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Cytotoxic activity of *Holothuria tubulosa* (Echinodermata) coelomocytes

Mirella Vazzana[a,1], Monica Celi[a,1], Marco Chiaramonte[a], Luigi Inguglia[a], Debora Russo[a], Vincenzo Ferrantelli[b], Dominique Battaglia[a], Vincenzo Arizza[a,*]

[a] Dept. STEBICEF, Università degli Studi di Palermo, Via Archirafi, 18, 90123, Palermo, Italy
[b] Istituto Zooprofilattico Sperimentale della Sicilia, via G. Marinuzzi, 3, 90129, Palermo, Italy

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ABSTRACT

The immune system of marine invertebrates, in particular that of holothurians, still requires further study. Our research showed that coelomocyte cells contained in the coelomic fluid of the sea cucumber, *Holothuria tubulosa,* are able to lyse, *in vitro*, red blood cells in rabbits and sheep.

A plaque-forming assay showed spherule cells to be the effector cells, able to release cytotoxic molecules after xenogenic cell contact. The coelomocyte lysate supernatant, analysed by polyacrylamide gel electrophoresis overlay technique, using rabbit and sheep erythrocytes, showed two different haemolytic protein patterns: one calcium dependent and the other calcium independent. The fractions of each pattern were resolved on a polyacrylamide gel and calcium-dependent and independent coelomocyte lysate patterns were compared.

1. Introduction

The phylum Echinodermata, containing approximately 7000 species, includes, on a taxonomic level, basal deuterostomes [1] and is the invertebrate phylum most closely related to chordates. Appearing at the dawn of the Cambrian Period, echinoderms have a rich fossil history and are well represented by many bizarre groups, most of which now extinct. Over their long history of evolution, they have developed a range of highly effective strategies to protect themselves against attack from various pathogens and environmental stresses. This arose undoubtedly in response to the fact that they live in the intertidal zone and are, therefore, directly exposed to potentially pathogenic microorganisms, developing a defence system mainly based on natural immune response [2–9].

The Echinoderms since they are the most ancestral phylum of deuterostome clade, they are good models for the study of the evolution of the immune system within deuterostomes.

Like all invertebrates, Echinoderms base their defence exclusively on a complex, innate, non-myeloid immune system with specialized cellular and humoral components. These components form a network of transcription factors and defence proteins called in mammalian "defensins", which then eliminate pathogens and potentially toxic chemicals [10].

Defence activity results in a non-specific inflammatory response, which is mediated, to a large extent, by circulating cells (coelomocytes) or their products. The main cell-mediated immune activity, in addition to phagocytosis, is cytotoxic activity.

Coelomocyte populations seem to be essential; they perform functions such as phagocytosis [11], ROI production [12–15], cytotoxicity [16,17], synthesis and release of antimicrobial substances [18–23], inflammatory reactions [5,24,25], including C3-like expression after activation with LPS [7,26], phenoloxidase activity [27–30], capsule formation and graft reaction [2,31–34].

In vertebrates, cell killing by non-specific cytotoxic cells (NCC) is effective against the proliferation of transformed cells, virus-infected cells, parasites, other foreign invaders and allografts. They exert their effector function without prior sensitization by killing virally infected cells and tumour cells, and by producing immune-regulatory cytokines and chemokines [35,36]. This killing function is integrated by the involvement of multiple receptor-ligand systems, such as the Toll-like receptor system, in which the various isoforms recognize different molecular patterns associated with pathogens [37,38].

The existence of NCC cytotoxicity, probably appearing early in the evolutionary history of the immune system, has been demonstrated in various invertebrate groups [39]. In the sipunculid worms (a group containing about 300 marine species) like *Themiste petricola*, killer leukocytes reacted against xenogenic erythrocytes. This reaction required effector-to-target cell contact and divalent calcium ions [40–42].

Abbreviations: RE, rabbit erythrocytes; SE, sheep erythrocytes; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; E, effector cells; T, target cell; CCA, coelomocyte cytotoxic assay; PFC, plaque forming cell assay

* Corresponding author.
E-mail address: vincenzo.arizza@unipa.it (V. Arizza).
[1] Equally contributed authors.

