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# Intestinal granular cells of a cartilaginous fish, thornback ray *Raja clavata*: Morphological characterization and expression of different molecules

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ABSTRACT

This investigation aims to fill gaps in our understanding of the intestinal immune cells of elasmobranchs. Whole digestive tracts of fifteen thornback ray *Raja clavata* were provided by a trawl fleet from the Gulf of Asinara (Sardinia, western Mediterranean Sea). Histochemical, immunohistochemical and ultrastructural observations were conducted on the spiral intestine. Three types of granular cells were identified; type I in epithelium, types II and III in lamina propria-submucosa, with each of them containing cytoplasmic granules with different ultrastructural characteristics. Data on size and density of each granular cell type are provided. Immunostaining of intestinal sections showed the reactivity of the granular cells: type I cells were positive for lysozyme, mast cell tryptase and tumor necrosis factor-α based on antibody staining; type III cells were immune-reactive to anti-interleukin 6 antibody, whilst type II cells were negative to all the antibodies used. Comparison of each granular cell type with immune cells of teleosts or mammals and an hypothesis on their nature and function are reported. A potential role for granular cells in intestinal cellular immunity is also discussed with respect to type I and type III cells having similarities to Paneth cells and neutrophils, respectively.

## 1. Introduction

Phylogenetically, fishes are the oldest vertebrate group representing more than one-half of the vertebrates on the planet [1]. Thus understanding the immune system of fish is of great relevance as it provides information on the evolution of immunity in vertebrates [2]. All sharks, skates and rays are cartilaginous fish and, fall into the group called the elasmobranchs. The rays (Rajiformes) form the largest elasmobranch order [3]. Elasmobranchs have a low incidence of disease and their immune cells have been identified as possible sources of novel tumour cell inhibitors [4].

In all vertebrates, the alimentary canal represents one of the main entry points for pathogen invasion of the host body [5]. This canal possesses an effective local immune system due to well-developed chemical and physical barriers, in addition to an efficient mucosal immune system [6–8]. The basic anatomical structure of the elasmobranch gut is similar to that of other vertebrates, with a striking exception being the presence of a spiral intestine which provides an enlarged surface area for digestion and absorption of food by means of spiral folds [9]. The wall of the spiral intestine is composed of mucosa, submucosa, muscularis and serosa layers, but the internal ring is formed only by mucosa and submucosa [9]. Above description of the internal ring met very closely histology of spiral valve of actinopterygians as mentioned in Argyrious et al. [10].

In the present evaluation of *R. clavata* spiral intestine we identified different types of granular cells. Older light microscopical and histochemical studies reported the presence of acidophilic granular cells in the intestinal epithelium of Chondrichthyes as possible Paneth-like cells [11–13]. In mammal intestine, the role of Paneth cells as a first line of defence is well known, particularly for their antibacterial activity relying on lysozyme and defensins [14–16]. Existence of Paneth cells in fish is not confirmed yet.

In teleosts three types of immune cells are very active in the innate immune response: macrophages, neutrophils and mast cells (MCs). Macrophages have emerged as a key cell type across all vertebrate classes and reside in virtually all animal tissues, representing one of the main professional phagocyte populations [17,18]. Macrophages show a plethora of functional roles pertaining to homeostasis and host immune defense and are largely governed by their respective tissue niches and microenvironments [18].

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Both MCs and neutrophils are morphologically, histochemically and functionally similar to their mammalian counterparts (respectively [19] and [20]). MCs are present in all vertebrate classes [21]. Their ancient origins suggest an essential importance in immunity, and indeed they are involved in modulation of the immune system, tissue repair [22,23] and angiogenesis [24]. With reference to cartilaginous fish, there are only two studies on MCs and their histochemical properties in some species of elasmobranchs [25,26] and thus virtually nothing is known about MC ontogenesis [21].

Neutrophils are highly motile phagocytic cells and are crucial for the innate immune response [27–29]. Neutrophils follow numerous signals to reach sites of infection and injury [30] and they are the first immune cells to migrate from the blood and other marginal pools into the focus of inflammation [31]. There are a few records on morphology of neutrophils in blood and spleen of rays [32] and in blood of dogfish [33] and in other elasmobranch species [34,35].

Innate immunity of all vertebrates relies on anti-microbial proteins including lysozyme [36]. In fish lysozyme genes are expressed in cells of myeloid origin [37] and this enzyme has been found in tissues, especially those rich in leucocytes, and secretions [38]. The intercellular communication necessary to mount and orchestrate the immune response is carried out by cytokines, small proteins which are well conserved among the vertebrates [39]. Cytokines can be divided into interferons, interleukins (ILs), tumor necrosis factors (TNFs), colony stimulating factors, and chemokines [40]. All the major cytokine families exist in both bony and cartilaginous fishes [41–44] and play important roles in haematopoiesis, inflammation and adaptive immunity [44].

During recent years considerable study has been made on the immune system of teleosts [7,8,17], whereas little effort has been directed towards immunity in elasmobranchs [45]. Most data on immunity of the elasmobranchs principally refers to lymphoid organs [45,46] and blood cells [32,47]. The lack of knowledge on immune cells in the intestine of elasmobranchs prompted us to carry out this preliminary investigation. Our results are the first to provide direct evidence on the presence of different granular cell types in the spiral intestine of a ray, and on their histochemistry, immunohistochemistry and ultrastructural features.

