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Histamine regulates the inflammatory response of the tunicate *Styela plicata*

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ABSTRACT

Histamine is stored inside hemocytes of the tunicate *Styela plicata* (Chordata, Tunicata, Ascidiacea), but no evidence on its role in the regulation of the immune response of this species has been reported. We examined whether histamine participated in the regulation of inflammation and host defense in *S. plicata*. The presence of histamine inside *S. plicata* hemocytes was confirmed by flow cytometry, and histamine release was detected by ELISA, after *in vitro* hemocyte stimulation with different PAMPs. *In vitro* hemocyte treatment with histamine, or specific histamine-receptor agonists, reduced their phagocytic ability. Injection of histamine into the tunic recruited hemocytes to the site of injection. Systemic injection of histamine, or the histamine-releasing agent compound 48/80, decreased the phagocytic ability of hemocytes. Histamine promoted the constriction of tunic hemolymph vessels *in vivo*, having a direct effect on vasoconstriction in tunic explants. These results provide for the first time clear evidence for the involvement of histamine in the regulation of inflammation and host defense in tunicates.

1. Introduction

Histamine is a biogenic amine playing a role in muscle contraction, neural transmission, and the regulation of gastric secretions, both in vertebrate and non-vertebrate organisms (Reite, 1972). Moreover, histamine has a key role in the regulation of the inflammatory process in vertebrates, modulating blood flow and vascular permeability (Eto et al., 2001), in addition to a number of cellular mechanisms of host defense (Jutel et al., 2006; O'Mahony et al., 2011). In vertebrates, both innate and adaptive immunity are regulated by histamine (Jutel et al., 2006). Histamine has been found to negatively-regulate the functions of phagocytes (e.g. monocytes, macrophages, and neutrophils), including phagocytosis, chemotaxis, the respiratory burst, exocytosis, and the production of cytokines, among others (Azuma et al., 2001; Burde et al., 1989; Mulero et al., 2007; Radermecker et al., 1989).

The large variety of inflammatory and host-defense mechanisms regulated by histamine is partly explained by the existence of various histamine receptors (H1–H4), which are differentially expressed in numerous cell types and tissues. In the context of the vertebrate immune system, histamine and its four receptors represent a complex regulatory system (Jutel et al., 2006; O'Mahony et al., 2011), involving the selective recruitment of effector cells into the tissues, the regulation of cellular maturation and activation, and the regulation of multiple effector functions influencing the balance between pro- and anti-inflammatory responses (Jutel et al., 2006; O'Mahony et al., 2011).

The correlation between histamine storage inside mast cells, and its ability to regulate vertebrate inflammation and immunity has been firmly established (Crivellato and Ribatti, 2010; Mulero et al., 2007; Reite and Evensen, 2006). Interestingly, it has been shown that histamine is stored inside granular hemocytes of the tunicate *Styela plicata* (de Barros et al., 2007), and also inside *S. plicata* test cells (Cavalcante et al., 2000). The fact that histamine was stored inside *S. plicata* hemocytes and test cells (a cell type thought to perform defensive functions in oocytes of this organism), prompted us to investigate the possibility that histamine could have a role in the regulation inflammation and host defense, akin

Abbreviations: c48/80, compound 48/80; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FSC, forward scatter; H1-H4, histamine receptors; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; SSC, side scatter; VaDNA, *Vibrio anguillarum* DNA; MFI, Mean Fluorescence Intensity; PAMPs, pathogen-associated molecular patterns; T.S., tunicate saline.

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to its role in higher vertebrates. The presence of histamine inside *S. plicata* hemocytes and histamine release after *in vitro* hemocyte stimulation with different PAMPs were confirmed. The ability of both histamine and histamine-releasing agent compound 48/80 (c48/80) to reduce hemocyte phagocytic ability was also observed. Finally, injection of histamine into the tunic recruited hemocytes to the site of injection and promoted the constriction of tunic hemolymph vessels. These results provide for the first time clear evidence for the involvement of histamine in the regulation of inflammation and host defense in tunicates.

2. Materials and methods

2.1. Animals

Adult individuals of *S. plicata* (Chordata, Tunicata, Ascidiacea) were collected at Puerto Deportivo Juan Montiel (Águilas, Murcia, Spain). Animals were maintained in filtered sea water, in an aerated aquarium at 19 °C until used.

2.2. Isolation of hemocytes

Hemocytes were extracted by injecting ice-cold tunicate saline (T.S., 49 mM $MgSO_4$, 11.3 mM $CaCl_2$, 490 mM NaCl, 20 mM HEPES, pH 7.3) into the atrium, lavaging several times, and collecting the diluted hemolymph by aspiration. Diluted hemolymph was passed through a nylon mesh (70 μm pore size) to remove large clumps of cells. Cells were centrifuged at 600×*g* for 5 min, resuspended in cold T.S. at 1×10^7 cells/mL, and kept on ice until use.

