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.

Key words: elasmobranch; spiral intestine; lysozyme; cytokines; transmission electron microscopy

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].

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 1-1.5 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 4ºC 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), Hæmatoxylin 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 μm^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 2x5 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 x 5 min at 500 W in 0,01 M citrate buffer, pH 6.0) for the antigen retrieval. Some sections were treated with buffer without antibodies as negative controls.

Slides processed with rabbit polyclonal antibodies were then washed twice in TBS-T for 5 min, and treated with biotinylated goat anti-rabbit immunoglobulins (Vector Lab., USA) diluted 1:200 in TBS for 60 min. The sections incubated with mouse monoclonal antibodies were washed as above, and treated with goat anti-mouse immunoglobulins (Vector Lab., USA) diluted 1:200 in TBS for 60 min. Afterwards, all slides were washed twice in TBS-T for 5 min and treated with the streptavidin-biotin/horseradish-peroxidase complex (Vectastain® ABC Kit, Vector Labs., USA) for 60 min at R.T.. After a brief washing step in TBS, slides were incubated with a freshly prepared 0.04% diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA) solution, in TBS containing 0,1 ml of 3% H_2O_2 for 7-14 min, and counterstained with Mayer's Haematoxylin. Slides were de-hydrated and mounted with Eukitt (Sigma-Aldrich, USA). Sections of mammal tissues (human, swine, rat and mouse) were used as positive controls, and they gave the expected results. The number of granular cells positive to each antibody was evaluated at 40X magnification in ten randomly selected tissue areas from each fish (N. areas = 150) and expressed as percentage (%) of positively stained cells.

3 - Results

Typical of most elasmobranch the intestine of *R. clavata* is subdivided into a short duodenum, a spiral intestine and a narrow colon. The data presented in this study referred to the spiral intestine of *R. clavata* because it is the largest portion of the intestine, internally coiled with a spiral ring.

3.1. Light microscopy

The wall of the spiral intestine is composed of mucosa (epithelium and lamina propria), submucosa, muscularis and serosa layers, but the internal ring is formed only by epithelium and lamina propria-submucosa (Fig. 1a). The intestinal mucosa is lined by a simple columnar epithelium consisting of enterocytes with microvilli, mucous cells, entero-endocrine cells and granular cells at the base of the epithelium in close vicinity to the basement membrane (Fig. 1b). Beneath the epithelium there is the lamina propria. Due to the lack of a muscularis mucosae, lamina propria is not clearly discernible from the submucosa. For this reason, in the whole manuscript the term "lamina propria-submucosa" was adopted to indicate the connective tissues between the epithelium and the muscularis layer. Correlation of light and electron microscope observations combined with the tinctorial properties found in the spiral intestine, led to the identification of three distinct types of granular cell. Density and dimensions (major and minor axes) of each of the three types of granular cell are reported in Table 2.

The type I granular cells are large ovoid cells located at the base of the epithelium (Fig. 1b,c,d) adhering to the basement membrane. These cells were attached to surrounding epithelial cells. Type I granular cells possessed numerous acidophilic and PAS negative fine granules, which occupied the whole cytoplasm. The cytoplasm of these granular cells stained uniformly and intensely with eosin (Fig. 1b).

The type II granular cell**s** are round-oval shaped cells, located in the lamina propria-submucosa (Fig. 1d,e), mainly in the tissue and sometimes inside the vessels. The cytoplasm of type II granular cells was filled with large, round granules (Figs. 1d,e). The granules of this cell type were strongly acidophilic and PAS negative. Degranulation of type II cells was observed (Fig. 1e).

The type III granular cells, were elongated and densely populated the (see Table 2) lamina propria-submucosa (Fig. 1d, e, f,g), either in tissue or inside the capillaries. They possess numerous fine granules weakly acidophilic and PAS negative. The cytoplasm of these cells is whitish to whitish gray (Fig. 1f).

