Evaluation of *in vivo* Hemocyte Phagocytosis of Microsphere Beads in *Litopenaeus vannamei* Utilizing Flow Cytometry Following Administration of Bacterial Lipopolysaccharides

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Summary and Implications

Carboxylate modified microspheres were injected into shrimp and phagocytosis of these particles was measured using flow cytometry following treatment with microbial lipopolysaccharides. This is the first time these methods have been used to assess innate immune responses in shrimp.

Introduction

In addition to an inducible anti-microbial peptide response, invertebrates rely on other innate defense systems. Recent research assessing the role of different pathways of innate immunity within invertebrates has demonstrated that antimicrobial peptide production is less immediately important than other responses, such as phagocytosis (Haine *et al.* 2008 and Bartholomay *et al.* 2004). Microsphere beads injected *in vivo* and measured with flow cytometry provides a useful tool to investigate the role phagocytosis plays in the immune response of shrimp to pathogens.

Materials and Methods

SPF juvenile *L. vannamei* acquired from Shrimp Improvement Systems (SIS) were injected with filter sterilized lipopolysaccharide (*Escherichia coli* 0111:B4) suspended in shrimp saline at 8 µg per gram of body weight 24 hours prior to bead injection, one receiving LPS 1 hour prior to bead injection, and one receiving an equivalent volume of shrimp saline one hour before injection. 50 µL of 1 µm diameter red-fluorescent carboxylate-modified latex microsphere beads (Molecular Probes) were injected into the ventral sinus of each group with recovery after 1 hour incubation. Cells were then immediately fixed and stained with the fluorescent nucleic acid stain Syto 16.

Results and Discussion

Samples were examined utilizing the BD FACSCanto flow cytometer (BD Biosciences). A phagocytic index (the proportion of the total number of hemocytes that have phagocytosed at least one bead) was calculated for each sample by drawing separate electronic gates around non-phagocytic and phagocytic hemocytes to determine their relative number (Figure 1) Phagocytic indices (PI) were then calculated for subpopulations of hemocytes categorized by forward and side scatter profiles and degree of nuclear staining. Significant differences were present in the PI of cells with high nucleus to cytoplasm (N:C) ratios. Significant differences in PI were present between the 24 hour treatment group and the 1 hour and saline control groups of all hemocyte types (Table 1). These results indicate the administration of LPS significantly increases the proportion of hemocytes that have phagocytosed beads after 24 hours. In conclusion, this technique provides an *in vivo* tool to further examine the role hemocyte phagocytosis plays in the innate immunity of shrimp.

Figure 1. Hemocytes scatter plot by Syto 16 (x-axis) and red fluorescent bead signal.

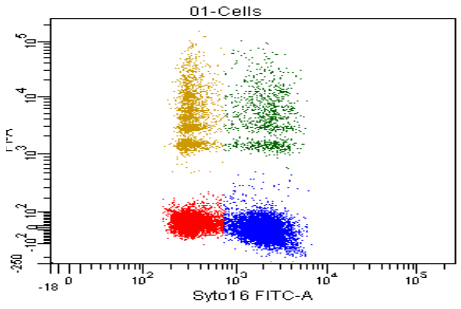

Table 1. Phagocytic indices of all hemocyte types.

LPS 24	LPS 1H	Saline 1h
0.441319	0.191476	0.369191
0.286895	0.279136	0.346256
0.415777	0.174028	0.212447
0.401906	0.141243	0.099299
LPS24 – LPS1		0.005894