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NCC cytotoxic activity modulated by IL-2 was observed in the mollusc *Planorbarius corneus* [43]. Wittke and Renwrantz [44] demonstrated that circulating cells (immunocytes) from *Mytilus edulis* are able to produce cytotoxic substances which lyse human erythrocytes. In oligochaetes, studies on allogeneic cytotoxicity by coelomocytes in *Eisenia fetida* and *Lumbricus terrestris* showed that preliminary close contact between target cells and *Lumbricus* effector cells is necessary to perform lysis [45]. Among the invertebrate deuterostome clade, a cell type, the granulocyte present in all species studied, seems to be strongly involved in cytotoxic activity. Granulocytes appear to contain several granules of various sizes. These haemocytes, after contact with foreign cells, may release the granules outside and cause cell lysis. In the Tunicate as the solitary ascidian *Ciona intestinalis* [46] or the colonial one *Botryllus schlosseri* [47], possesses granulocytes that play a key role in ascidian immunobiology. Indeed, they take part in a variety of biological functions of immunological relevance, such as haemolymph clotting, tunic synthesis, phenoloxidase activity and were able to lyse mammalian cells, releasing soluble lysins, following effector-target cells contact encapsulation of foreign bodies [48–53]. The ascidian effector cells can be attributed to a morula cell, a granular haemocyte characterized by the presence of several small or large vacuoles. In *C. intestinalis* the effector cell is the univacuolar haemocytes. It seems to be derived from a morula cell after the granules have fused together [54,55]. This haemocytes, besides containing phenoloxidase [56,57], also contains antimicrobial peptides [58,59] and cytotoxic molecules [46,60].

NCC activity and cytotoxic coelomocytes in echinoderms has been well demonstrated and characterized. Bertheussen [11] revealed a contact-dependent cytotoxic reaction in allogeneic mixtures of coelomic cells from the echinoid *Strongylocentrotus droebachiensis*, where both xenogeneic and allogeneic cells were killed in the same way. Moreover, the same author [11] demonstrated in the case of the allogeneic co-cultures, approx. 70% of cell combinations gave cytotoxicity, while the frequency of reactivity increased to over 90% when cells from different species were mixed. A recent study showed coelomocyte cytotoxic activity in the echinoidea *Paracentrotus lividus*, where the effector cells were the uncoloured spherulocytes; it is interesting to note that activity of uncoloured spherulocytes, isolated by Iodixinol centrifuged gradient, increased when mixed with the phagocyte fraction [61].

Canicattì et al. [62] demonstrated that *Holothuria polii* contained coelomocytes that were able to lyse sheep erythrocytes and were inhibited by sphingomyelin [63–65].

In Holothurians, coelomocyte classification has not yet been established; as yet classification lacks standardization based on results of existing studies from various researchers and regarding different species on descriptions of coelomocyte typing. Lymphocytes, morula cells, amoebocytes, crystal cells, fusiform cells and vibratile cells, however, have been successfully described [3,66–68]. In addition to phagocytosis [69], holothurian coelomocytes exhibit brown body formation in response to multicellular parasites. These are pigmented aggregates of phagocytes and spherule cells that encapsulate parasites which are too large to be phagocytized [31,33,70]. Spherule cells probably degranulate to chemically kill and/or degrade the invader [71], while other cells within the brown bodies possess active phenoloxidase resulting in melanization [32].

