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RESEARCH REPORT

Granulocytes of sea anemone *Actinia equina* (Linnaeus, 1758) body fluid contain and release cytolysins forming plaques of lysis

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Abstract

The Cnidaria phylum includes organisms that are among the most poisonous animals. The exact composition of cnidarian bioactive molecules is not known in detail, but little is known on the cells that produce the toxins. Here we have shown that the presence of cytolysins is not exclusive of nematocysts. A plaque-forming assay was carried out with cell populations extracted from the percoled body fluid showed for the first time that anthozoan granulocytes are able to form plaque of lysis. We have partitioned the total population of free cells into three distinct discrete bands by discontinuous Percoll gradient, and we have identified six small different types cells: morular granulocytes; cells with large or small peripherical granules, granulocytes with irregular shape containing blue and red granules, cells showing one fine red granule of uniform size and, finally, cells with elongated shape and small dispersed granules. Cell lysate of each cellular band resulted cytolytic toward different erythrocytes types. SDS page analysis of the lysate cell fraction showed a predominant of 20 kDa that corresponds to the weight of the cytolytic equinatoxin. The nature of equinatoxins-related activity was demonstrated by inhibition experiments using bovine sphingomyelin.

Key Words: cytolysin; *Actinia equina*; granulocytes; plaque of lysis; sphingomyelin

Introduction

Cnidarians are the rich source of bioactive molecules like antimicrobial peptides, cytolytic proteins and neurotoxins. Sea anemones (Actininaria, Cnidaria) are benthic sessile species that depend on their toxic venom for survival, providing for defense and predation.

Among last decades three classes of toxins, 20 kDa pore-forming cytolytic hemolysins (Kem, 1988; Anderluh and Macek, 2002, Bakrac and Anderluh, 2010), 3-5 kDa sodium channel toxins (Kem, 1988; Norton, 1991) and 3.5 - 6.5 kDa potassium channel toxins (Castaneda *et al*., 1995; Schweitz *et al*., 1995; Cotton *et al*., 1997; Gendeh *et al*., 1997; Diochot *et al*., 1998; Minagawa *et al*., 1998), have been isolated and well characterized from a great number of sea anemones.

The strongest *Actinia equina* cytolysins, equinatoxins (EqII), a pore forming protein of the actinoporin family of approximately 20 kDa (Shon *et al*., 2008; Kristan et al., 2009; Bakrac and Anderluh, 2010) have been extensively studied for their chemical and pharmacological properties. It exhibits a wide range of pharmacological activities, including platelet aggregation (Teng *et al*., 1988), cardiotoxicity (Sket *et al*., 1974), cytotoxicity (Batista *et al*., 1990) and an ability to cause pulmonary edema (Lafranconi *et al*., 1984). At a cellular level, its most important outcome is the formation of pores in lipid membranes thank to cytolytic and cytotoxic effects on different types of cells.

Cytolysins may have several isoforms and in *Actinia equina* five sequences of equinatoxins have been found to show a multigenic family (Anderluh *et al.,* 1999).

Regarding EqII, protein and sequence analysis (Simpson *et al*., 1990; Belmonte *et al*., 1994; Anderluh *et al*., 1996), pores formation (Mavek et al., 1994; Bonev *et al*., 2003), crystallographic and solution structure resolution (Zhang *et al*., 2000; Athanasiadis *et al*., 2001; Hinds *et al*, 2002) have been investigated.

Despite these studies at the molecular and structural level, is not yet known if other cells, in addition to the nematocysts, contain or release cytolysins, what is their localization and characterization.

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As others invertebrates, *A. equina* possess various morphological categories of amebocytes, such as phagocytes and non granular type (Hutton and Smith, 1996).

Research on cnidarian venom and toxins focused on the nematocysts, recognized as the venomous apparatus in sea anemones and jellyfish. It is widely accepted that the venom of cnidarians is produced in the nematocytes and is injected via the nematocysts upon encounter (Kass-Simon and Scappaticci, 2002). Some studies have shown that the nematocyst-derived fractions were often less toxic than whole tentacle extracts, suggesting the possibility that toxins may also reside in non-nematocyst compartments (Xiao *et al*., 2009).

