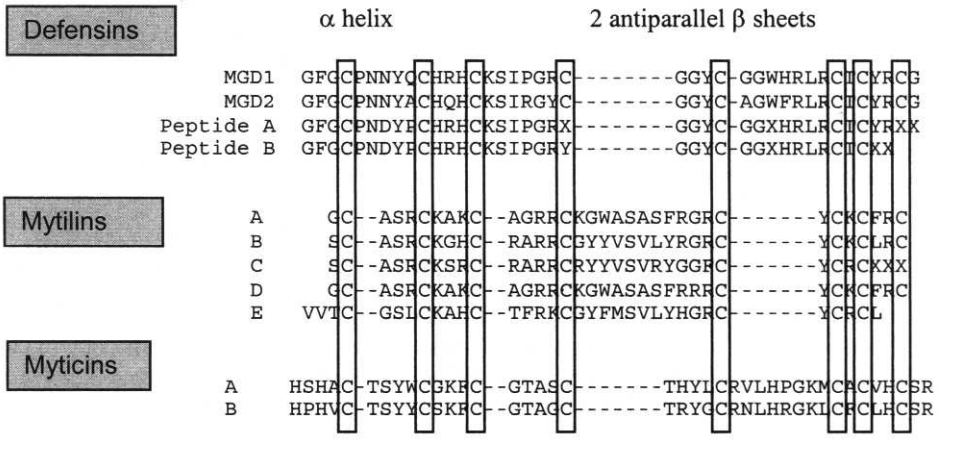

Fig. 3: Amino acid sequence alignments emphasizing in cysteine patterns.

Table 1: Arbitrary classification of antimicrobial peptides into four groups.

1 – Without cysteine, linear and mostly helical
cecropins, andropin, ceratotoxins, bombinin, dermaseptin, magainins, cathelin-like ...

2 – Without cysteine but with high proportion of certain residues
- proline-arginine : apidaecins, drosocin ...
- proline-glycine : attacins, sarcotoxin, diptericin ...
- proline : metchnikowin ...
- tryptophan : indolicidin ...

3 – With cysteines engaged in intramolecular bonds and then cyclics
- One disulfide bond, often in C-term : bactenecin, brevinins, ranalexin ...
- Several disulfide bonds with also β-sheets : α and β-defensins, insect and mussel defensins, penaedins (proline-rich), protegrins, tachyplesins ...

4 – Larger polypeptides with probably other functions
derived from gastric inhibitory polypeptide, derived from diazepam-binding inhibitor ...

5. Biological activities of mussel peptides

Immediate remark is the diversity of the *Mytilus* antimicrobial peptides that is questionable on the biological significance of such a system. One possible explanation arose from establishment of the activity spectrum of the different molecules [39-42]. Defensins and myticins are essentially active against Gram-positive bacteria, including some pathogens for marine invertebrates and are much less active against Gram-negative bacteria or fungi. Mytimycin is strictly antifungal and no one is active against protozoa. The different peptides and isoforms possess complementary properties that might permit an increase in antimicrobial capabilities of mussels. For instance, mytilin B and C share a high degree of homology in their primary structure. However, they display different activities, especially against the fungus *Fusarium oxysporum* and against the Gram-negative bacteria *Vibrio splendidus* [40].

All mussel peptides possess bactericidal effect but with strong differences between isoforms. *In vitro* tested against Gram-positive *Micrococcus luteus*, defensin A and mytilin C killed all the bacteria in less than 3 min of contact [40]. On the opposite, myticin A required 2 h of contact and mytilin E more than 6 h, revealing different kinetics of activity [41]. In conclusion, the different families and the different isoforms possess complementary activities, both in terms of target specificity and kinetics.

6. Structure and expression regulation of mussel antimicrobial genes

Complete structure of the genes encoding defensin and mytilin precursors was established [44]. The Southern blot analysis suggests that defensin gene is present as a single copy in the genome. Nevertheless, three different cDNAs sharing a high degree of homology were recorded, suggesting that such differences in the cDNA sequences were probably the result of genetic polymorphism. In the opposite, at least two copies of mytilin gene seemed to exist in the genome, probably encoding one of the numerous isoforms observed. Both defensin and mytilin genes shared the same organization (Fig. 4). The four exons are interrupted by three large introns. Moreover, exons contain precursor domains that fulfil different functions: the second exon of both genes contains the cDNA sequence encoding a N-term prosequence

required to address the precursor to the lumen of the endoplasmic reticulum, and the third exon contains the sequence encoding the mature peptide.

Defensin, mytilin and myticin cDNA structures are also similar (Fig. 4). In all cases, a signal sequence coding for a peptide of 20 to 22 residues is followed by the sequence coding for the active peptide (34 to 40 residues). Originally, an additional anionic C-term (21 to 48 residues) was found. This region may function as another addressing signal or interact with a cationic part of the active peptide to prevent unsuitable membrane interaction [45]. *Drosophila* defensin does not possess such C-term extension but consists in a pre-propeptide [46]. Only tachyplesin precursor from the horseshoe crabs *Tachypleus tridentatus* is arranged similarly [47].

One possible explanation of the similarities between genes, cDNAs and precursors, is that they all derived from a common ancestor, the different domains of preexisting genes having been combined (by recombination) to create a new gene enlarging the target spectrum. Such hypothesis is re-enforced by the presence of eight cysteines arranged in a close pattern (Fig. 3).

Mussel antimicrobial peptides are present only in granules of hemocytes, in contrast to insect peptides, which are synthesized in the fat body and surface epithelia [48-49]. As they can be purified as mature peptides from these granules, they must be processed inside the hemocytes and stored as active forms [44, 45]. Only 15 % of the circulating hemocytes were not labeled by either antidefensin or antimytilin antibodies. Meanwhile, 48 % contained defensins whatever they were alone or associated with mytilins (Fig. 5). Remarkable was the fact that 69 % were positive for mytilins suggesting the importance of such peptide family [50]. Involvement of the peptides in antimicrobial response is also original. Continuously synthesized even in the absence of challenge, the different peptides are engaged in the destruction of bacteria inside phagocytes (1 to 5 h), before being released into hemolymph (1 to 3 days) [51]. Such involvement in anti-infectious response is apparently more related to those of mammalian phagocytes than to those of insect hemocytes.

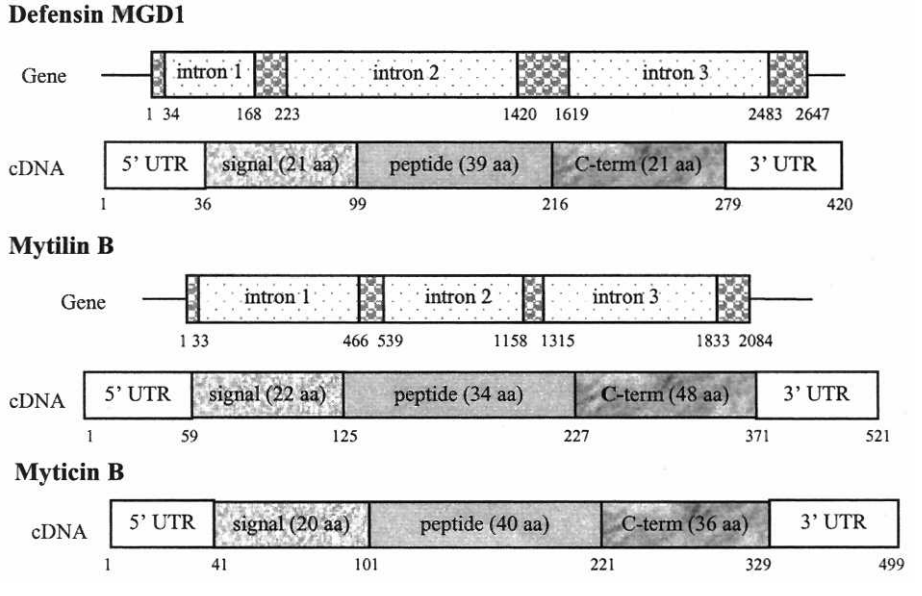

Fig. 4: Similar gene, cDNA and precursor structures.

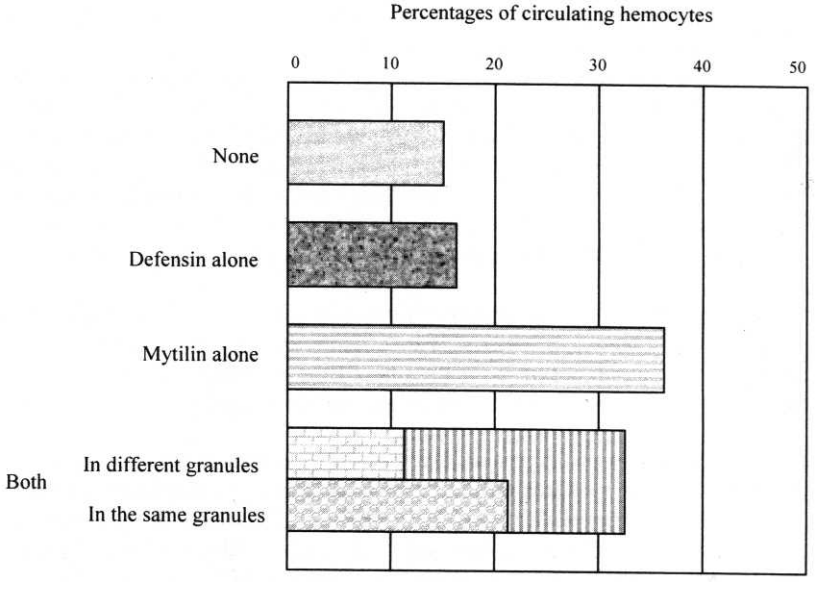

Fig. 5: Relative percentages of defensin and mytilin positive circulating hemocytes.

7. *Black boxes for the future*

Among future directions of investigations, the signaling system of invertebrates in general, and earthworms and mussels in particular, represents certainly the next challenge. Cell-cell interactions were demonstrated in both animals, numerous indirect evidences were reported using functional assay [52-53] or antibody crossreactivity [54], but no signaling molecules were isolated so far.

Immunoglobulin synthesis takes two weeks to develop. Innate immune response occurred within some hours. Earlier response might be through neuromediators (proenkephalin-derived or endocannabinoids), some fragments being hemocyte stimulating/recruiting agents and possessing antibacterial capacities [55, 56].

Investigating the early phase response will help to understand the recognition process and, consequently, which are the receptors or triggering molecules. Due to the complete sequence of the *Drosophila* genome, we discovered that antimicrobial peptide genes are regulated by distinct pathways and in different tissues according to the stimulus [57, 58]. Finally, we may approach the meaning of specificity when applied to invertebrate immunity.

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References

[1] E.E. Metchnikoff, Leçons sur la pathologie comparée de l'inflammation, Masson, Paris 1892.

[2] G.R. Cameron, Inflammation in earthworm, *J. Pathol. Bacterial* **35** (1932) 933-972.

[3] E.L. Cooper and L.M. Rubilotta, Allograft rejection in *Eisenia foetida, Transplantation* **8** (1969) 220-223.

[4] Ph. Roch *et al.*, Response of earthworm leukocytes to Con A and transplantation antigens, *Adv. Exp. Med. Biol.* **64** (1975) 45-53.

[5] J. Toupin and G. Lamoureux, Coelomocytes of earthworms: phytohemagglutinin (PHA) responsiveness. In: R.K. Wright and E.L. Cooper (Eds.), Phylogeny of thymus and bone marrow-bursa cells, Elsevier/North Holland, Amsterdam, 1976, pp. 19-26.

[6] P. Valembois and Ph. Roch, Identification par autoradiographie des leucocytes stimulés à la suite de plaies ou des greffes chez un ver de terre, *Biol. Cell.* **28** (1977) 81-82.

[7] Ph. Roch, Leukocyte DNA-synthesis in grafter lumbricids: an approach to study histocompatibility in invertebrates, *Dev. Comp. Immunol.* **3** (1979) 417-428.

[8] E.A. Stein *et al.*, Agglutinins in the earthworm *Lumbricus terrestris*: naturally occurring and induced. *Dev. Comp. Immunol.* **6** (1982) 407-421.

[9] A. Wojdani *et al.*, Agglutinins and proteins in the earthworm *Lumbricus terrestris* before and after injection of erythrocytes, carbohydrate and other materials, *Dev. Comp. Immunol.* **6** (1982) 613-624.

[10] E.L. Cooper and Ph. Roch, Second-set allograft responses in the earthworm *Lumbricus terrestris*: kinetics and characteristics. *Transplantation* **41** (1986) 514-520.

[11] E.L. Cooper and Ph. Roch, Earthworm leukocytes interactions during early stages of graft rejection, *J. Exp. Zool.* **232** (1984) 67-72.

[12] L. Du Pasquier and P. Duprat, Aspects humoraux et cellulaires d'une immunité naturelle non-spécifique chez l'oligochète *Eisenia fetida, C.R. Acad. Sci.* Paris **266** (1968) 538-541.

[13] Ph. Roch, Protein analysis of earthworm coelomic fluid: I- Polymorphic system of the natural hemolysin of *Eisenia fetida andrei*, *Dev. Comp. Immunol.* **3** (1979) 599-608.

[14] L. Tuckova *et al.*, Lytic activities in the coelomic fluid of *Eisenia foetida* and *Lumbricus terrestris, Dev. Comp. Immunol.* **10** (1986) 181-189.

[15] W. Mohrig *et al.*, Proteolytic activities in the coelomic fluid of earthworms, *Zool. Jb. Physiol.* **93** (1989) 303-317.

[16] A. Milochau *et al.*, Purification, characterization and activities of two hemolytic and antibacterial proteins from coelomic fluid of the annelid *Eisenia fetida andrei*, *Biochim. Biophys. Acta* **1337** (1997) 123-132.

[17] Ph. Roch *et al.*, Interactions between earthworm hemolysins and sheep red blood cell membranes, *Biochim. Biophys. Acta* **983** (1989) 193-198.

[18] M. Lassègues *et al.*, Sequence and expression of an *Eisenia-fetida*-derived cDNA clone that encodes the 40-kDa fetidin antibacterial protein, *Eur. J. Biochem.* **246** (1997) 756-762.

[19] Y. Sekizawa *et al.*, Molecular cloning of cDNA for lysenin, a novel protein in the earthworm *Eisenia foetida* that causes contraction of rat vascular smooth muscle, *Gene* **191** (1997) 97-102.

[20] M. Bilej *et al.*, Cellular expression of the cytolytic factor in earthworms *Eisenia foetida, Immunol. Lett.* **60** (1998) 23-29.

[21] J. Toupin and G. Lamoureux, Coelomocytes of earthworms, the T-cell-like rosette, *Cell. Immunol.* **26** (1976) 127-132.

[22] Ph. Roch *et al.*, Isolation of agglutinins from lysins in earthworm coelomic fluid by gel filtration followed by chromatofocusing, *J. Chromatogr.* **290** (1984) 231-235.

[23] E. Kauschke and W. Mohrig, Comparative analysis of hemolytic and hemagglutinating activities in the coelomic fluid of *Eisenia foetida* and *Lumbricus terrestris, Dev. Comp. Immunol.* **11** (1987) 331-341.

[24] E.A. Stein and E.L. Cooper, *In vitro* agglutinin production by earthworm leukocytes, *Dev. Comp. Immunol.*, **12** (1988) 531-548.

[25] Ph. Roch *et al.*, Genetic and biochemical polymorphism of earthworm humoral defences. In: E.L. Cooper *et al.* (Eds.), Progress in Clinical and Biological Research, vol. 233, Developmental and Comparative Immunology,. Alan R. Liss Inc. New York, 1987, pp. 91-102.

[26] M. Wittke and L. Renwrantz, Quantification of cytotoxic hemocytes of *Mytilus edulis* using a cytotoxicity assay, *J. Invert. Pathol.* **43** (1984) 248-253.

[27] H. Steiner *et al.*, Sequence and specificity of two antibacterial proteins involved in insect immunity, *Nature* **292** (1981) 246-248.

[28] H.G. Boman, Peptides antibiotics and their role in innate immunity, *Annu. Rev. Immunol.* **13** (1995) 61-92.

[29] Ph. Bulet *et al.*, Antimicrobial peptides in insects: structure and function, *Dev. Comp. Immunol.* **23** (1999) 329-344.

[30] A.M. Cole and T. Ganz, Human antimicrobial peptides: analysis and application, *Biotechniques* **29** (2000) 822-831.
[31] D.T. Fearon and R.M. Locksley, The instructive role of innate immunity in the acquired immune response, *Science* **272** (1996) 50-54.
[32] J.A. Hoffmann *et al.*, Phylogenetic perspectives in innate immunity, *Science* **284** (1999) 1313-1318.
[33] M. Bilej *et al.*, Antimicrobial defense in the earthworm, *Folia Microbiol.* **45** (2000) 283-300.
[34] M. Leippe and L. Renwrantz, Release of cytotoxic and agglutinating molecules by *Mytilus* hemocytes, *Dev. Comp. Immunol.* **12** (1988) 297-308.
[35] F. Hubert *et al.*, Cytotoxic and antibacterial properties of *Mytilus galloprovincialis, Ostrea edulis* and *Crassostre gigas* (bivalve molluscs) hemolymph, *Aquat. Living Resour.* **9** (1996) 115-124.
[36] F. Hubert *et al.*, Structure and differential target sensitivity of the stimulable cytotoxic complex from hemolymph of the Mediterranean mussel *Mytilus galloprovincialis*, *Biochim. Biophys. Acta* **1361** (1997) 29-41.
[37] Ph. Roch *et al.*, Present knowledge on the molecular basis of cytotoxicity, antibacterial activity and stress response in marine bivalves, *Ital. J. Zool.* **63** (1996) 311-316.
[38] C. Hétru and Ph. Bulet, Strategies for the isolation and characterization of antimicrobial peptides of Invertebrates. In : W.M. Shafer (Ed.), Methods in Molecular Biology, vol 78, Antibacterial Peptide Protocols, Humana Press Inc., Totowa, NJ (USA), 1997, pp. 35-49.
[39] F. Hubert *et al.*, A member of the arthropod defensin family from edible Mediterranean mussels (*Mytilus galloprovincialis*), *Eur. J. Biochem.* **240** (1996) 302-306.
[40] G. Mitta *et al.*, Involvement of mytilins in mussel antimicrobial defense, *J. Biol. Chem.* **275** (2000) 12954-12962.
[41] G. Mitta *et al.*, Myticin, a novel cysteine-rich antimicrobial peptide isolated from hemocytes and plasma of the mussel *Mytilus galloprovincialis*, *Eur. J. Biochem.* **265** (1999) 71-78.
[42] M. Charlet *et al.*, Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis, J. Biol. Chem.* **271** (1996) 21808-21813.
[43] Y-S. Yang *et al*, Solution structure and activity of the synthetic four-disulfide bond Mediterranean mussel defensin (MGD-1), *Biochemistry* **39** (2000) 14436-14447.
[44] G. Mitta *et al.*, Mytilin B and MGD2, two antimicrobial peptides of marine mussels: gene structure and expression analysis, *Dev. Comp. Immunol.* **24** (2000) 381-393.
[45] G. Mitta *et al.*, Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge, *J. Cell Sci.* **112** (1999) 4233-4242.
[46] J-L. Dimarcq *et al.*, Characterisation and transcriptional profiles of a *Drosophila* gene encoding an insect defensin, *Eur. J. Biochem.* **221** (1994) 201-209.
[47] T. Shigenaga *et al.*, Antimicrobial tachyplesin peptide precursor cDNA cloning and cellular localisation in the horseshoe crab, *Tachypleus tridentatus, J. Biol. Chem.* **265** (1990) 21350-21354.
[48] H. Boman *et al.*, Cell-free immunity in *Cecropia*. A model system for antibacterial proteins, *Eur. J. Biochem.* **201** (1991) 23-31.
[49] P. Tzou, Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia, *Immunity* **13** (2000) 737-748.
[50] G. Mitta *et al.*, Differential distribution and defence involvement of antimicrobial peptides in mussel, *J. Cell Sci.* **113** (2000) 2759-2769.
[51] G. Mitta *et al.*, Original involvement of antimicrobial peptides in mussel innate immunity, *FEBS Lett.* **486** (2000) 185-190.
[52] T.K. Hughes *et al.*, Interactions of immunoactive monokines (interleukin 1 and tumor necrosis factor) in the bivalve *Mytilus edulis, Proc. Natl. Acad. Sci.* USA **87** (1990) 4426-4429.
[53] E. Ottaviani *et al.*, Immunomodulation by recombinant human interleukin-8 and its signal transduction pathways in invertebrate hemocytes, *Cell. Mol. Life Sci.* **57** (2000) 506-513.
[54] E. Ottaviani *et al.*, Presence of several cytokine-like molecules in molluscan hemocytes, *Biochem. Biophys. Res. Comm.* **195** (1993) 984-988.
[55] M. Salzet *et al.*, Comparative biology of the endocannabinoid system: possible role in the immune response, *Eur. J. Biochem.* **267** (2000) 4917-4927.
[56] M. Salzet and A. Tamienski, Involvement of pro-enkephalin-derived peptides in immunity, *Dev. Comp. Immunol.* **25** (2001) 177-185.
[57] J.A. Hoffmann and J-M. Reichhart, *Drosophila* immunity, *Trends Cell Biol.* **7** (1997) 309-316.
[58] Y. Engstrom, Induction and regulation of antimicrobial peptides in *Drosophila, Dev. Comp. Immunol.* **23** (1999) 345-358.

A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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The prophenoloxidase activating system in invertebrates: a short review

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Abstract. Invertebrate animals do not have antibodies and therefore have to rely on innate immune systems, but still they have to be able to recognize foreign materials and respond to it so that appropriate measures are initiated to combat and destroy invading microorganisms. The ways in which invertebrate animals recognize and respond to nonself particles or molecules are beginning to be understood at the molecular level. The prophenoloxidase activating system (the proPO system) has been shown in many invertebrates as a nonself recognition and defense system. The activation of the proPO system in crustacean immunity comprises three processes - recognition, activation of proPO and amplification of the system - and this system has been studied intensively in the freshwater crayfish, *Pacifastacus leniusculus*. Similar molecules involved in this system have been characterized from another crustacean species, the black tiger shrimp, *Penaeus monodon*. In this review, we will discuss about this system in invertebrates, with an emphasis in crustaceans.

1. Innate immunity

A key feature of innate immunity is the ability to limit the infectious challenge in the early hours after the infection occurs. It has been suggested that studies of innate immunity will lead to the discovery of common molecular mechanisms used for host defense in plants, invertebrates and vertebrates. The recognition of conserved molecular patterns characteristic of pathogens is a property of the innate immune system, which is instrumental in initiating and regulating the adaptive immune response [1]. The target recognition of innate immunity is the so-called "Pathogen –Associated Molecular Patterns" (PAMPs) shared among groups of pathogens. Host organisms have developed the response to these PAMPs by a set of receptors referred to as "Pattern Recognition Proteins or Receptors" (PRPs or PRRs) [2]. These patterns include the lipopolysaccharides (LPS) of Gram-negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of Gram-positive bacteria, the mannans of yeast, the β-1,3-glucan of fungi and double-stranded RNAs of viruses [3].

In insects, the innate defense system has been studied intensively in *Drosophila melanogaster* [3]. Components such as transcription factors, antimicrobial defensins, and cecropins, binding proteins and putative members of innate immune cascades have been isolated by homology cloning, or by the empirical criterion of upregulation upon immune challenge. Toll/NFκB pathway is conserved between insects and mammals to activate nonspecific defense mechanisms in both cases. Toll has been shown to induce the synthesis of antifungal and antibacterial peptides in *Drosophila* [4], while in mammals, Toll induces signals required for the activation of the adaptive immune response [5]. Recent experiments indicate that mammalian Toll-like receptors are critical in LPS-mediated signaling in association with an LPS-binding protein (LBP) and CD14 [5,6].

Proteolytic cascades triggered by nonself recognition molecules have major roles in innate immunity. Examples are the complement cascade in mammals [7], hemolymph coagulation in

horseshoe crab [8] and the phenoloxidase-mediated melanization in crustaceans and insects [9, 10]. The complement cascade is activated directly (via alternative and lectin pathways) or indirectly (via classical pathway) by microorganisms and results in their opsonization for phagocytosis, chemotaxis or lysis by the assembly on their surface of a pore-forming membrane attack complex [11]. The lectin pathway requires the mannose-binding protein (MBP) [12]. MBP recognizes sugar moieties on microbe surfaces and results in the activation of the MBP-associated serine proteases, MASP-1 and MASP-2, which in turn activate the C3 convertase [13]. Recent cloning of MASPs in lamprey [14] and tunicates [15], C3-like molecules in tunicates [16] and sea urchins [17], and related thioester-containing protein (TEP) in *D. melanogaster* [18] and *Anopheles gambiae* [19] leads to the prediction that the lectin pathway of mammalian complement system seems to be ancient. An earlier link between recognition of microbial molecular patterns, proteolytic cascades and activation of host defense came from studies of the clotting cascade in the horseshoe crab, *Limulus polyphemus* [8, 20]. The proteins participating in the horseshoe crab clotting system all reside in the hemocytes and, upon activation, they are released from the cytoplasmic L-granules into the hemolymph through rapid exocytosis. Gram-negative bacteria and fungi invading the horseshoe crab hemolymph activate factor C and factor G, respectively, which results in the formation of an insoluble coagulin gel that limits the infection [8, 20]. Factor C in this cascade has five short consensus repeats (SCR, also called CCP or the sushi domain) [21] that are found in mammalian complement proteins, suggesting an early common origin of the complement and coagulation cascades. The prophenoloxidase activating system (the proPO system) is an enzymatic cascade reported in many invertebrates and large amount of information about this system has come from work done on crustaceans - the freshwater crayfish, *Pacifastacus leniusculus* [for reviews 10, 22]. The activation of the proPO system is brought about by an extremely low amount (pg/L) of microbial cell wall components such as LPS and β-1,3-glucans. Activation of the proPO system not only leads to the synthesis of melanin, but also initiates several biological molecules responsible in the defense system of the crayfish. Recently, Nagai and Kawabata [23] showed that *Tachypleus tridentatus* clotting enzyme and activated factor B are capable to functionally transform hemocyanin to phenoloxidase without proteolytic cleavage suggesting that the two host defense systems of blood coagulation and proPO activation are evolutionary related protease cascades.

