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Vertebrate cytokines interleukin 12 and gamma interferon, but not interleukin 10, enhance phagocytosis in the annelid *Eisenia hortensis*

Sheryl L. Fuller-Espie *

Science Department, Cabrini College, Iadarola Center for Science, Education and Technology, 610 King of Prussia Road, Radnor, PA 19087-3698, USA

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ABSTRACT

Phagocytosis assays employing class I [interleukin 12 (IL-12)], and class II [gamma interferon (gIFN) and IL-10] human recombinant cytokines were carried out to determine the biological effects of these molecules on innate immune responses in the earthworm *Eisenia hortensis*. Coelomocytes from *E. hortensis* were pre-incubated with the cytokines for 16–20 h *in vitro* followed by introduction of *Escherichia coli* expressing green fluorescent protein (*E. coli*/GFP). The pro-inflammatory cytokines IL-12 and gIFN stimulated statistically significant ($p \leqslant 0.05$) enhanced phagocytosis of *E. coli*/GFP by hyaline amoebocytes as determined by flow cytometry; 10 out of 21 earthworms (48%) responded to IL-12, while eight out of 21 (38%) responded to gIFN. In contrast, the anti-inflammatory cytokine IL-10 neither stimulated nor inhibited phagocytosis in nine earthworms tested. These results demonstrate that vertebrate pro-inflammatory cytokines influence invertebrate cellular responses of immune cells causing enhanced phagocytic activity in earthworm coelomocytes.

1. Introduction

Cytokines and cytokine-related molecules comprise a long list of entities that mediate both immunological and physiological processes in immune and non-immune organs and systems, with many exhibiting pleiotropicity and functional redundancy (Ozaki and Leonard, 2002). These processes include the induction and enhancement of apoptosis, proliferation, differentiation, chemotaxis, cytoxicity, energy metabolism, regulation of neural growth, viral resistance and others. Although best characterized in vertebrate systems, a plethora of evidence shows that cytokine-like molecules (also known as macrokines, a term coined by Beck, 1998) also operate in various invertebrate taxa including *Tunicata*, *Mollusca*, *Insecta*, *Echinodermata*, *Nematoda* and *Annelida* (reviewed in Ottaviani et al., 2004). For example, in the earthworm *Eisenia foetida*, immunocytochemical approaches have revealed the presence of IL-1 α-, tumor necrosis factor (TNF)-α-, platelet-derived growth factor (PDGF)-AB- and transforming growth factor (TGF)-β1-like molecules in immunocytes (Cooper et al., 1995; Franchini et al., 1996). In addition, *E. foetida* possess a humoral factor, coelomic cytolytic factor 1 (CCF-1), which is a cytokine-like cytolytic protein that is functionally analogous to TNF-α (Bilej et al., 1995) despite the absence of gene homology between the two (Beschin et al., 2004). More recently, a putative helical cytokine has also been reported in *Drosophila melanogaster* (Malagoli et al., 2007; Cheng et al., 2009).

Exogenous vertebrate cytokines stimulate *in vitro* cellular responses in a variety of invertebrates. It has been demonstrated that IL-1α, IL-2, TNF-α, IL-8, PDGF-AB and TGF-β1 affect immunocyte migration, bacterial phagocytosis, and nitric oxide synthase activity in molluscs (Ottaviani et al., 1995, 1997, 2000). In the earthworm *Eisenia hortensis*, IL-1β, TNF-α, granulocyte–macrophage colony stimulating factor (GM-CSF) and IL-2 enhance phagocytosis by hyaline amoebocytes (Fuller-Espie et al., 2008). Although the exact mechanism by which xenogeneic cytokines stimulate these cellular responses has not been elucidated, it is possible that an ancestral cytokine receptor on the invertebrate cell surface induces a receptor-mediated response, or, alternatively, a cytokine-receptor-independent pathway (e.g. aggregation) may be involved.

