Arylsulfatase in coelomocytes of *Holothuria polii*

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Summary. Holothuria polii coelomocytes possess arylsulfatase enzymes. Two pH optima were found for arylsulfatase activity in cell lysate preparations, one at pH 5.0 and the other at pH 5.8. Both increased after injection of zymosan particles or formalinized sheep red blood cells (fSR-BC), indicating an active role of the enzymes during phagocytosis of particulate substances.

Under a light microscope, the acid hydrolase arylsulfatase were localized in the granules of spherula cells, and therefore considered lysosomal in nature.

Key words. Holothuria polii; coelomocytes; lysosomal markers; arylsulfatase.

Arylsulfatases are lysosomal hydrolases that catalyze the hydrolysis sulfate bound by a variety of synthetic substrates including p-nitrocatechol-sulfate[2]. In mammalians, biochemical evidence indicates that mast-cells and many other cells contain large amounts of arylsulfatase[3]. This type of lysosomal enzyme could also be cytochemically localized in basophils[4]. Lysosomal enzymes are known to play a role in granulocyte functions including inflammation[5]. The latter is defined as a protective response against many injurious agents always present in the environment[6]. Some features of this mechanism are present in invertebrates[7–9] where, as demonstrated in molluscs[10], lysosomal enzymes play a central role. In *Holothuria polii* (Echinodermata), the inflammatory response occurs after stimulation with particulate antigens. It includes: recruitment of a large number of coelomocytes, phagocytosis by amebocytes, formation of a complex active structure, the brown body, through which the phagocytic residual products are cleared[11] and, finally, engagement of reticulo-endothelial-like organs[12].

Very little is know about molecular events accompanying the inflammatory response in *H. polii*. An enhancement of the hemolytic activity was demonstrated by Canicattí and Parrinello[13] after injection of formalinized sheep erythrocytes (fSRBC). The rosette formation of circulating coelomocytes after fSRBC injection is most likely induced by hemolysins which could represent the recognition factors mediating the first step of phagocytosis in *H. polii*[11].

No data are available on the role of lysosomal enzymes during holothuroid inflammation, and since arylsulfatase could represent one typical marker, studies were designed to explore whether coelomocytes possess arylsulfatase and, if so, whether the enzyme can be cytochemically localized in one or several of the circulating coelomocytes in *H. polii*.

Materials and methods. The coelomic fluid coelomocytes were obtained by longitudinal incision of the wide coelomic cavity of *H. polii*. The collected coelomic fluid was centrifuged at 400 × g for 15 min at 4 °C. The pelleted coelomocytes were washed in 0.1 M EDTA, 0.3 M Hepes in 0.5 M NaCl (Hepes-NaCl) and resuspended in 2 ml of the same medium. Cell counts were then performed in a Thomas' chamber.

Suspensions of 1.4×10^6 cell/ml were then prepared, pelleted by centrifugation, resuspended in 1 ml of distilled water and subjected to sonication in a Branson Sonifer model 15B at 50% duty cicle at 0 °C. When complete lysis was obtained (4 min), the sonicate was centrifuged for 30 min at 12,000 × g. The supernatant was separated and used for biochemical determination of arylsulfatase. The latter were also biochemically evaluated in PBS, zymosan and fSRBC-injected animals to demonstrate an eventual increase of their content.

Arylsulfatase determinations were carried out according to Franklin et al.[14]. 20 µl of coelomocyte lysate (1.4×10^6 cell/ml) were mixed with 80 µl of 6.2 mM p-nitrocatechol-sulfate (Sigma) in 0.2 M acetate buffer solutions at different pHs (4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 5.2, 5.4, 5.6, 5.8 and 6.0). Incubation took place in a water bath at 37 °C for 1 h and was terminated by the addition of 100 µl of 5 M NaOH. The orange color that developed was spectrophotometrically read at 515 nm. Inactivation of the enzyme activity was obtained by preincubating the 20 µl coelomocyte lysate samples at 56 °C or by adding 80 µl of 12.5 mM sodium sulfate (Na_2SO_4) or sodium sulfite (Na_2SO_3).