2. Pattern recognition proteins

PRPs recognize and respond to microbial invaders by the presence of signature molecules on the surface of the intruders. PRPs in mammals, a LPS-binding protein (LBP) [24] and the cellular receptor CD14 have been well characterized and play roles in stimulating macrophages to produce cytokines [1]. Besides microbial cell wall components, dsRNA has also been reported to behave as PAMP [25, 26]. DsRNA is an inducer of type I interferon (IFN) which plays a critical role in antiviral response [27, 28] as well as other cytokines including IL-6 and IL-12 [2, 29, 30]. Cella *et al.* [25] showed that dsRNA as well as viral injection induced the activation and rapid maturation of human dendritic cells with upregulation of MHC, adhesion and costimulatory molecules.

A number of invertebrate PRPs have been isolated and characterized and some of them contain common motifs for example, bacterial glucanase-like [31-37], bacteriophage lysozyme-like [38, 39] and immunoglobulin-like [40] motif in their primary structures. Some of them are lectins that can agglutinate a variety of vertebrate blood cells [41, 42]. Three molecules - which have been isolated from the coelomic fluid of the earthworm, *Eisenia*

foetida (CCF-1, [35]), the hemocytes of crayfish, *P. leniusculus* (LGBP, [36]), and *D. melanogaster* (DGNBP1, [37]) -showed affinity to both β-1,3-glucans and LPS. CCF-1 and LGBP have both been shown to be involved in the activation of the proPO system. In *Drosophila*, binding of DGNBP1 to either LPS or β-1,3-glucan induces the synthesis of antimicrobial peptides [37]. Recently, it was shown that a masquerade-like protein, a serine protease homologue [43], isolated from *P. leniusculus*, through proteolytic processing, can bind to LPS, Gram-negative bacteria, and yeast and subsequently participates in bacterial clearance [44].

So far, β-1,3-glucan-binding proteins (BGBPs) have been cloned from many arthropods – the horseshoe crab, *Tachypleus tridentatus* [45], the freshwater crayfish, *P. leniusculus* [33], the moth, *Manduca sexta* [34], the silkworm, *Bombyx mori* [32], and the black tiger shrimp, *Penaeus monodon* [46]. Although invertebrate BGBPs have glucanase-like motif, none has been shown to exhibit glucanase activity suggesting that the BGBPs have developed from a primitive glucanase and then evolved into proteins without glucanase activity, but instead after binding to glucans, operate as elicitors of defense responses. Alignment of glucanase-motifs contained in invertebrate PRPs reveals a high homology in the N-terminal region of all sequences suggesting that this region is involved in recognition of the microorganisms, which has been shown to be the case for BGBP of *B. mori* [32].

Several recognition proteins have been identified in penaeid shrimp, but none of them has been cloned so far. BGBPs from *Penaeus californiensis, P. stylirostris and P. vannamei* were reported [47-49]. They have the same characteristics as that of crayfish BGBP, since they are 100-kDa monomeric proteins and they share similar amino acid composition and N-terminal sequence [33]. Shrimp BGBP is involved in the activation of the proPO system and it was found that it is the same protein as LP1, a lipid transport protein found in another penaeid shrimp, *P. semisucultus* [50]. In *P. leniusculus*, BGBP and LGBP were not significantly changed when the animals were challenged with microbial components such as laminarin (unpublished data). This is in agreement with the studies done in *P. monodon* [46] suggesting that these proteins are constitutively expressed. In one insect, *M. sexta*, the level of its β-1,3-glucan recognition protein (GRP) mRNA in fat body did not increase significantly after larvae were injected with bacteria or yeast [34]. In contrast, PRPs expression from other insects, Gram-negative bacteria-binding protein [36] and BGBP from *B. mori*, GNBP from *Hyphantria cunea* [51], and *A. gambiae* [52] were all shown to be inducible upon microbial challenges. At present, five Gram-negative bacteria-binding proteins (GNBPs) have been discovered - three in insects, one in the earthworm and one in a crustacean [31, 35, 36, 37, 40, 52, 53]. These binding proteins from insects appear to be functionally similar by having affinity to the Gram-negative bacterial cell walls and are inducible during injury or infection.

Insect peptidoglycan recognition protein (PGRP) has been reported to be conserved from insects to human [54]. Upon binding to peptidoglycan (PG), PGRP in *B. mori* mediates the activation of the proPO system in the plasma fraction of the silkworm hemolymph and its mRNA expression is induced upon bacterial challenge [38, 39]. Insect PGRPs cloned from *B. mori* and *Trichoplusia ni* [54] are homologous proteins to bacteriophage lysozyme, although they do not contain the amino acid residues necessary for catalytic action of the enzyme [55].

3. Prophenoloxidase and phenoloxidase

It has long been recognized that defense reactions in many invertebrates are often accompanied by melanization. In arthropods, melanin synthesis is involved in the process of sclerotization, pigmentation and wound healing of the cuticle as well as in defense reactions. During the formation of melanin, toxic metabolites are formed which have fungististic activity

[56-59]. The proPO system is considered to be a nonself recognition and defense system in many invertebrates [22]. The susceptibility of *Rhodinus prolixus* to *Trypanosoma rangeli* infection might be related to the suppression of the activation of proPO in the presence of this flagellate [60]. Injection of the nonpermissive fungus, *Entomophaga aulicae* into lepidopteran insect, *Lymantria dispar* resulted in an increase of insect phenoloxidase activity when compared to injection of a permissive strain, *E. maimaiga* [61] suggesting that the activation of proPO continues during a brief survival of this fungus in a nonpermissive host. So far, proPOs have been cloned from fifteen invertebrate species, two crustaceans and thirteen insects. Several isoforms encoded by different genes have been found in insects [62, 63], but it is yet not known if they have different functions. The schematic drawing for the activation of the proPO cascade in crayfish is shown in Fig. 1.

The active enzyme, phenoloxidase (PO; monophenol, L-dopa:oxygen oxidoreductase; EC 1.14.18.1) is responsible for the well-known melanization reaction, which is generally observed in a wounded area or during an immune response in invertebrates. PO is a bifunctional copper-containing oxidase catalyzing the oxidation of phenolic substance into quinones, which are further converted to melanin [64]. PO has been detected in the hemolymph (blood) or coelom of both protostomes and deutereostomes, as well as the cuticle of arthropods. PO is found in different sizes, monomers, homodimers, heterodimers and homotetramers. However, the subunits from different species fall in the range between 71-83 kDa on a SDS-PAGE gel [22]. Arthropod proPOs have high similarity to arthropod hemocyanins, but rather remote relationship to vertebrate tyrosinases. Tyrosinase found in ascidians, *Halocynthia roretzi*, resembles vertebrate tyrosinases rather than arthropod proPOs [65], since this enzyme has a signal peptide and a transmembrane domain like vertebrate tyrosinases. By comparison of amino acid sequences, arthropod proPOs can be classified into two major groups, insect and crustacean proPOs, respectively [66, 67]. The highly conserved parts of primary sequences of arthropod proPOs are around two copper binding sites, CuA and CuB. In crayfish, it was shown that these sites are active and bind Cu^{2+} [68]. Recently, hemocyanins from two arthropod species – tarantula, *Eurypelma californicum* and horseshoe crab, *T. tridentatus* – were shown able to be converted to phenoloxidase [23, 69]. It is suggested that hemocyanins may switch to function as phenoloxidase at the site of injury to prevent microbial invasion or at the growing phase of the animal to harden the exoskeleton after molting or sclerotization [23, 69].

A thioester-like motif present in the complement components – C3, C4 and α2-macroglobulins – was also observed in invertebrate proPOs, although it is not known whether it is a functional thioester motif or not. In vertebrates, proteolytic activation of C3 leads to covalent attachment of a C3 cleavage product through a thioester bond to the pathogen [7]. Thioester-containing proteins have been described in several protostomes and they appear to exhibit α2-macroglobulin-like protease inhibitory activity [70, 71]. Recently, thioester-containing protein-1 (TEP-1) isolated from *A. gambiae* was shown to have a function that resembles that of vertebrate complement in promoting phagocytosis [19].

The activation of the proPO cascade is exerted by microbial cell wall components such as LPS, β-1,3-glucans or PG. The recognition of these nonself molecules by endogenous PRPs subsequently leads to degranulation of hemocytes. Several components and associated factors of the proPO system have also been found to play several important roles in the defense reaction of the freshwater crayfish [22]. Under physiological conditions, arthropod proPOs require a proteolytic cleavage by a specific protease for activation. For instance, the inactive proPO in the freshwater crayfish with a molecular mass of 76 kDa is converted into an active form with a molecular mass of 62 kDa by the proPO-activating enzyme (ppA) [68, 72]. A proppA becomes activated by the presence of PRPs [73]. ProppAs cloned from insects and crustaceans have been shown to be homologous to *Tachypleus* clotting enzyme and activated

factor B as well as to *Drosophila* easter [74-79]. The common feature of arthropod ppA enzymes are that they are serine proteinases and have clip-like domains [76-79]. The clip-like domain seems to play several biological functions. The clip-domain of clotting enzyme and factor B in horseshoe crab is proposed to mediate the functional conversion of hemocyanin to phenoloxidase [23]. Wang *et al.* [79] have recently shown that the recombinant peptide from a clip-like domain (defensins) of crayfish proppA has an antibacterial activity *in vitro*.

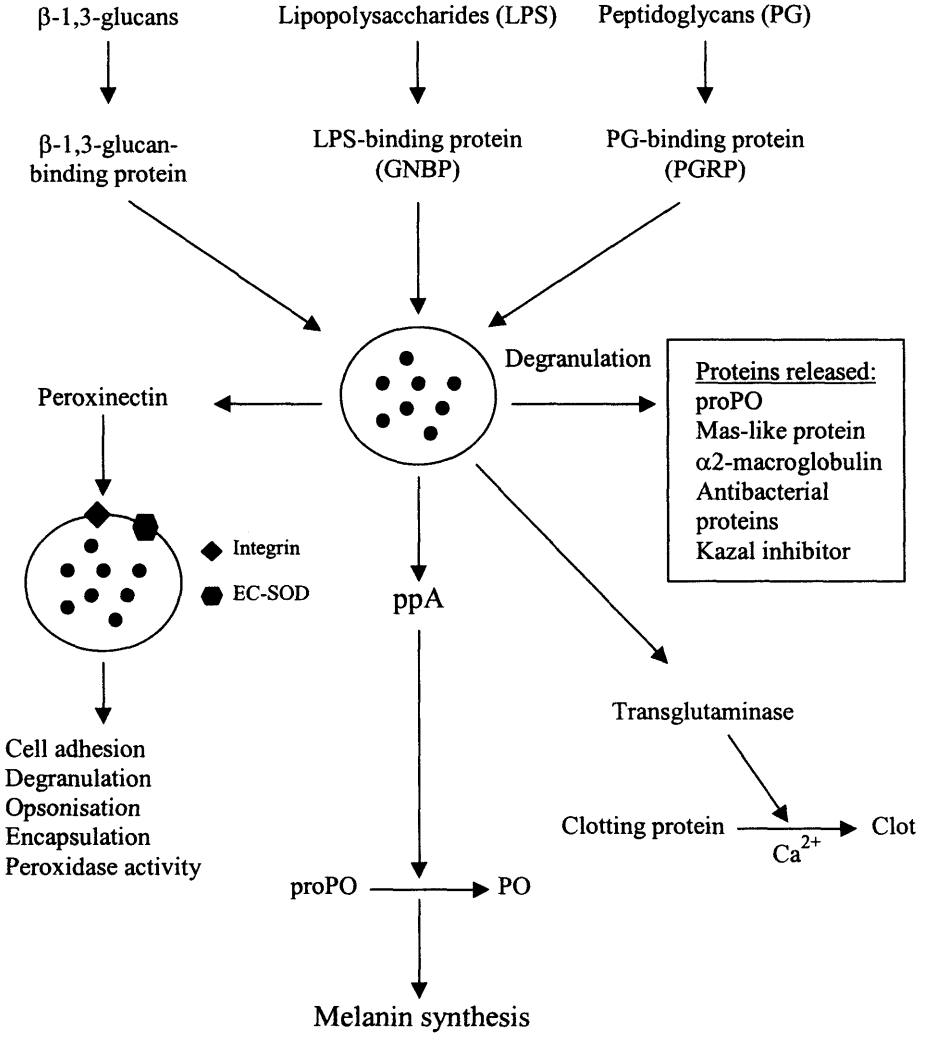

Fig. 1: The prophenoloxidase activating system in crustaceans.
: granule-containing hemocytes

4. Peroxinectin, an associated factor of the proPO system

Several cell adhesion molecules have been discovered and characterized during the past few years in invertebrates and have been shown to participate in immunological processes. These processes include cell attachment and spreading, nodule formation, encapsulation, agglutination (or aggregation) and phagocytosis [80]. So far, a few blood cell adhesion molecules in arthropods have been cloned (Table 1).

Peroxinectin is proposed to be involved in the amplification process of the proPO system. After binding to its receptor, it causes degranulation of hemocytes and thus amplifies the release of the proPO system. Upon activation of the proPO system in crayfish, peroxinectin, a cell adhesion factor with peroxidase activity is generated [81, 82]. Crayfish peroxinectin is synthesized in the blood cells, stored in secretory granules of granular hemocytes in an inactive form, released in response to stimuli, and activated outside the cells to mediate attachment and spreading. Besides having cell adhesion and peroxidase activities, crayfish peroxinectin also acts as a degranulation factor, an encapsulation-promoting factor and an opsonin [for reviews 22, 93]. However, it is important to emphasize that the peroxidase activity is not a prerequisite for the other biological activities of peroxinectin [81]. Crossreactive proteins with similar activities have been isolated from the insect, *Blaberus craniifer* [88], from the hemocytes of the shore crab, *Carcinus maenas* [87] and from the hemocytes of the black tiger shrimp, *Penaeus monodon* [83]. The sequence of a *Drosophila* peroxinectin-related molecule (accession no. AAF78217) has been reported which shows high similarity to crustacean peroxinectins within the peroxidase domain [83]. However, the function of this molecule is yet unknown. Thus, it is suggested that peroxinectin is widely distributed among arthropod species.

The deduced amino acid sequence of crustacean peroxinectins [81] has high similarity to both invertebrate and vertebrate peroxidases including human myeloperoxidase (MPO) (32% identity) [94]. Isolated primary human leukocytes and differentiated myeloid (HL-60) cells have been shown to adhere to MPO, whereas undifferentiated cells did not [95]. Taken together, cell adhesion may thus be a conserved function of animal peroxidases, in addition to producing a potent microbicidal agent [96]. Crustacean peroxinectin also has high similarity to *Drosophila* peroxidasin, which is a multidomain protein that combines an enzymatically functional peroxidase domain with motifs that typically occur as parts of cellular matrix proteins including four immunoglobulin (Ig) loops and six leucine rich repeats (LRR) [97]. The combination of Ig and LRR loop structures suggests that peroxidasin may mediate adhesion of cells to the extracellular matrix although this molecule has not yet been shown to exhibit cell adhesion activity. Thus, it is plausible that molecules containing peroxidase domains and having other biological activities as well as peroxidase activity such as crustacean peroxinectin, human myeloperoxidase and *Drosophila* peroxidasin, are likely to be widely distributed amongst animal species. Recently, a human peroxidasin homologue was found and shown to be upregulated in p53-dependent apoptotic cells [98].

The adhesive function of peroxinectin is likely to be mediated by the integrin-binding motifs, KGD or RGD [99]. A synthetic peptide derived from the sequence containing KGD triplet was found to mimic the adhesion activity of the entire protein [81]. Holmblad *et al.* [100] also reported the presence of an integrin β-subunit on surfaces of the crayfish hemocytes. Peroxinectin also binds to a peripheral blood cell surface CuZn-superoxide dismutase (EC-SOD) [101], which also contains integrin-binding motif. Therefore, it is plausible that peroxinectin first bind to SOD, which in turn is anchored to the integrins inside the hemocyte membrane. It was suggested that peroxinectin might produce hypohalic acid from hydrogen peroxide produced by SOD and as a consequence, function as an efficient microbicidal attack system against invading microorganisms [102].

Table 1: Arthropod blood cell adhesion molecules

Species	proteins	cloned	Reference	cell adhesion activity*	Reference
Pacifastacus leniusculus	Peroxinectin	Yes	[81]	Yes	[82]
	Masquerade-like protein	Yes	[43]	Yes	[43]
Penaeus monodon	Peroxinectin	Yes	[83]	Yes[1]	[83]
Penaeus paulensis		No		Yes[1]	[84]
Limulus polyphemus	Limunectin	Yes	[85]	No	
	Limulus agglutination-aggregation factor (LAF)	Yes	[86]	Yes	[86]
Carcinus maenus		No		Yes	[87]
Blaberus craniifer		No		Yes	[88]
Bombyx mori	Hemocytin	Yes	[89]	Yes	[89]
Drosophila melanogaster	Croquemort	Yes	[90]	Yes	[90]
Pseudoplusia includens	Plasmatocyte spreading peptide (PSP1)	Yes	[91]	Yes[2]	[92]

* cell adhesion activity detected from purified protein.

[1] cell adhesion activity detected in hemolymph

[2] cell adhesion activity detected in recombinant protein expressed in baculovirus vector

5. Regulation of the proPO system

To avoid deleterious effects, the proPO system has to be controlled and regulated. Several inhibitors of the proPO system have been reported in insects for example, low molecular mass proteinase inhibitors (LCIM I and LCIM II) from the locust, *Locusta migratoria* [103], a 4.2-kDa dopa-containing peptide from the housefly, *Musca domestica* [104], and a serpin from *M. sexta* [105]. The inhibitors of Kunitz family from hemolymph of *M. sexta* [106], *Sacrophaga bullata* [107], and *B. mori* [108] were also found to interfere with proPO activation.

A proteinase inhibitor in the plasma of the freshwater crayfish, *P. leniusculus*, has been shown to be the most effective inhibitor of the proPO system [109]. It was first shown that this 155-kDa protein has inhibitory activities toward trypsin, chymotrysin and elastase [109]. Pacifastin has a unique structure by having two peptide subunits, a 44-kDa light chain and a 105-kDa heavy chain coded from two different mRNAs and which are covalently linked [110]. The heavy chain has a significant similarity to transferrins from other animals [111-113] and two of the transferrin lobes were demonstrated to bind iron, whereas the light chain contains nine homologous cysteine-rich domains with significant similarities to three proteinase inhibitors from *L. migratoria* [103, 114-116]. An α-macroglobulin was shown to inhibit proPO activation, but to a lesser extend [109]. These proteinase inhibitors of the proPO system have only been characterized in detail in crayfish, not in other crustaceans.

6. Conclusion

Although much is known about the proPO system and its role in immunity still the details of its activation and how many proteinases and regulating factors are components or associated with the system is unknown. About twenty years ago, I published a review in which I proposed that this proPO system and the alternative pathway of complement is similar [9]. Today, we know that in principal the functions of these two systems are similar, but the structures of the proteins seem to be different and no real complement-like domains are present in ppA or proPO, although a thioester-like motif is present in several proPO's. Another innate immune system the horseshoe crab clotting system though contains proteinases with true complement domains (sushi) [8]. The recent finding in insects of several genes coding for thioester motifs is certainly of interest and may indicate that complement-like factor is present in protostomes and have function in immune reactions as that in deutereostomes in which several complement-like factors have been found [16]. Thus, it remains to be known whether other proteinases of the proPO system than ppA may have such domains and whether any of this so far unknown proteinases are involved in the induction of antibacterial synthesis through cleavage of Spätzle and subsequent binding of processed Spätzle to Toll.

References

[1] R. Medzhitov and C.A. Janeway, Innate immunity: The virtues of a nonclonal system of recognition, *Cell* **91** (1997) 295-298.

[2] C.A. Janeway Jr., Approaching the asymptote? Evolution and revolution in immunology, *Cold Spring Habour Symp. Quant. Biol.* **54** (1989) 1-13.

[3] J.A. Hoffmann *et al.*, Phylogenetic perspectives in innate immunity, *Science* **284** (1999) 1313-1318.

[4] B. Lemaitre *et al.*, The dorsoventral regulatory gene cassette spatzle/Toll/cactus control the potent antifungal response in *Drosophila* adults, *Cell* **86** (1996) 973-983.

[5] R. Medzhitov and C.A. Janeway, Innate immunity: The virtues of a nonclonal system of recognition, *Cell* **91** (1997) 295-298.

[6] F.L. Rock *et al.*, A family of human receptors structurally related to *Drosophila* Toll, *Proc. Natl. Acad. Sci.* USA **95** (1998) 588-593.
[7] J.E. Volanakis, Overview of the complement system. In: J.E. Volanakis and M.M. Frank (Eds.), The human complement system in health and disease, Marcel Dekker, Inc., New York, 1998, pp. 9-32.
[8] S. Kawabata *et al.*, Clotting cascade and defense molecules found in the hemolymph of horseshoe crab. In: K. Söderhäll *et al.* (Eds.), New Directions in Invertebrate Immunology, SOS Publications, Fair Haven, 1996, pp. 255-284.
[9] K. Söderhäll, Phenoloxidase activating system and melanization- a recognition mechanism of arthropods? A review, *Dev. Comp. Immunol.* **6** (1982) 601-611.
[10] K. Söderhäll *et al.*, The prophenoloxidase activating system and its role in invertebrate defense, *Ann. N. Y. Acad. Sci.* **712** (1994) 155-161.
[11] D.T. Fearon and R.M. Locksley, The instructive role of innate immunity in the acquired immune response, *Science* **272** (1996) 50-54.
[12] J. Epstein *et al.*, The collectins in innate immunity, *Curr. Opin. Immunol.* **8** (1996) 29-35.
[13] M. Matsushita and T. Fujita, Cleavage of the third component of complement (C3) by mannose-binding protein-associated serine protease (MASP) with subsequent complement activation, *Immunobioogy.* **194** (1995) 443-448.
[14] M. Matsushita *et al.*, Complement-related serine proteases in tunicates and vertebrates, *Curr. Opin. Immunol.* **10** (1998) 29-35.
[15] X. Ji *et al.*, Ancient origin of the complement lectin pathway revealed by molecular cloning of mannan binding protein-associated serine protease from urochordate, the Japanese ascidian, *Halocynthia roretzi*, *Proc. Natl. Acad. Sci.* USA **94** (1997) 6340-6345.
[16] L.C. Smith *et al.*, Complement systems in invertebrates. The ancient alternative and lectin pathways, *Immunopharmacology* **42** (1999) 107-120.
[17] W.Z. Al-Sharif *et al.*, Sea unchin coelomocyte specifically express a homologue of the complement component C3, *J. Immunol.* **160** (1998) 2983-2997.
[18] M. Lagueux *et al.*, Constitutive expression of a complement-like protein in Toll and JAK gain-of-function mutants of *Drosophila*, *Proc. Natl. Acad. Sci.* USA **97** (2000) 11427-11432.
[19] E.A. Levashina *et al.*, Conserved role of a complement-like protein in phagocytosis by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*, *Cell* **104** (2001) 709-718.
[20] S. Iwanaga *et al.*, New types of clotting factors and defense molecules found in horseshoe crab hemolymph: Their structures and functions, *J. Biochem.* **123** (1998) 1-15.
[21] T. Muta *et al.*, *Limulus* factor C: An endotoxin-sensitive serine protease zymogen with a mosaic structure of complement-like, epidermal growth factor-like, and lectin-like domains, *J. Biol. Chem.* **266** (1991) 6554-6561.
[22] K. Söderhäll and L. Cerenius, Role of the prophenoloxidase activating system in invertebrate immunity, *Curr. Opin. Immunol.* **10** (1998) 23-28.
[23] T. Nagai and S.I. Kawabata, A link between blood coagulation and prophenoloxidase activation in arthropod host defense, *J. Biol. Chem.* **275** (2000) 29264-29267.
[24] R.R. Schumann *et al.*, Structure and function of lipopolysaccharide binding protein, *Science* **249** (1990) 1429-1431.
[25] M. Cella *et al.*, Maturation, activation and protection of dendritic cells induced by double-stranded RNA, *J. Exp. Med.* **189** (1999) 821-829.
[26] W.M. Chu *et al.*, JNK2 and IKKβ are required for activating the innate response to viral infection, *Immunity* **11** (1999) 721-731.
[27] U. Müller *et al.*, Functional role of type I and type II interferons in antiviral defense, *Science* **264** (1994) 1918-1921.
[28] R. Manetti *et al.*, Polyinosinic acid: polycytidylic acid promotes T helper type 1-specific immune responses by stimulating macrophage production of interferon-alpha and interleukin-12, *Eur. J. Immunol.* **25** (1995) 2656-2660.
[29] H.E. Gendelman *et al.*, A selective defect of interferon alpha production in human immunodeficiency virus-infected monocytes, *J. Exp. Med.* **172** (1990) 1433-1442.
[30] R.M. Verdijk *et al.*, Polyriboinosinic polyribocytidylic acid (poly (I:C)) induces stable maturation of functionally active human dendritic cells, *J. Immunol.* **163** (1999) 57-61.
[31] W.J. Lee *et al.*, Purification and molecular cloning of an inducible Gram-negative bacteria-binding protein from the silkworm *Bombyx mori*, *Proc. Natl. Acad. Sci.* USA **93** (1996) 7888-7893.
[32] M. Ochiai and M. Ashida, A pattern recognition protein for β-1,3-glucan. The binding domain and the cDNA cloning of β-1,3-glucan recognition protein from the silkworm, *Bombyx mori*, *J. Biol. Chem.* **275** (2000) 4995-5002.

[33] L. Cerenius *et al.*, A β-D-glucan binding protein in crustacean blood. Structure and biological activity of a fungal recognition protein, *J. Biol. Chem.* **269** (1994) 29462-29467.

[34] C. Ma and M. Kanost, A β-1,3-glucan recognition from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade, *J. Biol. Chem.* **275** (2000) 7505-7514.

[35] A. Beschin *et al.*, Identification and cloning of a glucan- and lipopolysaccharide-binding protein from *Eisenia foetida* earthworm involved in the activation of prophenoloxidase cascade, *J. Biol. Chem.* **273** (1998) 24948-24954.

[36] S.Y. Lee *et al.*, A lipopolysaccharide- and β-1,3-glucan binding protein from hemocytes of the freshwater crayfish, *Pacifastacus leniusculus*. Purification, characterization and cDNA cloning, *J. Biol. Chem.* **275** (2000) 1337-1343.

