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Expression analyses of interferon gamma, tumor necrosis factor alpha and inducible nitric oxide synthase in the hemocyte morphotypes of two commercially important Indian molluscs

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ABSTRACT

Bellamya bengalensis and *Lamellidens marginalis* are the two common species of molluscs of Indian subcontinent which bear immense aquacultural prospect. These edible varieties of freshwater molluscs have nutritional, ecological, ethnomedicinal and industrial significance. Report of nonspecific immunological status of them is grossly inadequate in the current scientific literature. Hemocytes, the circulating blood cells of invertebrate molluscs play a pivotal role in cell mediated immunity. Information of existence of cytokines like IFNγ, TNFα and enzyme iNOS is very limited and controversial in molluscan hemocytes. We detected IFNγ, an immunomodulatory molecule and TNFα, a tumoricidal and apoptosis inducing cytokine in hemocyte morphotypes of molluscs *B. bengalensis* and *L. marginalis* by flow cytometry, iNOS had been detected in the hemocyte morphotypes of *B. bengalensis* and not in *L. marginalis*. We report the existence of IFNγ in molluscs for the first time. Comparative expression of IFNγ, TNFα and iNOS in hemocytes would provide a better information base to understand biology, evolution and immunological role of these molecules in molluscs and related metazoans.

1. Introduction

Hemocyte is established as chief effector cell of molluscan immunity. Information of hemocyte mediated immunological response in molluscs is limited in comparison to vertebrate immunocytes. We reported existence of hemocyte morphotypes with potentialities to perform phagocytosis and generation of superoxide anion and nitric oxide as nonspecific innate immune responses in *Bellamya bengalensis* (Gastropoda: Prosobranchia) and *Lamellidens marginalis* (Bivalvia: Eulamellibranchiata) (Ray et al., 2013a) and toxin induced modulation of hemocyte density, morphology, lysosomal fragility and apoptosis of hemocyte in same species (Ray et al., 2013b). In this present communication, we report morphotype specific existence of intracellular cytokines like interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) and an intracellular enzyme inducible nitric oxide synthase (iNOS) in the freshly collected unstimulated live hemocytes of *B. bengalensis* and *L. marginalis* employing flow cytometry. Flow cytometric detection of immunoactive molecules in hemocytes stained with labeled mammalian antibodies is an accepted method which provided novel immunological informations in invertebrates (Engelmann et al., 2002). Existing reports showed that hemocyte function can be modulated by human recombinant IFNγ (h-rIFNγ) in the hemocytes of mussel *Mytilus galloprovincialis* (Canesi et al., 2003) indicating possible existence of IFNγ-receptor in the mollusc. This report prompted us to investigate the possible existence of IFNγ in hemocytes of Indian gastropod and bivalve molluscs. TNFα is an immunoregulatory and inflammatory cytokine released by mammalian macrophages and monocytes in response to stimulation by bacterial lipopolysaccharide which induces apoptosis, sepsis, inflammation and necrosis of tumors. It is an effector of cell mediated immunity against bacteria, parasites and well characterized in mammals with very limited information in invertebrates (Engelmann et al., 2002). We report the expression of TNFα in Indian molluscs for the first time. Inducible nitric oxide synthase is an intracellular enzyme that catalyzes the reaction generating nitric oxide from L-arginine and is inducibly expressed within phagolysosomes of mammalian macrophages in response to pathogens, cytokines and lipopolysaccharide. Nitric oxide is reported to mediate cytotoxic response against tumors and pathogens within macrophages and bears very limited report in molluscs (Conte and Ottaviani, 1995). Hemocyte morphotypes

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of *B. bengalensis* and *L. marginalis* are reported to generate nitric oxide (Ray et al., 2013a). This led us to investigate the existence of iNOS in the hemocytes of *B. bengalensis* and *L. marginalis.* Expression of IFNγ in the hemocyte morphotypes of *B. bengalensis* and *L. marginalis* is probably the first time report of this cytokine in molluscs. TNFα and iNOS were detected in Indian molluscs for the first time.

