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ORIGINAL ARTICLE

Antibacterial properties of hemerythrin of the sand worm *Nereis diversicolor*

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Abstract

OBJECTIVES: To investigate the immune defense of the annelid *Nereis diversicolor* and the key role of a oxygen-binding protein, the metalloprotein MPII animals were subjected to bacteria infection.

METHODS AND RESULTS: Using RACE-PCR, we have cloned the complete cDNA coding for the MPII related to the hemerythrin family in the sand worm *Hediste diversicolor*. This cDNA (883 pb) codes for a polypeptide of 119 amino acid residues with no signal peptide. Previous works have identified this protein as a cadmium scavenger. We here clearly demonstrated that this protein is also involved in the worm defence towards bacteria growth by its iron scavenger ability. This protein is expressed and produced in a haematopoietic center that floats freely in the coelomic fluid before stored in a particular hemocyte type: the granulocyte type 1. During bacterial challenge, this protein contained in these cells is discharged into the blood stream 3-4 hours after the infection and remains active for approximately 10 hours. This time period blocks progression of the pathogen and its attachment to tissues.

CONCLUSION: These results reflect that MPII in conjunction with others partners like lysozyme act as defence molecule for the sand worm.

* Equal contribution

Introduction

During the course of evolution, annelids have developed immunodefense strategies against various aggressors *e.g*. bacteria, living in water or in soil or associated with feeding or introduced into the body as a consequence of injuries [1–15]. In this phylum, the sand worm, *Nereis diversicolor* lives in estuary sediments and is therefore subjected to aggressions by biological (bacteria, parasites) and toxic (heavy metals) agents resulting from pollution.

Sand worms are characterized by the presence of two compartments that contain free cells i.e. the blood system with hemocytes and the coelom containing several populations of other leukocytes (granulocytes and eleocytes) [2]. Three types of granulocytes have been described. Type I granulocytes, referred to as G1 cells are large, fusiform and contain two types of cytoplasmic granules and bundles of microfilaments that extend throughout the cells [16]. Type II granulocytes (G2 cells) have been frequently observed in several families of polychaetes, first referred to as granular amoebocytes [16] without microfilaments. Cytoplasmic granules are more numerous than in type I cells. Abundant phagocytic vacuoles are distributed in the cortical cytoplasm. *In vitro*, G2 granulocytes produce hyaline veil-like pseudopodia, and adhere to substrata by means of radially distributed actin fibers. Type III granulocytes or G3 cells are small [16], have a high nucleoplasmic ratio, contain a few round granules and do not adhere to glass. Eleocytes are characterized by their abundant nutritive inclusions [17]. These cells show strong analogy with the chloragogen cells of oligochaetes. Viewed together, granulocytes and eleocytes play a role in both cellular and humoral several immune responses.

The coelomic fluid is also the source of other components. Previous work on *Nereis* by Dhainaut and co-workers demonstrated the presence in the coelomic fluid of a cadmium binding protein that is involved in fluid detoxification, [18]. Demuynck et al. [19, 20], have further established the complete primary structure of this Cd-binding protein, called MPII (metalloprotein type II). MPII is related to the hemerythrin/myohemerythrin family first discovered in sipunculids [20], in other annelids [22] including oligochaetes [23], leeches [24, 25]. This protein is a non-hemic oxygen binding protein involved as a vitellogenin in leeches [24], and as a detoxification protein for heavy metals in oligochaetes and polychaetes, [18, 19, 22, 24, 25].