[37] Y.S. Kim *et al.*, Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and β-1,3-glucan that mediates the signaling for the induction of innate immune genes in *Drosophila melanogaster* cells, *J. Biol. Chem.* **275** (2000) 32721-32727.

[38] H. Yoshida *et al.*, Purification of a peptidoglycan recognition protein from the hemolymph of the silkworm, *Bombyx mori*, *J. Biol. Chem.* **271** (1996) 13854-13860.

[39] M. Ochiai and M. Ashida, A pattern recognition protein for peptidoglycan: cloning the cDNA and the gene of the silkworm, *Bombyx mori*, *J. Biol. Chem.* **274** (1999) 11854-11858.

[40] S.C. Sun *et al.*, Hemolin: an insect immune protein belonging to the immunoglobulin superfamily, *Science* **250** (1990) 1729-1732.

[41] P. Kopácek *et al.*, Isolation and characterization of a hemagglutinin with affinity for lipopolysaccharides from plasma of the crayfish *Pacifastacus leniusculus*, *Dev. Comp. Immunol.* **17** (1993) 407-418.

[42] F. Vargas-Albores *et al.*, A lipopolysaccharide binding agglutinin isolated from brown shrimp (*Penaeus californiensis* Holmes) haemolymph, *Comp. Biochem. Physiol.* **104B** (1993) 407-413.

[43] T.S. Huang *et al.*, A cell adhesion protein from the crayfish, *Pacifastacus leniusculus*, a serine proteinase homologue similar to *Drosophila* masquerade, *J. Biol. Chem.* **275** (2000) 9996-10001.

[44] S.Y. Lee and K. Söderhäll, Characterization of a pattern recognition protein, a Masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*, *J. Immunol.* **166** (2001) 7319-7326.

[45] N. Seki *et al.*, Horseshoe crab (1,3) β-D-glucan-sensitive coagulation factor G. A serine protease zymogen heterodimer with similarities to β-glucan-binding proteins, *J. Biol. Chem.* **269** (1994) 1370-1374.

[46] K. Sritunyalucksana *et al.*, A β-1,3-glucan binding protein from the black tiger shrimp, *Penaeus monodon*, *Dev. Comp. Immunol.* **26** (2002) 237-245.

[47] F. Vargas-Albores *et al.*, A plasma protein isolated from brown shrimp (*Penaeus californiensis*) which enhances the activation of prophenoloxidase system by β-1,3-glucan, *Dev. Comp. Immunol.* **20** (1996) 299-306.

[48] F. Vargas-Albores *et al.*, Purification and comparison of β-1,3-glucan binding protein from white shrimp (*Penaeus vannamei*), *Comp. Biochem. Physiol.* **116** (1997) 5453-458.

[49] G.M. Yepiz-Plascencia *et al.*, Shrimp plasma HDL and β-glucan binding protein (BGBP): comparison of biochemical characteristics, *Comp. Biochem. Physiol.* **121B** (1998) 309-314.

[50] E. Lubzens *et al.*, Isolation and characterization of the high-density lipoproteins from the hemolymph and ovary of the penaeid shrimp, *Penaeus semisulcatus* (de Haan): apoproteins and lipids, *J. Exp. Zool.* **278** (1997) 339-348.

[51] S.W. Shin *et al.*, Isolation and characterization of immune-related genes from the fall webworm, *Hyphantria cunea*, using PCR-based differential display and substractive cloning, *Insect Biochem. Mol. Biol.* **28** (1998) 827-837.

[52] G. Dimopoulos *et al.*, Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites, *Proc. Natl. Acad. Sci.* USA **94** (1997) 11508-11513.

[53] S. Natori and T. Kubo, Roles of lectin in development and morphogenesis in insects. In: K. Söderhäll *et al.* (Eds.), New Directions in Invertebrate Immunology, SOS Publications, Fair Haven, 1996, pp. 175-187.

[54] D. Kang *et al.*, A peptidoglycan recognition protein in innate immunity conserved from insects to humans, *Proc. Natl. Acad. Sci.* USA **95** (1998) 10078-10082.

[55] X. Cheng *et al.*, The structure of bacteriophage T7 lysozyme, a zinc amidase and an inhibitor of T7 RNA polymerase, *Proc. Natl. Acad. Sci.* USA **91** (1994) 4034-4038.

[56] K. Söderhäll and R. Ajaxon, Effect of quinone and melanin on mycelial growth of *Aphanomyces* spp. and extracellular protease of *Aphanomyces astaci*, a parasite of crayfish, *J. Invert. Pathol.* **39** (1982) 105-109.

[57] M. St. Leger *et al.*, The effect of melanization of *Manduca sexta* cuticle on growth and infection by *Metarhizium anisopliae*, *J. Invert. Pathol.* **52** (1988) 459-470.

[58] A.F. Rowley *et al.*, Possible involvement of the prophenoloxidase system of the locust, *Locusta migratoria*, in antimicrobial activity, *J. Invert. Pathol.* **56** (1990) 31-38.

[59] A. Nappi and E. Vass, Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions, *Pigm. Cell Res.* **6** (1993) 117-126.
[60] E.A. Gregorio and N.A. Ratcliffe, The prophenoloxidase system *in vitro* interaction of *Trypanosoma rangeli* with *Rhodnius prolixus* and *Triatoma infestans* hemolymph, *Parasite Immunol.* **13** (1991) 551-564.
[61] M.J. Bidochka and A.E. Hajek, A.E. A nonpermissive entomophthoralean fungal infection increases activation of insect prophenoloxidase, *J. Invert. Pathol.* **72** (1998) 231-238.
[62] M. Ashida and P.T. Brey, Role of the integument in insect defense: prophenoloxidase cascade in the cuticular matrix, *Proc. Natl. Acad. Sci.* USA **92** (1995) 10698-10702.
[63] H.M. Müller *et al.*, A hemocyte-like cell line established from the malaria vector *Anopheles gambiae* express six prophenoloxidase genes, *J. Biol. Chem.* **274** (1999) 11727-11735.
[64] M. Sugumaran, Roles of the insect cuticle in host defense reactions. In: K. Söderhäll *et al.* (Eds.), New Directions in Invertebrate Immunology, SOS Publications, Fair Haven, 1996, pp. 355-374.
[65] S. Sato *et al.*, Ascidian tyrosinase gene: its unique structure and expression in the developing brain, *Dev. Dyn.* **208** (1997) 363-374.
[66] K. Sritunyalucksana *et al.*, Molecular cloning and characterization of prophenoloxidase in the black tiger shrimp, *Penaeus monodon*, *Dev. Comp. Immunol.* **23** (1999) 179-186.
[67] T. Burmester, Molecular evolution of the arthropod hemocyanin superfamily, *Mol. Biol. Evol.* **18** (2001) 184-195.
[68] A. Aspán *et al.*, cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation, *Proc. Natl. Acad. Sci.* USA **92** (1995) 939-943.
[69] H. Decker and T. Rimke, Tarantula hemocyanin shows phenoloxidase activity, *J. Biol. Chem.* **273** (1998) 25889-25892.
[70] H.G. Hergenhahn *et al.*, Purification and characterization of an α2-macroglobulin-like proteinase inhibitor from plasma of the crayfish *Pacifastacus leniusculus*, *Biochem. J.* **255** (1987) 801-806.
[71] P.B. Armstrong and J.P. Quigley, Immune function of α2-macroglobulin in invertebrates, *Prog. Mol. Subcell. Biol.* **15** (1996) 101-130.
[72] A. Aspán and K. Söderhäll, Purification of phenoloxidase from crayfish blood cells and its activation by endogenous serine proteinase, *Insect Biochem.* **21** (1991) 363-373.
[73] A. Aspán *et al.*, Purification and characterization of a prophenoloxidase activating enzyme from crayfish blood cells, *Insect Biochem.* **20** (1990) 709-718.
[74] T. Muta *et al.*, Proclotting enzyme from horseshoe crab hemocytes. CDNA cloning, disulfide locations, and subcellular localization, *J. Biol. Chem.* **265** (1990) 22426-22433.
[75] T. Muta *et al.*, Horseshoe crab coagulation factor B. A unique serine protease zymogen activated by cleavage of an Ile-Ile bond, *J. Biol. Chem.* **268** (1993) 21384-21388.
[76] S.Y. Lee *et al.*, Molecular cloning of cDNA for pro-phenol-oxidase-activating factor I, a serine protease is induced by lipopolysaccharide or 1,3-beta-glucan in coleoptera insect, *Holotrichia diomphalia* larvae, *Eur. J. Biochem.* **257** (1998) 615-621.
[77] H. Jiang *et al.*, Pro-phenoloxidase activating proteinase from an insect, *Manduca sexta*: a bacteria-inducible protein similar to *Drosophila* easter, *Proc. Natl. Acad. Sci.* USA **95** (1998) 12220-12225.
[78] D. Satoh *et al.*, Prophenoloxidase-activating enzyme of the silkworm, *Bombyx mori*: purification, characterization and cDNA cloning, *J. Biol. Chem.* **274** (1999) 7441-7453.
[79] R. Wang *et al.*, Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*, *Eur. J. Biochem.* 268 (2001) 895-902.
[80] M.W. Johansson, Cell adhesion molecules in invertebrate immunity, *Dev. Comp. Immunol.* **23** (1999) 303-315.
[81] M.W. Johansson *et al.*, Peroxinectin, a novel cell adhesion protein from crayfish blood, *Biochem. Biophys. Res. Commun.* **216** (1995) 1079-1087.
[82] M.W. Johansson and K. Söderhäll, Isolation and purification of a cell adhesion factor from crayfish blood cells, *J. Cell Biol.* **106** (1988) 1795-1803.
[83] K. Sritunyalucksana *et al.*, Peroxinectin, a cell adhesive protein associated with the proPO system from the black tiger shrimp, *Penaeus monodon*, *Dev. Comp. Immunol.* **25** (2001) 353-363.
[84] L.M. Perrazolo and M.A. Barracco, The prophenoloxidase activating system of the shrimp *Penaeus paulensis* and associated factors, *Dev. Comp. Immunol.* **21** (1997) 385-395.
[85] T. Liu *et al.*, Limunectin, *J. Biol. Chem.* **266** (1991) 14813-14821.
[86] N. Fujii *et al.*, Isolation, cDNA cloning, and characterization of an 18-kDa hemagglutinin and amebocyte aggregation factor from *Limulus polyphemus*, *J. Biol. Chem.* **267** (1992) 22452-22459.
[87] P.O. Thörnqvist *et al.*, Opsonic activity of cell adhesion proteins and β-1,3-glucan-binding proteins from two crustaceans, *Dev. Comp. Immunol.* **18** (1994) 3-12.

[88] J. Rantamäki *et al.*, Isolation of a 90 kDa protein from haemocytes of *Blaberus craniifer* which has similar properties to the 76 kDa protein from crayfish haemocytes, *J. Insect Physiol.* **37** (1991) 627-634.
[89] E. Kotani *et al.*, Cloning and expression of the gene hemocytin, an insect humoral lectin with is homologous with the mammalian von Willebrand factor, *Biochim. Biophys. Acta* **1260** (1995) 245-258.
[90] N.C. Franc *et al.*, Croquemort, a novel *Drosophila* hemocyte/macrophage receptor that recognizes apoptotic cells, *Immunity* **4** (1996) 431-443.
[91] K.D. Clark *et al.*, Plasmatocyte spreading peptide is encoded by an mRNA differentially expresses in tissues of the moth *Pseudoplusia includens*, *Biochem. Biophys. Res. Commun.* **250** (1998) 479-485.
[92] K.D. Clark *et al.*, Isolation and identification of a plasmatocyte-spreading peptide from the hemolymph of the lepidopteran insect *Pseudoplusia includens*, *J. Biol. Chem.* **272** (1997) 23440-23447.
[93] K. Sritunyalucksana and K. Söderhäll, The proPO and clotting system in crustaceans, *Aquaculture* **191** (2000) 53-69.
[94] K. Morishita *et al.*, Chromosomal gene structure of human myeloperoxidase and regulation of its expression by granulocyte colony-stimulating factor, *J. Biol. Chem.* **262** (1987) 15208-15213.
[95] M.W. Johansson *et al.*, Myeloperoxidase mediates cell adhesion via the $\alpha_M\beta_2$ integrin (Mac-1, CD11b/CD18), *J. Cell Sci.* **110** (1997) 1133-1139.
[96] S.J. Klebanoff, Myeloperoxidase: occurrence and biological function. In: J. Everse *et al.* (Eds.), Peroxidase in chemistry and biology, CRC Press, Boca Raton, 1991, pp. 2-35.
[97] R.E. Nelson *et al.*, Peroxidasin: a novel enzyme-matrix protein of *Drosophila* development, *EMBO J.* **13** (1994) 3438-3447.
[98] N. Horikoshi *et al.*, Isolation of differentially expressed cDNAs from p53-dependent apoptotic cells, Activation of the human homologue of the *Drosophila* peroxidasin gene, *Biochem. Biophys. Res. Commun.* **261** (1999) 864-869.
[99] E. Ruoslahti, RGD and other recognition sequences for integrins, *Annu. Rev. Dev. Cell Biol.* **12** (1996) 697-715.
[100] T. Holmblad *et al.*, Identification and cloning of an integrin β subunit from hemocytes of the freshwater crayfish *Pacifastacus leniusculus*, *J. Exp. Zool.* **277** (1997) 255-261.
[101] M.W. Johansson *et al.*, A cell-surface superoxide dismutase is a binding protein for peroxinectin, a cell-adhesive peroxidase in crayfish, *J. Cell Sci.* **112** (1999) 917-925.
[102] T. Holmblad and K. Söderhäll, Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity, *Aquaculture* **172** (1999) 111-123.
[103] E. Kromer *et al.*, Cloning of a *Locusta* cDNA encoding a precursor peptide of two structurally related proteinase inhibitors, *Insect Biochem. Mol. Biol.* **24** (1994) 329-331.
[104] A.C. Daquinag *et al.*, A novel endogenous inhibitor of phenoloxidase from *Musca domestica* has a cystine motif commonly found in snail and spider toxins, *Biochemistry* **38** (1999) 2179-2188.
[105] H. Jiang and M.R. Kanost, Characaterization and functional analysis of twelve naturally occurring reactive site variants of serpin-1 from *Manduca sexta*, *J. Biol. Chem.* **272** (1997) 1082-1087.
[106] M. Sugumaran *et al.*, Endogenous protease inhibitors prevent undesired activation of prophenoloxidase in insect hemolymph, *Biochem. Biophys. Res. Commun.* **132** (1985) 1124-1129.
[107] T. Sasaki, Amino acid sequences of two basic chymotrypsin inhibitors from silkworm larval hemolymph, *Biol. Chem. Hoppe Seyler* **369** (1988) 1235-1241.
[108] Y. Aso *et al.*, Inhibition of pro-phenoloxidase activating enzyme from *Bombyx mori* by endogeneous chymotrypsin inhibitors, *Biochem. Mol. Biol. International* **33** (1994) 751-758.
[109] A. Aspan *et al.*, The effect of endogeneous proteinase inhibitors on the prophenoloxidase activating enzyme, a serine proteinase from crayfish haemocytes, *Insect Biochem.* **20** (1990) 485-492.
[110] Z. Liang *et al.*, Pacifastin, a novel 155 kDa heterodimeric proteinase inhibitor containing a unique transferrin chain, *Proc. Natl. Acad. Sci.* USA **94** (1997) 6682-6687.
[111] N.S. Bartfeld and J.H. Law, Isolation and molecular cloning of transferrin from the tobacco hornworm, *Manduca sexta*. Sequence similarity to the vertebrate transferrins, *J. Biol. Chem.* **265** (1990) 21684-21691.
[112] R.C. Jamroz *et al.*, Transferrin in a cockroach: molecular cloning, characterization, and suppression by juvenile hormone, *Proc. Natl. Acad. Sci.* USA **90** (1993) 1320-1324.
[113] T. Kurama *et al.*, Molecular characterization of an insect transferrin and its selective incorporation into eggs during oogenesis, *Eur. J. Biochem.* **228** (1995) 229-235.
[114] R. Biogegrain *et al.*, Insect Immunity: two proteinase inhibitors from hemolymph of *Locusta migratoria*, *Biochem. Biophys. Res. Commun.* **189** (1992) 790-793.
[115] N. Nakakura *et al.*, Isolation and structural determination of three peptides from the insect *Locusta migratoria*. Identification of a deoxyhexose-linked peptide, *Eur. J. Biochem.* **204** (1992) 147-153.
[116] C. Kellenberger *et al.*, Serine protease inhibition by insect peptides containing a cysteine knot and a triple-stranded -β-sheet, *J. Biol. Chem.* **270** (1995) 25514-25519.

Mitogenic, Lectin and Cytokine-like Activities

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A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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Mitogenic factors in *Eisenia foetida* coelomic fluid as regulatory molecules

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Abstract. Free coelomocytes that are derived from mesenchymal lining of the coelomic cavity are able to proliferate under certain conditions but the proliferation rate is low. Mitogenic factors evidenced in the coelomic fluid act on mammalian cells. Their role in earthworm physiology and immunity remains unclear but they may have regulatory functions.

1. Introduction

Burnet's clonal theory [1] suggesting that differentiation of immunocytes to the effector functions is inseparable from their proliferation is undoubtedly accepted in the vertebrate immunology. The level of proliferative activity is often used as a valuable indicator of the cell differentiation. Until now, Burnet's dogma has not been directly confirmed in the immunology of invertebrates, but some available data do not exclude the role of proliferation in immunocyte differentiation. In particular in annelids there is evidence that free coelomocytes are able to proliferate under certain conditions although the proliferation rate is rather low. Moreover, some soluble factors controlling the proliferation of particularly vertebrate cells have been detected suggesting the presence of humoral control of cellular processes in invertebrates.

2. The origin of free coelomocytes

The existence of a leukopoietic organ was evidenced in megascolecid earthworms [2, 3], but, surprisingly, the stem-cell function of this organ was not proven. Mesenchymal lining of the coelomic cavity is believed to be the primary and main source of free coelomocytes (chloragocytes and amoebocyte coelomocytes having primarily defense functions)[4, 5]. The precursor role of the mesenchymal lining was experimentally substantiated when tissue explants were cultivated *in vitro*. Within few days free cells released from the lining were detected into the medium and identified as chloragocytes and round-shaped less differentiated cells [6, 7].

3. Proliferative capacity of free coelomocytes

The basal *in vitro* level of coelomocyte proliferation is very low and not more than 8 % of cells incorporate ^{3}H-thymidine [8]. However, the basal proliferation can increase upon different stimuli. Roch [9, 10] compared the proliferative response of *Eisenia foetida* coelomocytes to allo- and xenografts and he found increased DNA synthesis in nonadherent cells with the maximum on day 4 after grafting. Similarly, Parry [11] followed coelomocyte proliferation after grafting and during wound healing. While chloragocytes remained always mitotically inactive, increased proliferation of amoebocytic coelomocytes was recorded during the graft rejection and even during autografting. In contrary, no ^{3}H-thymidine incorporation was detected in free coelomocytes after a body wall injury and during wound healing. This observation is in good agreement with the results of Burke [12] who described the crucial role of basal cells of the epidermis in wound healing and graft rejection in *E. foetida*.

Surprisingly, the *in vitro* proliferative activity of coelomocytes decreased after parenteral protein prestimulation *in vivo* and incerased only after a second contact with the same antigen [8]. Precursor cells in the mesenchymal lining of the coelomic cavity responded to the protein stimulation immediately. These results support the opinion that foreign stimuli might induce the proliferation and differentiation of precursor cells that then can enter the coelomic cavity and fullfil their defense functions. These "primed" cells undergo further division only after the second contact with same stimulus (protein).

Coelomocytes respond by increased proliferation to phytomitogens. Though all coelomocytes bind concanavalin A, only a small portion (less than 1 %) incorporates ^{3}H-thymidine even at the time when the scintillation measurement reaches maximum values [10, 13]. Similarly, increased ^{3}H-thymidine incorporation by coelomocytes was detected after phytohemagglutinin stimulation [14].

4. Mitogenic factors

Proliferation of earthworm coelomocytes can be controlled either directly upon binding of *e.g.* phytomitogens and regulated by soluble factors. Actually we have only scarce evidence for the existence of such souluble mediators until now. Moreover, available data are restricted to the mitogenic effect of such factors on vertebrate cells. Indeed, mitogenic activity of the coelomic fluid of *Lumbricus terrestris* earthworms on murine and human lymphocytes was described already in 1984 by Wojdani *et al.* [15]. They found that the coelomic fluid collected from earthworms prestimulated with rabbit erythrocytes exhibits significantly higher mitogenic activity as compared to the coelomic fluid from nontreated individuals. This might reflect the nonspecific increase of coelomic fluid proteins after injection [16]. Interestingly, primarily T cells were activated and neutralization of coelomic fluid agglutinins had no effect on proliferation [15].

Another earthworm mitogen was detected in a glycolipoprotein complex G-90 isolated from the whole *E. foetida* tissue extract [17, 18]. Mitogenic effect of G-90 on mouse melanoma and fibrosarcoma cells in serum-free conditions seems to be associated with insulin-like proteins. These proteins crossreact with anti-insulin antibodies and display molecular mass of 38, 39 and 56 kDa. The presence of insulin-like growth factors was proven in various invertebrates and thus the existence of similar growth factor in earthworms is highly expected.

More recently a 60-kDa coelomic mitogenic factor (CMF) was isolated and partially characterized [19]. It was found that both coelomic fluid and CMF-enriched fraction block

concanavalin A-induced murine spleen cell proliferation while exhibiting a synergistic effect on LPS-induced proliferation. Inhibition of concanavalin A-induced spleen cell proliferation results from a direct interaction of CMF with the lectin. CMF is present in the coelomic fluid as a trimer of a 20-kDa protein. N-terminal amino acid sequence reveals partial sequence homology with phospholipase A2, and moreover, CMF-enriched coelomic fluid fraction exhibits phospholipase activity. The mechanism of mitogenic activity of CMF has not been identified so far. Preliminary FACS analyses of blast formation indicated that the main proliferating subpopulation are T cells. On the other hand, activation of purified T-cells was not observed. These data suggest that cooperation of T and B cell subsets or macrophages, respectively, is crucial for the mitogenic effect of CMF. The synergistic effect of LPS and CMF on spleen cell proliferation indicates that while LPS triggers B-cell proliferation, CMF stimulates the production of cytokines activating T-cell proliferation. We cannot overlook the possibility that CMF might affect cell membrane fluidity due to the phospholipase A2 enzyme activity [20].

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References

[1] F.M. Burnet, The Clonal Selection Theory of Acquired Immunity, Cambridge University Press, London, 1959.

[2] J.E. Kindred, The leucocytes and leucocytopoietic organs of an oligochaete, *Pheritima indica* (Horst), *J. Morphol. Physiol.* **47** (1929) 435-478.

[3] M.M. Friedman and L. Weiss, The leukocytic organ of the megascolecid earthworm, *Amynthas diffringens* (*Annelida, Oligochaeta*), *J. Morphol.* **174** (1982) 251-268.

[4] G.R. Cameron, Inflammation in earthworms, *J. Pathol. Bacteriol.* **35** (1932) 933-972.

[5] E. Liebmann, The coelomocytes of *Lumbricidae*, *J. Morphol.* **71** (1942) 221-249.

[6] V. Janda and P. Bohuslav, Sur l'explantation du tissu de la paroi intestinale et des amibocytes de *Lumbricus terrestris* L. et des cellules d'épithelium intestinal d'*Anodonta cygnea* L., *Publ. Fac. Sci. Univ. Charles* **133** (1934) 1-23.

[7] M. Bilej *et al.*, A new approach to *in vitro* studies of antigenic response in earthworms, *Dev. Comp. Immunol.* **18** (1994) 363-367.

[8] M. Bilej *et al.*, Repeated antigenic challenge induces earthworm coelomocyte proliferation, *Immunol. Lett.* **32** (1992) 181-184.

[9] Ph. Roch, Leukocyte DNA-synthesis in grafted lumbricids: an approach to study histocompatibility in invertebrates, *Dev. Comp. Immunol.* **3** (1979) 417-428.

[10] Ph. Roch *et al.*, Response of earthworm leukocytes to concanavalin A and transplantation antigen, In W.H. Hildemann and A.A Benedict (Eds.), Immunologic Phylogeny, Plenum Publishing Corporation, New York, 1975, pp.45-54.

[11] M.J. Parry, Evidence of mitotic division of coelomocytes in the normal, wounded and grafted earthworm *Eisenia foetida*, *Experientia* **32** (1976) 449-450.

[12] J.M. Burke, Wound healing in *Eisenia foetida* (*Oligochaeta*). I. Histology and ^{3}H-thymidine radioautography of the epidermis. *J. Exp. Zool.* **188** (1974) 49-63.

[13] Ph. Roch and P. Valembois, Evidence for concanavalin A-receptors and their redistribution on lumbricid leukocytes, *Dev. Comp. Immunol.* **2** (1978) 51-63.

[14] J. Toupin and G. Lamoureux, Coelomocytes of earthworms: phytohemagglutinin (PHA) responsiveness, In R.K. Wright and E.L. Cooper (Eds.) Phylogeny of Thymus and Bone Marrow-Bursa Cells, Elsevier, Amsterdam, 1976, pp. 19-27.

[15] A. Wojdani *et al.*, Mitogenic effect of earthworm (*Lumbricus terrestris*) coelomic fluid on mouse and human lymphocytes, *Immunobiology* **166** (1984) 157-167.

[16] L. Tuckova *et al.*, Response to parenteral stimulation in earthworms *L. terrestris* and *E. foetida*, *Dev. Comp. Immunol.* **12** (1988) 287-296.

[17] T. Hrzenjak *et al.*, A new source of biologically active compounds - earthworm tissue (*Eisenia foetida, Lumbricus rubelus*), *Comp. Biochem. Physiol.* **102A** (1992) 441-447.

[18] M. Hrzenjak *et al.*, Mitogenicity of the earthworm's (*Eisenia foetida*) insulin-like proteins, *Comp. Biochem. Physiol.* **104B** (1993) 723-729.

[19] R. Hanusova *et al.*, Identification of a coelomic mitogenic factor in *Eisenia foetida* earthworm, *Immunol. Lett.* **65** (1999) 203-211.