Recently, Vazzana et al. [68], characterized cellular types for *Holothuria tubulosa*. At least five cells populations were found: 1) phagocyte (32.1% ± 8.1), that can be found in two forms petaloid and filopodial with a *ratio* of 4:1. The first one is roundish with a bladder cytoplasm containing large vacuoles. The single nucleus is large and spheroidal. The second one has the cytoplasm extended in long filiform pseudopodia, which radiated in any direction from the central endoplasmic mass of the cell. 2) Morula cell (9.7% ± 4.4) has a diameter of approximately 16 μm, and it is characterized by a spherical mulberry shape. The cytoplasm is filled with large, uniform-size granules, 4 μm in diameter. The nucleus is not evident because is hidden by the granules. Spherulocyte (29.2% ± 9.1), with spherical shape and a size of approximately 9 μm in diameter and a cytoplasm typically filled with numerous regular and irregular granules. The round nucleus is usually eccentrically placed. 3) acidophilic spherulocytes (7.2% ± 2.8) showed the same characteristics as described for spherulocytes with a diameter of approximately 10 μm with the cytoplasm full of acidophilic granules. 4) progenitor cells (21.2% ± 3.4) the smallest coelomocytes of the population of *H. tubulosa* with a dimension that ranged from 4 μm to 5 μm. They were spherical, with a single round nucleus filling almost the whole volume of the cytoplasm generally placed on a side of the cell, surrounded by a thin rim of cytoplasm.

Although the interest in cytotoxic reactions in invertebrates has increased in recent years, it remains unclear whether the NCC recognize and destroy foreign particles in the same way of vertebrate NK cells. One way to investigate cytotoxic activity in vertebrates is to study the biology of NK-like cells and extend this research to other species. This will increase the understanding of mammalian host defences and the mechanisms employed by pathogens in an attempt to evade these defences [72].

This study aims at extending our knowledge on the immune system of H. tubulosa and its cytotoxic defence mechanisms by examining cytotoxic activity of *H. tubulosa* coelomocytes against rabbit and sheep blood cells and the coelomocyte population responsible for cytotoxic activity. Cytotoxic protein molecules responsible for cytotoxic activity were characterized by overlay and SDS-PAGE assays.

2. Materials and methods

2.1. Animals, bleeding procedure and coelomocyte collection

Healthy adults of *Holothuria tubulosa* (about 120 animals; body length: 9–13 cm; weight: 80–120 g), namely those that did not undergo visceral ejection and showed no skin ulceration, as described by Deng et al. [73] [29], were periodically collected (December–August) by diving in the Gulf of Palermo (Mongerbino: 38°06.00′ N; 13°30.00′ E). The animals were collected at a depth of 10 m, close to a *Posidonia oceanica* meadow, and kept until use in 360-L aquaria filled with artificial sea water (0.425 M NaCl; 9 mM KCl; 9.3 mM $CaCl_2{\cdot}2H_2O$; 0.0255 M $MgSO_4{\cdot}7H_2O$; 0.023 $MgCl_2{\cdot}6H_2O$; 2 mM $NaHCO_3$ pH 8.0) (ASW). The Aquaria were fitted with a sand filter and sand bed on the bottom, and maintained at 15 ± 2 °C. The animals were kept in aquaria for a week for acclimation before beginning assays and fed regularly with commercial invertebrate food (Alga-Mac 3000, Bio-Marine, Hawthorne, CA.).

Coelomic fluid (CF) was collected in separate beakers containing ISO-EDTA (0.5 M NaCl, 20 mM Tris-HCl, 30 mM EDTA; pH 8.0). An incision measuring 3–5 cm was made along the ventral side, taking care not to injure the internal organs, and the CF was then transferred to test tubes and placed at 4° C until analysed. Cell suspension and CF were separated by centrifugation at 400*g* for 10 min at 4 °C and cells washed twice in ISO-EDTA. Cell pellets were then suspended again in the same buffer at a concentration of 1 × 10^6 cells/ml. To observe the morphology of living cells and evaluate total cell counts, 10 μl of freshly collected coelomic fluid was drawn into a Neubauer haemocytometer and examined under a light microscope using a magnification of 20–40 ×. Dead cells were evaluated through the eosin-y exclusion assay (0.5% in ISO-EDTA).

2.2. Coelomocyte lysate supernatant (CLS)

Coelomocytes (5 × 10^7/ml) were suspended in 1 ml of ISO containing 20 mM of Ca^{2+} (ISO- Ca^{2+}) and 0.5% v/v protease inhibitor cocktail (pepstatin A, E−64, bestatin, leupeptin, aprotinin, and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), sonicated (Branson Sonifier, Model B-15 Danbury, CT USA) for 1 min (1 pulse per second, 70% duty cycle) at 0 °C and centrifuged at 27,000 *g* for