3.2. Electron microscopy

Observations of numerous grids of spiral intestine revealed that type I granular cells were oval in shape, within the depth of the intestinal fold and in close proximity to the basement membrane (Fig. 2a) or were attached to it (Fig. 2b). Type I granular cells were voluminous relative to surrounding cells, irregular in shape with a central or eccentric nucleus and prevalently heterochromatic (Fig. 2c), and contained numerous granules within the cytoplasm (Fig. 2a,c). Granules were spherical or oval in shape and showed a finely granular electron-dense material (Fig. 2c). In addition the granules were

frequently arranged in a scroll pattern (Fig. 2d). Round or ovoid mitochondria were noticed near the nucleus (Fig. 2e). The presence of rough endoplasmic reticulum (RER) was very rare.

The type II granular cells were recognizable in the lamina propria-submucosa either within tissue (Fig. 3a) or inside the capillaries (Fig. 3b). They were ovoid in shape, with an eccentric heterochromatic nucleus (Fig. 3a). Cytoplasm was filled with big, round-oval and, frequently, finely electron-dense granules (Fig. 3b). Few ovoid mitochondria were present and no other cytoplasmic organelles were noticed.

The type III granular cells were the dominant granular cells in the spiral intestine of *R. clavata* (Tab. 2; Fig. 3c). Both the cell and the nucleus were irregular in shape (Fig. 3d). Two types of granules co-occurred in their cytoplasm as large, round electron-dense granules and elongated granules (Fig. 3d,e,f). The latter granules contained longitudinal fibrils that consolidated to form an axial rod-like inclusion (Fig. 3e). The type III cell had several ovoid mitochondria scattered among elongated granules (Fig. 3f) and few RER, no other cytoplasmic organelles were observed. In some instances, type I and type III granular cells were seen opposite each other on either side of the basement membrane of the epithelium (Fig. 2f).

3.3. Immunohistochemistry

Not all the granular cells of the same type were immunoreactive to the antibodies and the immunostaining intensities ranging from weak (light brown) to strong (dark brown). Accordingly, 82% of type I granular cells were positive to anti-lysozyme (Fig. 4a) with a strong staining intensity. Antibody staining in type I cells was also found as follows: anti-TNF-α (85 %; Fig. 4b), and anti-MC tryptase (65 %; Fig. 4c). With regard to the type III granular cells, 44% were weakly immunoreactive to anti-IL6 (Fig. 4d). Type II granular cells were negative to all the antibodies tested (Fig. 4d). The rabbit polyclonal anti-i-NOS was the only antibody that failed to stain any type of granular cell.

4 - Discussion

Elasmobranchs have a complex immune system typical of all jawed vertebrates [48]. Continued investigation of their immune system will be of great help in understanding the origins of specific immunity [41]. The mucosal surfaces of fish, with the intestine being the largest of the body's, constitute the first line of defence for the organism and are therefore highly active sites of immune responses [6]. The mucosal barriers are constituted by epithelial cells, neuroendocrine cells [49,50], mucous cells [51,52], and different types of immune cells [17,53,54]. With regard to latter, MCs

[7,8,55-57] and neutrophils [27,28,30,58] are two types of myeloid-derived cells which have received great attention in recent years.

The immune system cells of the spiral intestine of cartilaginous fish have received only passing interest. Indeed, very few studies are available on elasmobranchs gut innate immunity (see [43]). Ultrastructural analysis is believed to be the most reliable method for identification of fish cell types [59]. Electron microscopical examination of *R. clavata* spiral intestine did indeed identify three cell types in the present study. In addition to a morphological basis, our cell classification also relies upon immunohistochemical staining, both of which are discussed further for each new cell type below.