[20] C.E. Hack *et al.*, A role for secretory phospholipase A2 and C-reactive protein in the removal of injured cells, *Immunol. Today* **18** (1997) 111-115.

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TNF analogue in earthworms and the role of lectin-saccharide interactions in regulatory function of primitive cytokines

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Abstract. Based on the assumption that invertebrates, as vertebrates, possess factors regulating responses to infection or wounding, studies dealing with the evolution of immunity have focussed on the isolation and characterization of putative cytokine-related molecules from invertebrates. Until recently, most of our knowledge of cytokine- and cytokine receptor-like molecules in invertebrates relies on functional assays and similarities at the physicochemical level. As such, a phylogenetic relationship between invertebrate cytokine-like molecules and vertebrate counterparts could not be convincingly demonstrated. Recent data from annelids suggest that invertebrate cytokine-like molecules and vertebrate factors do not have the same evolutionary origin. However, the convergence of function of invertebrate cytokine-like molecules and vertebrate counterparts involved in innate immune defenses may be based on similar lectin-like activities.

1. Introduction

Invertebrates share many of the innate immune mechanisms of vertebrates although they lack the adaptive immune defenses that rely on antibodies or lymphocytes. Cellular defenses of invertebrate include wound repair, clotting and coagulation responses, phagocytosis and encapsulation reactions. Invertebrate lectin-like molecules [1] and "Pattern Recognition Receptors" [2] that recognize conserved microbial antigens, such as LPS, peptidoglycan or β-1,3-glucans, participate in non-self recognition during cellular immune reactions. In addition, humoral defenses also include a range of naturally occurring antimicrobial factors such as lysozyme-like proteins, proteases, cytolysins, antimicrobial peptides, phenoloxidase and metabolites of the prophenoloxidase-activating cascade [3, 4].

In vertebrates, cytokines are the major regulators of the host defense processes. These molecules secreted mainly by immunocytes are thought to mediate their activity mainly via interaction with specific cytokine receptors. Considerations of the functional parallels between innate (non-adaptive) host defenses in invertebrates and vertebrates led to the hypothesis that invertebrates possess soluble cytokine-like mediators that regulate inflammatory responses to infection or wounding [for review 5]. Accordingly, several putative functional analogues of inflammatory cytokines, particularly IL-1-, IL-2-, IL-6-, IL-

8, TGF-β- and TNF-like molecules were detected in a variety of invertebrates such as porifers, nematodes, annelids, mollusks, insects, echinoderms, and protochordates (Table 1). Moreover, several reports have suggested the existence of putative invertebrate IL-1, IL-6, IL-2 and IFN-γ cytokine receptor-like molecules. Collectively, these studies suggest the presence of functional analogues of cytokines and cytokine-receptors in invertebrates, opening the possibility that an invertebrate cytokine network is operative and regulates host defense mechanisms as in vertebrates. However, these studies did not convincingly demonstrate a common evolutionary origin for cytokine-like network interactions [for review 6]. Indeed, our current knowledge on invertebrate cytokine- and cytokine receptor-like molecules is mainly based on immunocytochemical methods and functional assays such as (i) the cross-reactivity of antibodies neutralizing the binding of vertebrate cytokines to their specific receptors and (ii) the sensitivity of invertebrate immunocytes to vertebrate cytokine action, or vice versa on the responsiveness of vertebrate immune cells to invertebrate factors. (iii) In some reports, the analogy of invertebrate and vertebrate cytokine- and cytokine receptor-like molecules was also based on biochemical similarities such as the molecular mass and the isoelectric point. Finally, marginal trials were performed to identify homologues of vertebrate cytokine and cytokine receptor genes in invertebrates. However, amino acid or gene sequence homology analyses of the putative invertebrate cytokine molecules are not conclusive in view of the limited length of homologous regions. Therefore, up to now, it has not been possible to demonstrate unequivocally a phylogenetic relationship between vertebrate cytokines and their invertebrate functional analogues. Consequently, although the lack of molecular evidence does not preclude the existence of true homologues of vertebrate cytokines and their receptors, the question of whether the first cytokine arose in invertebrates still remains open.

Table 1: Cytokine-like molecules identified in protostome and deuterostome invertebrates

Taxa	Cytokine-like molecules
Porifera	TNF
Nematoda	TGF-β
Annelida	IL-1, TNF
Mollusca	IL-1, TNF, LT, IL-6, IL-8, TGF- β, IL-2
Arthropoda	IL-1, TNF, TGF- β
Echinodermata	IL-1α/β, IL-1 precursor, TNF, IL-6, IL-2, TGF- β
Protochordata	IL-1, TNF, IL-2, TGF- β

Interestingly, up to now the antibodies used for cross-reactivity studies have mainly been antibodies that neutralize the activity of mammalian cytokines by impairing their binding to specific cytokine receptors. This approach is based on the assertion that mammalian cytokines exert their activity only through interaction with a specific cytokine receptor. As discussed in another paper in this issue (De Baetselier *et al.*), evidence is accumulating that this postulate undervalues the possible modes of actions of cytokines. Indeed, cytokines also exert biological functions in a cytokine receptor-independent way, which may shed a new light on the molecular basis of the functional analogies between vertebrate and invertebrate defense molecules.

2. Invertebrate cytokine-like molecules as lectins

Studies from our group suggest that cytokine-like molecules in protostome invertebrates and mammalian inflammatory cytokines share similar lectin-like activities. This saccharide-recognition activity may be responsible for the functional analogies evidenced between invertebrate cytokine-like factors and vertebrate counterparts as discussed below.

2.1. Coelomic cytolytic factor as an earthworm defense molecule

We have identified and cloned a 42-kDa protein named CCF (coelomic cytolytic factor, originally described as CCF-1) from the coelomic fluid of the earthworm *Eisenia foetida* [7, 8]. CCF is localized in the cells of chloragogenous tissue adjacent to the gut wall and in the translucent free large coelomocytes, i.e. in cells with macrophage-like function [9]. CCF shows homology with the saccharide-binding motif of bacterial and animal β-1,3-glucanases, with Gram-negative bacteria binding protein and β-1,3-glucan-recognition proteins of arthropods, and with glucan-sensitive factor G from the horseshoe crab *Limulus polyphemus* [for review 10]. Interestingly, although these proteins show high homology in the putative polysaccharide-binding domain and the catalytic sites of the bacterial glucanases, neither CCF nor its invertebrate homologues exhibit glucanase activity. It was proposed that these glucan-binding proteins developed from a primitive glucanase that evolved to proteins without enzymatic activity but that instead bind glucans and operate as elicitors of defense reactions by recognizing non-self [11]. CCF binds efficiently the O-antigen of LPS, muramyl dipeptide of peptidoglycan, β-1,3-glucans and *N,N'*-diacetylchitobiose (β-1,4-N-acetylglucosidic link) [8, 12]. Upon recognition of these cell wall components of bacteria or yeast, CCF triggers the activation of the prophenoloxidase (proPO) cascade, an important defense mechanism both in protostomian and deuterostomian invertebrates (Fig. 1). Generally, upon recognition of polysaccharides of microbial cell walls, serine proteinases cleave by limited proteolysis inactive proPO to its active state phenoloxidase. Subsequently, the active enzyme catalyses the *o*-hydroxylation of monophenols and oxidation of diphenols to quinones, which are subsequently, polymerized to melanin which exhibits cytotoxic and antibacterial properties [4]. The importance of CCF in the induction of proPO in *E. foetida* was confirmed showing that when CCF is removed from the coelomic fluid, the activation cascade is blocked. However exogenous supply of recombinant CCF restores the L-DOPA oxidation (substrate for proPO) in CCF-depleted coelomic fluid [8, 12]. These data point out for the existence of the proPO cascade in annelids.

The broad saccharide recognition pattern of CCF might result from the existence of distinct domains in the molecule. Thus attempts to identify the CCF lectin-like domains were initiated. Deletion mutants were tested for their ability to reconstitute the proPO cascade in *E. foetida* coelomic fluid depleted of native CCF in the presence of LPS, laminarin (β-1,3-glucan), *N,N'*-diacetylchitobiose and muramic acid. In addition, affinity chromatography on immobilized LPS, β-1,3-glucans or *N,N'*-diacetylchitobiose of CCF peptides was performed [12]. We found that *E. foetida* CCF contains two distinct saccharide-binding domains. A first domain showing homology with the saccharide-binding motif of β-1,3-glucanases and invertebrate defense molecules located in the central part of CCF, is implicated in interactions with LPS and β-1,3-glucans. The C-terminal domain mediates interactions of CCF with *N,N'*-diacetylchitobiose and muramic acid. These data provide evidence for the presence of spatially distinct lectin-like pattern recognition domains within an invertebrate defense molecule.

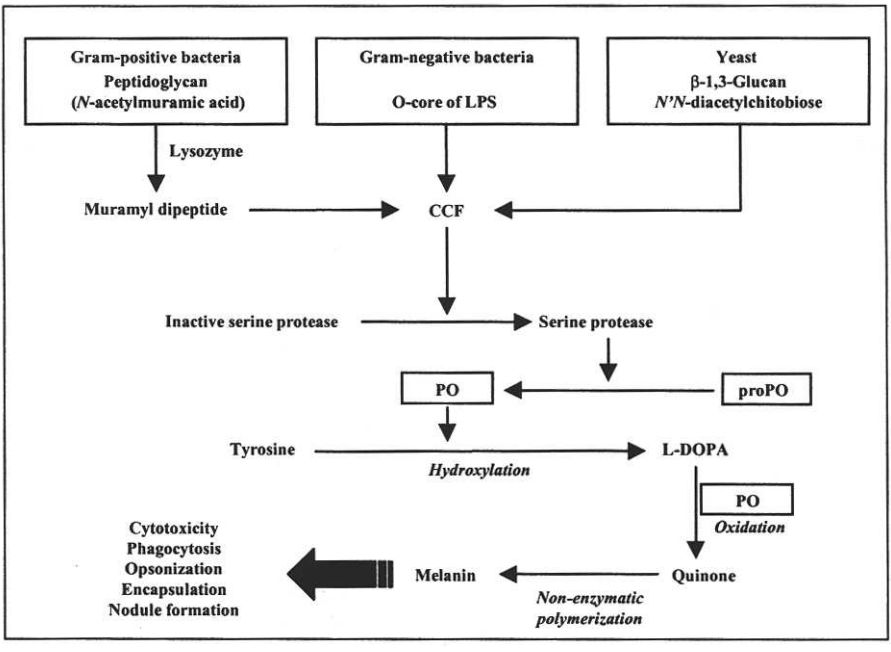

Fig. 1: Putative role of CCF in activation of the proPO cascade in *E. foetida* earthworm.

Melanin and its precursors involved in the prophenoloxidase activating system stimulate, besides antimicrobial properties, a wide range of other biological activities including phagocytosis and opsonization, capsule/nodule formation, and wound healing. In earthworm cytotoxic and antimicrobial activities are tightly connected to the aggregating, hemolytic and opsonizing activities present in the coelomic fluid [13-16]. To this regard, we observed that the cytotoxicity of *E. foetida* coelomocytes against coelomocytes from *L. terrestris* is blocked by anti-CCF monoclonal antibody, suggesting that CCF is involved in the cell-mediated cytotoxic reaction in earthworms [9]. Moreover, CCF agglutinates smooth but not rough Gram-negative bacteria or Gram-positive bacteria [8]. In addition, CCF was reported to be involved in the opsonizing properties of the coelomic fluid providing an efficient mechanism for phagocytosis during earthworm defense reactions [7]. More recently, CCF was proved to potentiate the lytic activity of the coelomic fluid on rat, mice and guinea pig red blood cells [17]. CCF is not hemolytic by itself, however present data suggest that CCF interacts with the H3 hemolysin present in *E. foetida* coelomic fluid [18]. The hemolytic activity of the coelomic fluid is impaired by removing CCF from the coelomic fluid or by preincubating the coelomic fluid with insoluble β-1,3-glucans. We hypothesize that CCF, by binding to saccharide moieties on red blood cells favors the interaction of hemolysins with erythrocyte membranes.

The pleiotropic activities of CCF suggest that this pattern recognition molecule plays a key role in innate defense mechanisms of *E. foetida* earthworm.

2.2. Coelomic cytolytic factor as a TNF analogue

Earthworms have provided for more than 30 years a useful model for comparative immunology. Their coelomic fluid exhibits a large variety of biological effects including bacteriostatic, hemolytic, proteolytic, and cytolytic activities that are involved in effective defense mechanisms against invaders [for review 10, 19, 20]. It was suggested that invertebrate molecules with tumorilytic activity might be analogous to the vertebrate cytokine TNF. CCF was originally identified in experiments aiming to characterize novel cytolytic factors from the coelomic fluid of *E. foetida*. We observed that the coelomic fluid lyses TNF-sensitive tumor L929 cells in a protease-independent way, and subsequent isolation of the lytic proteins led to the identification of the 42-kDa protein CCF [7]. In addition to the TNF-like lytic activity, CCF showed other similarities with this mammalian cytokine. CCF is secreted by macrophage-like coelomocytes upon LPS stimulation while TNF is produced by macrophages [9, 21]. Moreover, CCF is involved in the opsonizing properties of the earthworm coelomic fluid [7]. Similarly TNF was reported to provide opsonin-like signal that mediates the attachment of bacteria to macrophages [22]. Furthermore, CCF and TNF proteins bind β-1,3-glucans as well as *N,N'*-diacetylchitobiose via lectin-like interaction [8, 10, 23, 24]. In addition, murine monoclonal antibodies elicited against the lectin-like domain of TNF (TIP domain), that is spatially distinct from the TNF-receptor binding site [25], cross-react with CCF. Finally a monoclonal antibody elicited against CCF reacts with TNF. Together these data suggest that CCF and TNF share similar β-1,3-glucan and *N,N'*-diacetylchitobiose lectin-like activities/domains.

The lectin-like domain of TNF was shown to be involved in the killing of African and American trypanosomes by TNF [25-27]. So, in view of the similar *N,N'*-diacetylchitobiose lectin-like activity of CCF and TNF, the possible trypanolytic activity of CCF was investigated [24, 28]. The coelomic fluid of *E. foetida* as well as purified CCF display potent trypanolytic activity that can be inhibited not only by anti-CCF monoclonal antibodies but also by *N,N'*-diacetylchitobiose and anti-TIP TNF antibodies. Vice versa, anti-CCF antibodies neutralize TNF-mediated trypanolysis. In addition, using the ability of CCF to trigger the proPO cascade upon saccharide recognition in *E. foetida* coelomic fluid, the N-linked *N,N'*-diacetylchitobiose core of the variant-specific glycoprotein (VSG), that acts as a protective coat on bloodstream forms of the African trypanosome *T. brucei*, was identified as a possible target for CCF on the trypanosome surface during trypanolytic events.

More recently TNF was reported to play an important role in inflammatory process by increasing the membrane conductance in endothelial cells and peritoneal macrophages [29, 30]. This effect is independent of the TNF-receptor since it occurs in cells isolated from mice deficient in both types of TNF-receptors (p55 and p75). The ion channel gating effect of TNF was found to be mediated by the *N,N'*-diacetylchitobiose lectin-like domain of molecule. The increased ion permeability induced by TNF is also inhibited by amiloride, an inhibitor of sodium transport [30]. Therefore, the increase in the in- and outward current in mammalian cells was suggested to result from the binding of TNF to endogenous ion channel transporters or to proteins coupled to ion channels. Similarly, we documented that CCF activates an amiloride-sensitive cationic channel in murine endothelial cells and macrophages via its *N,N'*-diacetylchitobiose lectin-like domain [31]. CCF, as TNF treatment, increases the outward current in macrophages from mice lacking both types of TNF-receptors excluding the interaction of the CCF with the TNF-receptor.

Together, our data suggest that CCF and TNF share lectin-like activities that have been conserved as recognition mechanism in innate defense reactions in invertebrates and vertebrates respectively (Fig. 2). Importantly, despite their functional analogies, CCF and TNF do not show gene homology indicating a lack of common evolutionary origin [24].

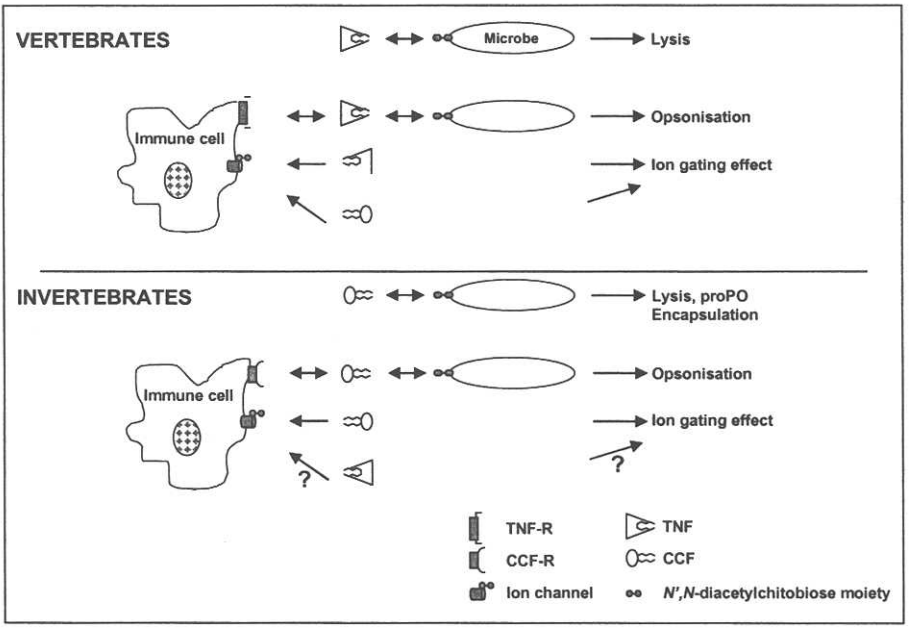

Fig. 2: Putative involvement of TNF and CCF in vertebrate and invertebrate innate defense mechanisms (reproduced from [6] with courtesy of Birkhäuser Verlag, Basel).

3. Conclusion

One of the challenges in comparative biology is to ascertain to what extent animals have homologous structures that derive from a common ancestor or whether any similarities are due to convergent evolution resulting from the need to perform similar biological functions. The striking superficial similarities between processes taking place in the invertebrates and vertebrates represents a great temptation to consider these two to be homologous, that is to have the same evolutionary origin. However, homology must not be based on functional similarities but on gene or protein sequence similarities [32].

Our comparative analysis shows that despite their functional analogies, CCF and TNF do not show gene or amino acid sequence homology [24]. As a consequence, CCF cannot be considered as an invertebrate cytokine homologue. In fact, the functional analogies between cytokines and their presumed invertebrate counterparts may actually not reflect homology, but rather result from molecular convergence [33] based on 3D-structural similarity of the lectin-like recognition domain. Thus, invertebrate defense molecules and mammalian cytokines may display a similar lectin-like activity that has been functionally conserved as recognition mechanism in innate immunity in invertebrates and vertebrates, respectively [24]. Importantly, the ion gating activity of TNF was recently shown to cause edema resorption, suggesting that the lectin-like activity of TNF may contribute to the resolution of inflammatory processes during innate immune response in vertebrates [34, 35]. Since various cytokines possess lectin-like properties, these data suggest that mammalian cytokines may exert innate immune function (recognition of microbes, down-regulation of inflammatory processes, etc) independently of the binding to their cytokine-specific receptor [for reviews 6,

36] that would have evolved to fulfil adaptive immune function. This may indicate that we are currently underscoring the biological relevance of cytokine lectin-like activities. This postulate is illustrated in the paper of De Baetselier *et al.* in this issue.

In conclusion, in view of the little amino acid or gene sequence information available, it remains premature to propose that the invertebrate cytokine or cytokine-receptor analogues described share a phylogenetic relation with their vertebrate counterparts. The question whether the immune system arises by analogy from various origins, by homology through diversification from a common ancestor, or by a combination of both remains open.

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References

[1] J.A. Olafsen, Lectins: models of natural and induced molecules in invertebrates, *Adv. Comp. Env. Physiol.* **24** (1996) 49-76.

[2] C.A. Janeway Jr., Approaching the asymptote? Evolution and revolution in immunology, *Cold Spring Harbor Symp. Quant. Biol.* **54** (1989) 1-13.

[3] E.L. Cooper *et al.*, Invertebrate immunity: another viewpoint, *Scand. J. Immunol.* **35** (1992) 247-266.

[4] M.W. Johansson and K. Söderhäll, The prophenoloxidase activating system and associated proteins in invertebrates, *Prog. Mol. Subcell. Biol.* **15** (1996) 46-66.

[5] G. Beck and G.S. Habicht, Cytokines in invertebrates. In: K. Söderhäll *et al.* (Eds.), New directions in invertebrate immunology, SOS Publications, Fair Haven, NJ, 1996, pp. 131-154.

[6] A. Beschin *et al.*, On the existence of cytokines in invertebrates, *Cell. Mol. Life Sci.* **58** (2001) 801-814.

[7] M. Bilej *et al.*, Identification of a cytolytic protein in the coelomic fluid of *Eisenia foetida* earthworms, *Immunol. Lett.* **45** (1995) 123-128.

[8] A. Beschin *et al.*, Identification and cloning of a glucan- and lipopolysaccharide-binding protein from *Eisenia foetida* earthworm involved in the activation of prophenoloxidase cascade, *J. Biol. Chem.* **273** (1998) 24948-24954.

[9] M. Bilej *et al.*, Cellular expression of the cytolytic factor in earthworms *Eisenia foetida*, *Immunol. Lett.* **60** (1998) 23-29.

[10] M. Bilej *et al.*, Antimicrobial defense of earthworm. *Folia Microbiol.* **45** (2000) 283-300.

[11] K. Söderhäll and L. Cerenius, Role of prophenoloxidase-activating system in invertebrate immunity, *Curr. Opin. Immunol.* **10** (1998) 23-28.

[12] M. Bilej *et al.*, Distinct carbohydrate recognition domains of an earthworm defense molecule recognize Gram negative and Gram positive bacteria, *J. Biol. Chem.* **276** (2001) 45840-45847.

[13] P. Roch, A definition of cytolytic response in invertebrates, *Adv. Comp. Env. Physiol.* **23** (1996) 115-150.

[14] P. Valembois *et al.*, Antibacterial activity of the hemolytic system from the earthworm *Eisenia fetida andrei*, *J. Invert. Pathol.* **40** (1982) 21-27.

[15] A. Milochau *et al.*, Purification, characterization and activities of two hemolytic and antibacterial proteins from coelomic fluid of the annelid *Eisenia fetida andrei*, *Biochim. Biophys. Acta* **1337** (1997) 123-132.

[16] M. Lassègues *et al.*, Sequence and expression of an *Eisenia fetida*-derived cDNA clone that encodes the 40-kDa fetidin antibacterial protein, *Eur. J. Biochem.*. **246** (1997) 756-762.

[17] M. Bilej *et al.*, Interaction of hemolytic and cytolytic molecules in *Eisenia fetida*, *Dev. Comp. Immunol.* **24** Supplement 1, (2000) S98.

[18] I. Eue *et al.*, Isolation and characterization of earthworm hemolysins and agglutinins, *Dev. Comp. Immunol.* **22** (1998) 13-25.

[19] M. Bilej, Cellular defense mechanisms. In: V. Vetvicka *et al.* (Eds.), Immunology of annelids, CRC Press, Boča Raton, 1994, pp. 167-200.

[20] M. Bilej, Humoral defense mechanisms. In: V. Vetvicka *et al.* (Eds.), Immunology of annelids, CRC Press, Boca Raton, 1994, pp. 245-162.

[21] B.B. Aggarwal, Human tumor necrosis factor. Production, purification and characterization, *J. Biol. Chem.* **260** (1985) 2345-2354.

[22] G. Luo *et al.*, Tumor necrosis factor-α binding bacteria: evidence for a high-affinity receptor and alteration of bacterial virulence properties, *Infect. Immun.* **61** (1993) 830-835.

[23] E.J. Olson *et al.*, Fungal β-glucan interacts with vitronectin and stimulates TNF-α release from macrophages, *Infect. Immun.* **64** (1996) 3548-3554.

[24] A. Beschin. *et al.*, Convergent evolution of cytokines, *Nature* **400** (1999) 627-628.

[25] R. Lucas *et al.*, Mapping the lectin-like activity of tumor necrosis factor, *Science* **263** (1994) 814-817.

[26] S. Magez *et al.*, Specific uptake of tumor necrosis factor-α is involved in growth control of *Trypanosoma brucei*, *J. Cell Biol.* **137** (1997) 715-727.

[27] E. Olivares Fontt *et al.*, Effects of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-α on *Trypanosoma cruzi* trypomastigotes, *Infect. Immun.* **66** (1998) 2722-2727.

[28] E. Olivares Fontt *et al.*, *Trypanosoma cruzi* is lysed by coelomic cytolytic factor-1, an invertebrate analogue of TNF, and induces phenoloxidase activity in the coelomic fluid of *Eisenia foetida foetida*, *Dev. Comp. Immunol.* **26** (2002) 27-34.

[29] F.G. van der Goot *et al.*, Membrane interaction of TNF is not sufficient to trigger increase in membrane conductance in mammalian cells, *FEBS Lett.* **460** (1999) 107-111.

[30] M. Hribar *et al.*, The lectin-like domain of tumor necrosis factor-α increases membrane conductance in microvascular endothelial cells and peritoneal macrophages, *Eur. J. Immunol.* **29** (1999) 3105-3111.

[31] A. Bloc. *et al.*, An invertebrate defense molecule activates membrane conductance in mammalian cells by means of its lectin-like domain, *Dev. Comp. Immunol.* **26** (2002) 35-43.

[32] J. Klein, Homology between immune responses in vertebrates and invertebrates: does it exist? *Scand. J. Immunol.* **46** (1997) 558-564.

[33] S. Conway Morris, Evolution: bringing molecules into the fold, *Cell* **100** (2000) 1-11.

[34] S. Rezaiguia *et al.*, Acute bacterial pneumonia in rats increases alveolar epithelial fluid clearance by a tumor necrosis factor-α-dependent mechanism, *J. Clin. Invest.* **99** (1997) 325-335.

[35] N. Fukuda *et al.*, Mechanisms of TNF-α stimulation of amiloride-sensitive sodium transport across alveolar epithelium, *Am. J. Physiol.. Lung Cell. Mol. Physiol.* **280** (2001) L1258-L1265.