The origin of neurotoxin and cytolytic substances remained unanswered despite the knowledge on their storage in nematocysts. Thus, for very few toxins has been shown that the origin of formation is the nematocysts (Lotan *et al*., 1996) and it was not known the cell compartment involved in the toxins production.

Moran *et al.,* (2009; 2013) reported that toxins are held also in ectodermal gland cells. In *Anemonia viridis* neurotoxins are associated with both nematocytes and ectodermal gland cells. Honma *et al.* (2008) described gigantoxins derived from unknown organelles other than nematocysts.

The aim of this research is to characterize *Actinia equina* body fluid percoled cell types and their cytotoxic activity, the presence of equinatoxin or other cytolytic molecules and the ability to lyse foreign cells.

Materials and methods

Collection of the Sea Anemone Actinia equina

The sea anemone *A. equina* (Linnaeus, 1758) was collected from a typical rocky shore of the intertidal zone of Capo Gallo coast (Palermo Italy). Specimens were maintained and powered in a closed-circuit aquarium at 20 °C with aerated sea water (150 L aquaria) and fed every second day with a marine invertebrate filter feeding diet (Kent Marine Inc. WI USA) until experimental use.

Cell extraction

Free cells were extracted from the body fluid percolate and mesenteric filaments by making an incision into the center of the pedal disc and excising the filaments into a sterile 15-ml plastic centrifuge tube (Hutton and Smirh, 1996). The filaments and liquid of internal cavity were mixed with Free Sea Water FSW (NaCl 0.5 M, KCl 8 mM, Na_2SO_4 30 mM, $NaHCO_3$ 2mM) with 10 mM EDTA (ethylendiamminotetraacetic acid) as an anticoagulant and vigorously pipetted to dissociate the cell. Free cell were separated from internal liquid-FSW EDTA by centrifuging (800×g) for 10 min at 4 °C, immediately suspended in ice cold FSW-EDTA and counted in a Neubauer chamber. After observation under a light microscope material was immediately used or stored at −20 °C.

Preparation of Percoll gradient

According to Cammarata *et al*. (1993), a 75.0, 55.0, 37.5 % discontinuous gradient of Percoll (Pharmacia, Uppsala, Sweden) in FSW with 10 mM EDTA and 10 mM cysteine, was formed in 6 ml round-bottom polyethylene tubes (du Pont de Nemours & Co. Inc. Instrument Product, Newton, CT, USA). Briefly, freshly collected cell suspension (approximately 4 ml of $6x10^7$/ml) diluted with FSW-EDTA was spun through a discontinuous gradient of equilibrated Percoll. The tube was centrifuged in a swing-out rotor (850xg, 30 min, 4 °C). Bands of cells were gently removed by aspiration from the gradients and washed twice before suspension in FSW. For microscopy observations, the cells were removed with a pipette from the gradients and washed twice in FSW before being deposited on a glass slide. Also each cellular band was removed gently and, after being washed with FSW, was employed for the lysis plaques assay.

Plaque-forming cell assay (PFC)

A PFC assay has been originally described by Cunningham and Szenberg (1968) for the human B cell/sheep red blood cells and subsequently modified for invertebrate blood cells (Parrinello *et al.,* 1996; Cammarata *et al,* 1997; Ballarin *et al.*, 2008). Fifty µl of hemocytes suspension ($1x10^6$/ml) in Marine Solution (MS:12 mM $CaCl_2$, 11 mM KCl, 26 mM $MgCl_2$, 45 mM Tris, 38 mM HCl, 0.45 M NaCl, pH 7.4) are mixed with 10 µl of suspension of 5 % rabbit erythrocytes in MS. The reaction mixture is rapidly layered into the slide chamber by capillary action. The chamber has been manufactured by placing three thin strips of double-stick tape placed between the borders and in the center of a coverslip and another glass coverslip is then suspended onto the three pieces of tape forming a double chamber. Each slide chamber can accommodate just under 0.1 ml on either side of the tape (0.2 ml per slide). After 15 min of incubation at 20 °C the cell mixture was examined under a phase contrast microscope. The control was prepared with 10 µl of erythrocytes and 50 µl of MS.