2. Materials and methods

Hemolymph was collected aseptically from *B. bengalensis* and *L. marginalis* by foot prodding (Renwrantz et al., 1981) and cardiac puncture (Sauve et al., 2002) methods respectively. Collected hemolymph was subjected to cold centrifugation (Hermle Z323K, Germany) by centrifuging the sample at 650*g* for 10 min at 4 °C. Hemocyte pellets were resuspended thrice in sterile snail saline (SSS: 4 mM HEPES, 37 mM NaOH, 36 mM NaCl, 2 mM KCl, 2 mM $MgCl_2.\ 2H_2O$, 4 mM $CaCl_2$, $2H_2O_2$, pH- 7.4) (SRL, India) (Adema et al., 1991). Viability and morphology of hemocytes were routinely examined microscopically (Olympus, BH 2) before immunostaining by staining the hemcytes with 0.2% trypan blue following the principle of dye exclusion. For IFNγ and TNFα immunostaining, monoclonal antibodies, mouse anti rat IFNγ- FITC, mouse anti human TNFα- FITC and isotype control mouse IgG1- FITC (mIgG1-FITC) were used. For immunostaining of iNOS, rabbit anti human iNOS as primary antibody, rabbit IgG (rIgG) as isotype control and goat anti rabbit IgG- FITC as secondary antibody, were used. All these antibodies were purchased from Abcam Plc., UK., Freshly isolated hemocytes (Ray et al., 2013a) were permeabilized in permeabilizing buffer containing 0.1% saponin for 15 min and stained with IFNγ- FITC, TNFα- FITC antibodies and mouse IgG1-FITC for 45 min at 25 °C in separete tubes for analyses of IFNγ and TNFα. Unstained cells were set as negative controls (Engelmann et al., 2002). For iNOS analysis, hemocytes were first incubated with primary antibody, rabbit anti iNOS antibody and isotype control, rIgG for 45 min at 25 °C and with secondary antibody, anti rabbit IgG-FITC for 30 min. Unstained cells and cells stained with only secondary antibody were set as negative controls. Post stained cells were fixed in 1% formalin and immediately processed for detection of green fluorescence in FL-1 channel (FITC) in the flow cytometer (FACS VERSA, BD Bioscience, USA). A total of 30,000 events were considered for each hemocyte sample analysis. Two parameter dot-plots of FSC and SSC were constructed to gate three hemocyte morphotypes designated as P1, P2 and P3 (Ray et al., 2013a). Dot-plots of FL1 were constructed for each gate seperately. Percent positive cells were then assessed for cells differentially stained with isotype control, IFNγ, TNFα and iNOS specific antibodies. Appropriate overlay histogram was constructed for isotype and antibody stained cells. Instrument calibration, statistical analyses and data representation were performed using FACSuite software attached with flow cytometer.