[36] R. Lucas *et al.*, Cytokines: multifunctional pathogen-specific mammalian lectins? In: E. Van Driessche *et al.* (Eds.), Lectins: biology, biochemistry, clinical biochemistry, Textop, Hellerup (Danemark), 1994, vol 10, pp. 244-249.

A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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The functional relevance of the lectin-like activities of cytokines: TNF as an illustrative example

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Abstract. Mammalian cytokines modulate the activity of the immune system. It is generally accepted that the action of cytokines results from their binding to specific receptors. However, many cytokines were shown to contain two domains. The first domain defines indeed the receptor-binding site. The second domain localized at the opposite to the receptor-binding site is necessary for the expression of the full biological activity of the cytokine. However, the exact function of the latter domain remains largely unknown. Recent evidences have shown that second domain of cytokines displays lectin-like activity allowing interaction of the cytokines with glycosaminoglycans on the cell surface of mammalian cells, thereby resulting in modulation of their biological activities. In addition, it has been suggested that the lectin-like domains of cytokines represent pathogen-specific recognition sites that can contribute to their elimination. This review focuses on the physiological relevance of the lectin-like activity of cytokines during the innate immune response in mammals, using TNF as an illustrative example.

1. Cytokine activities mediated by lectin-like interactions

Mammalian cytokines are usually produced and secreted by a various cells types and often exhibit multiple and sometimes synergistic or antagonistic functions on different target cells (pleiotropy). A particular cytokine displays its autocrine, paracrine or endocrine activities through binding to specific cell surface receptors. The coordinated regulation of expression of cytokines, as well as their corresponding receptors, underlies the concept of cytokine networks.

Many cytokines can also be considered as mammalian lectins. In this regard, IL-1α/β, IL-2 to IL-8, IL-10, IL-12, IFN-γ, TNF, TGF–β1, GM-CSF have been shown to interact with soluble glycosaminoglycans (GAG) or GAG-side chains of proteoglycans expressed on the cell surface of mammalian cells, thereby resulting in modulation of their biological activities [1-9]. For example, binding of cytokines to cell surface proteoglycans or to the extracellular matrix proteoglycans may provide a local tissue-bound reservoir of cytokines, facilitating their interaction with high affinity receptors and/or triggering receptor dimerization and biological responses [10, 11]. In turn, soluble GAG such as heparin or

heparan sulfate can modulate the activity of cytokines by competing with membrane receptors or by protecting the cytokines from proteolytic inactivation in the circulation [12, 13].

Certain cytokines, in particular IL-1, IL-2 and TNF, have also been shown to directly interact with various pathogens through *N,N'*-diacetylchitobiose and Man5/6GlcNAc2-R lectin-like interactions [14-19] (Table 1). IL-1 binds virulent *Escherichia coli*, acting as a growth factor for the bacteria. Similarly, IL-2 binds *Candida albicans* and acts as a growth factor for virulent *E. coli* and *Leishmamia mexicana*. In addition, TNF was shown to interact through lectin-like interactions with Gram-negative bacteria or fungal β-1,3 glucans. TNF also exhibits a direct lytic activity on bloodstream forms of the protozoan parasites *Trypanosoma brucei* by binding *N,N'*-diacetylchitobiose moieties on parasite surface components.

The carbohydrate-binding domains of cytokines are spatially distinct from the cytokine-receptor binding sites [20]. In the case of TNF, antibodies specific to classical mammalian cytokine receptors do not inhibit the interaction of cytokines with pathogens [14, 18, 19]. Moreover, the binding of many cytokines to GAGs does not interfere with the cytokine-cytokine receptor interactions [21]. It has been suggested that the lectin-like domains of cytokines represent pathogen-specific recognition sites that can contribute to the elimination of pathogens via opsonization and/or leukocyte activation [14, 16, 18, 21].

Table 1:Lectin properties of cytokines: specific recognition of microbes?

	Carbohydrate ligands	**Pathogen interaction**
IL-1	*N,N'*-diacetylchitobiose	Growth factor for virulent *E. coli*
	Man5/6GlcNAc2-R	Binding to *T. brucei* VSG
	Man core of VSG	
IL-2	*N,N'*-diacetylchitobiose	Binding to *Candida albicans*
	Man5/6GlcNAc2-R	Growth factor for virulent *E. coli*
		Growth factor for *Leishmania mexicana*
IL-6	β-1,3-glucan	opsonization of yeast
TNF	*N,N'*-diacetylchitobiose	opsonization of *Shigella*, *E. coli* and yeast
	Man5/6GlcNAc2-R	Lysis of African and American trypanosomes
	β-1,3-glucan	
GM-CSF	Glycosaminoglycan	Growth factor for virulent *E. coli*
BFGF	Heparin sulfate	Growth factor for African trypanosomes

2. *On the physiological relevance of the lectin-like activity of TNF: interactions with trypanosomes*

As mentioned above, the cytokine TNF was found to exhibit a direct trypanolytic activity on the protozoan parasite *T. brucei* and this activity relied on the carbohydrate specific domain of this cytokine. Subsequent studies showed that a similar interaction occurs between TNF and the protozoan parasite *T. cruzi* [22]. Since the trypanolytic activity of TNF on these parasites was recorded *in vitro* the question was addressed whether such activities are relevant *in vivo*. To tackle this important question, more extensive studies were carried out in the *T. brucei* model of infection, and the major observations and thereof-deduced conclusions are summarized below.

- Infections with *T. brucei* were extensively documented to cause a profound activation of macrophages as manifested by the secretion of pro-inflammatory cytokines including

TNF [23, 24]. Subsequently, the major cell surface component of the parasite namely the Variant Surface Glycoprotein (VSG), a GPI-anchored protein that forms an ever changing dense protective coat, was identified as the main TNF-inducing component and this activity was localized in the GPI-anchor of the molecule [25]. Hereby, it should be emphasized that African trypanosomes have adopted a mechanism of antigenic variation of their VSGs, and during oscillating waves of parasitemia large amounts of VSG molecules (estimated at 4 x 10^{15} molecules or an equivalent of 200 μg of protein) and hence of VSG-specific GPI moieties, are released in the circulation of the mammalian host. Consequently, during infections with African trypanosomes there is a chronic VSG/GPI-mediated production of TNF in the host. Similarly, American trypanosomes (i.e. *T. cruzi*) trigger via major GPI-anchored membrane components, such as mucin, host macrophages to produce TNF [26]. Recent studies suggest that GPI and related structures from parasitic protozoa anchors engage members of the Toll-like receptor family to trigger the synthesis of inflammatory cytokines by macrophages [26, 27]. Moreover, GPI-anchored antigens presented by CD1 molecules were shown to induce the proliferation of NKT cells [28].

- Based on the potential of TNF to exert a trypanolytic activity at least *in vitro*, the parasite-mediated (via GPI-linked molecules) induction of TNF could play a role in parasite control. This possibility was substantiated by analyzing *T. brucei* development in either mice treated with monoclonal antibodies specific for the lectin-like domain of TNF or in $TNF^{-/-}$ mice [19, 29]. In both experimental models (*in situ* neutralization of the lectin-like activity of TNF or total absence of TNF), a significant increase of the first wave of parasitemia was recorded, strongly suggesting that TNF contributes to parasite control at least during the early phase of infection. Hereby, it is appropriate to mention that whereas early bloodstream forms of *T. brucei* (i.e. before the ascent of the first peak of parasitemia) are resistant to TNF-mediated lysis *in vitro*, late bloodstream forms of *T. brucei* (i.e. at the peak of parasitemia) became fully TNF-sensitive. Furthermore, local rather than systemic TNF production may play a physiological role in parasite control. Indeed, peritoneal and liver macrophages from *T. brucei* infected animals exert a trypanocidal activity *ex vivo* and such activity was totally absent in macrophages derived from $TNF^{-/-}$ infected mice [30]. Collectively, these results, combined with a recent report showing that macrophage-derived TNF kills *T. gambiense* in mixed cell cultures [31], suggest that local production of TNF in inflamed tissues underlies an efficient killing mechanism *in vivo* that may account for early parasitemia control during African trypanosomosis.
- Further studies aimed at the identification of the ligand for TNF binding and uptake by trypanosomes. Intriguingly, VSG was identified as the major ligand for TNF and the binding activity was localized in the N-linked carbohydrate side chain, more specifically the conserved *N,N'*-diacetylchitobiose-oligomannose (GlcNAc2-Man5-9) moiety of VSG [30]. In fact, the VSG glycopeptide carrying the GlcNAc2-Man5-9 moiety potently inhibits TNF-mediated trypanosome killing of macrophages from *T. brucei*-infected animals. Hereby, it should be emphasized that the *N,N'*-diacetylchitobiose-oligomannose moiety of VSG is not exposed on the surface coat of the parasite but only accessible in the flagellar pocket where endocytosis takes place [32]. Accordingly, TNF binding on *T. brucei* was restricted to the flagellar pocket and upon binding prompt endocytotic intracellular uptake of the mammalian cytokine took place [19]. Subsequently, TNF-loaded endosomes fuse with lysosomes and the acidic pH of this organelle triggers TNF-mediated disruption of lysosomes (see 3), resulting in autolysis of the parasite.

To our knowledge, the accumulated results provide the first physiological link between a lectin-like cytokine domain, its carbohydrate ligand on a micro-organism and an effector function namely endocytosis-mediated lysis. Hereby, the parasite utilizes a single component, i.e. VSG, for the induction (via the GPI anchor) and the uptake (via the N-linked carbohydrate chains) of this trypanocidal molecule (Fig. 1). Furthermore, from the conceptional point of view, at least in the field of parasite research, our results suggest how trypanosomes may circumvent the necessity to express large arrays of specific protein receptors for the uptake of host molecules required for regulated survival and development. Indeed, taking into account that trypanosomes have to cope with a diversity of effector macromolecule analogues from different mammalian host species, the expression of few conserved but parasite-specific lectin-ligands would offer a great advantage over the expression of multiple specific protein receptors. The same argument may hold true for other protozoan parasites and urges for a broadened view within parasite-receptor research.

3. On the physiological relevance of the lectin-like activity of TNF: interaction with mammalian cells

Our studies on the interaction between TNF and trypanosomes illustrate clearly the capacity of a mammalian cytokine to exert via its lectin-like domain a microbicidal activity. One may wonder whether this innate immune function, most probably shared with other cytokines displaying similar lectin-like domains (Table 1), is solely restricted to interactions with microbes or enables also physiological relevant interactions with mammalian cells.

In fact, recent evidences suggest that the cytokine TNF contributes to inflammatory reactions in mammals through its lectin-like activity. Indeed, TNF was reported to increase the membrane conductance in endothelial cells, peritoneal macrophages and alveolar epithelial cells [34, 35]. This effect does not rely on interaction with both types of TNF membrane receptors (TNF-R1 and TNF-R2) since it also occurred in cells isolated from TNF-R1/2 gene-deficient mice. Hence, a TNF-R1/2-independent mechanism seemed to be implicated in this new physiological activity of TNF, and this ion channel gating activity was found to be mediated by the lectin-like domain of TNF. Furthermore, the TNF-mediated increased ion permeability could be inhibited by amiloride, an inhibitor of sodium transport, suggesting that the increase in whole cell current across the plasma membrane of mammalian cells resulted from the binding of TNF to an endogenous ion-channel transporter or to a protein coupled to ion channels. Of interest is the fact that the ion channel gating activity of TNF required an acidic environment [34, 35]. This is quite reminiscent to the trypanolytic activity of TNF that also required an acidic pH in lysosomal compartments in order to cause autolysis of the parasite. Hence, it is possible that in lysosomes, TNF triggers changes in ion permeability resulting in lysosome disruption. Finally, the TNF-induced change in sodium permeability in Type II alveolar epithelial cells, was suggested to be important in edema resorption during acute inflammatory responses [36, 37].

Fig. 1: Dual role of African trypanosomes VSG during host-parasite interactions.
At the peak stage parasitemia, African trypanosomes release large amounts of VSG (from their whole surface) in the infected hosts. The released VSG activate host macrophages to produce the cytokine TNF. In turn, TNF interacts via its lectin-like activity/domain with the flagellar pocket exposed N-linked carbohydrate chain of VSG, leading to VSG-mediated endocytosis and subsequent TNF-mediated trypanolysis. The structure of the GPI anchor of VSG [33] is detailed. Asp is the C-terminal residue of the protein.

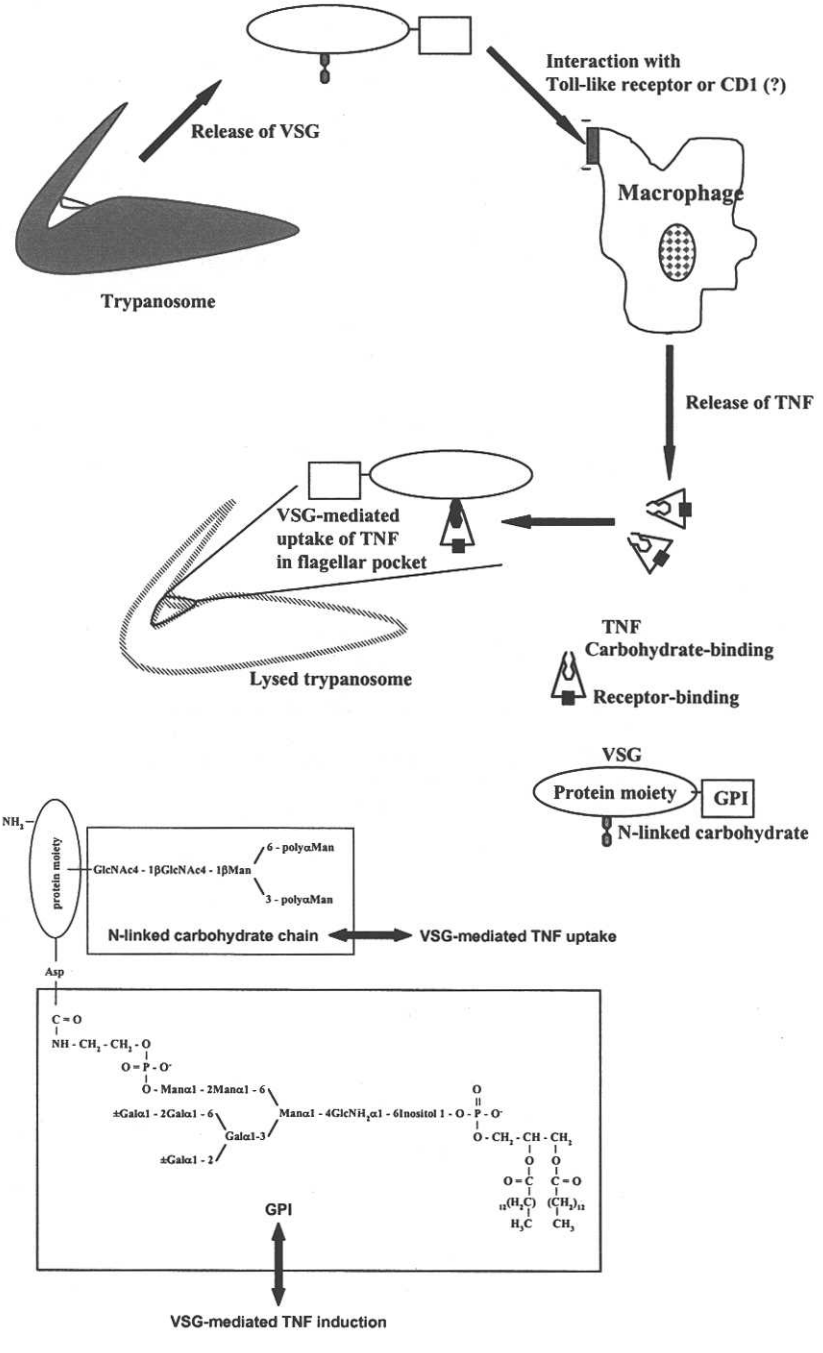

Fig. 1: Dual role of African trypanosomes VSG during host-parasite interactions.

In conclusion, at least in the case of TNF, the lectin-like activity/domain of the cytokine may contribute to both innate defense mechanisms against microbes and inflammatory reactions in vertebrates. As summarized in another chapter of this book (Beschin *et al.*), we have identified in the earthworm *Eisenia foetida* a defence molecule termed coelomic cytolytic factor (CCF) that represents a functional analogue of the TNF. Interestingly, the analogies between CCF and TNF are not based on primary sequence homology but are particularly based on a similar lectin-like activity/domain. Hence, from an evolutionary point of view, the lectin-like activity/domain of CCF and TNF may represent an ancient but essential recognition mechanism that has been functionally conserved during the innate immune response of invertebrates and vertebrates as a result of convergent evolution.

Acknowledgements

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References

[1] L. Ramsden and C.C Rider, Selective and differential binding of interleukin (IL)-1α, IL-1β, IL-2 and IL-6 to glycosaminoglycans, *The European Journal of Immunology* **22** (1992) 3027-3031.

[2] C. Rider, Many cytokines and interleukins bind to glycosaminoglycans, *Immunol. Today* **14** (1993) 615.

[3] D.P. Witt and A. D. Lander, Differential binding of chemokines to glycosaminoglycan subpopulations, *Curr. Biol.* **4** (1994) 394-400.

[4] J.A. Grimaud and H. Lortat-Jacob, Matrix receptors to cytokines: from concept to control of tissue fibrosis dynamics, *Pathol. Res. Pract.* **190** (1994) 883-890.

[5] H. Lortat-Jacob *et al.*, Human IL-4 is a glycosaminoglycan-binding protein, *Cytokine* **9** (1997) 101-105.

[6] R.J. Lipscombe *et al.*, Interleukin-5 binds to heparin/heparan sulfate. A model for an interaction with extracellular matrix, *J. Leuk. Biol.* **63** (1998) 342-350.

[7] D. Clarke *et al.*, Interaction of interleukin 7 (IL-7) with glycosaminoglycans and its biological relevance, *Cytokine* **7** (1995) 325-330.

[8] M. Lyon *et al.*, The interaction of the transforming growth factor-βs with heparin/heparan sulfate is isoform-specific. *J. Biol. Chem.* **272** (1997) 18000-18006.

[9] S. Salek-Ardakani *et al.*, Heparin and heparan sulfate bind interleukin-10 and modulate its activity, *Blood* **96** (2000) 1879-1888.

[10] Y. Tanaka *et al.*, Modulation of cytokine function by heparan sulfate proteoglycans: sophisticated models for the regulation of cellular responses to cytokines, *Proc. Assoc. Am. Physicians* **110** (1998) 118-125

[11] C. Cebo *et al.*, Recombinant human interleukins IL-1α, IL-1β, IL-4, IL-6, and IL-7 show different and specific calcium-independent carbohydrate-binding properties, *J. Biol. Chem.* **276** (2001) 5685-5691.

[12] W. Daubener *et al.*, Heparin inhibits the antiparasitic and immune modulatory effects of human recombinant interferon-γ, *Eur. J. Immunol.* **25** (1995) 688-692.

[13] R. Fernandez-Botran *et al.*, Binding of interferon γ by glycosaminoglycans: a strategy for localization and/or inhibition of its activity, *Cytokine* **11** (1999) 313-325.

[14] R. Lucas *et al.*, Cytokines: multifunctional pathogen-specific mammalian lectins? In: E. Van Driessche *et al.* (Eds.), Lectins: biology, biochemistry, clinical biochemistry, Textop, Hellerup (Danemark), 1994, vol 10, pp. 244-249.

[15] J.P. Zanetta *et al.*, Differential binding of lectins IL-2 and CSL to *Candida albicans* and cancer cells, *Glycobiology* **8** (1998) 221-225.

[16] G. Luo *et al.*, Tumor necrosis factor-α binding bacteria: evidence for a high-affinity receptor and alteration of bacterial virulence properties, *Infect. Immun.* **61** (1993) 830-835.

[17] E.J. Olson *et al.*, Fungal β-glucan interacts with vitronectin and stimulates TNF-α release from macrophages, *Infect. Immun.* **64** (1996) 3548-3554.

[18] R. Lucas *et al.*, Mapping the lectin-like activity of tumor necrosis factor, *Science* **263** (1994) 814-817.

[19] S. Magez *et al.*, Specific uptake of tumor necrosis factor-α is involved in growth control of *Trypanosoma brucei*, *J. Cell Biol.* **137** (1997) 715-727.
[20] J.P. Zanetta *et al.*, Lectin activities of cytokines and growth factors: function and implications for pathology, *Histol. Histopathol.* **11** (1996) 1101-1108.
[21] A.J. T. George, Surface-bound cytokines: a possible effector mechanism in bacterial immunity? *Immunol. Today* **15** (1994) 88-89.
[22] E. Olivares Fontt *et al.*, Effects of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-α on *Trypanosoma cruzi* trypomastigotes, *Infect. Immun.* **66** (1998) 2722-2727.
[23] S. Magez *et al.*, Murine tumour necrosis factor plays a protective role during the initial phase of the experimental infection with *Trypanosoma brucei brucei*, *Parasite Immunol.* **15** (1993) 635-641.
[24] R. Lucas *et al.*, A role for TNF during African trypanosomiasis: involvement in parasite control, immunosuppression and pathology, *Res. Immunol.* **144** (1993) 370-376.
[25] S. Magez *et al.*, The glycosyl-inositol-phosphate and dimyristoylglycerol moieties of the glycosylphosphatidylinositol anchor of the trypanosome variant-specific surface glycoprotein are distinct macrophage-activating factors, *J. Immunol.* **160** (1998) 1949-1956.
[26] M.A. Campos *et al.*, Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite, *J. Immunol.* **167** (2001) 416-423.
[27] I.C. Almeida *et al.*, Highly purified glycosylphosphatidylinositols from *Trypansosoma cruzi* are potent proinflammatory agents, *EMBO J.* **19** (2000) 1476-1485.
[28] L. Schofield L *et al.*, CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells, *Science* **283** (1999) 225-229.
[29] S. Magez *et al.*, Tumor necrosis factor α is a key mediator in the regulation of experimental *Trypanosoma brucei* infections, *Infect. Immun.* **67** (1999) 3128-3132.
[30] S. Magez *et al.*, A conserved flagellar pocket exposed high mannose moiety is used by African trypanosomes as a host cytokine binding molecule, *J. Biol. Chem.* **276** (2001) 33458-33464.
[31] S. Dalouède *et al.*, Human macrophage tumor necrosis factor (TNF)-α production induced by *Trypanosoma brucei gambiense* and the role of TNF-α in parasite control, *J. Infect. Dis.* **183** (2001) 988-991.
[32] P. Overath *et al.*, Endocytosis and secretion in trypanosomatid parasites: tumultuous traffic in a pocket, *Trends Cell Biol.* **7** (1997) 27-33.
[33] M.A. Fergusson *et al.*, Glycosyl-sn-1,2-dimyristyl phosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein, *J. Biol. Chem.* **260** (1985) 14547-14555.
[34] F.G. Van der Goot *et al.*, Membrane interaction of TNF is not sufficient to trigger increase in membrane conductance in mammalian cells, *FEBS Lett.* **460** (1999) 107-111.
[35] M. Hribar *et al.*, The lectin-like domain of tumor necrosis factor-α increases membrane conductance in microvascular endothelial cells and peritoneal macrophages, *Eur. J. Immunol.* **29** (1999) 3105-3111.
[36] S. Rezaiguia *et al.*, Acute bacterial pneumonia in rats increases alveolar epithelial fluid clearance by a tumor necrosis factor-α-dependent mechanism, *J. Clin. Invest.* **99** (1997) 325-335.
[37] N. Fukuda *et al.*, Mechanisms of TNF-α stimulation of amiloride-sensitive sodium transport across alveolar epithelium, *Am. J. Physiol. Lung Cell Mol. Physiol.* **280** (2001) L1258-L1265.

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Activities against Parasites

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A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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Invertebrate immune responses to eukaryotic parasites: reaction of coelomocytes of *Lumbricus terrestris* to native and non-native parasites

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Abstract. The oligochaete annelid *Lumbricus terrestris* is almost universally infected with a gregarine parasite belonging to the genus *Monocytsis*. The infection occurs in very large numbers in the earthworm's seminal vesicles; however, there is no consensus as to the degree of pathogenicity of this parasite. Moreover, the nature of the worm's immune response and the host-parasite relationship have never been thoroughly investigated. These preliminary experiments were designed to determine the nature and degree of immune responses to *Monocystis* and the steps necessary for further characterization of host-parasite relationships. We first determined if there is evidence for an immune reaction occurring within the seminal vesicles. Although coelomocytes were found in both preparations, there was no observed direct contact with parasites. We also attempted to determine if number and type of coelomocytes varied between heavily infected and less heavily infected worms. Though coelomocyte populations varied greatly, all of the worms were so heavily parasitized that it was difficult to establish any correlation. In a third study, we exposed earthworm immune cells to parasites by performing in vitro experiments culturing coelomocytes from *L. terrestris* together with its naturally occurring monocystid parasites, and then comparing this to reactions to a related parasite, *Cryptosporidium,* that does not normally infect earthworms. These in vitro studies showed rapid and significant reactions both to *Monocystis* and *Cryptosporidium,* but the nature of the reaction of cells, size of "granulomas" that formed, and rapidity of reaction and degranulation of cells differed between the two parasites. These differences are the most significant finding thus far, and are currently the focus of further investigations.

1. Introduction

Research on annelid (oligochaete) immune cell responses has focused primarily on the following areas: identification and classification of immune cells [1], reactions to transplantations [2, 3], phagocytic reactions to microorganisms [1], soluble factors [4, 5], reactions to vertebrate tumor lines [6] and effects on immune cells by toxins [7]. Polychaete responses to parasites have been studied by introducing them into the coelomic cavity [8-10]. However, though naturally occurring parasites have been known of for a long time and response has been attributed to an "encapsulation process", *in vitro* and *in vivo* immune responses to common parasites in oligochaete annelids have not been thoroughly investigated. The oligochaete annelid *Lumbricus terrestris* is almost universally infected with a gregarine parasite belonging to the genus *Monocytsis*. The infection occurs in very large numbers in the

earthworm's seminal vesicles; however, there is no consensus as to the degree of pathogenicity of this parasite. Although it is often referred to as completely non-pathogenic in textbook accounts [11], Goldova [12] recently stated that it is very pathogenic, interrupts the earthworms reproduction and can cause its death. Pizl [13] had previously reported that the application of herbicide zeazin 50 to the earthworm's soil resulted in a significant increase in the numbers of *Monocystis* in *L. terrestris*. This led to the conclusion that the earthworm's defense system was adversely affected and resulted in the increase in parasitism, indicating that these are probably not harmless symbionts.