Cell staining

For the cells characterization 100 µl of cell suspension in FSW-EDTA containing cysteine were placed on a glass slide treated with Poly-L-Lysine (Sigma Diagnostics Inc.) and left for 30 min at room temperature. The cells were fixed with Lavdowsky fixative (2.5 ml of 37 % formaldehyde, 1 ml acetic acid, 12.5 ml 96 % ethanol, 10 ml distilled water) for 30 minutes. After washing with PBS (0.1 M NaCl; 0.02 M KCl; KH_2PO_4 0.01 M; Na_2HPO_4 0.06 M) cells suspension was treated with Toluidine blu stain 0.1 % in PBS. After two times washing with PBS, the slide was closed with a solution of PBS and 20 % glycerol and observed under a microscope.

Cell cytotoxic assay

The rabbit erythrocytes (RBC) obtained by Istituto Zooprofilattico della Sicilia in Alsever solution (0.42 % NaCl; 0.08 % sodium citrate dihydrate, citric acid monohydrate 0.045 %, 2.05 % D-glucose pH 7.2) were washed three times with PBSE (KH_2PO_4 6mM; Na_2HPO_4 0.11 M; NaCl 30 mM; pH 7.4) and centrifuged at 1800 rpm for 10 min at 4 °C. The supernatant was removed by gentle aspiration and the above process was repeated two times. The

erythrocytes were finally resuspended in TBS gel (Tris-HCl 50 mM; NaCl 0.15 M; pH 7.4, gelatin 0.1 %) to make a 1 % final concentration.To perform cytotoxic assay 500 µl of hemocyte suspensions ($1x10^6$) unfractionated cells in MS or 500 µl of cellular band lysate was mixed with an equal volume of freshly prepared Sheep, Bovine, Pig or Rabbit erythrocyte suspensions ($8x10^6$ cells) in MS. Hemocyte counts were determined in the final volume of the reaction mixture. The mixture was incubated with continuous and moderate shaking at 25 °C for 1 h. At the end of the incubation, the supernatant was separated and the amount of the released hemoglobin (Hb) was estimated by reading the absorbance at 541 nm in a spectrophotometer. The degree of hemolysis was determined according to the equation:

$$\text{Percent hemolysis} = \frac{\text{measured release - spontaneous release}}{\text{complete release - spontaneous release}} \times 100$$

Complete hemolysis was obtained by preparing an erythrocyte suspension in distilled water. Control erythrocyte suspensions were also prepared in the same medium and incubated as reaction mixtures: spontaneous hemoglobin release never exceeded 5 % of the total release. For each experiment three samples were assayed.

Cell lysate from the Percoll cell bands

Each cell fraction recovered from the Percoll gradient was used to carry out cell release and extracts for rabbit erythrocytes cytotoxic assay. To get the released cell product, the cell fractions ($10x10^6$ cells) were removed from Percoll gradient, washed in MS and left in this solution (1 ml) for one hour. Then the suspension taken out was centrifuged at 800x*g*, 4 °C for 10 min and the supernatant was and used for the lysis assay. The cell from each fraction ($1.2x10^6$ cells) were washed and resuspended in TBS and placed in polycarbonate tubes to be sonicated at 4 °C for 60 s (Branson, model B15, Danbury, CT, USA). The cell lysate was centrifuged at 27,000 g for 20 min at 4 °C and the resulting hemocyte lysate supernatant was used for the assay. Sphingomyelin (Sigma-Chemicals) was dissolved in MS to obtain 0.25 and 250 ng/ml concentrations in reaction mixture. Due to insolubility stock solution Sphingomyelin was briefly sonicated (Vibra cell - sonics & materials, Inc. Dambury).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE)