Previous experiments revealed the presence of antibacterial substances in the coelomic fluid of polychaetes [3–5] as well as in oligochaetes [11, 14, 15]. In *Nereis*, we also demonstrated the presence of an antibacterial protein induced by lipopolysaccharide (LPS) injections into the coelomic cavity of this sand worm [26, 27]. Interestingly during its purification, this protein presented a biochemical behaviour that resembled MPII. Using antibodies (polyclonal and monoclonal) directed against MPII, we confirmed that the antibacterial protein was immunologically related to it [26]. Moreover, antibody neutralization experiments with anti-MPII in the cœlomic fluid previously stimulated by LPS showed an inhibition of the antibacterial activity, confirming its immunological identity [26, 27], yet additional indirect proof of a close relationship. Moreover, using a PCR strategy based on the first 33 amino acid residues of the MPII, a cDNA probe of 220 pb was generated and used for in *situ* hybridisation [28]. A strong labelling was found in longitudinal and oblique muscles of the worm, confirming the results obtained by Takagi and Cox [22] of the existence in this animal of a myohemerythrin. The probe is unable to distinguish between hemerythrin and myohemerythrin and consequently a labelling was also obtained at different levels in the sandworm, around the nerve cord [28].

In this context, we report here the complete cDNA sequence of the antibacterial protein and establish its homology with MPII and other hemerythrins found in annelids. Combining, *in situ* hybridization and immunocytochemistry, we demonstrated the presence of a haematopoietic center in *Nereis* from which hemocytes (granulocyte type 1) originate. MPII is then stored in these coelomocytes before it is released during bacterial infection. Protein labelling experiments coupled with bacterial injection experiments confirm the fact that this MPII is really implicated in host immunodefence due to its iron scavenger capability. Taken together, our data confirm the involvement of MPII protein in the sand worm's innate immune system.

Materials and methods

Sand worms and collection of cœlomic fluid

Nereis (*Nereis) diversicolor* (O. F. Müller) were collected in Petit-Fort-Philippe (France). Cœlomic fluid was withdrawn immediately by puncturing the body wall with sharpened Pasteur pipettes, then centrifuged at 10, 000 for 10 min at 4 °C to remove coelomocytes.

Growth inhibition tests

Sensitivities of bacteria (*Escherichia coli*) were determined in agar medium using a previously described disk method, [27]. The inhibition growth test in liquid was also used. Briefly, 10 μl of bacterial cultures (10 μl of OD of 0.7) are incubated with 700 μl of LB medium with or without the sample to be tested (20μl) as an indicator of concentration. In fact, maximal growth inhibition is given by the sample that received the same volume of sterile water. The percentage of surviving bacteria is given by the amount of anti-bacterial substance calculated according to the following formula:

$$\text{OD (culture + sample) x 100/OD (control)}$$

Bacteria

Bacterial strains used for immunization were *Escherichia coli*, *Vibrio algenolyticus* or *Micrococcus luteus*. Immunizations were performed by injecting 50 μl of sterile sea water containing 2 x 10^6 living bacteria into the coelom. Injection of bacteria occurred 24 hr before sampling the coelomic fluid. (Bacteria were a generous gift of Professor Catteau, Institut Pasteur, Lille).

Coelomocyte harvesting

Freshly collected coelomocytes were fractionated on 37 μm filters. In contrast to gametes, which are extremely large, coelomocytes go through the filters. They are then collected before subjecting them to a centrifugation for 5 min at 3000g. Coelomocytes were then washed in phosphate buffer 50 mM, NaCl 450 mM, pH 7.2.

Polyacrylamide gel electrophoresis and immunoblotting

The procedures were the same as used previously by Dhainaut *et al.*, [27].

ELISA Procedures

BUFFERS*:* For all subsequent procedures the following buffers were used:

Coating buffer (CB) 100 mM sodium carbonate pH 9.6 Blocking buffer (BB) 20 mM sodium phosphate, 50 mM NaCl, pH 7.4 (PBS); 2% bovine serum albumin (BSA) Washing buffer (WB) PBS, 0.05% Tween 20 Dilution buffer (DB) PBS, 0.05% Tween 20, 0.1% BSA.