The arylsulfatase activity on *H. polii* coelomocytes was localized by using Goldfischer's medium containing p-nitrocatechol-sulfate as substrate[15]. The smears of coelomocytes were air-dried, fixed for 30 min in 1.5% gluteraldehyde in 0.1 M sodium cocodylate buffer (pH 7.4) containing 1% sucrose, washed three times in acetate buffer pH 5.7 and then incubated in a darkplace at 30 °C for 2 h with Goldfischer's medium. After incubation, the cells were washed five times for 5 min in acetate buffer pH 5 supplemented with 7% sucrose. The reaction product was converted by incubation in 2% ammonium sulfide in acetate buffer pH 5.4 for 10 min and after several washes. examined under a light microscope. Control preparations were incubated without substrate or heated at 90 °C for 10 min before incubation.

Sheep erythrocytes (SRBC) and zymosan (purified cell walls from *Saccharomyces cerevisiae*) (Sigma) were used to elicit phagocytosis in the *H. polii* coelomocytes. After washing with phosphate buffer saline (PBS) pH 7.4, a suspension of SRBC was formalinized according to Csizmas[16]. The formalinized SRBC (fSRBC) were extensively washed in PBS and resuspended in the same buffer at a concentration of 6×10^8 cell/ml. Solutions of 10 mg/ml of zymosan were prepared in PBS pH 7.4. A single dose of 0.15–0.20 ml of these antigen suspensions was injected into the coelomic cavity of five animals. Controls were carried out by injecting 0.15–0.20 ml PBS. Uninjected animals were also examined.

Results and discussion. The *H. polii* coelomocyte lysate possesses arylsulfatase activity. As shown in figure 1, when the enzyme activity was tested at different pH values, two major peaks were detectable. The lower activity level was registered at pH 5.0, and the higher one at pH 5.8. It is evident from these data that there are at least two different types of arylsulfatase in *H. polii* coelomocytes. However, they appear to differ from analogous enzymes present in rat mast cells, named A and B by Lynch et al.[17], whose pH optima are 4.2 and 6.2, respectively.

The *H. polii* arylsulfatase are inactivated by heating at 56 °C. Moreover, the preincubation of the coelomocyte lysate samples with 12.5 mM Na_2SO_3 or Na_2SO_4 inhibited catalysis of the p-nitrocatechol-sulfate used as substrate. Sulfate and sulfite ions are competitive inhibitors of the relatively soluble arylsulfatase A and B[2]. Coelomocytes seem therefore to possess both A and B-like arylsulfatase but with different pH optima.

Since the levels of several lysosomal enzymes can be experimentally increased in hemocytes of molluscs during inflammation[10], it was useful to gather information about the pos-

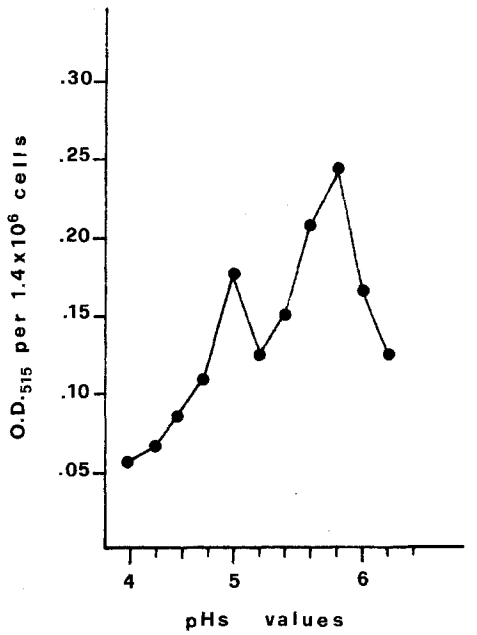

Figure 1. Arylsulfatase activity of 1.4×10^6 cell/ml of *Holothuria polii* at different pH values.