Because of the importance of earthworms to agriculture, and also their role as a model for studying effects of environmental toxins [14, 15], and as a model for studying innate immune responses from an evolutionary perspective [16, 17], the host-parasite relationship between *L. terrestris* and its most commonly occurring parasites requires investigation. The primary objective of this preliminary study is to determine the exact nature and degree of the immune response to *Monocystis*, and then to determine the steps necessary for further characterization of the host-parasite relationship.

2. *Background: Life Cycle of* Monocystis

Review of the literature yielded no recent studies on the life cycle of any of the *Monocystis* species. Accounts of the life cycle are found only in textbooks, and have absolutely no references. The life cycle of this organism purportedly begins when the worm consumes spores; the sporozoite emerges from the spore in the worm's gut, penetrates the gut and enters the dorsal blood vessel, travels through the hearts, exits into the coelom and penetrates the seminal vesicle. In the seminal vesicle it matures into a trophozoite (also called gamont) and feeds on developing sperm. The gametes produced by further development of this form fuse and form a spore (also called oocyst), each of which contains 8 sporozoites. The spores then exit the sperm duct into the soil, and are consumed by the next worm [18]. However, textbook accounts vary widely in terms of mode of transmission, degree of pathogenicity and many other aspects of the life cycle. It would definitely be worthwhile to do some studies to verify this life cycle, and particularly to establish the mode of transmission.

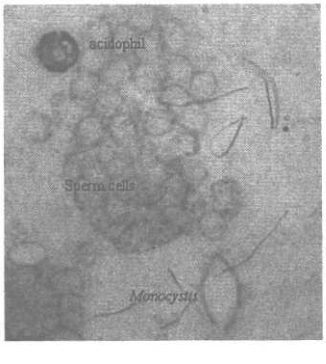

Fig. 1: Wright's stain of seminal vesicles showing *Monocystis*, sperm forming cells and acidophil (x 1000).

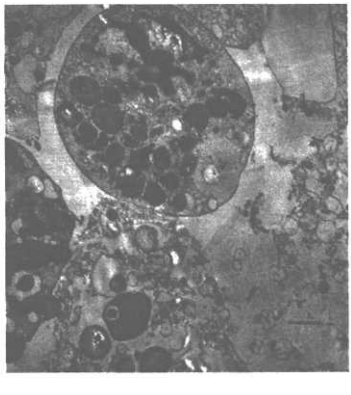

Fig. 2: Transmission electron micrograph of coelomocyte.

3. *Immune response to* Monocystis *within the seminal vesicles of* Lumbricus terrestris

Annelid response to organisms too large to be phagocytosed is accomplished in the coelom through a process of encapsulation [for review 19]. It begins with coelomocytes adhering to the foreign body and then degranulating, as described by Porchet-Henneré *et al.* in the polychaete *Nereis diversicolor* [8-10]. The process continues as subsequent layers of cells surround this reaction, eventually forming a large capsule or "brown body", which could eventually be eliminated. The attempt here was to discover whether *L. terrestris* coelomocytes actually enter the seminal vesicle and initiate any type of reaction against monocystid parasites. Light microscopic and transmission electron microscopic preparations were done on sections of infected seminal vesicles and examined for presence of coelomocytes. Fig. 1 shows a Wright's stained preparation of the seminal vesicle containing spores of *Monocystis sp.* Occasional coelomocytes were detected (approximately one per every 2-3 high power fields), though none appeared to be adhering to the spores. All of coelomocytes seen were either neutrophils or acidophils. Transmission electron micrographs also revealed occasional coelomocytes. However, no spores were seen in these, possibly due to difficulty in fixation of resistant spores. Fig. 2 shows a transmission electron micrograph of a coelomocyte obtained from a previous study in which coelomocytes were extruded and processed alone.

Subsequent experiments show that coelomocytes from *L. terrestris* do recognize these spores as foreign since they will react *in vitro* to their own *Monocystis.* Why does not this occur within the seminal vesicles? Is the seminal vesicle an "immunologically privileged site" as is the testis in vertebrates? If this is so, the mechanisms for this would be of great interest, since immunological privilege in the testis is associated with altered MHC expression [20], which supposedly is not possible in invertebrates. Repeat electron microscopic studies are planned to confirm these preliminary results.

Even if there is not active immune response within the seminal vesicles, the worms are probably constantly consuming new spores, so there could be evidence of coelomocyte populations differing between already heavily infected and less infected worms. Worms were obtained through Ward's Biology where they are collected, not raised. Coelomocytes were obtained by extrusion as described in [21]. Unfortunately, all worms examined appeared to be very heavily infected. The attempt to correlate differential counts with degree of *Monocystis* infection was therefore not possible. However, as Fig. 3 shows, there was an interesting variation in coelomocyte counts between worms, which cannot be accounted for here, nor has been previously described. It would be necessary to obtain uninfected worms in order to get some definitive correlations.

4. In vitro *response of* Lumbricus terrestris *coelomocytes to* Monocystis

In this set of *in vitro* experiments, coelomocytes from *L. terrestris* were cultured with the *Monocystis* parasites from the same worm. This was done to discover if the *Monocystis* is capable at all of triggering a reaction by the coelomocytes, or if it is tolerated as self and there is no reaction. All results shown here were obtained by using the worm's own parasites, in order to eliminate the possibility of reacting against foreign tissue from another worm. For purposes of comparison, coelomocytes were also cultured with a non-native parasite. The parasite chosen for this purpose was *Cryptosporidium parvum* because it is also a member of the phylum *Apicomlexa*, class *Sporozoa* and is easily obtained (from Waterborne, Inc. New Orleans). Additionally, it was shown that fluorescent antibodies used in testing for *Cryptosporidium* crossreact with *Monocystis* [22]. Also, this is a fairly ubiquitous parasite, and chances exist of the earthworm encountering it in nature.

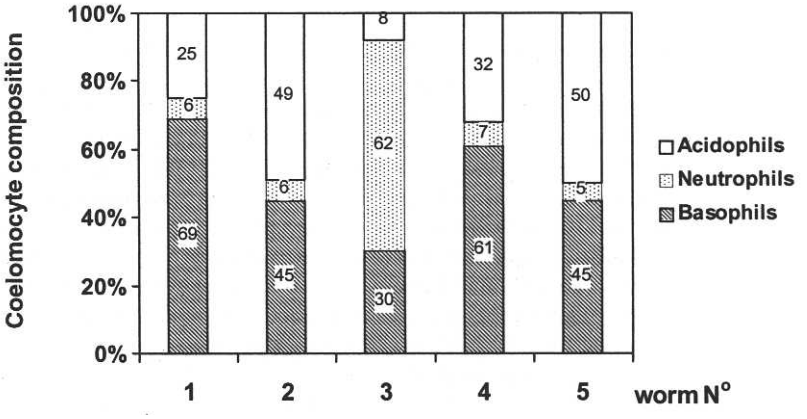

Fig. 3: Coelomocyte counts from 5 worms heavily infected with *Monocystis* (values are percentages of coelomocyte type out of 200 coelomocyte cells counted per worm).

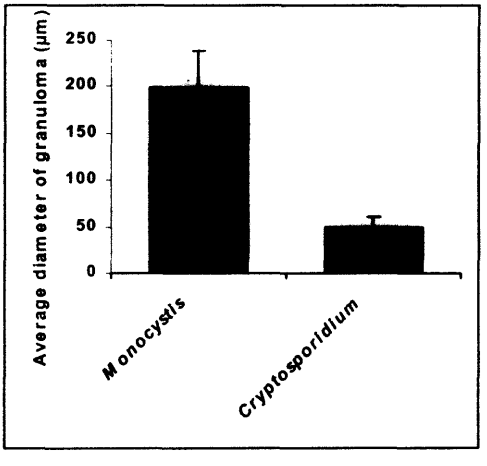

Fig. 4a: Size of granulomas formed around parasites (mean ± SD, n = 5).

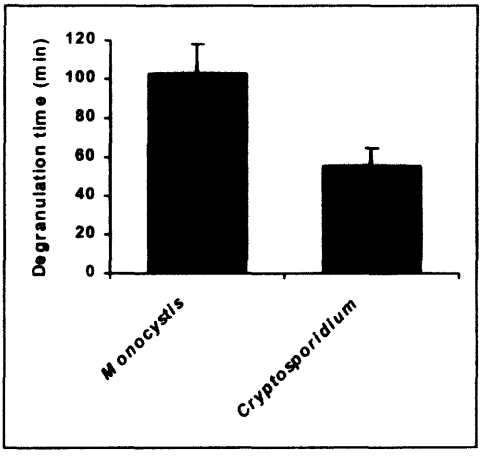

Fig. 4b: Time required for complete degranulation of coelomocytes (mean ± SD, n = 5).

Each of the parasites was co-cultured with coelomocytes, as described in [6]. The *Monocystis* were removed from the seminal vesicles, diluted to concentrations similar to those of the *Cryptosporidium* and cultured with coelomocytes from the same earthworm. Samples of the culture mixes were removed at certain time periods and examined both in wet preparations and Wrights stained preparations using light microscopy. It was observed that the coelomocytes reacted to both parasites by initially adhering and aggregating around the parasites, and then degranulating. This was significant because it showed that the earthworm's immune cells do respond to *Monocystis*, and therefore do not tolerate them as "self". Even more remarkable was that even though reactions to both *Monocystsis* and *Cryptosporidium*

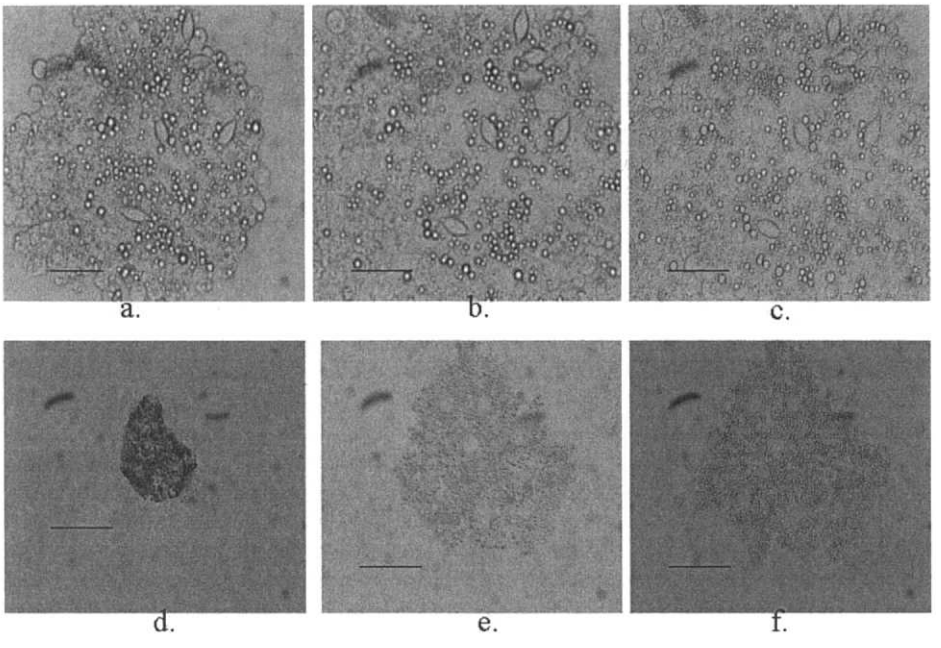

Fig. 5: a-c: Degranulation around *Monocystis* (60, 90, 120 min); d-f: Degranulation around *Cryptosporidium* (30, 45, 60 min); Bar = 25 µm.

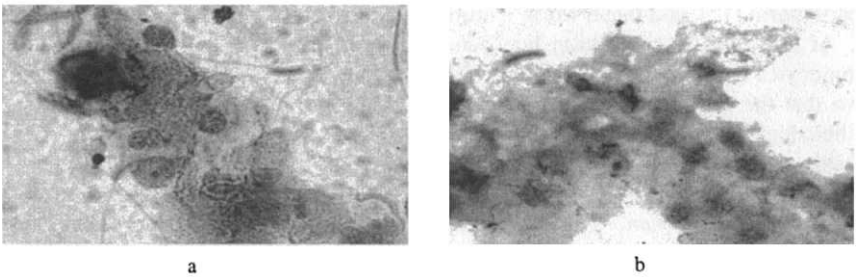

Fig. 6: Wright's stained granulomas (x 1000). a: *Monocystis* with acidophils and basophils; b: *Cryptosporidium* with basophils.

were vigorous and rapid, they differed significantly as to the size of "granulomas" (the aggregates of coelomocytes and parasites) formed, the speed at which degranulation proceeded, and the types of coelomocytes that participated in the response.

Fig. 4a compares the size of granulomas formed around *Monocystis* to those formed around *C. parvum*. The *Monocystis* granulomas were significantly larger, indicating that more coelomocytes aggregated. This could be a function of the fact that although there are certain similarities between the two parasites, there is a substantial difference in size between the two. Also, even though both parasites share some of the same surface antigens [22], other surface molecules might differ and stimulate different types of coelomocytes. Fig. 4b indicates that the coelomocytes attacking the *Cryptosporidium* degranulated faster than those around the *Monocystis*. Such differences could indicate that there is some degree of specialization by coelomocyte types for certain types of surface molecules. Fig. 5 shows the live preparations of the cells adhering, aggregating and degranulating around each of the parasites. Fig.5a-c and d-f shows the degranulation process for *Monocystis* and *Cryptosporidium*, respectively. The degranulation of the cells occurred much more rapidly in the *Cryptosporidium* cultures than in the *Monocystis* cultures. At 1 h incubation, the coelomocytes were almost completely degranulated in the *Cryptosporidium* culture, whereas the cells were still partially intact in the *Monocystis* cultures, and did not completely degranulate until greater than 1 h and 45 min. Fig. 6 shows Wright's stained preparations of each of the granulomas showing clearly that those cells surrounding the *Cryptosporidium* are more homogenous and of a basophilic nature; those around the *Monocystis* appear to be more heterogeneous and many are acidophilic.

5. Conclusion

The host-parasite relationship between *L. terrestris* and members of *Monocystis sp.* is intriguing in that little is known of the degree of pathogenicity, the nature of the immune response by the worm, and of any possible evasive mechanisms employed by the parasite. By searching the literature, there is an indication that the parasite may indeed be causing harm to the earthworm [12], and that a worm's immunity at least helps keep parasite numbers in check [13]. At this time, a correlation between degree of parasitism and numbers and types of coelomocytes could not be determined. However, preliminary *in vitro* experiments here have shown that the earthworm's coelomocytes will recognize *Monocystis* as foreign by adhering and then degranulating. A similar process has been described in the polychaete *N. diversicolor* by using latex beads introduced into the coelomic cavity [8-10]. The reaction to the *Monocystis*, however, was markedly different than that to the control parasite *Cryptosporidium parvum*. The cells that adhered to the *Cryptosporidium* were more homogenous, composed primarily of basophilic cells, and the degranulation process was complete by 1 h, whereas the adherent cells around *Monocystis* were of different cell types including many eosinophils, and the degranulation process was slower. We propose several possible reasons for these differences in the nature of the immune reactions to the two parasites: 1) difference in size of the parasites, 2) difference in antigenic nature of the two parasites along with the fact that each coelomocyte type will recognize different types or patterns of molecules, 3) resistant or evasive qualities of the parasites, or 4) degree of "tolerance" for the *Monocystis*.

These findings have raised many questions regarding the earthworm's immune responses to parasites. Further experiments characterizing the host-parasite relationship which are currently in progress include: 1) fluorescent and electron microscopic studies of the granulomas formed *in vitro* in order to determine if the parasites were damaged by the

degranulation, 2) *in vitro* experiments culturing the parasites with the coelomic fluid alone and with coelomocytes, 3) *in vivo* studies injecting *Monocystis* or *Cryptosporidium* into the coelomic cavities of earthworms, 4) injecting *Cryptosporidium* into the seminal vesicles, 5) incubating coelomocytes and parasites with vertebrate IgE to note any possible opsonization effects, 6) further investigation into variations in degree of parasite infestation, and correlation with coelomocyte numbers and sperm counts, 7) studies on adhesion and receptor molecules on the coelomocytes, along with similarities/differences in parasite surface molecules and, 8) further studies on *Monocystis* including genetics, life cycle, and pathogenicity.

References

[1] E.A. Stein *et al.*, The coelomocytes of the earthworm *Lumbricus terrestris* morphology and phagocytic properties, *J. Morphol.* **153** (1977) 467-476.

[2] E.L. Cooper, Phylogeny of transplantation immunity graft rejection in earthworms, *Transplant. Proc.* **3** (1971) 214-216.

[3] C.E.A. Lemmi and E.L. Cooper, Induction of coelomocyte proliferation by xenografts in the earthworm *Lumbricus terrestris*, *Dev. Comp. Immunol.* **5** (1981) 73-80.

[4] I. Eue *et al.*, Isolation and characterization of earthworm hemolysins and agglutinins, *Dev. Comp. Immunol.* **22** (1998) 13-25.

[5] E. Kauschke *et al.*, Immune proteins in earthworms, *Rec. Res. Dev. Comp. Biochem. Physiol.* 1 (2000) 105-122.

[6] A. Cossarizza *et al.*, Earthworm leukocytes that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines, *Exp. Cell Res.* **224** (1996) 174-182.

[7] J.B. Rodriguez-Graw *et al.*, Suppression of secretory rosette formation by PCBs in *Lumbricus terrestris*: an earthworm immunoassay for humoral immunocytotoxicity of xenotrophs, *Env. Toxicol. Chem.* **8** (1989) 1201-1207.

[8] E. Porchet-Henneré *et al.*, Ultrastructural study of the encapsulation response of the polychaete annelid *Nereis diversicolor*, *Cell Tissue Res.* **248** (1987) 463-471.

[9] E. Porchet-Henneré, Cooperation between different coelomocyte populations during the encapsulation response of *Nereis diversicolor* demonstrated by using monoclonal antibodies, *J. Invert. Pathol.* **56** (1990) 353.

[10] E. Porchet-Henneré and G. Vernet, Cellular immunity in an annelid (*Nereis diversicolor*, Polychaeta): production of melanin by a subpopulation of granulocytes, *Cell Tissue Res.* **260** (1992) 167-174.

[11] J.N. Farmer, 1980. *The Protozoa, Introduction to Protozoology*, C.V. Mosby Co, St. Louis.

[12] M. Goldova and M. Breza, Earthworms (*Lumbricidae*) from the viewpoint of veterinary parasitology, *Folia Vet.* **43** (1999) 154-157.

[13] V. Pizl, The effect of the herbicide Zeazin 50 on the earthworm infection by monocystid gregarines, *Pedobiologica* **28** (1985) 399-402.

[14] S.A. Reinecke *et al.*, The effects of dieldrin on the sperm ultrastructure of the earthworm *Eudrilus eugeniae* (*Oligochaeta*), *Env. Toxicol. Chem.* **14** (1995) 961-965.

[15] A. Dhainaut and P. Scaps, Immune defense and biological responses induced by toxics in *Annelida*, *Can. J. Zool.* **79** (2001) 233-253.

[16] E.L. Cooper, Earthworm Immunity, *Prog. Mol. Subcell. Biol.* **15** (1996) 10-45.

[17] K. Söderhäll K., Iwanaga, S. and Vasta G.R. (Eds.) 1996 *New Directions in Invertebrate Immunology*, SOS Publications, Fair Haven NJ.

[18] Cheng 1986. *General Parasitology* 2nd ed. Academic Press, Orlando

[19] M. Bilej, Cellular defense mechanisms. In V. Vetvicka *et al.*, (Eds.), Immunology of Annelids, CRC Press, Boca Raton, 1994, pp. 167-200.

[20] D. Fiszer and M. Kurpisz, Major histocompatibility complex expression on human, male germ cells: a review, *Am. J. Reprod. Immunol.* **40** (1998) 172-176.

[21] G.S. Eyambe *et al.*, A non-invasive technique for sequential collection of earthworm (*Lumbricus terrestris*) leukocytes during subchronic immunotoxicity studies, *Lab. Animals* **25** (1991) 61-67.

[22] S. Bull *et al.*, Cross-reaction of an anti-*Cryptosporidium* monoclonal antibody with sporocysts of Monocystis species, *Vet. Parasitol.* **77** (1998) 195-197.

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Summary and Perspectives

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A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications
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An overview of cytolytic factors in *Eisenia foetida* coelomic fluid

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Abstract. Coelomic fluid of *Eisenia foetida* earthworms exhibits cytolytic activities against different target cells. In general, there are six cytolysins identified till now in two main groups. The first group includes factors with antibacterial and hemolytic activity and usually with sphingolipid-binding specificity as fetidins, lysenin and lysenin-related proteins, eiseniapore and hemolysins H_1, H_2, H_3. The second group comprises cytolysins active to other cell types, particularly to tumor cells, coelomic cytolytic factor (CCF) and perforin-like molecule. Interestingly, calreticulin-like material was copurified with earthworm perforin suggesting the chaperoning function of calreticulin in earthworms.

1. Introduction

Coelomic fluid of annelids exhibits numerous biological activities. Cytolytic components secreted by coelomocytes into the coelomic cavity are of particular interest in view of their potential clinical applications. There are two main groups of such cytolytic factors. The first group includes factors displaying hemolytic activity that is accompanied by antibacterial/bacteriostatic effects against pathogenic soil bacteria [1-4]. Interestingly, all susceptible bacteria express at least one surface antigen that is common to vertebrate erythrocytes but distinct from the Forssman antigen [5]. The second group includes factors exerting lytic activity to other cell types, particularly tumor cells.

2. Overview of E. foetida *cytolysins*

To date, six cytolytic proteins or group of proteins have been identified in *Eisenia foetida*.

2.1. Fetidins

Fetidins are two hemolytic glycoproteins of 40- and 45-kDa secreted by chloragocytes that were originally described as the *E. foetida andrei* factor – EFAF [1, 5-7]. The 45-kDa protein, encoded by one non-polymorphic gene, shows a pI of 6.0, while the 40-kDa protein is encoded by a gene possessing four alleles, giving rise to four protein isoforms with a pI of 6.3, 6.2, 5.95, and 5.9. Besides the protein isoform of pI 6.0 present in all earthworms, each individual expresses one or two active proteins [1, 8]. The global amino acid sequence of the 40 and 45-kDa fetidins suggests homology between them [9], and the different molecular weight of these two proteins is mainly due to the extent of glycosylation [10]. By screening a cDNA library with a rabbit anti-45-kDa antiserum cross-reacting with the 40-kDa protein, a clone encoding a protein of 34140 Da, which corresponds to the size of the deglycosylated fetidins, was isolated [10, 11]. The amino acid sequence presents an N-glycosylation site (position 250-252), and homologies with peroxidase (position 52-62). Accordingly, fetidins display peroxidase activity. Fetidins lyse erythrocytes in absence of Ca^{2+} and Mg^{2+} and in a nonenzymic way [3, 12]. They polymerize upon binding to sphingomyelin causing 10-nm open channels through the lipid bilayer in sphingomyelin microvesicles or red blood cell membranes [5, 13].

Fetidins synthesis *in vivo* increases following an injection of pathogenic bacteria in the coelom of the worms [14]. Moreover, these proteins exhibit antibacterial activity on Gram-negative and positive bacteria [2, 11, 15], particularly on strains that are pathogenic for earthworms [3, 4, 8]. Interestingly, it was shown that the expression of a particular combination of the 45 and 40-kDa proteins in a worm individual influences the antibacterial capacity of the coelomic fluid.

2.2. Lysenin

Lysenin was first identified as a 41-kDa protein produced by coelomocytes that cause contraction of rat vascular smooth muscles [16]. Simultaneously, a lysenin-related protein with a molecular mass of 42-kDa and a weak contractive activity was found. A cDNA encoding lysenin was isolated [17]. The 297 amino acid sequence with a molecular mass 33440 Da displays one potential N-glycosylation site (position 248). Two cDNA clones encoding lysenin-related proteins of 300 amino acids were isolated [17]. The latter have respectively 76 and 90 % amino acid residues identical to those of lysenin. In addition, lysenin and lysenin-related protein reveal high homology with fetidins suggesting a close relationship between these lytic molecules (Table 1). Accordingly, as fetidins, lysenin do not show sphingomyelinase activity, but induces erythrocyte lysis by binding sphingomyelin on membranes [18]. Moreover, the presence of cholesterol in membrane increases the accessibility of sphingomyelin to lysenin and fetidins, facilitating hemolysis. It is therefore likely that lysenin cause membrane damage by forming aqueous pores in the membrane.

Fetidins and lysenins were described independently in France and in Japan and their entries to sequence databases were done in the same time. When the original papers have been released the high level of sequence homology of fetidins and lysenins became obvious and it might reflect that these two proteins are isoforms.

2.3. Eiseniapore

Eiseniapore cytolytic protein is a 38-kDa from *E. foetida* coelomic fluid that requires sphingomyelin or galactosylceramide to bind on red blood cell membranes to induce lysis [19]. As for fetidins and lysenins, the eiseniapore lytic activity toward sphingomyelin-containing vesicles is enhanced by cholesterol. The protein seems to be associated in the coelomic fluid with a natural inhibitor named eiseniapore-regulating protein [20]. The properties of eiseniapore are detailed in the contribution of Kauschke *et al.* in this issue.

2.4. H_1, H_2 and H_3 hemolysins

Eue *et al.* [21] described 3 hemolytic proteins in the coelomic fluid of *E. foetida*, H_1, H_2, and H_3, with respective molecular mass of 46, 43 and 40-kDa. It was observed that in contrast to H_1 and H_2, H_3 splits into two fragments of 18 and 21-kDa after SDS treatment. These hemolysins are described by Kauschke *et al.* in this issue.

2.5. Coelomic cytolytic factor

Coelomic cytolytic factor (CCF) was originally isolated as a 42-kDa protein responsible for proteinase-independent lysis of some TNF-sensitive cell lines [22]. However, the mechanism of the lytic action of CCF and TNF is different since the activity of CCF is not inhibited by anti-TNF neutralizing antibodies. A cDNA encoding CCF was isolated [23]. The 384 amino acid sequence does not display potential N-glycosylation site and shows high amino acid sequence homology with some pattern recognition receptors of invertebrates. CCF is the main topic of the article by Beschin *et al.* in this issue.