IMMUNOASSAYS: One hundred microliters of coelomic fluid were coated 24 hr at 4 °C under agitation. After being saturated with BB (200 ml/well), plates were rinsed twice with WB, then covered with parafilm and kept at 4 °C. All subsequent steps were performed under continuous agitation. After coating, wells added of 100 μl of a-MPII (polyclonal) at a dilution of 1/1000, and incubated 2hr, before rinsed three times with WB, followed by the addition of 100 ml peroxidase-conjugated goat anti-rabbit IgG (Pasteur production; diluted 1/10000 in DB) to each well. After a 2 h incubation, followed by two washes in WB and one in PBS, 100 ml of freshly prepared substrate (6 mg ortho-phenylene-diamine/12 ml of 0.1 M citrate buffer pH 5.5 containing 0.08% H_2O_2) was added to each well. The reaction was stopped with 100 ml of 1N HCl and absorbance was measured at 490 nm on a MR 250 microplate reader (Dynatech). All assays were conducted in duplicate.

RNA extraction

Total RNA was extracted from coelomocytes by the guanidium isothiocyanate procedure [30].

cDNA synthesis

First strand synthesis was performed on total RNA. Ten micrograms of RNA were first denatured for 10 min at 37 °C in the presence of 0.5 μg oligo $(dT)_{12–18}$ and 1μl of RNase inhibitor (Boehringer Mannheim), in a total volume of 10 μl. First strand synthesis was generated in a 30 μl/volume by adding 1.5 μl of each deoxynucleoside triphosphate (dNTP) at 10 mM, 1 μl of RNase inhibitor, 3 μl of 0.1 M dithiothreitol (DTT), 6 μl of 5X buffer (1X buffer is 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3mM $MgCl_2$) and 1 μl M-MLV-RT (18 U/μl) (Bethesda Research Laboratories, MD). Incubation was done at 37 °C for 30 min then 1 μl of M-MLV-RT was added and incubation was performed for another 30 min. RNA was hydrolyzed at 65 °C for 10 min in the presence of 40 μl NaOH 0.7 M and EDTA 45 mM. The resulting cDNA was precipitated with 2.5 V ethanol and NaAc 0.3 M, resuspended in 2.5 μl of 10 mM Tris-HCl, 1 mM EDTA pH 7.5 and used as template for the PCR.

DNA amplification

PCR reactions were performed in 100 μl mixtures containing 2 μl of cDNA in 50 mM KCl, 10 mM Tris pH 8.3, 2 mM $MgCl_2$, 0.01% (w/v) gelatin, each primer at 1 μM, each dATP, dCTP, dGTP, dTTP, (Pharmacia) at 100 μM and 2 units of Taq DNA polymerase (Perkin-Elmer Cetus). Samples were overlaid with 75 μl of mineral oil. 30 cycling reactions were performed in a Biorad DNA thermal cycler. Each cycle consisted of denaturation at 94 °C for 1 min, annealing at 47 °C for 1.5 min and extension at 72 °C for 1.5 min.

Cloning and Sequencing

The PCR product was purified by electrophoresis on 1% agarose gel followed by DNA recovery according to Maniatis *et al.* [31]. The purified fragment was subsequently digested with the appropriate restriction enzymes (Xba I and Pst I), then ligated with XbaI-Pst I digested pUC 13 vector (Promega) and used to transform *E coli* RR1 DM15 (K12, RR1, lac z DM15, F' lac I ^{q} ZDM15 pro A) (ATCC 35102) using a complementation system. Clones containing an insert were isolated and DNA sequence of the insert was determined by the dideoxy chain-termination method [29] using the Sequenase kit (U.S. Biochemical).