Lysed and released cell products were analyzed by 10 % SDS-PAGE according to the method of Laemmli (1970) under reducing conditions (5 % mercaptoethanol). After electrophoresis, protein bands were stained with Coomassie Brilliant Blue R250 (Sigma). Relative molecular weights of the protein bands were determined by using molecular weight markers: albumin, bovine serum (66.0 kDa), ovalbumin (45.0 kDa), glyceraldehyde-3- phosphate dehydrogenase (36.0 kDa), carbonic anhydrase (29.0 kDa), trypsinogen (24.0 kDa), and alpha lactalbumin (14.2 kDa).

Results

Separation on a Percoll discontinuous density gradient and characterization of A. equina *percoled body fluid cells*

The percoled body fluid of *A. equina* cell suspension was obtained and separated into the Percoll gradient. The total population of hemocytes was separated into three distinct discrete bands (B1-B3). The cells removed from density gradient separated bands were examined and identified for their morphology. Differential count of the hemocytes from each set was performed (at least 200 cells/slide).

Six small types of different cells have been identified, as reported in Figure 1: granulocytes (A) that assume a morular appearance, considered as globular cells with a variable vacuoles stained with Toluidine blue 0.1 % in PBS; B type cells have a globular shape and contain a variable number of large

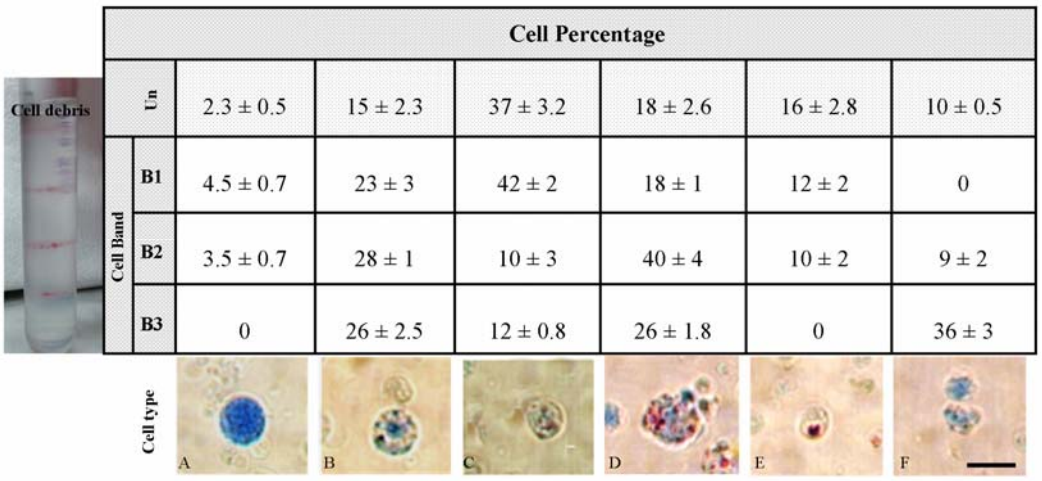

Fig. 1 Characterization of *A. equina* cell types present in percoled body fluid separated by Percoll gradient. Cell differential distribution (%) in bands obtained from a discontinuous Percoll density gradient (B1, B2, B3) and in the unfractionated cells (UN). Identification of cell types (A-F) was carried out with Toluidine Blue 0.1 % in PBS stain. Bar = 10 µm.