## 2. Materials and methods

### 2.1. Animals

In February 2017, 15 specimens of the thornback ray *Raja clavata* were caught in the Gulf of Asinara (Sardinia, western Mediterranean Sea), by commercial trawl fishing during a haul at 100–150 m depth. Fish ranging from 1 to 1.5 kg were immediately eviscerated and the whole digestive tract was promptly fixed in 10% neutral buffered formalin while still on board. After landing the samples were transported to the Department of Veterinary Medicine of the University of Sassari, and processed within 24 h post-fixation. The fixed digestive tracts were rinsed in several changes of 4 °C 70% ethanol. Afterward, different parts of the intestine were sliced into small pieces, stored in the same medium and sent to the University of Ferrara for embedding process and light and electron microscopy investigations.

### 2.2. Histology, histochemistry and electron microscopy

The fixed tissues were dehydrated through an alcohol series and then paraffin wax embedded using a Shandon Citadel 2000 Tissue Processor (Shandon, UK). After blocking out, sections (5 μm thick) were stained with either Giemsa, alcian blue 8 GX pH 2.5 and periodic acid Schiff's (AB/PAS), Haematoxylin and Eosin (H&E), AB/H&E and photographed using a Nikon Microscope ECLIPSE 80i (Nikon, Tokyo, Japan).

The numbers and dimensions of the three types of granular cells were evaluated on Giemsa-stained slides at 400X magnification via light microscopy (Nikon Eclipse 80i; Tokyo, Japan), using a computerized image analysis software (Nis Elements AR 3.0). The cell density was determined in twelve tissue areas from each fish (N. areas = 180) and expressed as mean number of each cell type ± standard deviation in 25.000 $\mu m^2$ of tissue (epithelium or lamina propria-submucosa). The major axis (height) and minor axis (width) of all three types of granular cells were also measured (N. cell measured = 170 for each cell type).

For electron microscopy, representative pieces (7 × 7 mm) of spiral intestine of *R. clavata* arrived at our laboratory in 70% ethanol and were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 3 h at 4 °C before being post-fixed in 1% osmium tetroxide in the same buffer for 3 h. The samples were then dehydrated through a graded acetone series before being embedded in epoxy resin (Durcupan™ ACM, Fluka, Sigma-Aldrich, Saint Louis, Mo). Semi-thin sections (*i.e.* 1.5 μm) were cut on a Reichert Om U 2 ultramicrotome (Reichert, Vienna, Austria) using glass knives and then stained with Toluidine Blue. Ultra-thin sections (*i.e.* 90 nm) were stained with a 4% uranyl acetate solution in 50% ethanol and Reynold's lead citrate and examined using a Hitachi H-800 electron microscope (Hitachi Ltd, Tokyo, Japan).

### 2.3. Immunohistochemistry (IHC)

Due to the unavailability of commercial elasmobranch-specific antibodies we chose antibodies that are routinely used for the identification of innate immune cells in mammals and in teleosts.

The following anti-bodies were applied on sections of spiral intestine: inducible-nitric oxide synthase (i-NOS), interleukin-6 (IL6), lysozyme, mast cell tryptase (MC tryptase), tumor necrosis factor-α (TNF-α). This panel of antibodies are direct against pro-inflammatory molecules to investigate the chemical nature of the cytoplasmic granulation of the three types of granular cells.

Different tissue sections were re-hydrated, washed twice in 0.05 M Tris-HCl, 0.15 M NaCl containing 0.1% Triton-X 100 (TBS-T) for 2 × 5 min, and treated with 1% $H_2O_2$ (Sigma-Aldrich, USA) in TBS for 20 min to block the endogenous peroxidase. Afterwards, slides were washed in TBS-T, placed in a humid chamber, and re-covered with 1:20 goat normal serum for 30 min to block non-specific staining. Sections were then incubated with the polyclonal rabbit or monoclonal mouse antibodies (Table 1) for 24 h at room temperature (R.T.). As suggested by antibody manufacturer, for anti-TNF-α and anti-mast cell tryptase, the sections were pre-treated with two microwave cycles (2 × 5 min at

**Table 1**
Antibodies used on sections of spiral intestine of the *Raja clavata*.

| Clonality | Host | Antibody | Source | Code | Working dilution |
|---|---|---|---|---|---|
| Polyclonal | Rabbit | inducible-nitric oxide synthase (i-NOS) | SantaCruz Biotechn., Inc., Santa Cruz, CA, USA | sc-651 | 1:10 |
| Monoclonal | Mouse | interleukin-6 (IL6) | SantaCruz Biotechn., Inc., Santa Cruz, CA, USA | sc-28343 | 1:50 |
| Polyclonal | Rabbit | lysozyme | Dako[a],Glostrup, Denmark | A0099 | 1:100 |
| Monoclonal | Mouse | mast cells tryptase (MC tryptase) | Dako[a],Glostrup, Denmark | M7052 | 1:100 |
| Polyclonal | Rabbit | tumor necrosisfactor-α (TNF-α) | Abcam,Cambridge, UK | ab6671 | 1:200 |

[a] Dako now is a part of Agilent Technologies Solutions, Santa Clara, CA, USA.