In Situ *hybridization experiments*

Parts of worms (several segments) were fixed 4 hr in 2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.0. After washes in sodium phosphate buffer including 15% sucrose, tissues were embedded in mounting media (Tissue-Tek) and kept at –20 °C until sectioning. Sections (10 μm) were cut on a cryostat, mounted on gelatinized slides (1% gelatin, 1% chrome-alum) and stored at –70 °C. The probe was labeled by using the random priming kit (Boehringer Mannheim) with [$_a{}^{35}$S] dATP (1200 Ci/mmol, Amersham). Before hybridization, the cDNA probe was denatured by boiling for 5 min and then chilled on ice. Sections were prehybridized for 5 min at 20 °C in 4X SSC, 1X Denhardt solution and then for 45 min in the same solution. In order to improve the probe penetration, tissue-sections were pre-treated 30 min with 0.25% glacial acetic acid in 0.1M triethanolamine, 4X SSC pH 8 buffer. The slides were then dehydrated and air-dried. Hybridization was performed in 4X SSC, 50% formamide, 1X Denhardt, 120mM sodium-phosphate at pH 7.2, 1% N-Lauryl-Sarcosinate, 100mM DTT and a final concentration of probe at 0.5 ng/20 μl. After 16 hr of hybridization at 42 °C, slides were rinsed in 2 x SSC, 10 mM DTT, then washed twice at 20 °C, in 2 x SSC (5 min then 45 min) and finally at 42 °C in 1 x SSC for 5 min then for 45 min. In order to detect possible specific hybridization reactions, we used as control a probe containing the same concentration of labelled probe to which a ten-fold unlabelled probe was added. Slides

were finished by dipping in 50% aqueous solution of photographic emulsion NTB 2 (Kodak), exposed for 2 weeks at 4°C in dark-tight boxes. They were developed with D.19 developer (Kodak) for 2 min at 16°C, washed in water for 30 sec, fixed 5 min at 16°C with Kodak Fixer and contrasted with 1%Azur Blue.

Results

Cloning of the antibacterial protein

Based on biochemical sequence determination [19, 20], sets of primers that allowed nesting of the coding region were performed. Using PCR and RACE-PCR techniques, the entire coding region and the 5'–3' non-coding regions were obtained. This allowed establishing the complete cDNA sequence (Fig. 1). Blast databank researches revealed no sequence homology with other known nucleotide sequence. Whereas the deduced amino acid one revealed 95%, 80% and 63% identity with respectively the cadmium-protein MPII [19, 20], the *N. diversicolor* myohemeryhrin [22] and the leech *Theromyzon tessulatum* ovohemeryhrin [24] (Table 1).

Biological activity

We injected sand worms with different concentrations of *Micrococcus kristinae* from 10^6 to 10^7 bacteria/ml. Inhibition of growth areas were detected and correlated with the dose of injected bacteria to each worm (data not shown). From these inhibition areas, the amount of antibacterial proteins released in the medium can be estimated. This amount ranged between 18 nM/100 μl of stimulated coelomic fluid (SCF) to 80 nM/100μl SCF corresponding to the 50 fold of the lethal dose (LD_{50}=1.6 10^7 bacteria). Moreover, since we demonstrated that the presence of antibacterial proteins displaying antibacterial activity towards *E. coli* and *Micrococcus kristinae* (Fig. 2), we completed the spectrum of pathogens by testing the antibacterial activity towards *Vibrio algenolyticus* (Fig. 2), a specific pathogen of mussels and all sand animals. Sand worms quickly respond to the bacteria challenge by releasing in the first 3 hours antibacterial substances that peak at 4 hours post-injection, 80-nM/100 μl of coelomic fluid (CF) for *E. coli vs.* 50nM/100 μl for *V. alginolyticus,* (Fig.2). Antibacterial protein levels decreased 10 hours post-injection. Moreover, injury also stimulated the antibacterial proteins released (30 nM/100 μl CF).

Preadsorption of the SCF with an antibody rose against purified MPII revealed a complete lack of antibacterial activity (Fig. 3). Moreover, addition of iron to previous active SCF also blocked the antibacterial activity like the addition of phenanthroline (Fig. 3). Tests with purified MPII confirmed the antibacterial properties of the protein. Addition of iron to the purified MPII provoked a lack of activity confirming the hypothesis that MPII would act as an iron scavenger like lactoferrin.