4.1. Type I granular cells

The type I granular cells encountered in the intestine of *R. clavata* are hereby referred to as epithelial granular cells because they occur only in the epithelium and because of the close relationship with the basement membrane and the other epithelial cells. Similar cells characterized by the same location (i.e. the intestinal epithelium) and by eosinophilic granules in the cytoplasm were first reported in elasmobranchs in the 1930s [11-13]. These authors postulated that they are very similar to mammalian Paneth cells, specialized immune epithelial cells, and have a defensive function. The epithelial granular cells of *R. clavata* express lysozyme and display immunoreactivity to anti-mast cell tryptase and anti- TNF-α. Some of these molecules or their homologues have been found in mammalian Paneth cells. It is well established that Paneth cells act as a first line of defense because they contain lysozyme and defensins, which have antibacterial activity [14-16]. In fish the kidneys have the highest lysozyme levels, followed in descending order by the alimentary tract, liver, spleen, skin mucus and finally gills [60].

Tryptase is highly conserved among vertebrates [61] and is the main type of serine protease stored in MC granules. However little is known regarding tryptase in fish cells, with only one histochemical study available demonstrating the presence of this substance in zebrafish MCs [62]. Van Dussen et al. [63] reported that Paneth cells express the gene of another type of tryptase i.e. tryptase ɣ1.

The type I granular cells also contain TNF-α, a key cytokine involved in pathological and physiological processes [64]. Occurrence of TNF-α has been related to epithelial cell proliferation and inflammatory response [65-67]. Among the functions of TNF-α are the modulation of cytokine production and the regulation of immune cells [68]. TNF-α is produced chiefly by activated macrophages but may be expressed in multiple cell populations of fish, including epithelial cells (e.g.,

epithelial granular cell of *R. clavata*) [68]. With regard to mammals, Paneth cells are also a source of TNF-α as has been documented in humans [69], in mice [70], and in rats [65].

Based on the evidence presented in this paper, it is reasonable to suppose that type I granular cells are involved in the first line defense mechanisms of the ray's spiral intestine. Nevertheless, the preliminary study presented here does not permit the suggestion that type I granular cells of *R. clavata* are the counterparts, or possibly ancestors,of mammalian Paneth cells but it is worthy of noting that there are some important analogies between them, such as location, morphology and expression of lysozyme and TNF-α.

4.2. Type II granular cells

Type II granular cells were found in the lamina propria-submucosa of *R. clavata* spiral intestine. Type II granular cells have morphological similarities with the intestinal MCs of some teleosts, for example *Salmo trutta* [71] and *Silurus glanis* [8]. The similarities referred to ovoid shape of the cell, an eccentric heterochromatic nucleus and several big, round-oval electron-dense cytoplasmic granules. MCs are motile cells [8,20,23,71,72] and are derived from the bone marrow [73] but mature from MC progenitors in peripheral tissues [74]. The intestine is an exceptional organ in that it shows a greater density of cells with MCs capacity than the bone marrow [74].

It is also notable that there are no available studies on the contents of MCs in cartilaginous fish. Conversely, there is an extensive literature dealing with the molecular composition of teleost MC granules which indicates the presence of various phospholipids, acid mucopolysaccharides, alkaline phosphatases, acid phosphatases, arylsulphatase, 5-nucleotidase [75], histamine [7, 76], host defense peptides, which include the piscidins [77-81], lysozyme [82], 5-HT [83-86] and met-enkephalin [87]. In our study type II granular cell**s** failed to react with any of the 5 antibodies used, an unexpected finding as some of the molecules (i.e. MC tryptase, lysozyme) are expressed by MCs of fish. Thus based solely on morphology, it is at present inadvisable to label the type II granular cells as "putative MCs" or "MCs".