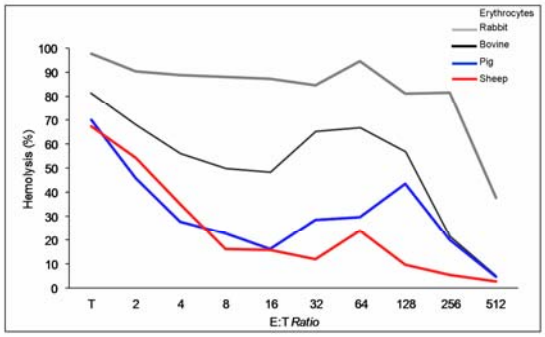

Fig. 2 *A. equina* cells cytotoxic activity against mammals erythrocytes. Typical lytic activity of *A. equina* cell assayed in vitro against different erythrocytes at various effecter/target ratios. Cell lysates are serially diluted twofold and incubated with an equal volume of 1 % rabbit erythrocyte suspension for 1 h at 37 °C. After centrifugation the absorbance of the supernatants was determined at 545 nm. The value were at least the mean of three experiments, each performed in duplicate.

large granules distributed at the periphery of cells; C type cells are distinguished by a granular inclusions and a blue less staining than in other classes; D cells are known as granular cells with irregular shape that contain blue and red granules with variable dimensions; cells of category E contain in their cytoplasm one fine red granule of uniform size; F type cells with very large nuclei appear with round or elongated shape and small granules dispersed in the peripheral regions of the cell bodies.

Gradient centrifugation allows separating cell debris in the one band located on the top of the Percoll tube (Fig. 1) where no whole cell has been identified through microscopic observation.

As indicated in Figure 1, B1 band mainly contained cells of B and C category, respectively 23 % and 42 % and, to a lesser extent, granulocytes (D) and round small cells with a red nucleus (E). B2 band consisted primarily of ~40 % granular cells (D), ~28 % granulocytes, an equal percentage of C and E categories and ~9 % of cells with large nuclei of F category. Instead, this last type of cells is predominant in the B3 followed by the presence of B and D type granulocytes with percentage of 26 %.

Cytotoxic activity at various effector:target cell ratios

Toxic activity of effector cells has been clarified by examining the effector:target cell *ratio*. As reported in Figure 2, the curve of hemolysis *in vitro* to rabbit, sheep, bovine and pig erythrocytes is similar in shape but higher towards rabbit erythrocytes. The highest value of the undiluted sample (T) decreases until the last dilution of 1:512, although it showed an increase at the dilutions between 1:32 and 1:64 for all the used targets.

Plaque of lysis are induced by granulocytes

A plaque-forming assay was carried out with cell populations separated into three bands by Percoll gradient to investigate which cell type was responsible for the cytotoxic activity and plaque formation. For the first time, anthozoan granulocytes that form plaque of lysis are shown (Fig. 3). In three distinct experiments, by using rabbit erythrocytes, plaques constituted 10 - 20 % of the effector cells. On the contrary, plaques were not found when sheep erythrocytes were used as a target (Fig. 3 cnt).