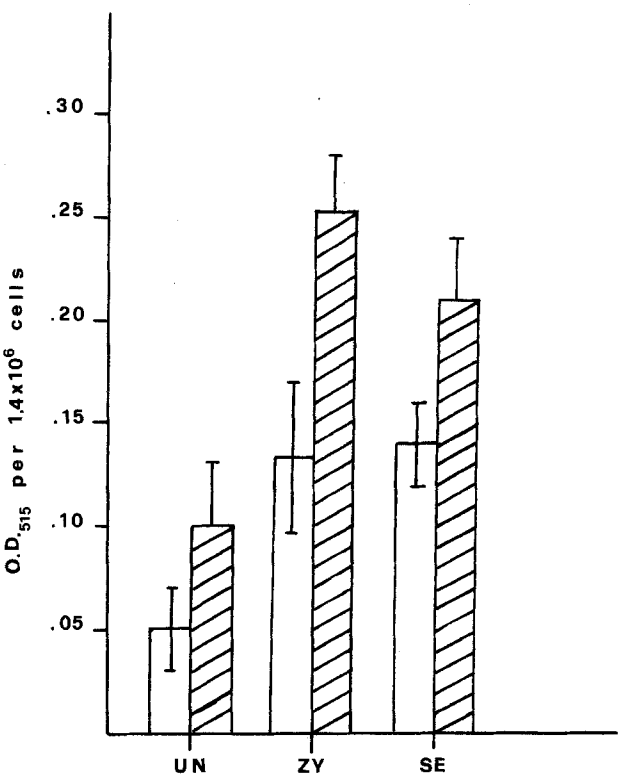

Figure 2. Mean arylsulfatase activity of 1.4×10^6 cell/ml lysate samples of five undisturbed (UN), zymosan (Zy) and fSRBC (SE) injected *Holothuria polii* specimens. In all samples, the enzyme activity was tested at pH 5.0 (■■) and pH 5.8 (■■). Vertical bars = SE.

sibility to elevate the arylsulfatase levels in *H. polii* coelomocytes during phagocytic stimuli. Figure 2 shows an evident increase, compared to non-injected controls, in the amount of both, pH 5.0 and 5.8 arylsulfatase activities associated with the cells of *H. polii* having been injected with zymosan or fSRBC. These results indicate a probable active role of the enzymes during the sea cucumber response to injection of particulate substances. As previously demonstrated [11], intense phagocytosis occurred 24 h after injection of fSRBC in the coelomic cavity of *H. polii*. Phagocytosis was also stimulated by zymosan (Canicatti, unpublished). However, whether or not such substances are subsequently digested by lysosomal enzymes has not yet been determined. The enhancement of arylsulfatase activity which could be considered a marker of lysosomal activity, favors the idea of its possible role in the degradation of endocytosed substances. Hypersynthesis of lysosomal enzymes was demonstrated in molluscs [10]. Cell lysosomal lipase [18], aminopeptidase [19], acid phosphatase [20] can be experimentally elevated following bacteria injection into the body of certain molluscan species. In these animals, an enzyme release was also observed in the serum. In *H. polii* it was noted that normal and 24-h injected cell-free coelomic fluids did not contain arylsulfatase activities. This is interpreted in the way that no release of this enzyme occurs, even in post-challenged animals.

Cytochemical evidence for the presence of arylsulfatase in *H. polii* coelomocytes was obtained using Goldfischer's method [15]. After incubation of the cells with Goldfischer's medium, the dense reaction product, lead sulfite, was localized in the granules of spherula cells (fig. 3). Heated 90 °C and control smears did not show any positive reaction. These results indicate that granules of spherula cells have a lysosomal origin. Surprisingly, the phagocytic amebocytes were not positive. This fact could depend on negligible reaction products not visible under a light microscope. On the other hand, it is possible that amebocytes contain arylsulfatase in a latent form. Histochemical latency has been demonstrated in mammalian neutrophils [21] and eosinophils [22]. Ultrastructural localization studies are in progress to clarify these problems.