This paper describes an investigation aimed at determining the *in vitro* effects of human recombinant cytokines on immune function in invertebrates; specifically the impact of pro-inflammatory cytokines interleukin 12 (IL-12) and gamma interferon (gIFN), and the anti-inflammatory cytokine IL-10, on phagocytosis was analyzed in the earthworm *E. hortensis*. The enhancing effect of gIFN on phagocytosis in vertebrate macrophages is well documented (Schroder et al., 2004), but few studies have investigated the effect of gIFN in invertebrates. Reports by Parker and Ourth (1999), (2000), and Ourth and Parker (2005) have shown that

* Fax: +1 610 902 8285
E-mail address: sfuller-espie@cabrini.edu

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human gIFN binds specifically to particulates from the protocerebrum and hemolymph of *Manduca sexta* (tobacco hornworm larvae), and confers significant protective resistance to *Galleria mellonella* nuclear polyhedrosis virus.

IL-12 also enhances phagocytosis in vertebrates, but via an indirect pathway. IL-12, also known as NK cell stimulatory factor, mediates many biological functions of T cells and NK cells. It plays a key role in regulating innate and adaptive immunity. It promotes the differentiation and expansion of Th1 cells, is produced mainly by macrophages, dendritic cells and B cells when challenged by bacterial and parasitic antigens, and also stimulates the production of gIFN by NK cells and T cells (Trinchieri, 2003; Watford et al., 2003). A study by Bermudex et al. (1995) demonstrated the indirect effect of IL-12 on macrophages; IL-12 had no direct stimulatory effect on mycobacterial-infected macrophages, however, the culture supernatant of IL-12-treated NK cells stimulated macrophages and caused inhibition of intracellular growth of *Mycobacterium avium*.

IL-10, first described as cytokine synthesis inhibitory factor, regulates many functions of hemopoietic cells in vertebrates including its principal function of containment and termination of inflammatory response through inhibiting effector functions of T cells, monocytes, and macrophages. It also regulates growth and differentiation of T regulatory cells, B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes and endothelial cells (reviewed by Moore et al., 2001). Laichalk et al. (1996) showed that IL-10 inhibited phagocytic activity of neutrophils, attenuated microbicidal activity, and suppressed the respiratory burst *in vitro* as evidenced by decreased production of superoxide. In the blue mussel *Mytilus edulis*, IL-10 was shown to inhibit microglial egress in excised ganglia. The amoeboid immunocytes also became rounded and immobile, suggesting an inhibitory activity of IL-10 in invertebrates (Stefano et al., 1999).

This study shows that the pro-inflammatory cytokines IL-12 and gIFN, when added exogenously to *in vitro* cell cultures of earthworm coelomocytes, significantly increased phagocytic activity as detected by a flow cytometric procedure. The "prey" was *Escherichia coli* expressing green fluorescent protein (*E. coli*/GFP), and analysis was restricted to the major phagocytic subpopulation of cells, the hyaline amoebocytes, also commonly referred to as the large coelomocytes. Of those earthworms exhibiting statistically significant results ($p \leqslant 0.05$ by student's *t*-test), 48% of earthworms responded to IL-12, while 38% responded to gIFN. IL-10, in contrast, did not significantly affect phagocytosis by hyaline amoebocytes of *E. hortensis*.

2. Materials and methods

2.1. Cell culture supplies and chemical reagents

Chemical reagents and tissue culture plasticware were purchased from Fisher Scientific. All cell culture reagents and phosphate-buffered saline (PBS) were purchased from Invitrogen unless otherwise noted. Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 10% fetal calf serum, 100 μg ml^{-1} ampicillin (Shelton Scientific), 10 μg ml^{-1} kanamycin (Shelton Scientific), 10 μg ml^{-1} tetracycline, 5 μg ml^{-1} chloramphenicol (Fluka Biochemika), 1× penicillin/streptomycin/amphotericin B, 1× non-essential amino acids and 1× L-glutamine to comprise Super DMEM (SDMEM).