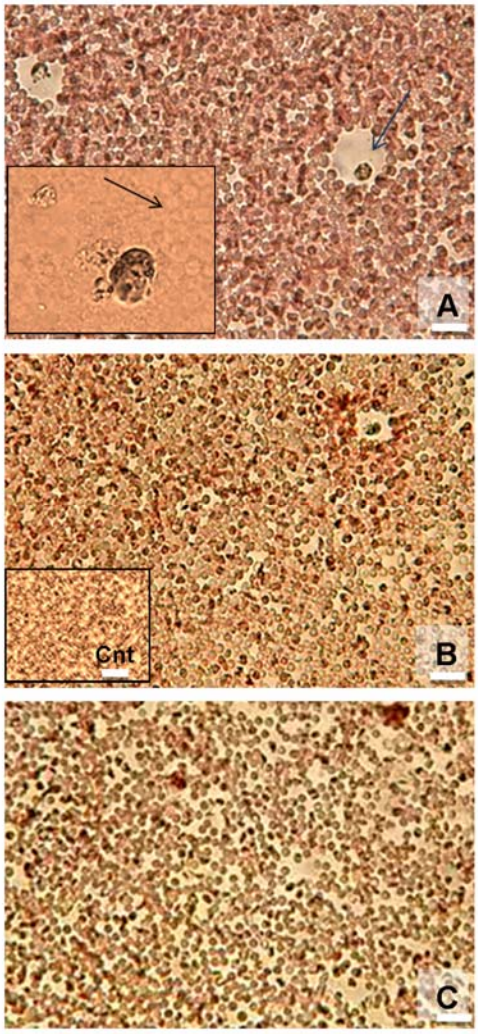

Fig. 3 Plaque lysis assay of cells from separated bands from a Percoll gradient**.** Plaque of lysis of *A. equina* cells fractionated in B1, B2 and B3 bands after separation on Percoll density gradient were observed by phase-contrast microscope. Figure 3A shows a plaque of lysis formed by cells isolated from the band B1. Two typical plaques of lysis against rabbit erythrocytes with the effector cell at the center of the plate are shown. Arrowed indicate the ghosts of erythrocytes. Bar = 20 µm. Inset A is a magnification of the cell (Type D) responsible for cytotoxicity. Bar = 60 µm. Figure 3B shows a plaque of lysis from the band B2 cell. Bar = 20 µm. Cnt: monolayer of rabbit erythrocytes. Bar = 20 µm. In Figure 3C small plaques from B3 are shown.

Plaques were observed when cells present in B1 and B2 fraction were placed in contact with the erythrocyte suspension in a Cunningham - Szenberg chamber (Figs 3A, B), in which a clear granulocyte is present at the center as an effector releasing cell. In the band that contains cells debris the lysis process is very fast and takes place immediately after contact between the cells and the erythrocytes used. Cells of the B3 (Fig. 3C) not showed plaques of lysis although in the bands lysate cytotoxicity has indicated a moderate lytic activity. The addition of sphingomyelin at 250 and 25 µg/ml inhibited the plaque formation, whereas there was no effect on cell or erythrocyte suspensions used as controls.

Cell bands are cytolytic towards various erythrocyte targets and lysis is inhibited by sphingomyelin

A. equina cells lysate was assayed for its hemolytic activity (CLC) using the rabbit erythrocytes. All the data were obtained using aliquots of the same cell population bands lysate. The results were reported in Figure 4A. The samples caused 75 % and 88 % hemolysis respectively in band of cells debris and in the cell fraction of B1. Cell component from the density Percoll gradient of bands 2 and 3, B2 and B3, show a 65 % and 57 % of CLC, respectively. Percentage of CLC is clearly higher in the unfractionated samples (94 %). With the aim of examining the cytolytic mechanism of an enriched population of effector cells, the interaction between erythrocytes membrane lipids and lysins was examined by inhibition experiments. Rabbit erythrocytes have been utilized for the values of the hemolysis *ratio* where the higher respect to another target. As shown in Figure 4B, sphingomyelin inhibits the cytotoxic activity of unfractionated, band1 and 2 cells at 250 µg/ml and at various E/T ratios. Percentage of *Actinia equina* cell mortality is always less than 4 % in the presence of sphingomyelin. The Figure 5 reported the SDS page stained with Coomassie Brilliant blue that showed a pattern of total protein in the lysate cell fraction. Many bands were recorded with different molecular weights ranging from 66 to 14.2 kDa. The predominant band is the one with an apparent molecular weight of 20 kDa that corresponds to the equinatoxin MW, a more intense band is visible in B1 and B2 and in unfractionated sample.