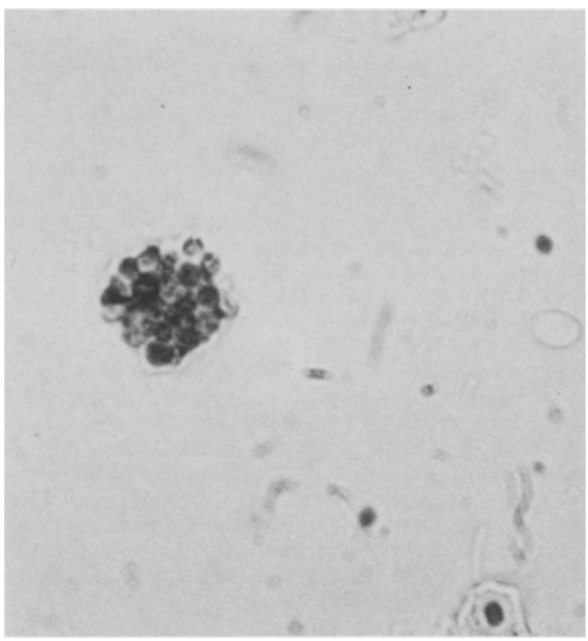

Figure 3. Photomicrograph of *Holothuria polii* spherula cell containing dark-staining, arylsulfatase-positive granules. No counterstaining. Magnification × 1000.

Moreover, since spherula cells are massively present in the brown bodies[11], it will be useful to study the role of the arylsulfatase in these coelomic cavity structures formed following antigenic stimuli.

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In vitro proliferation of human large granular lymphocytes with *v-raf/v-myc* recombinant retrovirus[1]

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Summary. The effect of infection with a retrovirus carrying *v-raf/v-myc* oncogenes (J2 virus) on the in vitro proliferation of human large granular lymphocytes (LGL) was investigated. LGL infected with J2 virus (J2LGL), unlike uninfected cells, grew with a proliferation peak eight days after infection. Such cells retained the morphology and functional properties typical of LGL. Furthermore, 5% of J2LGL produced virus the day after infection, whereas non-virus production was detectable five days later. These data indicate that J2 virus provides a transient mitogenic signal for LGL.
Key words. Large granular lymphocytes; retrovirus; infection; cell proliferation.

Natural killer (NK) cells have been described as a subpopulation of lymphoid cells with spontaneous cytotoxic activity versus a variety of target cells, including tumor cells, virus-infected cells and some normal cells[3]. Most of the NK activity has been recently associated with a subpopulation of $CD3^-$ lymphocytes, morphologically distinct from typical lymphocytes, which are known as large granular lymphocytes (LGL) because of their large size and the presence of characteristic azurophilic granules in their cytoplasm[4]. $CD3^-$ LGL, representing about 5% of mononuclear cells in the blood or spleen, express some cell surface markers characteristic of T cells, but also some markers associated with monocytes or granulocytes[4,5]. In addition, LGL can be grown in vitro in the presence of interleukin 2 (IL-2)[3,6]. Recently, numerous experimental evidence has shown that oncogenes, besides being involved in neoplastic transformation, also play a central role in cellular proliferation and/or differentiation[7–11]. For example, it has been reported that fresh tissues can be immortalized in vitro by appropriate recombinant retrovirus. Blasi et al.[12] found that in vitro infection of fresh murine bone marrow cells with the recombinant retrovirus J2 carrying *v-raf/v-myc* oncogenes results in selective immortalization of macrophage-like cells. Furthermore, it has been shown that infection of IL-2 or IL-3-dependent cell lines with a recombinant retrovirus, expressing the avian *v-myc* oncogene, abrogates the requirement for growth factors and suppresses *c-myc* expression[13].

In this report, we investigated the effects of infection with J2 virus on in vitro proliferation of human LGL.

Materials and methods. Isolation of LGL. LGL were obtained from buffy coats of blood donations of normal healthy volunteers, as previously described[4,6]. Briefly, mononuclear cells were separated by centrifugation on a Ficoll-Hypaque gradient (Pharmacia Fine Chemical, Uppsala, Sweden) and depleted of monocytes by adherence on a plastic surface. Nonadherent cells were applied onto a nylon-wool column, and eluted cells were then fractionated on a 7-step discontinuous gradient of Percoll (Pharmacia) at concentrations ranging from 40 to 60%[4]. LGL were collected from the low-density fractions (2 and 3 counting from the top). Contaminant T lymphocytes were further removed by rosetting technique with SRBC at 29 °C for 1 h[6]. Cell preparations contained an average of 85–90% LGL, as determined by morphological analysis of Giemsa-stained cytopreparations, and were $>60\%$ $OKM1^+$ and $<5\%$ $OKT3^+$ cells, as determined by flow cytometry.

Culture conditions. Culture medium was RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% human