2.2. Earthworm husbandry

E. hortensis (European nightcrawlers) were purchased from Vermitechnology Unlimited, Orange Lake, Florida, USA, who imports *E. hortensis* from Star Food, Holland, Scherpenzeelseweg 95, 3772ME Barneveld, The Netherlands. Species identity was determined by the United States Department of Agriculture, USDA Permit #52262 (Vermitechnology, personal communication). Short-term colonies were maintained at room temperature in the dark on moistened autoclaved pine woodchips sprinkled with Single Grain Rice Cereal or Rice with Bananas Cereal (Gerber) and covered with autoclaved, shredded and moistened paper towels. Habitats were changed twice weekly. Animals were euthanized by freezing at −20 °C.

2.3. Extrusion of coelomocytes

Prior to experimentation, earthworms were first washed with distilled water on paper towels using a water bottle to remove wood chip fragments or food particles. They were then placed overnight on paper towels moistened with 2.5 μg ml^{-1} Fungizone (Fisher Scientific) in 100 mm petri dishes to minimize fecal contamination during the extrusion process, and remove further any surface contaminants. To collect coelomocytes, earthworms were subjected to the basic extrusion procedure according to Engelmann et al. (2004) with some modifications. Briefly, earthworms were placed in a 100 mm Petri dish containing 3 ml ice cold extrusion buffer (71.2 mM NaCl, 5% v/v ethanol, 50.4 mM guaiacol-glyceryl-ether, 5 mM EGTA, pH 7.3). The coelomocytes were then transferred to 1 ml Accumax (Innovative Cell Technology) for a 5 min incubation period at room temperature to reduce aggregation of cells. Finally, 5 ml PBS was added and the sample was centrifuged immediately at 150*g*, 5 min at 4 °C. After decanting the supernatant, the coelomocyte pellet was gently mixed by flicking the bottom of the centrifuge tube, and coelomocytes were resuspended in 1 ml SDMEM. Enumeration was carried out using a hemacytometer. Only hyaline amoebocytes (large coelomocytes) and hyaline granulocytes (small coelomocytes) were included in the cell count; eleocytes were not counted but did factor into a quality score. Samples with large numbers of eleocytes compared to large and small coelomocytes were not used in phagocytosis assays. Samples were adjusted to 5×10^5 coelomocytes ml^{-1} in SDMEM.

2.4. Cytokines

Recombinant human cytokines gIFN, IL-12 and IL-10 were purchased from ProSpec-Tany TechnoGene Ltd. (CYT-206, CYT-362 and CYT-335, respectively). All lyophilized cytokines were reconstituted in ultrapure water at 10 ng μl^{-1} and stored in aliquots at −20 °C. For consistency and based on the phagocytosis assays of Fuller-Espie et al. (2008) who tested the effects of other vertebrate cytokines (IL-1 beta, IL-2, GM-CSF and TNF-alpha) on phagocytosis in *E. hortensis*, these experiments used gIFN, IL-12 and IL-10 at 20 ng ml^{-1} and 2 ng ml^{-1}. Only two concentrations of cytokine were employed owing to the limited number of coelomocytes (less than 10^6 $earthworm^{-1}$) obtained from each earthworm and the numerous controls required. Pooling of coelomocyte populations was never conducted to avoid possible allogeneic responses.

2.5. Bacteria for phagocytosis assays

E. coli/GFP: *E. coli* HB101 transformed with pGLO (BioRad) and expressing GFP were grown on tryptic soy agar containing 100 μg ml^{-1} ampicillin and 0.2% (w/v) arabinose at 32 °C for 24 h. After washing the cells once in PBS, they were fixed chemically with 4% (v/v) paraformaldehyde in PBS, 1 h at room temperature with periodic mixing, followed by three PBS washes. Centrifugation was carried out at 3273*g* for 5 min at 4 °C. The final cell pellet was resuspended in PBS, bacteria were enumerated using a hemacytometer, and then stored in the dark at 4 °C.