3. Result

The detection of IFNγ, TNFα and iNOS were carried out in hemocyte morphotypes of *B. bengalensis* and *L. marginalis*. The P1, P2 and P3 gates in the dot-plot presentation along FSC and SSC axes represent three hemocyte morphotypes namely agranulocytes, semigranulocytes and granulocytes respectively (Fig. 1Aa1 and Aa2). The flow cytometric distribution of hemocytes was carried out on the basis of size (FSC) and granularity (SSC) (Ray et al., 2013a). Hemocytes of P1, P2 and P3 gates of *B. bengalensis* and *L. marginalis* expressed strong positive reactivity upon staining with IFNγ- FITC antibody, as revealed by dot-plot analysis with percent positive cells (Fig. 1B, C) and overlay histogram representation (Fig. 1D) of FACS analysis. In *B. bengalensis,* percent positive cells recognizing IFNγ antibody in agranulocytes (P1), semigranulocytes (P2) and granulocytes (P3) morphotypes were 4.12, 3.54 and 0.28 respectively as compared to negligible binding of isotype mouse IgG1-FITC. Similarly, in *L. marginalis*, strong positive binding of IFNγ antibody by agranulocytes (P1), semigranulocytes (P2) and granulocytes (P3) morphotypes were recorded as 6.46%, 4.04% and 1.06% respectively. Agranulocyte (P1) and semigranulocyte (P2) morphotypes of both molluscs exhibited strong responsiveness to binding by IFNγ antibody in comparison to granulocytes of P3 gate which exhibited lowest binding response. IFNγ antibody bound more strongly with *L. marginalis* hemocytes than that of *B. bengalensis* hemocytes. For TNFα, dot-plot and overlay histogram analyses showed that agranulocytes (P1) and semigranulocytes (P2) of *B. bengalensis* and *L. marginalis* presented strongest binding of TNFα-FITC antibody compared to isotype mouse IgG1-FITC (Fig. 2A–C). Percent positive cells for agranulocytes (P1) and semigranulocytes (P2) morphotypes in *B. bengalensis* and *L. marginalis* were recorded as 3.13% (P1), 4.79% (P2) and 7.41% (P1), 5.28% (P2) respectively. Granulocytes (P3) recognized the TNFα antibody at a lowest level as revealed by TNFα positive cells as 0.97% and 1.57% in *B. bengalensis* and *L. marginalis* respectively. When compared between two species, *L. marginalis* hemocytes exhibited stronger binding with TNFα antibody than *B. bengalensis* hemocytes. Substantial expression of IFNγ, TNFα and iNOS enzyme were detected in the morphotypes of *B. bengalensis* hemocytes. Binding of iNOS antibody by agranulocytes (P1), semigranulocytes (P2) and granulocytes (P3) of *B. bengalensis* were 2.80%, 3.94% and 0.93% respectively whereas binding of isotype, rabbit IgG were 0.30% (P1), 0.49% (P2) and 0.10% (P3) respectively (Fig. 3A–C). In *B. bengalensis*, agranulocytes (P1) and semigranulocytes (P2) exhibited strongest binding of iNOS antibody as compared to granulocytes (P3). Recognition of iNOS antibody by agranulocytes (P1), semigranulocytes (P2) and granulocytes (P3) of *L. marginalis,* was negligible as revealed by percent positive cells presented in dot-plot analyses. A stronger positive reactivity of iNOS antibody by *B. bengalensis* hemocytes was recorded in comparison to *L. marginalis* hemocytes which are assumed to be a species specific response.

4. Discussion

Study of cytokine network is of current importance in the field of comparative immunology (Malagoli, 2010). Unlike vertebrates, information of cellular distribution and biological roles of cytokines like IFNγ, TNFα and enzyme like iNOS in invertebrates is very limited or absent. There are limited reports of cytokine like molecules like IL-1α, IL-1β, IL-2, IL-6 TNFα in the hemocytes of molluscs *Planorbarius corneas* and *Viviparus ater* (Ottaviani et al., 1993). IFNγ is a reported immunoregulatory cytokine of vertebrate innate immunity which provides protective immunity against viral and parasitic infection (Sieger et al., 2009a) is not reported in molluscs. IFNγ stimulates the release of reactive oxygen species and nitric oxide and thus acts as a major activator of cytotoxicity. In this communication, we report the existence of IFNγ in three principal morphotypes of hemocytes of two species of gastropod and bivalve molluscs. Agranulocytes of P1 gate exhibited highest expression of IFNγ in comparison to semigranulocytes (P2) and granulocytes (P3). Existence of IFNγ in molluscan hemocytes can be explained on the basis of ability of hemocyte to generate cytotoxic molecules. Savan et al. (2009) constructed evolutionary history of IFNγ and proposed that it evolved more than 450 million years ago in fish, a lower vertebrate. According to them, structural and functional conservation of IFNγ in vertebrate series indicate evolution of innate immunity and natural killer (NK) cell activity in lower vertebrates. Hemocytes of sea urchin, a deuterostomic