2.3. Analysis of hemocyte histamine content by flow cytometry

S. plicata hemocyte histamine was analyzed by flow cytometry, by preforming an intracellular anti-histamine staining. Hemocytes (1×10^6 cells in 100 μL) were fixed overnight with 2% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC, Sigma–Aldrich) in T.S. Hemocytes were permeabilized by adding 100 μL of 0.2% saponin in phosphate buffered saline (PBS). After 30 min on ice, cells were centrifuged at 1500×*g* for 3 min. After completely removing the supernatant by aspiration, cells were resuspended in 500 μL PBS+4% fetal bovine serum (FBS, Life Technologies) and incubated on ice for 1 h. After this blocking step, cells were centrifuged at 1500×*g* for 3 min, and resuspended in 100 μL PBS+4% FBS containing the anti-histamine antibody (Rabbit polyclonal; Sigma–Aldrich, catalog number H 7403) at a 1:200 dilution. After a 1 h incubation on ice, cells were washed with 1 mL PBS+4% FBS, centrifuged at 1500×*g* for 3 min and, resuspended in 100 μL of PBS+4% FBS containing 10 μg/mL Alexa Fluor 488 Goat Anti-Rabbit IgG (Life Technologies, catalog number A-11008). Cells were kept on ice for 1 h, washed as above and finally resuspended in 100 μL 1% paraformaldehyde in PBS. In selected experiments hemocytes were stimulated with the indicated concentrations of the histamine releasing agent c48/80 (Sigma–Aldrich, catalog number C2313) before EDAC fixation (Mulero et al., 2007). Experimental replicates were performed for all samples. Cells were analyzed by flow cytometry, using a FACSCalibur flow cytometer (BD Biosciences).

2.4. Analysis of hemocyte histamine release

Histamine release by *S. plicata* hemocytes was analyzed using a commercial competitive enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's specifications (Cayman Chemical, catalog number A05890). Histamine was detected in the supernatant of hemocytes (2×10^6 cells per sample in 200 μL T.S.) stimulated with the indicated concentrations of LPS from *Escherichia coli* 0111:B4 (Sigma–Aldrich, L2630), *Vibrio anguillarum* (strain R-82, serotype 01) DNA (VaDNA) (prepared as described (Pelegrin et al., 2004)), Poly I:C (InvivoGen, catalog number tlrl-pic), PAM3CSK4 (InvivoGen, catalog number tlrl-pms) or c48/80. After 45 min of stimulation, cells were centrifuged at 2000×*g* for 5 min, and the supernatant recovered to perform the assay. Experimental replicates were performed for all samples. Because histamine release was measured using a competitive ELISA assay, data were expressed as the reciprocal of light absorbance ($1/A_{405}$) ± SEM for clarity.

2.5. Phagocytosis assays

The phagocytic ability of *S. plicata* hemocytes was analyzed using heat-killed FITC-stained *V. anguillarum* (Esteban et al., 1998) as phagocytic targets. Hemocytes (1×10^6 in 100 μL T.S.) were mixed with 1×10^8 bacterial targets (suspended in 10 μL PBS; 1:100 hemocyte to target ratio), centrifuged at 600×*g* for 5 min to maximize cell-bacteria contacts, and incubated at either 4 °C or 19 °C. After 1 h incubation cells were centrifuged, and resuspended in 50 μL ice-cold PBS+0.025% trypsin+2 mM EDTA to detach non-ingested bacteria. After 10 min incubation on ice, cells were mixed with 100 μL of ice-cold 4% FBS in T.S., centrifuged at 600×*g* for 5 min, and resuspended in 2% paraformaldehyde in PBS. In some experiments hemocytes were treated with the indicated concentrations of histamine, the H1-receptor agonist 2-(2-Pyridyl)ethylamine (Sigma–Aldrich catalog number A55306), the H2-receptor agonist dimaprit (Sigma–Aldrich catalog number D7571), LPS (10 μg/mL), Poly I:C (25 μg/mL), or VaDNA (50 μg/mL); which were added together with the phagocytic targets. External fluorescence was quenched using a solution of 0.4% trypan blue in PBS, added immediately before flow cytometry analysis. Experimental replicates were performed for all samples. Phagocytosis is evidenced as an increase in the Mean Fluorescence Intensity (MFI) value of cells incubated with bacteria at 19 °C, with respect to the MFI values of cells kept at 4 °C throughout the phagocytosis assay. Data were expressed MFI ± SEM. In some experiments MFI values were normalized against 4 °C controls, or against the MFI values of cells from control conditions (i.e. no histamine or histamine receptor agonists), from the same individual. Phagocytosis was then expressed as percentage of control ± SEM.

2.6. Analysis of the tunic inflammatory-like reaction

An inflammatory-like reaction (De Leo et al., 1997; Di Bella and De Leo, 2000) in the tunic of *S. plicata* was induced as previously described (de Barros et al., 2007) by injecting 100 μL of T.S. close to the tunic–mantle interface, or 100 μL of T.S. containing 10 mM Histamine, 10 μg/mL LPS, 25 μg/mL Poly I:C, or 50 μg/mL VaDNA. After 4-h incubation, the site of injection was dissected, and fixed in 4% paraformaldehyde for 18 h. An additional section of the tunic, distal to the site of injection, was dissected from each animal and used as control to evaluate individual variations in hemocyte number under resting conditions. After fixation, tunic sections were dehydrated in a graded ethanol series to 100% ethanol and embedded in paraffin wax as described (de Barros et al., 2007). Five micrometer sections were obtained, mounted on glass slides and stained with hematoxylin and eosin (2 and 3 min, respectively). The number of hemocytes in the tunic was evaluated microscopically (600× magnifications) as previously described (de Barros et al., 2007). Hemocytes were counted in 30 randomly-selected microscope fields, using three serial microtome sections separated by at least 15 μm. Quantitation was performed dividing the number of hemocytes per microscope field at the site of injection, by the number of hemocytes present in normal (not-injected) tunic