Cellular localization of the antibacterial protein

Immunocytochemical analyses performed using a polyclonal antibody raised against the purified protein revealed a specific staining in sand worm immunocytes at the periphery of a cluster of cells that float freely in the coelom (Fig. 4). These cells stained by this antibody are large and fusiform and contained two types of cyto-

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ATCCACACCCCCAGTCAATTCTGCCCAAATCGTTCTTTCCATATAAGTTAATGAAAGTTGGATTCCAATCACCAA
                                                      K  L  D  S  N  H  Q

CTGTCAATTAGTACATTTGTGACTCGGGTTCAGCGCAGCTCAGCCACCTTACAATACAAGATGGGTTTCGAAATT
 L  S  I  S  T  F  V  T  R  V  Q  R  S  S  A  T  L  Q  Y  K  M  G  F  E  I

CCAGAACCATATAAGCAGGACGAGTCCTTCCAGGTCTTCTACGAGAAGCTTGATGAAGAACACAAGCAAATCTTC
 P  E  P  Y  K  Q  D  E  S  F  Q  V  F  Y  E  K  L  D  E  E  H  K  Q  I  F

AATGCCATCTTCGCCCTTTGCGGAGGAAACAACGCTGACAACCTGAAGTCTCTCGTTGACGTCACTGCCAACCAC
 N  A  I  F  A  L  C  G  G  N  N  A  D  N  L  K  S  L  V  D  V  T  A  N  H

TTCGCCGAAGAGGAGGCCATGATGAAGGCATCTGGTTCCTACGGAGACTTCGATTCCCACAAGAAGAAGCACGAG
 F  A  E  E  E  A  M  M  K  A  S  G  S  Y  G  D  F  D  S  H  K  K  K  H  E

GACTTCTTGGCCGTCATCCGTGGCCTTGGAGCCCCAGTGCCCCAGGACAAGATCGACTACGCCAAGGAATGGTTG
 D  F  L  A  V  I  R  G  L  G  A  P  V  P  Q  D  K  I  D  Y  A  K  E  W  L

GTCAACCACATCAAGGGAACCGACTTCGGCTACAAGGGAAAGTTGTAGACCTCGCGCGCCATGACATGTAGCGCC
 V  N  H  I  K  G  T  D  F  G  Y  K  G  K  L  *