4.3. Type III granular cells

Type III cells were the dominant population of granular cells in spiral intestine of *R. clavata.* Type III cells possess morphological features found in the neutrophils of bony fish [53,88]. These similarities referred to the same shape of cells and of their nuclei and the ultrastructural characteristics of the cytoplasmic granules. Specifically, the presence of numerous axial rod-like inclusions within

the granules was the main evidence for supporting a putative neutrophil identity. Of the limited studies published on the structure of elasmobranch granulocytes [32,59] none have dealt with immunohistochemical aspects of these cells. Indeed, Hine and Wain [32], studying the composition and ultrastructure of granulocytes of seven species of ray, stated that "…. the neutrophils and eosinophilic granulocytes were morphologically similar and it appeared that neutrophilic granulocytes develop to eosinophilic granulocytes by increase in granule eosinophilia". Little information was provided by these authors to substantiate this conclusion. However, it is very unlikely that neutrophilic granulocytes can develop to eosinophilic granulocytes. Our results regarding putative neutrophils within the spiral intestine of *R. clavata* do not agree with the inaccurate cell descriptions provided by Hine and Wain [32]. The latter authors reported neutrophilic granulocytes with weakly basophilic granules whilst in intestine of *R. clavata* all the type III cells contain acidophilic granules. Moreover, no evidence of granule transformation from basophilic to eosinophilic nor occurrence of different forms of neutrophils were noticed in our study.

Neutrophils are highly motile phagocytic cells and are the first type of immune cell to migrate to the site of inflammation [27-31]. Teleost neutrophils were demonstrated to have as their mammalian counterpart, a critical role in the resolution of inflammation by discharge of proresolving lipid mediators, which modulate macrophage and neutrophil functions [89]. The gastrointestinal tract of vertebrates is "physiologically" challenged by many pathogens and opportunistic organisms that elicit strong countermeasures from an ontogenetic and phylogenetic point of view [90]. Thus, neutrophils are normally present along the gastrointestinal tract to cope with these physiological challenges [57,91]. Furthermore it is well established that physiological migration of neutrophils into the gastrointestinal lumen occurs [91,92].

Type III granular cells of *R. clavata* were positively stained by anti-IL6 antibody. The classical pro-inflammatory cytokines IL1β and IL6 are present, with multiple paralogues in most fish species [44]. IL6 mediates the innate and adaptive immune responses [93]. IL6 is not synthesized under normal conditions, but is rapidly and transiently up-regulated during viral, or bacterial pathogenic invasion or by pro-inflammatory cytokines [94]. In mammals, IL6 is produced by neutrophils and many other cell types [95-97]. In spite of this, biopsy specimens taken in macroscopically and microscopically unaffected areas of intestinal mucosa from patients with Crohn's disease, provide evidence of sustained IL6 expression even in the absence of patent inflammation [98]. Moreover it should be stressed that IL6 has dual effects, protecting against inflammation to some extent but also being proinflammatory in the course of chronic inflammation. Therefore its weak expression in about

half of the type III granular cells from apparently normal intestinal tissue of *R. clavata* may account for a protective anti-inflammatory activity in the physiologically challenged tissue of spiral intestine.

Conclusion

Based on cell distribution, morphological/ultrastructural criteria and reactions with immuno-histochemical stains three granular cell types in the spiral intestine of *R. clavata* have been identified and compared with similar immune cells of teleosts and/or mammals. In the current study, the intensity of immunochemical staining within types of granular cell was not uniform, suggesting that the antigen (i.e. lysozyme, MC tryptase, TNF- α, IL6) concentrations differ among cells. Further studies are necessary in view of the dearth of information on the cells involved in the intestinal mucosal immunity in elasmobranchs. The isolation of cells and the analysis of their molecular markers could help in accurate identification of these cells.

