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Flow cytometry analysis of softness syndrome effects on hemocytes of the tunicate *Halocynthia roretzi*

Dong-Lim Choi [a], Nam-Sil Lee [a], Myoung Sug Kim [a], Jung Soo Seo [a], Myoung Ae Park [a], Jin Woo Kim [b], Jee Youn Hwang [a,*]

[a] Division of Fish Pathology, Fisheries Research and Development Institute, 408-1 Gijang, Busan 619-705, Republic of Korea
[b] Aquatic Animal Disease Control Center, NFRDI, 408-1 Gijang, Busan 619-705, Republic of Korea

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ABSTRACT

The mortality of the tunicate *Halocynthia roretzi* due to softness syndrome, which exhibits tunic thinning as well as loss of muscle elasticity, has caused serious problems for the aquaculture industry in Korea. The effects of softness syndrome can be better understood by studying the defense mechanisms of *Halocynthia roretzi*. This study used flow cytometry to assess hemocyte parameters in *H. roretzi*. Flow cytometry provided a means to rapidly quantify tunicate defense mechanisms by measuring temperature and zymosan-dependent effects on phagocytosis activity.

Also, immune parameters, such as morphology of cell sub-populations (size and granularity), total hemocyte counts (THC), hemocyte viability, and phagocytosis activity using flow cytometry analysis, of normal tunicates and softness syndrome tunicates were compared. The results showed that, in normal tunicates, the phagocytic rate and hemocyte viability are higher than those of tunicates with softness syndrome. Total hemocyte numbers in softness syndrome tunicates were 4 times higher than that of normal tunicates.

It is suggested that the tunicate immune system is associated with softness syndrome and that flow cytometry is a powerful tool for characterizing immune response.

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1. Introduction

The tunicate *Halocynthia roretzi* belongs to the subphylum Urochordata and occupies a phylogenetic position between vertebrates and invertebrates. *H. roretzi* is commercially cultured in the southern coast of Korea. Over the last 20 years, large numbers of tunicates have died in this farming area, with victims characterized by thinning and soft and dissolving muscle tissue (Cho et al., 2008; Choi et al., 2006; Hirose et al., 2009). As a result, the combination of these symptoms has been referred to as 'softness syndrome'. The cause of mortality is unclear. Researchers have proposed several possible syndrome-inducing factors, including environmental pollutants, lack of plankton, genetic factors (due to selection effects in the breeding process), and infectious diseases caused by parasites (e.g. intrahemocytic paramyxean parasite) or viruses (e.g. birna virus) (Cho et al., 2008; Choi et al., 2006; Kitamura et al., 2010; Song et al., 2009).

Generally, it is believed that tunicates' ability to react to disease or environment stress depends upon their defense system (Fournier et al., 2001; Gauthier-Clerc et al., 2006; Ordas et al., 2007), which relies on hemocytes of a primitive open vascular system. Hemocytes are an excellent target for studies of softness syndrome because some of these hemocytes are able to phagocytose invading microorganisms (Raftos and Nair, 2004). Researchers have used a luminol-enhanced chemiluminescence (CL) test to understand the immune system in tunicates (Azumi et al., 2002; Ballarin and Cima, 2005). However, there is a demand for techniques that are able to measure hemocyte function quickly and accurately. Flow cytometry has been a valuable tool for these purposes. Flow cytometry has previously been used to characterize marine invertebrate immune systems associated with pathology or environmental stress, such as hemocyte populations in *Ruditapes spp.* clams (Allam et al., 2001), the Pacific oyster *Crassostrea gigas* (Lambert et al., 2003), and the tunicate *Pyura stolonifera* (Pearce et al., 2001). However, suitable methods have not been developed yet to use flow cytometry in the study of tunicate hemocyte parameters.

The objective of this study is to apply flow cytometry to the study of the tunicate hemocyte population and to determine the relative proportions of the hemocyte sub-populations. Furthermore, flow cytometry analysis is used to accurately and quantitatively evaluate the effect of temperature on hemocyte phagocytic ability. Lastly, this study uses flow cytometry to examine the effects of softness syndrome on the cellular defense system in the tunicate *Halocynthia roretzi*.

* Corresponding author. Tel.: +82 51 720 2494; fax: +81 51 720 2498.
E-mail address: jyhwang@nfrdi.go.kr (J.Y. Hwang).

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2. Materials and methods

2.1. Tunicate samples and collection of hemocytes

Halocynthia roretzi tunicates (ranging from 74 ± 11 g in weight and 71 ± 5.4 mm in length) were collected from hanging culture at an aquaculture farm in Tongyoung, on the south coast of Korea, from January 2007 to September 2007. To acclimate to experimental conditions, tunicates were transported in coolers maintained at 4 °C and, over the next 24 hrs, were kept in a constant flow of seawater at 15 °C in the laboratory.

Collected tunicates were classified visually for softness syndrome symptoms. Using a 1 ml plastic syringe fitted with a 25-gauge needle, hemocytes were withdrawn from the muscle of 3~5 individuals, filtered through 80 μm mesh, and held on ice to reduce the rate of hemocyte aggregation. To minimize individual variability, 10 to 30 tunicates were used for each experimental condition.