ATCTAGCTGTTAAGTGTGGTATATCAGCTTGTGAGAGCGACGCTCCTGGTGGTCAACAACAAAACTAAGTTGTGT
TACCTGTTTGAAAAGACTGTCCTTGTGTTAGTCGTTTTTAAGTGTCTCGTCAGCTTCTGCAACGGTATATGCACA
AGGCAGCTTGTGTTATTTTCTCAGCCGAATAGTATTAAGAACTTGTCAAGTTCGTCAATTACACATTGGTTTATG
TTTGGAAATTGTAAAATCATGTGTGTTACCTTTTAATAAACTCTGCTTTTTCTAATTTCCAAAAAAAAAAAAAAA
AAAAGGGATCC
```

Fig. 1: Nucleotide and deduced amino acid sequences of MPII. Nucleotides and amino acid residues are numbered on the right column. Amino acids are numbered from the first methionine residue and identified with the single letter code. The peptide signal is underlined and an asterisk indicates the stop codon (TAA) of the open reading frame. Possible polyadenylation signals are double-underlined.

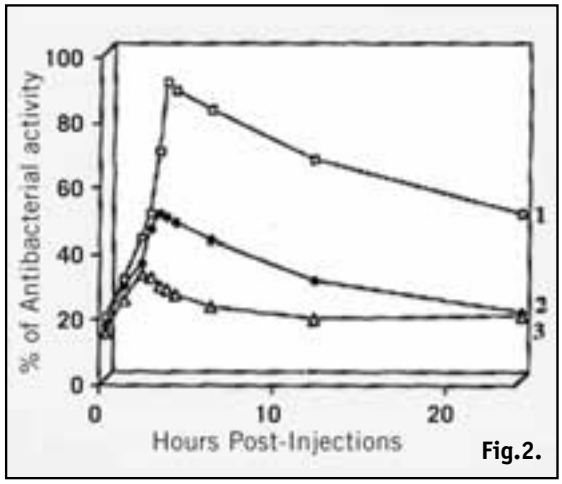

Fig.2.

Fig.2: Time course antibacterial response after injection (10 µl bacteria solution at a OD of 0.7) of (1) *E. coli,* (2) *M. luteus* or V *algenolyticus* (3).

Fig. 3: bacteria growth curves in presence SCF preadsorpbed with anti-MPII (a), iron 100 µg/ml SCF (b) or not (c). Inset of photographs represent a western blot analysis of SCF before (1) predsorption by anti-MPII (2) and antibacterial tests of purified MPII (1) or SCF presorbed with anti-MPII (2).

Fig. 4: Immunocytochemical studies with anti-MPII at the level a cluster of cell free in the coelom (A) or G1 cells (B).

Fig. 5: Detection of MPII messages in *H. diversicolor* sections were hybridized with ^{35}S-labeled cDNA probe followed as described in the Materials and Methods. Note that not all cells were stained in the medullar part of the cluster.

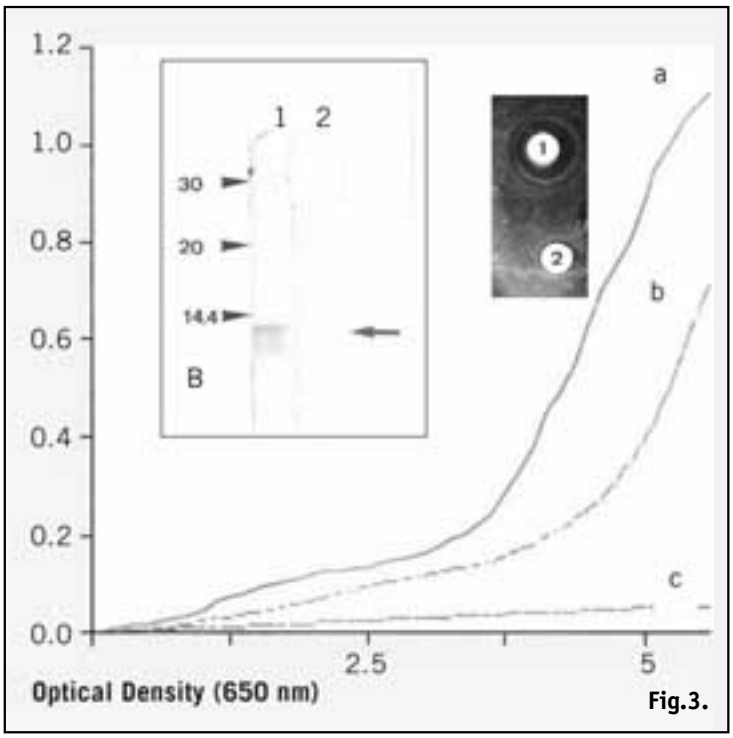

Fig.3.

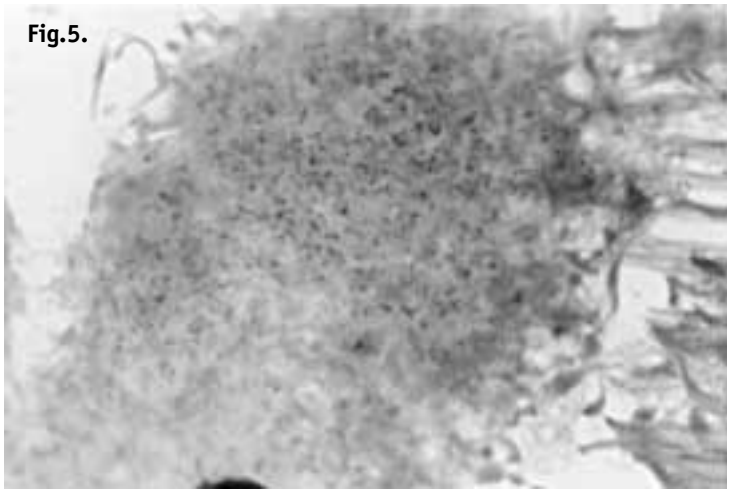

Fig.5.

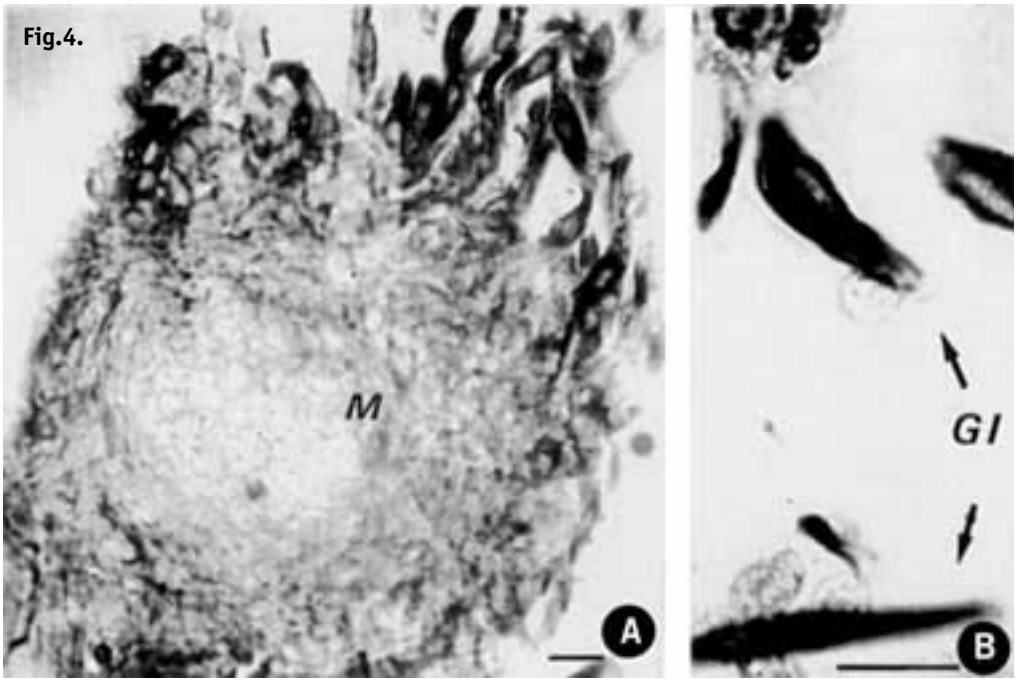

Fig.4.

plasmic granules and bundles of microfilaments that extend throughout the cells. These are the granulocytes 1 [30], (Fig. 4). *In situ* hybridization performed with the MPII cDNA fragment on *N. diversicolor* on G1 cells and the floating cell cluster, revealed that only the core of the cluster was positively hybridized with the probe whereas neither the cortical region of the cluster nor the G1 cells were recognized (Fig.5). These results allow suggesting that the floating cell cluster may be the site of genesis of G1 granulocytes. The mRNA encoding MPII is high at this site. By contrast, when the G1 are released into the coelomic cavity, the mRNA expression of MPII disappeared in the mature G1, despite bacterial challenge (Fig. 5). This contrasts with the immunocytochemical results that revealed a high content of MPII in G1 cells