2.6. Perforin-like protein

It was shown that earthworm small coelomocytes exhibit lytic activity against K562 target cells. The lytic effect seems to be mediated by a perforin-like factor of molecular mass of about 70 kDa. Moreover, anti-perforin antibodies cross-react with small coelomocytes and perforin-like mRNA was evidenced in earthworm coelomocytes by RT-PCR and Southern blotting [24, 25 and Komiyama *et al.* in this issue].

3. Relationships between E. foetida cytolysins

Based on biochemical data we can anticipate that hemolysins are related proteins (Table 1). Indeed they display similar molecular mass around 40-kDa and similar pI. Moreover, comparison of the available cDNA sequences clearly shows that fetidins and lysenins share strong amino acid homologies. H_3 is however different from all the other lysins since it is the only dimer. Concerning lipid specificity, eiseniapore is quite different from lysenin and possibly from fetidins, since eiseniapore does not only bind sphingomyelin but also galactosylceramide. Inhibition experiments revealed that the hemolytic activity of the coelomic fluid is partially inhibited by acetylated or methylated carbohydrates such as N-acetyl-D-glucosamine, α-methyl-D-mannopyranoside, N-acetyl-D-galactosamine and N-acetyl-neuraminic acid [5, 21]. However, neither zymosan, inulin or LPS, nor hydrazine or methylamine inhibit the coelomic fluid hemolytic activity, suggesting that *E. foetida* hemolysins are not related to the C3 or the C3b complement component [13]. The mode of action of the eiseniapore may be analogous to that of the membrane attack complex of complement or perforin since heparin, vitronectin and lysophosphatidylcholine prevent its binding to erythrocytes but do not affect the membrane insertion and pore formation [26].

Table 1: Comparison of selected data available on cytolytic protein in E. foetida earthworms

Protein	Molecular mass (kDa)	Glycosylation	Accession no.	Homology with
Fetidin	40	+	AAB67727	Lysenin
	45	+		Lysenin-related protein 1
				Lysenin-related protein 2
Lysenin	41	+	BAA21518	Fetidin
				Lysenin-related protein 1
				Lysenin-related protein 2
Lysenin-related protein 1	42	–	BAA21520	Fetidin
				Lysenin
				Lysenin-related protein 2
Lysenin-related protein 2	42	–	BAA21519	Fetidin
				Lysenin
				Lysenin-related protein 1
Eiseniapore	38	not known	not cloned	
H_1	46	not known	not cloned	
H_2	43	not known	not cloned	
H_3	40 (18 + 21)	not known	not cloned	
Coelomic cytolytic factor (CCF)	42	–	AAC35887	Invertebrate pattern recognition proteins, β-1,3-glucanases
Perforin	68, 70, and 72	not known	not cloned	

4. Is calreticulin chaperoning in earthworms?

Secreted lytic molecules often require molecular chaperones preventing autolysis and increasing the yield of correctly folded proteins. One of the most important lectin-like chaperone necessary for glycoprotein maturation was described as calreticulin. It was first isolated as a 60-kDa Ca^{2+}-binding protein from the muscle sarcoplasmic reticulum [27] and cloned in 1989 [28, 29, for review 30, 31]. Calreticulin is encoded by a single gene, which has been identified in both vertebrate and invertebrate animal species and in some higher plants. Protein product consists of three distinct domains with different functions. The highly conserved N-terminal domain is proceeded by the signal sequence targeting the protein to the endoplasmic reticulum. The N-domain binds Zn^{2+} ions, contains potential phosphorylation site, exerts autokinase activity, and interacts with steroid receptor and integrins. The proline-rich P-domain (position 170-285 in mouse) shows amino acid sequence homology with calnexin and calmegin, binds Ca^{2+} with high affinity, strongly interacts with perforin and with proteins of endoplasmic reticulum. The C-domain is rich in acidic amino acids and binds Ca^{2+} with high capacity.

As mentioned above calreticulin acts as molecular chaperone preventing the aggregation of partially folded proteins and increasing the yield of correctly folded proteins and their intermediates by recruiting other folding enzymes. In the last years, the role of lectin-saccharide interaction in chaperoning function of calreticulin has been envisaged and calreticulin has been defined as a lectin-like chaperone with GlcManGlcNAc specificity [for reviews 31, 32]. Furthermore, it has been found that human neutrophils can be activated by

a synthetic antimicrobial peptide derived from insect sapecin (a potent antimicrobial protein from flesh fly *Sarcophaga peregrina*) via cell surface calreticulin [33]. Using an anti-calreticulin antibody it has been suggested that calreticulin is localized on the surface of neutrophils, interacts with the synthetic peptide, transmits a signal into cells via G-protein and thus activates neutrophils to generate superoxide anion.

Very recently, Kauschke *et al.* [25] has analyzed by MALDI-MS a peptide digest of *E. foetida* coelomocyte lysate that cross-reacts with an anti-perforin monoclonal antibody. They have found four peaks with peptide masses matching the sequence of calreticulin from the rape *Brassica napus* (Table 2). While the peptides AAFDEIEK and SEVSEETAEK are located in the lowly conserved C-terminal part of rape calreticulin, the two other peptides fit to the more conserved part. Interestingly, the peptide KPDGYDDIPK is located in the P-domain involved in perforin-binding. Although not conclusive, the data suggest that a calreticulin homologue was copurified with a perforin-like material. The role of calreticulin as molecular chaperone in earthworms may be important only for certain cytolytic molecules. Indeed, chaperoning function of calreticulin seems to be mediated by lectin-saccharide interaction with GlcManGlcNAc specificity that would exclude the interaction with nonglycosylated molecules as CCF or lysenin-related protein 1.

Table 2: Amino acid sequence of calreticulin from rape *Brassica napus* (AAB70919). The peptides identified by MALDI analysis of coelomocyte lysate are in bold.

MAKLTLASSP WFLSVSWRSL PPLLSSRNAL MMDGRTDGLN QSGRKRTSLL
GSGATLLEIG LVMLTIKVSR PARTTDSTPS QLSSLNSATR TKTLVCQFSV
KHEQKLDCGG GYMKLLSGDV DQKKFGGDTP YSIMFGPDIC GYSTKKVHAI
LTYNDANHLI KKDVPCETDQ LTHVYTFILR PDATYTILVD NVEKQTGSLY
SDWDLLPPKK IKDPSAKKPE DWDEQEYIPD PEDK**KPDGYD** **DIPK**EIPDTD
AKKPEDWDEE EDGEWTAPTI PNPEYMGEWK PKQIKNPNYK GKWEAPEIDN
PDFK**DDPELY** **VFPK**LKYVGI ELWQVKSGSL FDNVLICDDP DYAKKLAEET
WGKLKDAEK**A** **AFDEIEK**KKE EEESKDAPAE TDGEDEAEED EGEESDTESK
TEAK**SEVSEE** **TAEK**DATAHD EL

Molecular mass	921.44	AAFDEIEK
Molecular mass	1107.49	SEVSEETAEK
Molecular mass	1146.56	KPDGYDDIPK
Molecular mass	1221.59	DDPELYVFPK

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References

[1] P. Roch, Protein analysis of earthworm coelomic fluid. I. Polymorphic system of the natural hemolysins of *Eisenia foetida andrei, Dev. Comp. Immunol.* **3** (1979) 599-608.

[2] P. Valembois *et al.*, Antibacterial activity of the hemolytic system from the earthworm *Eisenia fetida andrei, J. Invert. Pathol.* **40** (1982) 21-27.

[3] P. Valembois *et al.*, Antibacterial molecules in annelids, In M. Brehèlin (Ed.), Immunity in Invertebrates, Springer-Verlag, Berlin, Heidelberg, New York, 1986 pp. 74-93.

[4] P. Roch *et al.*, Antibacterial activity of *Eisenia fetida andrei* coelomic fluid: III. Relationship within the polymorphic hemolysins, *Dev Comp Immunol.* **15** (1991) 27-32.
[5] P. Roch *et al.*, Protein analysis of earthworm coelomic fluid. II. Isolation and biochemical characterization of the *Eisenia foetida andrei* factor (EFAF), *Comp. Biochem. Physiol.* **69B** (1981) 829-836.
[6] L. Du Pasquier and P. Duprat, Aspects humoraux et cellulaires d'une immunité naturelle non spécifique chez l'oligochète *Eisenia fetida* (*Lumbricidae*), *C. R. Acad. Sci. Paris* **266** (1968) 538-542.
[7] P. Roch *et al.*, Isolation of agglutinins and lysins from earthworm coelomic fluid by gel filtration followed by chromatofocussing, *J. Chromat.* **290** (1984) 231-235.
[8] P. Roch *et al.*, Genetic and biochemical polymorphism of earthworm humoral defenses, *Dev. Comp. Immunol.* **11** (1987) 91-102.
[9] P. Roch *et al.*, Amino-acid compositions and relationships of five earthworm defense proteins, *Comp. Biochem. Physiol.* **85B** (1986) 747-751.
[10] M. Lassègues *et al.*, Sequence and expression of an *Eisenia fetida*-derived cDNA clone that encodes the 40-kDa fetidin antibacterialprotein, *Eur. J. Biochem.* **246** (1997) 756-762.
[11] F. Hirigoyenberry *et al.*, Antibacterial activity of *Eisenia fetida andrei* coelomic fluid: immunological study of the two major antibacterial proteins, *J. Invert. Pathol.* **59** (1992) 69-74.
[12] M. Lassègues *et al.*, Mise en évidence des protéines hémolytiques et hémagglutinantes spécifiques de l'albumen des cocoons du lombricien *Eisenia fetida andrei*, *C. R. Acad. Sci Paris* **299** (1984) 691-696.
[13] P. Roch *et al.*, Interactions between earthworm hemolysins and sheep red blood cell membranes, *Biochim. Biophys. Acta.* **983** (1989) 193-198.
[14] M. Lassègues *et al.*, Antibacterial activity of *Eisenia fetida andrei* coelomic fluid: evidence, induction and animal protection, *J. Invert. Pathol.* **53** (1989) 1-6.
[15] M. Lassègues *et al.*, Action de quelques souches bactériennes telluriques sur le lombricien *Eisenia fetida andrei*, *C. R. Acad. Sci. Paris* **292** (1981) 731-734.
[16] Y. Sekizawa *et al.*, A novel protein, lysenin, that causes contraction of the isolated rat aorta: its purification from the coelomic fluid of the earthworm, *Eisenia fetida, Biomed. Res.* **17** (1996) 197-203.
[17] Y. Sekizawa *et al.*, Molecular cloning of cDNA for lysenin, a novel protein in the earthworm *Eisenia fetida* that causes contraction of rat vascular smooth muscle, *Gene* **191** (1997) 97-102.
[18] A. Yamaji *et al.*, Lysenin, a novel sphingomyelin-specific binding protein, *J. Biol. Chem.* **273** (1998) 5300-5306.
[19] S. Lange *et al.*, Interaction of eathworm hemolysin with lipid membranes requires sphingolipids, *J. Biol. Chem.* **272** (1997) 20884-20892.
[20] W. Mohrig *et al.*, Inhibitor controlled hemolytic activity in the coelomic fluid of earthworms, *Dev. Comp. Immunol.* **21** (1997) 116.
[21] I. Eue *et al.*, Isolation and characterization of earthworm hemolysins and agglutinins, *Dev. Comp. Immunol.* **22** (1998) 13-25.
[22] M. Bilej *et al.*, Identification of a cytolytic protein in the coelomic fluid of *Eisenia foetida* earthworms *Immunol. Lett.* **45** (1995) 123-128.
[23] A. Beschin *et al.*, Identification and cloning of a glucan- and LPS-binding protein from *Eisenia foetida* earthworms involved in the activation of prophenoloxidase cascade, *J. Biol. Chem.* **273** (1998) 24948-24954.
[24] K. Komiyama *et al.*, Identification of perforin gene and its protein in the earthworm coelomocytes, *Dev. Comp. Immunol.* **21** (1997),115.
[25] E. Kauschke *et al.*, Evidence for perforin-like activity associated with earthworm leukocytes, *Zoology* **104** (2001) 13-24.
[26] S. Lange *et al.*, Biochemical characteristics of eiseniapore, a pore-forming protein in the coelomic fluid of earthworms, *Eur. J. Biochem.* **262** (1999) 547-556.
[27] T.J. Ostwald and D.H. MacLennan, Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum, *J. Biol. Chem.* **249** (1974) 974-979.
[28] M.J. Smith and G.L. Koch, Multiple zones in the sequence of calreticulin (CRP55, calregulin, HACBP), a major calcium binding ER/SR protein, *EMBO J.* **8** (1989) 3581-3586.
[29] L. Fliegel *et al.*, Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum, *J. Biol. Chem.* **264** (1989) 21522-21528.
[30] K.H. Krause and M. Michalak, Calreticulin, *Cell* **88** (1997) 439-443.
[31] M. Michalak *et al.*, Calreticulin: one protein, one gene, many functions, *Biochem. J.* **344** (1999) 281-292.
[32] H.-P. Hauri *et al.*, Lectins and traffic in the secretory pathway, *FEBS Lett.* **476** (2000) 32-37.
[33] J.-H. Cho *et al.*, Activation of human neutrophils by a synthetic antimicrobial peptide, KLKLLLLLKLK-NH_2, via cell surface calreticulin, *Eur. J. Biochem.* **266** (1999) 878-885.

Bibliography

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Bibliography of Edwin L. Cooper emphasizing on earthworms (1966-2002)

Original articles

Cooper E.L.: Algunos aspectos de inmunidad en invertebrados, peces, y anfibios. *Acta Med.* **2**: 1-5, 1966.

Cooper E.L. and Garcia-Herrera F.: La organización de un laboratorio de investigación en embriologia e inmunologia comparada. *Acta Med.* **3**: 59-67, 1967.

Cooper E.L.: Transplantation immunity in Annelids. I. Rejection of xenografts exchanged between *Lumbricus terrestris* and *Eisenia foetida*. *Transplantation* **6**: 322-337, 1968.

Cooper E.L.: Multinucleate giant cells, granulomata and "myoblastomas" in annelid worms. *J. Invertebr. Pathol.* **11**: 123-131, 1968.

Cooper E.L. and Baculi, B.S.: Degenerative changes in the annelid *Lumbricus terrestris*. *J. Gerontol.* **23**: 375-381, 1968.

Cooper E.L.: Chronic allograft rejection in *Lumbricus terrestris*. *J. Exp. Zool.* **171**: 69-73, 1969.

Bailey S., Miller B.J., **Cooper E.L.**: Transplantation immunity in annelids. II. Adoptive transfer of the xenograft reaction. *J. Immunol.* **21**: 81-86, 1971.

Hostetter R.K. and **Cooper E.L.**: Coelomocytes as effector cells in earthworm immunity. *Immunol. Commun.* **1**: 155-183, 1972.

Hostetter R.K. and **Cooper E.L.**: Cellular anamnesis in earthworms. *Cell. Immunol.* **9**: 384-392, 1973.

Linthicum D.S., Stein E.A., Marks D.H., **Cooper E.L.**: Electron microscopic observations of normal coelomocytes from the earthworm *Lumbricus terrestris*. *Cell and Tissue Res.* **185**: 315-330, 1977.

Linthicum D.S., Marks D.H., Stein E.A. **Cooper E.L.**: Graft rejection in earthworms: an electron microscopic study. *Eur. J. Immunol.* 7: 871-876, 1977.

Marks D.H. and **Cooper E.L.**: *Aeromonas hydrophila* in the coelomic cavity of the earthworms *Lumbricus terrestris* and *Eisenia foetida*. *J. Invertebr. Pathol.* **29**: 382-383, 1977.

Stein E., Avtalion, R.R., **Cooper E.L.**: The coelomocytes of the earthworm *Lumbricus terrestris*: morphology and phagocytic properties. *J. Morphol.* **153**: 467-477, 1977.

Marks D.H., Stein E.A., **Cooper E.L.**: Chemotactic attraction of *Lumbricus terrestris* coelomocytes to foreign tissue. *Dev. Comp. Immunol.* **3**: 277-286, 1978.

Stein E.A. and **Cooper E.L.**: Cytochemical observations of coelomocytes from the earthworm, *Lumbricus terrestris*. *Histochem. J.* **10**: 657-678, 1978.

Marks D.H., Stein E.A., **Cooper E.L.**: Acid phosphatase changes associated with response to foreign tissue in the earthworm, *Lumbricus terrestris*. *Comp. Biochem. Physiol.* **80A**: 681-685, 1981.

Stein E.A. and **Cooper E.L.**: The role of opsonins in phagocytosis by coelomocytes of the earthworm, *Lumbricus terrestris*. *Dev. Comp. Immunol.* **5**: 15-25, 1981.

Stein E.A., Wojdani A., **Cooper E.L.**: Agglutinins in the earthworm *Lumbricus terrestris*: naturally occurring and induced. *Dev. Comp. Immunol.* **6**: 407-421, 1982.

Wojdani A., Stein E.A., **Cooper E.L.**, Alfred L.J.: Agglutinins and proteins in the earthworm, *Lumbricus terrestris*, before and after injection of erythrocytes, carbohydrates and other materials. *Dev. Comp. Immunol.* **6**: 613-624, 1982.

Roch P., **Cooper E.L.**, Eskinazi D. P.: Serological evidences for a membrane structure related to human β_2-microglobulin expressed by certain earthworm leukocytes. *Eur. J. Immunol.* **13**: 1037-1042, 1983.

Stein E.A. and **Cooper E.L.**: Carbohydrate and glycoprotein inhibitors of naturally-occurring and induced agglutinins from the earthworm, *Lumbricus terrestris*. *Comp. Biol. Chem.* **76B**: 197-206, 1983.

Cooper E.L. and Roch P.: Earthworm leukocyte interactions during early stages of graft rejection. *J. Exp. Zool.* **232**: 67-72, 1984.

Wojdani A., Stein E.A., Alfred L.J., **Cooper E.L.**: Mitogenic effect of earthworm (*Lumbricus terrestris*) coelomic fluid on mouse and human lymphocytes. *Immunobiol.* **166**: 157-167, 1984.

Cooper E.L.: Leukocyte activity during earthworm inflammatory responses. *Int. J. Tiss. Reac.* **8**: 175-184, 1986.

Cooper E.L. and Roch, P.: Second-set allograft responses in the earthworm *Lumbricus terrestris*: Kinetics and characteristics. *Transplantation* **41**: 514-520, 1986.

El Amir A., Saad A.H., El Deeb S., Wahby A.F., Soliman A.W., **Cooper E.**: Serological evidence for a Thy-1 homolog in earthworms. *Proc. Zool. Soc. A.R. Egypt* **12**: 287-302, 1986.

Stein E.A., Younai S., **Cooper E.L.**: Bacterial agglutinins of the earthworm *Lumbricus terrestris*. *Comp. Biochem. Physiol.* **84**: 409-415, 1986.

Goven A.J., Venables B.J., Fitzpatrick L.C., **Cooper E.L.**: An invertebrate model for analyzing effects of environmental xenobiotics on immunity. *Clin. Ecol.* **4**: 150-154, 1988.

Stein E.A. and **Cooper E.L.**: *In vitro* agglutinin production by earthworm leukocytes. *Dev. Comp. Immunol.* **12**: 531-547, 1988.

Rodriguez-Grau J., Venables B.J., Fitzpatrick L.C., Goven A.J., **Cooper E.L.**: Suppression of secretory rosette formation by PCBs in *Lumbricus terrestris*: an earthworm assay for humoral immunotoxicity of xenobiotics. *Env. Toxicol. Chem.* **8**: 1201-1207, 1989.

Stein E.A., Morovati A., Rahimian P., **Cooper E.L.**: Lipid agglutinins from coelomic fluid of the earthworm *Lumbricus terrestris*. *Comp. Biochem. Physiol.* **94B**: 703-707, 1989.

Saad A.H. and **Cooper E.**: Evidence for a Thy-1 like molecule expressed on earthworm leukocytes. *Zool. Sci.* 7: 217-222, 1990.

Stein E.A., Younai S., **Cooper E.L.**: Separation and partial purification of agglutinins from coelomic fluid of the earthworm *Lumbricus terrestris*. *Comp. Biochem. Physiol.* **97B**: 701-705, 1990.

Cooper E.L.: Evolutionary development of the neuroendocrineimmune system. *Adv. Neuroimmunol.* **1**: 83-96, 1990.

Chen S.C., Fitzpatrick L.C., Goven A.J., Venables B.J., **Cooper E.L.**: Nitroblue tetrazolium dye reduction by earthworm (*Lumbricus terrestris*) coelomocytes: an enzyme assay for nonspecific immunotoxicity of xenobiotics. *Env. Toxicol. Chem.* **10**: 1037-1043, 1991.

Eyambe G.S., Goven A.J. Fitzpatrick L.C., Venables B.J., **Cooper E.L.**: A non-invasive technique for sequential collection of earthworm (*Lumbricus terrestris*) leukocytes during subchronic immunotoxicity studies. *Lab. Animals.* **25**: 70-76, 1991.

Roch P. and **Cooper E.L.**: Cellular but not humoral antibacterial activity of earthworms is inhibited by Aroclor 1254. *Ecotoxicol. Environ. Safety* **22**: 283-290, 1991.

Cooper E.L., Rinkevich B., Uhlenbruck G., Valembois P.: Invertebrate Immunity: another viewpoint. *Scand. J. Immunol.* **35**: 247-266, 1992.

Cooper E.L. and Roch P.: The capacities of earthworms to heal wounds and to destroy allografts are modified by polychlorinated biphenyls (PCB). *J. Invert. Path.* **60**: 59-63, 1992.

Cooper E.L., Leung M.K., Stefano G.B., Cadet P., Suzuki M.M.: An enkephalin-like molecule in earthworm coelomic fluid modifies leukocyte behavior. *Dev. Comp. Immunol.* **17**: 201-209, 1993.

Goven A.J., Eyambe G.S., Fitzpatrick L.C., Venables B.J., **Cooper E.L.**: Cellular biomarkers for measuring toxicity of xenobiotics: Effects of polychlorinated biphenyls on earthworm *Lumbricus terrestris* coelomocytes. *Environ. Toxicol. Chem.* **12**: 863-870, 1993.

Ville P., Roch P., **Cooper E.L.**, Masson P., Narbonne J-F.: PCBs Increase molecular-related activities (lysozyme, antibacterial, hemolysis, proteases) but inhibit macrophage-related functions (phagocytosis, wound healing) in earthworms. *J. Invertebr. Pathol.* **65**: 217-224, 1995.

Suzuki M.M. and **Cooper E.L.**: Killing of intrafamilial leukocytes by earthworm affected cells. *Immunol. Lett.* **44**: 45-49, 1995.

Suzuki M.M. and **Cooper E.L.**: Allogeneic killing by earthworm effector cells. *Nat. Immun.* **14**: 11-19, 1995.

Suzuki, M.M., and **Cooper, E.L.** 1995. Spontaneous cytotoxic earthworm leukocytes kill K562 tumor cells. *Zool. Sci.* 12: 443-451.

Suzuki M.M., **Cooper E.L.**, Eyambe A.J., Fitzpatrick L.C., Venables B.J.: Polychlorinated biphenyls (PCBs) depress allogeneic natural cytotoxicity by earthworm coelomocytes. *Environ. Toxicol. Chem.* **14**: 1697-1700, 1995.

Cooper E.L., Cossarizza A. Suzuki M.M., Salvioli S., Capri M., Quaglino D., Franceschi C.: Autogeneic but not allogeneic earthworm effector coelomocytes kill the mammalian tumor target K562. *Cell. Immunol.* **166**:113-122, 1995.

Cossarizza A., **Cooper E.L.**, Quaglino D., Salvioli S., Kalachnikova G., Franceschi C.: Mitochondrial mass and membrane potential in coelomocytes from the earthworm *Eisenia foetida*: Studies with fluorescent probes in single intact cells. *Biochem. Biophys. Res. Comm.* **214**: 503-510, 1995.

Cooper E.L., Arizza V., Cammarata M., Pellerito L., Parrinello N.: Tributyltin affects phagocytic activity of *Ciona intestinalis* hemocytes. *Comp. Biochem. Physiol.* **112C**: 285-289, 1995.

Cossarizza A., **Cooper E.L.**, Suzuki, M. M., Salvioli S., Capri M., Gri G., Quaglino D., Franceschi C.: Earthworm leukocytes that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines. *Exp. Cell Res.* **224**: 174-182, 1996.

Quaglino D., **Cooper E.L.**, Salvioli S., Capri M., Suzuki M.M., Pasquali-Ronchetti I., Franceschi C. Cossarizza A.: Earthworm coelomocytes *in vitro*: cellular features and "granuloma" formation during cytotoxic activity against the mammalian tumor cell target K562. *Eur. J. Cell Biol.* **70**: 278-288, 1996.

Kauschke E., Pagliara P., Stabili L., **Cooper E.L.**: Characterization of proteolytic activity in coelomic fluid of *Lumbricus terrestris* L. (Annelida, Lumbricidae). *Comp. Biochem. Physiol.* **116B**: 235-242, 1997.

Ville P., Roch P., **Cooper E.L.**, Narbonne J-F.: Immuno-modulatror effects of carbaryl and 2,4D in the *earthworm Eisenia fetida andrei*. *Environ. Contam. Toxicol.* **32**: 291-297, 1997.

Lange S., Nüssler F., Kauschke E., Lutsch G., **Cooper E.L.**, Herrmann A.: Interaction of earthworm hemolysin with lipid membranes requires sphingolipids. *J. Biol. Chem.* **272**: 20884-20892, 1997.

Roch P., Ville P., **Cooper E.L.**: Characterization of a 14 kDa plant-related serine protease inhibitor and regulation of cytotoxic activity in earthworm coelomic fluid. *Dev. Comp. Immunol.* **22**: 1-12, 1998.

Eue I., Kauschke E., Mohrig W., **Cooper E.L.**: Isolation and characterization of earthworm hemolysins and agglutinins. *Dev. Comp. Immunol.* **22**: 13-25, 1998.

de Eguileor M., Tettamanti G., Grimaldi A., Boselli A., Scari G., Valvassori R., **Cooper E.L.**, Lanzavecchia G.: Histopathological changes after induced injury in leeches. *J Invertebr Pathol* **74**: 14-28, 1999.