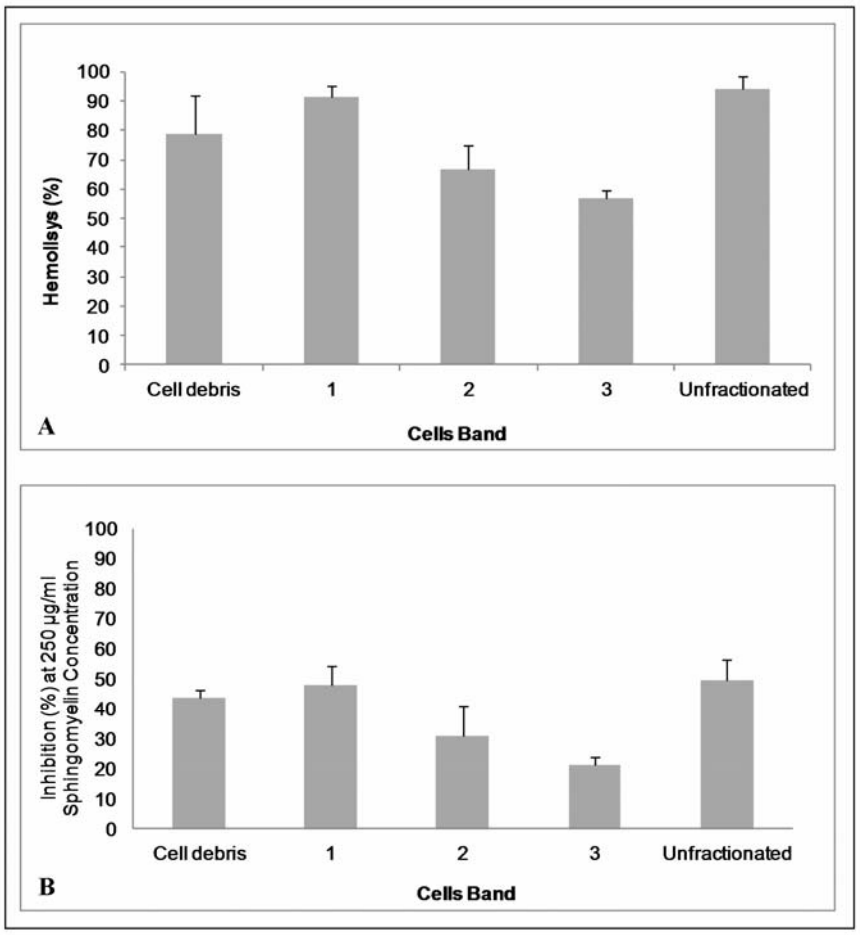

Fig. 4 Cell lysate cytotoxicity from a Percoll gradient cell band and sphingomyelin inhibition. A) Percentage of hemolysis towards rabbit erythrocytes of *A. equina* cell lysate from bands B1, B2, B3, unfractionated cell and debris. B) Inhibitory effect of sphingomyelin on cytotoxicity towards RE at the concentration of 250 µg/ml. Vertical bars represented the mean ± SE (N = 3). Significant differences across control were indicated with an asterisk at $p < 0.05$ and two at $p < 0.01$.

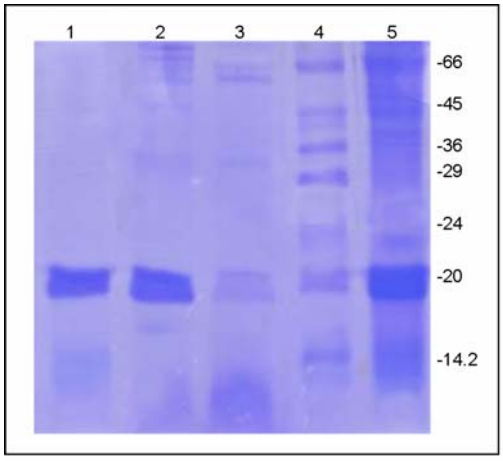

Fig. 5 Electrophoretic analysis of *A. equina* cell lysates. SDS-PAGE gel of *A. equina* cell lysate from B1, B2 and B3 bands obtained from Percoll density gradient stained with Coomassie blue. Lane 1 - 3: electrophoretical profile of B1, B2 and B3 cell bands. Lane 4: Protein molecular weight standards. Lane 5: Unfractionated cell lysate.

Discussion

Anthozoans are important modulators of marine habitats in benthic communities. From *A. equina*, one of the conspicuous species of intertidal rocky shore areas, have been isolated many bioactive molecules (Frazão *et al*., 2012) but few is known about source of these.