Discussion

Using a RT-PCR following a RACE PCR strategy, we cloned the complete cDNA encoding for an antibacterial protein, so called MPII from the sand worm *Nereis diversicolor*. This protein is present at the periphery (cortex) of a cluster of cells that float freely in the coelomic fluid and in granulocytes type 1 cells. By contrast, expression of MPII mRNA was only found in the center, the medullary region. No MPII mRNA detection was observed in the cortical region or in G1 cells. This allowed suggesting that this protein is accumulated in G1 cells.

The MPII presents antibacterial properties due to its ability to scavenge iron like lactoferrin [32] or fetidin in oligochaeta [13–15]. Furthermore, this protein may also be associated with the immune response by inhibiting pathogenic tissue fixation caused by antibacterial activity like lactoferrin [32]. This will prevent the dissemination of pathogens. After bacterial challenge, MPII as antimicrobial peptides found in invertebrates [33] is released 3–4 hours later in the coelomic fluid from G1 cells. MPII mRNAs are detected in the medullary region of the floating cell cluster in the coelomic fluid. We suggest that this center acts as a haematopoietic site from which G1 cells are generated. These cells are filled with this protein before being released into the circulation thus preventing bacterial infection.

Granulocytes are known to be involved in the clearance of bacteria from the coelom. In Arenicolidae, *Micrococcus* appears to be engulfed by phagocytic cells, which revealed a strong phosphatase activity [3]. In the same family, Fitzgerald and Ratcliffe [6] observed a reduction in the number of free circulating coelomocytes and the appearance of cellular aggregates after inoculation by bacteria Gram $^{+}$ and Gram $^{-}$. In *Nereis*, *V. alginolyticus*, previously labeled by incorporation of tritiated thymidine, was injected into the coelomic cavity. Radioactivity of the coelomic fluid dropped rapidly after inoculation. Simultaneously, the number of circulating granulocytes decreased whereas the number of eleocytes showed no variation. Electron microscopy revealed that bacteria were phagocytosed by granulocytes [16]. In this context, we propose that in contact with bacterial LPS, the G1 cells release proteins involved in inhibiting bacterial proliferation into the coelomic cavity.