Lange S., Kauschke E., Mohrig W., **Cooper E.L.**: Biochemical characteristics of Eiseniapore, a pore-forming protein in the coelomic fluid of earthworms. *Eur. J. Biochem.* **262**: 547-556, 1999.

Cikutovic M.A., Fitzpatrick L.C., Goven A.J., Venables B.J., Giggleman M.A., **Cooper E.L.**: Wound healing in earthworms *Lumbricus terrestris*: a cellular-based biomarker for assessing sublethal chemical toxicity. *Bull. Environ. Contam. Toxicol.* **62**: 508-514, 1999.

de Eguileor M., Grimaldi A., Tettamanti G., Valvassori R., **Cooper E.L.**, Lanzavecchia G.: Different types of response to foreign antigens by leech leukocytes. *Tissue Cell.* **32**: 40-48, 2000.

de Eguileor M., Grimaldi A., Tettamanti G., Valvassori R., **Cooper E.L.**, Lanzavecchia G.: Lipopolysaccharide-dependent induction of leech leukocytes that cross-react with vertebrate cellular differentiation markers. *Tissue Cell.* **32**: 437-445, 2000.

Kauschke E., Komiyama K., Moro I., Eue I., König S., **Cooper E.L.**: Evidence for perforin-like activity associated with earthworm leukocytes. *Zoology* **32**: 13-24, 2001.

Chapters and contributions to proceedings and monographs

Cooper E.L.: Neoplasia and transplantation immunity in annelids. *J. Nat. Cancer Inst.* **31**: 655-669, 1969.

Cooper E.L.: Earthworm coelomocytes: role in understanding the evolution of cellular immunity. I. Formation of monolayers and cytotoxicity. In J. Rehacek, D. Blaskovic, W.F. Hink (Eds.), *Proc. III International Colloquium on Invertebrate Tissue Culture*, Slovak Academy of Sciences, Bratislava, 1973 pp. 381-404.

Cooper E.L.: Evolution of cellular immunity. In W. Braun and J. Ungar (Eds.), *Symposium on Non-Specific Factors Influencing Host Resistance*, S. Karger, Basel, Switzerland, 1973 pp. 11-23.

Cooper E.L.: Foreword and Introduction to: *Invertebrate Immunology. Contemporary Topics in Immunobiology*, Vol. 4. Plenum Press, New York, 1974 pp.1-12.

Cooper E.L.: Phylogeny of leucocytes: earthworm coelomocytes *in vitro* and *in vivo*. In K. Lindahl-Kiessling and D. Osoba (Eds.), *Lymphocyte Recognition and Effector Mechanisms. Proc. Eighth Leukocyte Culture Conf.*, Academic Press, New York, 1974 pp. 155-162.

Cooper E.L., Lemmi C.A., Moore T.C.: Agglutinins and cellular immunity in earthworms. *Ann. N.Y. Acad. Sci.* **234**: 34-50, 1974.

Hostetter R.K. and **Cooper E.L.**: Earthworm coelomocyte immunity. In E.L. Cooper (Ed.), *Contemporary Topics in Immunobiology*, Vol. 4, Plenum Press, New York, 1974 pp. 91-107.

Lemmi C. A., **Cooper E.L.**, Moore T.C.: An approach to studying evolution of cellular immunity. In E.L. Cooper (Ed.), *Contemporary Topics in Immunobiology*, Vol. 4, Plenum Press, New York, 1974 pp. 109-119.

Cooper E.L.: Cellular recognition of allografts and xenografts in invertebrates. In J.J. Marchalonis (Ed.), *Phylogeny of Immune Responses*, Blackwell, Oxford, 1975 pp. 36-79.

Cooper E.L.: Characteristics of CMI and memory in annelids. In W.H. Hildemann and A.A. Benedict (Eds.), *Immunologic Phylogeny. Advances in Experimental Medicine and Biology*, Vol. 64, Plenum Press, New York, 1975 pp. 127- 136.

Cooper E.L. and Winger L.A.: Transplantation immunity in annelids. III. Effects of temperature on xenograft rejection in earthworms. *Amer. Zool.* **15**: 7-11, 1975.

Cooper E.L.: The earthworm coelomocyte: a mediator of cellular immunity. In R.K. Wright and E.L. Cooper (Eds.), *Phylogeny of Thymus and Bone Marrow-Bursa Cells*, Elsevier/North-Holland, Amsterdam, 1976 pp. 9-18.

Cooper E.L.: Phylogenetic aspects of transplantation. In: J.W. Masshoff (Ed.), *Handbuch der Allgemeinen Pathologie VI/8. Transplantation*, Springer-Verlag, Berlin, 1977 pp. 139-167.

Cooper E.L.: How the immune system evolved. *J. Madurai Kamaraj Univ.* **7**: 59-68, 1978.

Cooper E.L.: Cell-mediated memory in invertebrates. In M.J. Manning (Ed.), *Phylogeny of Immunological Memory*, Elsevier/North-Holland, Amsterdam, 1980 pp. 35-46.

Cooper E.L.: First Course Workshop, Comparative Immunology, Verona, Italy, *Dev. Comp. Immunol.*, **5**: 167-178, 1981.

Cooper E.L.: Immunity in Invertebrates. *CRC Crit. Rev. Immunol.* **2**: 1-32, 1981.

Cooper E.L. and Lemmi C.A.E.: Invertebrate humoral immunity. *Dev. Comp. Immunol.* **5** Suppl. 1: 3-21, 1981.

Cooper E.L. and Stein E. A.: Leucocytes of Annelids: The Oligochaeta. In N.A. Ratcliffe and A.F. Rowley (Eds.), *Invertebrate Blood Cells*, Academic Press, New York, 1981 pp. 75-140.

Cooper E.L., Stein E.A., Tochinai S., Wojdani A., Lemmi C.A.E.: Earthworms, immunology and aging. In M. Appelhof (Compiler), *Workshop on the Role of Earthworms in the Stabilization of Organic Residues*, Beech Leef Press of Kalamazoo Nature Center, 1981 pp. 449-571.

Cooper E.L., Stein E.A., Wojdani A., Lemmi C.A.E. Tochinai S.: Induction of earthworm-anti-erythrocyte agglutinins. In: *Aspects of Developmental and Comparative Immunology I*, J. B. Solomon (Ed.), Pergamon Press, New York, 1981 pp. 17-22.

Lemmi C.A.E. and **Cooper E.L.**: Induction of coelomocyte proliferation by xenografts in the earthworm, *Lumbricus terrestris*. *Dev. Comp. Immunol.* **5** Suppl. 1: 73-80, 1981.

Cooper E.L.: Invertebrate defense systems: An overview. In N. Cohen and M. M. Sigel (Eds.), *Ontogeny and Phylogeny of the Reticuloendothelial System*, Plenum Press, New York, pp. 1-35.

Cooper E.L., Roch P. G., Wright R. K.: Phylogeny of mononuclear phagocytes. In D. Mizuno, Z.A. Cohn, K. Takeya, N. Ishida (Eds.), *Self-Defense Mechanisms: Role of Macrophages*, University of Tokyo Press, Tokyo, Japan, 1982 pp. 3-14.

Stein E.A. and **Cooper E.L.**: Agglutinins as receptor molecules: a phylogenetic approach. In E.L. Cooper and M.A.B. Brazier (Eds.), *Developmental Immunology: Clinical Problems and Aging*, Academic Press, New York, 1982 pp. 85-98.

Wojdani A. and **Cooper E.L.**: The influence of ambient temperature on antibody production and aging. In E.L. Cooper and M.A.B. Brazier (Eds.), *Developmental Immunology: Clinical Problems and Aging*, Academic Press, New York, 1982 pp. 277-289.

Roch P.G. and **Cooper E.L.**: A β_2-microglobulin-like molecule on earthworm (*L. terrestris*) leukocyte membranes. *Dev. Comp. Immunol.* 7: 633-636, 1983.

Stein E.A. and **Cooper E.L.**: Inflammatory responses in annelids. *Am. Zool.* **23**: 145-156, 1983.

Cooper E.L.: Cell mediated immunity and aging in annelids. In D.H. Mitchell and T.E. Johnson (Eds.), *Invertebrate Models in Aging Research*, CRC Press, Boca Raton, 1984 pp. 121-142.

Cooper E.L. and Du Pasquier L.: Workshop 209. Immunity in lower vertebrates and invertebrates. 5th International Congress of Immunology, Kyoto, Japan. *Dev. Comp. Immunol.* **8**: 237-244, 1984.

Cooper E.L., Stein E.A., Wojdani A.: Recognition receptors in annelids. In E. Cohen (Ed.), *Recognition Proteins, Receptors and Probes: Invertebrates*, Alan R. Liss, Inc., New York, pp. 43-54, 1984.

Stein E.A., Wright R.K., **Cooper E.L.**: Agglutinins of annelids and tunicates. *Dev. Comp. Immunol* **8** Suppl. 3: 165-171, 1984.

Cooper E.L. and Brehelin M.: Invertebrate Immunology Conference. *Dev. Comp. Immunol.* **9**: 159-182, 1985.

Cooper E.L.: Overview of humoral factors in invertebrates. *Dev. Comp. Immunol.* **9**: 577-583, 1985.

Cooper E.L.: Comparative immunology. Past Presidential Address. *Am. Zool.* **25**: 649-664; 1985.

Cooper E.L.: Evolution of histoincompatibility. In M. Brehelin (Ed.), *Immunity in Invertebrates (Cells, Molecules and Defense Reactions)*, Springer Verlag, Berlin, 1986 pp. 139-150.

Cooper E.L., Wright R.K., Stein E.A., Roch P. and Mansour M.H.: Immunity in earthworms and tunicates with special reference to receptor origins. In A.H. Greenberg, (Ed.), *Invertebrate Models, Cell Receptors and Cell Communication*, Karger, N.Y., 1987 pp. 79-103.

Stein E.A., Younai S., **Cooper E.L.**: Hemagglutinins and bacterial agglutinins of earthworms. In E.L. Cooper, C. Langlet, J. Bierne (Eds.), *Developmental and Comparative Immunology*, Alan R. Liss, N.Y., 1987 pp. 79-89.

Cooper E.L.: New animal models in immunobiology research. In J.J. Marchalonis and C.L. Reinisch (Eds.), *Defense Molecules*, Wiley-Liss, New York, 1990 pp. 255-260.

Cooper E.L.: Phylogenie du systeme immunitaire. In P.-P. Pastoret, A. Govaerts, H. Bazin (Eds), *Immunologie Animale*, Medicine-Sciences, Flammarion, Paris, 1990 pp. 181-185.

Fitzpatrick L.C., Goven A.J., Venables B.J., Rodriguez-Grau J., **Cooper E.L.**: Earthworm immunoassay for evaluating biological effects of exposure to hazardous materials. In S. Sandh (Ed.), *In situ evaluation of Biological Hazards of Environmental Pollutants* Plenum Press N. Y.,1990 pp. 119-129.

Cooper E.L.: Overview of immunoevolution. *Boll. Zool.* **59**: 119-128, 1992.

Cooper E.L.: Perspectives in neuroimmunomodulation: Lessons from the comparative approach. *Anim. Biol.* **1**: 169-180, 1992.

Cooper E.L.: Innate immunity. In I.M. Roitt and P.J. Delves (Eds.), *Encyclopedia of Immunology*, Harcourt Brace Jovanovich, Inc. 1992 pp.867-870.

Cooper E.L.: Comparative Immunology: The value of annelids. In V. Vetvicka, P. Sima, E.L. Cooper, M. Bilej, P. Roch,.*Immunology of Annelids*, CRC Press Boca Raton, 1993 pp. 1-12.

Cooper E.L. and Roch P.: Immunological profile of annelids: Transplantation Immunity. In V. Vetvicka, P. Sima, E.L. Cooper, M. Bilej, P. Roch,.*Immunology of Annelids*, CRC Press Boca Raton, 1993 pp. 201-243.

Cooper E.L.: Specificity and memory in invertebrates In G. Beck, G.S. Habicht, E.L. Cooper, J.J. Marchalonis (Eds.), *Primordial Immunity*, New York Academy of Sciences, New York, 1994 pp. 245-259.

Cooper E.L., Zhang Z. Raftos D.A., Habicht G.S., Beck G., Connors V., Cossarizza A., Franceschi C., Ottaviani E., Scapigliati G., Parrinello N.: When did communication in the immune system begin? *Int. J. Immunopath. Pharmacol.* **3**: 203-217, 1994.

Cooper E.L.: Invertebrates can tell us something about senescence. *Aging Clin. Exp. Res.* **6**: 5-23, 1994.

Roch P., Ville P., **Cooper E.L.**, Narbonne J-F.: Differential modulation of earthworm cellular and humoral immune-related functions by polychlorinated biphenyls (PCB). In: J.S. Stolen, T.C. Fletcher, C.J. Bayne, C.J. Secombes, J.T. Zelikoff, L.E. Twerdok, D.P. Anderson (Eds.), Modulators of immune responses: The evolutionary trail. SOS Publications, Fair Haven NJ, 1996 pp 515-522.

Cooper E.L., Suzuki M. M., Cossarizza A., Franceschi C.: Cytotoxic reactions in invertebrates: the earthworm model. In: New Directions In K. Söderhäll, S. Iwanaga, G.R. Vasta (Eds) Invertebrate Immunology. SOS Publications, Fair Haven NJ.,1996 pp 23-42.

Cooper E.L.: Earthworm Immunity. In B. Rinkevich and W.E.G. Müller (Eds.), Invertebrate Immunology Springer Verlag, Heidelberg, 1996 pp. 10-45.

Cooper E.L.: Diversity of Immune Systems. *Ital. J. Zool.* **63**: 295-302, 1996.

Cooper E.L., Cossarizza A., Kauschke E., Franceschi C.: Cytotoxicity in earthworms. In K. Maramorosch and M. Loeb (Eds), *Proc. World Congress on In Vitro Biology*. 1997 pp.53-58.

Cooper E.L.: Ecotoxicology: Compromising immunodefense of aquatic and terrestrial species. In J Zelikoff (Ed.), *Proceedings. Symposium on Ecotoxicology: Responses, Biomarkers and Risk Assessment.* SOS Publications, Fairhaven NJ., 1997 pp.109-126.

Cooper E.L., Kauschke E., Cossarizza A., Franceschi C.: Cell adhesion and the immune system: A case study using earthworms. *Microscopy Res. Tech.* **44**: 237-253, 1999.

Cooper E.L.: Immune Response: Phylogeny. In *Encyclopedia of Life Sciences (Article 519)* Macmillan Reference Limited, Grove's Dictionaries Inc. (In press)

Kauschke E., Eue I., Lange S., Mohrig W., **Cooper E.L.**: Immune proteins in earthworms. *Recent. Res. Dev. Comp. Biochem. Physiol.* **1**: 105-122, 2000.

Cooper E.L. and Bosch T.C.G.: Evolution of the Immune system. *Exp. Clin. Immunogenetics.* **17**: 77-82, 2000.

Cooper E.L., Kauschke E, Cossarizza A.: Annelid humoral immunity: cell lysis in earthworms. In G. Beck, M. Sugumaran, E.L. Cooper (Eds.), *Phylogenic Perspectives on the Vertebrate Immune System.* Advances in Experimental Medicine and Biology, Vol. 484, 2001 pp. 169-184.

Books and edited symposium volumes

Hildemann W.H. and **Cooper E.L.** (Eds.) *Phylogeny of Transplantation Reactions. Transplant. Proc.* **2**: 179-341, 1970.

Acton R.T., Evans E.E., Weinheimer P.F., **Cooper E.L.**, Campbell R.D., Prowse R.H., Bizot M., Stewart J.E., Fuller G.M. *et al. Invertebrate Immune Defense Mechanisms.* MSS Information Corp., New York, 204 pp., 1972.

Cooper E.L. (Ed.) Invertebrate Immunology. *Contemporary Topics in Immunobiology* Vol. 4. Plenum Press, New York, 299 pp., 1974.

Cooper E.L. (Ed.) Developmental Immunology. *Am. Zool.* **15**: 35, 1975.

Cooper E.L.: *Comparative Immunology*, Prentice-Hall, Englewood Cliffs, NJ 338 pp., 1976, (Translated into Russian, 1980).

Wright R.K. and **Cooper E.L.** (Eds.) *Phylogeny of Thymus and Bone Marrow-Bursa Cells.* Elsevier/North Holland, Amsterdam, 325 pp., 1976.

Gershwin M.E. and **Cooper E.L.** (Eds.): *Animal Models of Comparative and Developmental Aspects of Immunity and Disease.* Pergamon Press, New York, 396 pp., 1978.

Azzolina L.S., Tridente G., **Cooper E.L.** (Eds.) *Dev. Comp. Immunol.* **5** Suppl. 1: 1-180 pp., 1981.

Cooper E.L.: *General Immunology*, Pergamon Press, New York, 343 pp., 1982.

Cooper E.L. and Brazier M.A.B. (Eds.) *Developmental Immunology: Clinical Problems and Aging.* UCLA Forum in Medical Sciences, Vol. 25, Academic Press, New York, 321 pp., 1982.

Cooper E.L. (Editor-in-Chief) and van Miuswinkel W.B. (Guest Editor) *Immunology and Immunization of Fish. Dev. Comp. Immunol.* **6** Suppl. 2: 255 pp., 1982.

Cooper E.L. and Wright R.K. (Eds.) Aspects of Developmental and Comparative Immunology II. *Proc. 2nd Int. Congr. Int. Soc. of Dev. Comp. Immunol., Suppl.* 3, Pergamon Press, New York, 280 pp., 1984.

Cooper E.L., Langlet C., Bierne, J. (Eds.) *Developmental and Comparative Immunology*, Alan R. Liss, NY, 180 pp., 1987.

Cooper E.L.: *General Immunology* (Japanese Translation) Nishimura Co. Ltd., Japan, 324 pp., 1990.

Cooper E.L. (Ed.) Comparative Immunology. *Bioscience* **40**: 720-768, 1990.

Vetvicka V. Sima P., **Cooper E.L.**, Bilej M. Roch P.:. *Immunology of Annelids*, CRC Press, Boca Raton, 300 pp., 1993.
Beck G., Habicht G.S., Cooper E.L., Marchalonis J.J. (Eds.) *Primordial Immunity*, Foundations for the Vertebrate Immune System, New York Academy of Sciences, New York, 376 pp., 1994.
Cooper E.L. (Ed.) *Invertebrate immune responses: Cells and Molecular Products*. In *Adv. Comp. Environ. Physiol.* **23**: 1-216, 1996.
Cooper E.L.(Ed.) *Invertebrate immune responses: Cell Activities and the Environment*. In *Adv. Comp. Environ. Physiol.* 24: 1-249, 1996.
Beck G, Sugumaran M, **Cooper E.L.**. (Eds) Phylogenic Perspectives on the Vertebrate Immune System. Advances in Experimental Medicine and Biology vol. 484. pp.1-383. 2001.

Brief communications

Cooper E.L.: Specific tissue graft rejection in earthworms. *Science* **166**: 1414-1415, 1969.
Cooper E.L., Acton R.T., Weinheimer P., Evans E. E.: Lack of a bactericidal response in the earthworm *Lumbricus terrestris* after immunization with bacterial antigens. *J. Inverterbr. Pathol.* **14**: 402-406, 1969.
Cooper E.L. and Rubilotta L. M.: Allograft rejection in *Eisenia foetida*. *Transplantation* **8**: 220-223, 1969.
Cooper E.L.: Phylogeny of transplantation immunity. Graft rejection in earthworms. *Transplant. Proc.* **3**: 214-216, 1971.
Cooper E.L.: Transplantation immunity in annelids. IV. Influence of earthworm size on rejection. *J. Invertebr. Pathol.* **24**: 260-261, 1974.
Toupin J., Marks D.H., **Cooper E.L.**, Lamoureux G.: Earthworm coelomocytes *in vitro*. *In Vitro* **13**: 218- 222, 1977.
Cooper E.L.: Comparison by species. In C. Bishop (Ed.), *Overview of Blood*, BLDIS, Dual Printing, Inc., Buffalo, 1978 pp. 75-78.
Stein E.A., Marks D.H., **Cooper E.L.**: Lack of acid phosphatase release during *in vitro* phagocytosis by coelomocytes of the earthworm, *Lumbricus terrestris*. *J. Invert. Pathol.* **39**: 116-118, 1982.
Cooper E.L.: Immune diversity throughout the animal kingdom. *Bioscience* **40**: 720-722, 1990.

Brief communications for meetings

Hildemann W. H. and **Cooper E.L.**: Foreword to: Phylogeny of transplantation reactions. *Transplant. Proc.* **2**: 179, 1970.
Cooper E.L.: Introduction to: Phylogeny of transplantation reactions. *Transplant. Proc.* **2**: 180, 1970.
Cooper E.L.: Introduction to: Developmental Immunology. *Am. Zool.* **15**: 3-5, 1975.
Cooper E.L. and Sigel M. M.: Evolution of transplantation immunity. *Transplant. Proc.* 7: 875-876, 1975.
Wright R.K. and **Cooper E.L.**: Preface and concluding remarks to: *Phylogeny of Thymus and Bone Marrow-Bursa Cells*. Elsevier/North-Holland, Amsterdam, 1976 pp. xvii-xix, 295-300.
Cooper E.L.: Preface, Symposium on Invertebrate Immunity. In L. A. Bulla and T. C. Cheng (Eds.), *Comparative Pathobiology*, Vol. 3, Plenum Press, New York, 1977 pp. ix-xii.
Cooper E.L. and Hildemann, W.H.: Workshop #5, Phylogeny, Progress in Immunology III. In: *Proc., 3rd Internat. Cong. Immunol*, Australian Academy of Science, 1978 pp. 738-740.
Gershwin M.E. and **Cooper E.L.**: Comparative immunology: to whither or whether. In: *Animal Models of Comparative and Developmental Aspects of Immunity and Disease*, Pergamon Press, 1978 pp. ix-x.
Cooper E.L.: Properties of coelomocyte membranes. In H. Peeters (Ed.), *Protides of Biological Fluids*, 27th Colloquium, Pergamon Press, New York, 1979 pp. 581-584.
Cooper E.L., MacDonald H.R., Sordat B.: Separation of earthworm coelomocytes by velocity sedimentation. In W. Muller Ruchholtz and H.K. Muller-Hermelink (Eds.), *Function and Structure of the Immune System*, Plenum Press, New York, 1979 pp. 101-106.
Cooper E.L.: Welcome address. In J.B. Solomon (Ed.), *Aspects of Developmental and Comparative Immunology I*, Pergamon Press, New York VII-VIII, 1981.
Cooper E.L.: Workshop on Phylogeny, Invertebrate Section, Paris 1981. *Dev. Comp. Immunol.* **5**: 179-181, 1981.
Cooper E.L. and Tridente G.: Summary. A comparative approach to immunopathology. *Dev. Comp. Immunol.* **5** Suppl. 1: 171-175, 1981.
Cooper E.L.: Introduction. In E.L. Cooper and M.A.B. Brazier (Eds.), *Developmental Immunology: Clinical Problems and Aging*, Academic Press, New York, 1982 pp. 3-5.

Cooper E.L. and Brazier M.A.B.: Preface. In E.L. Cooper and M.A.B. Brazier (Eds.), *Developmental Immunology: Clinical Problems and Aging*, Academic Press, New York, 1982 pp. xv-xvi.

Cooper E.L. and Walford R. L.: New perspectives on aging and immunity: lower animals, ontogeny, phylogeny and immunoendocrinology. *Dev. Comp. Immunol.* **6**: 391-393, 1982.

Cooper E.L. and Logdberg L.: Summary, plenary session, phylogeny of the major histocompatibility complex. *Dev. Comp. Immunol.* **8** Suppl. 3: 19-21, 1984.

Cooper E.L.: Greetings from the President. In E.L. Cooper, C. Langlet, J. Bierne (Eds.), *Developmental and Comparative Immunology*, Alan R. Liss, N.Y. 1987 p. xv.

Cooper E.L.: Introductions of the President-elect, Margaret Manning, In E.L. Cooper, C. Langlet, J. Bierne, (Eds.), *Developmental and Comparative Immunology*, Alan R. Liss, N.Y., 1987 pp. xxi.

Cooper E.L.: New directions in evolutionary immunobiology. A NSF workshop organized by J.J. Marchalonis and G.W. Warr. *Dev. Comp. Immunol.* **11**: 461-464, 1987.

Cooper E.L., Franchini A., Ottaviani E.: Earthworm coelomocytes possess immunoreactive cytokines and POMC-derived peptides. *Anim. Biol.* **4**: 25-29, 1995.

Cooper E.L.: The immunodefense system. In Cooper E.L. (Ed.) Invertebrate immune responses: Cells and Molecular Products. *Adv. Comp. Environ. Physiol.* 23: 1-5, 1996.

Cooper E.L.: Introduction. In Cooper E.L. (Ed.) Invertebrate immune responses: Cells and Molecular Products. *Adv. Comp. Environ. Physiol.* **24**: 1-5, 1996.

Popular articles

Cooper E.L., Beller J., Hayes S. A.: A successful medium for maintaining earthworms. *Lab. Anim.* **4**: 25-28, 1970.

Cooper E.L.: What is the origin and function of the immune system? *Trends Biochem. Sci.* **2**: N130-N132, 1977.

Cooper E.L.: Earthworms and immunology. *Trends Biochem. Sci.*, 4: 295-296, 1979.

Cooper E.L.: L'evolution de l'immunité. *La Recherche* **103**: 824-833, 1979.

Cooper E.L.: Phylogeny of cytotoxicity. *Endeavor* **4**: 160-165, 1981.

Younai S., Stein E.A., **Cooper E.L.**: Bacterial agglutination by earthworm lectins. UCLA Undergraduate Science Journal **1**: 41-56, 1985.

Cooper E.L.: 40 years of Pergamon Press and 10 of Developmental and Comparative Immunology. In E. Maxwell (Ed.), *Robert Maxwell and Pergamon Press: 40 Years Service to Science, Technology and Education*, Pergamon Press, N.Y., 1988 pp. 271-276.

Cooper E.L.: The immunology of earthworms and tunicates. *Lab Anim.* **25**: 38-43, 1996.

Journal *Developmental and Comparative Immunology*

Cooper E.L.: *Developmental and Comparative Immunology*, Vols. **1-16**, (52 issues) Pergamon Press, New York. Founding Editor and Editor-in Chief; 1977-1992.

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