In this work morphology characterization of *A. equina* cell from percolated animal body fluid after Percoll gradient density separation has been carried out. Previous histological studies indicated Anthozoa as very simply organized with a body built up of sheets of tissue, above the mesoglea, and on whose surface is present an epithelium (Gadelha *et al*., 2012). Here, six cells categories distributed into three cell bands have been characterized. For the first time we showed that *A. equina* cells able to form plaque of lysis versus sheep erythrocytes. Cytotoxic activity was different for the different cell bands depending from the cell types composition. Cells from debris band were not recognized even though there is a strong cytotoxic activity, probably due to lytic factors rapidly released after the contact with the targets. Most likely the cell responsible for lytic activity are the granulocytes in B1 and B2 as seen from low magnification of a cell of B1 band regardless of the percentage of cells calculated for each fraction (Fig. 3A). It is not excluded combination of lytic factor present in the sample to determine the cytotoxic results. The component capable of mediating the cytotoxic response has been investigated analyzing supernatant obtained by cell release or cell lysate.

SDS PAGE analysis of cell lysate component showed similar electrophoretic profile. The predominant band is the one with a molecular weight of 20 kDa, molecular mass characteristic of numerous toxins of type II inhibited by sphingomyelin. These cytolysins are also called Actinoporins due to their ability to hold the membrane phospholipids domains of the host organism, oligomerizing and forming cation selective pores (Kem, 1988). They belong to the unique family of the α-pore-forming toxins (PFTs) (Monastyrnaya *et al*., 2010).

The most representative of cytolytic anthozoan Actinoporins is Equinatoxin (Macek and Lebez, 1988; Anderluh and Macek, 2002), a mixture of five isoforms of which Equinotoxin II is the most abundant one, exclusively found in sea anemones (Anderluh *et al.,* 2009).

Studies about the cytotoxicity activity of living sea anemones or isolated toxins showed that 20 kDa cytolysins are able to destroying the tissues of not symbiotic fishes (Anderluh and Macek, 2002).

One of the hallmarks of Actinoporins is their sphingomyelin specificity, as they efficiently make pores in lipid membranes containing this lipid (Kristan *et al*., 2009). Therefore, here we wanted to assess the interaction between erythrocytes membrane lipids and lysins by inhibition experiments carried out using rabbit erythrocytes. Sphingomyelin inhibits the cytotoxic activity at 25 and 250 µg/ml and at various E/T ratios particularly in band 2.

In higher metazoans the defense responses are mainly based on hemocyte types that release humoral factors (Iwanaga and Lee, 2005; Loker *et al*., 2004) or display cell-linked activities (Parrinello, 1996; Parrinello *et al.,* 2003). Cellular recognition has been attributed to proteins that are located on the cell surface. The ability of cnidarians to distinguish between self and non-self has previously been shown to occur in anthozoans and hydrozoans (Rinkevich 2004; Bosch, 2008). The anti-erythrocyte cytotoxic activity examined in this paper resides in different cell bands, able to recognize erythrocyte target and appear dependent from Equinatoxin presence.

Many studies focused interest to the employment of cytolysins as model proteins to study protein-lipid membrane interaction (Anderluh and Macek, 2002) and to study the eradication of tumour cells and parasites (Tejuca *et al*., 2009), cardio-stimulating, dermatonecrotic and antihistamine properties (Klyshko *et al*., 2004). Equinatoxin II has been also been studied as an alternative permeabilizing agent that lyses the limiting membrane of *Plasmodium falciparum* infected and uninfected RBCs (Jackson *et al*., 2007).

Here we have attempted a characterization of *A. equina* cells that evidently contain and release bioactive molecules. Thus, nematocysts are not the only structure capable of releasing toxic substances. It would like to pursue studies to give more description of cell-target interaction, and to investigate the biological properties of the released cytotoxic molecules.

Acknowledgements

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