MPII along with lysozyme are unique in possessing antimicrobial activity. In fact, at the present time, no cationic antibacterial peptides have been found in the coelomic fluid after bacterial challenge in sand worms. However, we cannot exclude those discovered in molluscs [34–36]. Here the cationic antimicrobial peptides are contained in certain hemocytes and are released into phagolysosomic vesicles during engulfment by phagocytic cells as in mammalian and molluscan immunocytes. Moreover, experimental studies of encapsulation performed in sand worms using implantation of micro carriers [37], reveal cooperation between different coelomocyte populations. In the minutes following implantation of a foreign body, the first cells that came into contact with the beads were G3 cells. They completely and immediately lysed after implantation on the bead's surface. The nucleus, organelles and dense granules are extruded and formed a coating around the implant. Several hours after implantation, numerous granulocytes, mainly G2 cells, are recruited and then are flattened and stacked as concentric sheets around the foreign body. This process resembles the granuloma like formation following the killing of cancer cells during cooperation in vitro between small and large coelomocytes of *Eisenia* (Quaglino, et al, 1996). G2 cells are linked by numerous desmosomes. Degranulation occurs in the inner sheets of coelomocytes and gives rise to a large amount of electron-dense fluid, which runs in the intercellular spaces and flows on the surface of the implant. The material produced by the G2 during the immune reaction of encapsulation is brown due to its relationship to melanin. Moreover, the G2 produced phenoloxidase enzyme known to trigger melanin biosynthesis [38]. The G2 cells can be compared to a melanocyte in which melanin is not stored as in mammalian cells, but produced and extruded following synthesis in the form of a thick fluid. In this context, we propose that because G2 releases melanotic material that requires oxygen to catalyze the reaction, MPII by its ability to bind oxygen also participates in this reaction.

Taken together, all of the results reveal that annelids like other invertebrates possess an innate immune response whose cellular component (the source of antimicrobial molecules) is important as it is in certain mollusks [mussels [39]] or in crustaceans [shrimp [40]].

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