2.2. Cellular parameters of hemocytes using flow cytometry

300 μl subsamples of hemocytes were fixed by adding 300 μl of 6% formalin solution in filtered seawater (FSSW) for measurements of hemocyte size and complexity. Before flow cytometric analysis, those samples were incubated with SYBR green I (Molecular probe, 10× final concentration) at room temperature for 30 mins. A single-laser FACScan flow cytometer (Beckman Coulter FC500) was used to analyze fluorescently stained hemocytes. Using a 488~615 nm band-pass filter, fluorescence emissions of 10,000 cells per sample were analyzed. Gates were set to eliminate debris. Parameters (photo-multiplier tube voltage, scatter, fluorescence gain) were kept constant within any one experiment.

2.3. Total hemocyte counts (THC) and hemocyte viability

Based on the flow rate (usually 60 μl/min) and the number of events counted in 1 min, total hemocyte counts (THC) were evaluated in each subsample.

Hemocyte viability was assayed according to a two-color fluorescent labeling protocol. With stains from a Live/Dead Viability assay kit (Invitrogen), 300 μl subsamples were incubated at room temperature for 30 min before flow cytometric analysis. Flow cytometric analysis to measure hemocytes was described previously and each experiment was reproduced at least in triplicate.

2.4. Effects of temperature and zymosan concentration on phagocytosis

Cell concentrations were adjusted to 1×10^6 hemocytes/ml by addition of FSSW without centrifugation for all experiments. Zymosan A bioparticles labeled with fluorescein (Molecular Probes) were used for the phagocytosis assay with a protocol adapted from the supplier's manuals.

Three hundred microliter subsamples of hemocytes from normal tunicates were mixed with different ratios of zymosan particles (1:0.5~1:20) to optimize the phagocytosis assay. After 30 min incubation at 20 °C, hemocytes were fixed with 300 μl of 6% formalin solution in FSSW and analyzed by flow cytometry as described previously. The phagocytosis activity of the hemocytes was estimated as the percentage of hemocytes that had engulfed more than one zymosan particle.

To determine the influence of temperature on phagocytosis activity, 300 μl subsamples of hemocytes were tested across a range of temperatures (10, 15, 20, 25 and 30 °C). Each temperature was maintained throughout the experiment (during both incubation and counting). After 30 min incubation at 20 °C, hemocytes were fixed with 300 μl of 6% formalin solution in FSSW and analyzed by flow cytometry as described formerly. The ANOVA test was used to carry out statistical comparisons to compare results from different treatments. Differences were considered significant at the $p<0.05$.

2.5. Phagocytic activity of hemocytes from normal and softness syndrome tunicates

Hemocyte subsamples of 300 μl from normal and softness syndrome tunicates were adjusted to 1×10^6 hemocytes/ml, mixed with zymosan particles (1:1), incubated 30 min at 20 °C and analyzed by flow cytometry as described earlier.

3. Results

3.1. Flow cytometric visualization of tunicate hemocytes

All SYBR green I stained cells were visualized by log side scatter (SS, cell complexity) on a logarithmic scale cytogram and forward scatter (FS, cell size) on a liner scale cytogram. Sub-populations were easier to distinguish in fixed hemocytes than non-fixed hemocytes (Fig. 1).

3.2. Effect of temperature and zymosan concentration on tunicate phagocytosis

To evaluate the functional immune responses of hemocytes, effects of temperature and zymosan to hemocyte incubation ratios on

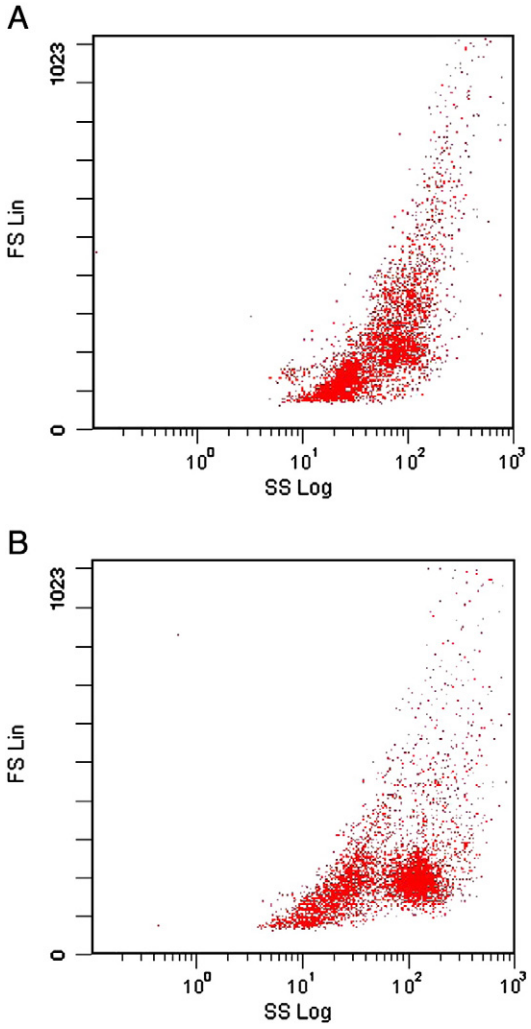

Fig. 1. *Halocynthia roretzi.* Flow cytometry plots show distributions of log side scatter (SS) and forward scatter (FS) of (A) non-fixed hemocytes and (B) formalin-fixed hemocytes.

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