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Figure captions

Fig 1. Histological sections through spiral intestine of *Raja clavata*. **(a)** Transverse section shows screw-like aspect of the intestine. The intestinal wall is composed of four layers: M = mucosa, SM, submucosa, Mu = Muscularis, S = serosa. The arrow indicates an internal ring composed only by epithelium and lamina propria-submucosa, Alcian-Blue and Haematoxylin & Eosin (AB/H&E), scale bar = 1 mm. **(b)** Two adjacent intestinal folds formed by single columnar epithelium. Note the type I granular cells (arrows) in the epithelium in close proximity to basement membrane (arrowheads), (H&E), scale bar = 20 μm. **(c)** Occurrence of several type I granular cells (arrows) at the base of epithelium and in close vicinity to basement membrane (arrowheads), Giemsa, scale bar = 20 μm. **(d)** Micrograph shows type II granular cells (curved arrows) and type III granular cells (thick arrows) in lamina propria-submucosa; the granular aspect of type II cells is remarkable. Note the presence of type I granular cells (arrows) at epithelial base, Giemsa, scale bar = 10 μm. **(e)** Occurrence of type II granular cells (curved arrows) and some in degranulation (arrowheads) are evident in lamina propria-submucosa. Numerous type III granular cells are also present (thick arrows), Giemsa, scale bar = 10 μm. **(f)** Type III granular cells (arrows) are visible in lamina propria-submucosa, Giemsa, scale bar = 10 μm. **(g)** Semithin section of lamina propria-submucosa showing several type III granular cells (arrows) with numerous little granules, Toluidine Blue, scale bar = 10 μm.

Fig 2. Transmission electron micrographs of spiral intestine of *R. clavata* showing type I granular cells. **(a)** Type I granular cells (arrows) in close proximity to the basement membrane (arrowheads), note the nuclei of enterocytes (asterisks), scale bar = 3.33 μm. **(b)** Two granular cells belonging to type I (arrows) in contact with basement membrane (arrowheads). Asterisks indicate the nuclei of enterocytes, scale bar = 2.86 μm. **(c)** A type I granular cell with eccentric and heterochromatic nucleus (asterisk), the cell is in contact with basement membrane (arrowheads), scale bar = 1.43 μm. **(d)** High magnification of type I granular cell: the granules contain finely granular electron-dense material. Note scroll pattern of some granules (asterisks), scale bar = 0.67 μm. **(e)** Mitochondria (arrows) near nucleus (asterisk) and scattered among the granules (white asterisks) are visible, scale bar = 0.25 μm. **(f)** Occurrence of epithelial granular cell (arrow) and type III granular cell (thick arrow) in opposite sides of basement membrane (arrowheads) can be seen, scale bar = 2.50 μm.

Fig 3. Types II and III granular cells in spiral intestine of *R. clavata*. **(a)** High magnification of type II granular cell in lamina propria-submucosa with eccentric heterochromatic nucleus (asterisk). Note

the presence of big, round or oval electron-dense granules, scale bar = 1.83 μm. **(b)** Type II granular cell (arrow) inside a capillary in the thickness of lamina propria-submucosa; the cytoplasm is filled with numerous round-oval electron-dense granules, scale bar = 2.69 μm. **(c)** Numerous type III granular cells (arrows) with eccentric nuclei are visible in lamina propria-submucosa, scale bar = 5.0 μm. **(d)** Two types of electron-dense granules in type III granular cells: small round and large elongated electron-dense granules filled the cell cytoplasm, scale bar = 2.0 μm. **(e)** Micrograph shows elongated granules (arrows) with axial rod-like inclusions inside the cytoplasm of a type III granular cell, scale bar = 0.25 μm. **(f)** Numerous small round mitochondria (arrows) near nucleus (asterisk) and among electron-dense granules (white asterisks) of a type III granular cell, scale bar = 0.33 μm.

Fig 4. Immunoreactivity of granular cell types in the spiral intestine of *Raja clavata*. **(a)** Several type I granular cells (thin arrows) strongly immunoreactive (IR) to anti-lysozyme at the depth of epithelium and in close proximity to basement membrane, scale bar = 50 μm. **(b)** Numerous type I granular cells (thin arrows) immunoreactive to anti-TNF-α at the base of the intestinal epithelium, scale bar = 50 μm. **(c)** Some type I granular cells (arrows) IR to the anti-MC tryptase at the depth of the intestinal epithelium, scale bar = 50 μm. **(d)** A type III granular cell (thick arrow) weakly IR to anti-IL6 close to a blood vessel in the connective tissue of the intestinal wall. Two type I granular cells (thin arrows) in the basal epithelial region are negative to anti-IL6, scale bar = 20 μm.