Fish & Shellfish Immunology 52 (2016) 206–209

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Extracellular trap formation in kuruma shrimp (*Marsupenaeus japonicus*) hemocytes is coupled with c-type lysozyme

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ARTICLE INFO

Article history:
Received 31 December 2015
Received in revised form
14 March 2016
Accepted 18 March 2016
Available online 21 March 2016

Keywords:
Extracellular traps (ETs)
c-type lysozyme
Shrimp
Hemocytes

ABSTRACT

In invertebrates, hemocytes play an important role in immune responses. Recently, a novel form of innate immune mechanism called extracellular traps (ETs) was identified in shrimps, where DNA and antimicrobial peptides form complex structure to entrap the invading microbes. In this study, we detected the formation of ETs from hemocytes of kuruma shrimp in response to various stimulations, including phorbol myristate acetate (PMA), lipopolysaccharide (LPS), peptidoglycan (PGN) and *Escherichia coli*. *E. coli* cells were also found to be trapped by ET fibers. Fluorescence imaging revealed that c-type lysozyme proteins were released around the ET complex after *E. coli* stimulation, suggesting the presence of a coupled antimicrobial immune response involving ET formation and AMP release.

1. Introduction

In mammals, the discovery of extracellular trap (ETs) formation seen in neutrophils (NETs) described as a new antimicrobial mechanism that releases net-like traps made of chromatin fibers equipped with histones and antimicrobial peptides [1–3]. The fiber-like structures entangles pathogens, and then kills pathogens with the combination of antimicrobial activities by histone proteins, granular components, and other cytoplasmic proteins released from neutrophils [2,4]. It has been discussed that NETs are the forefront of the innate immune system where aside from the entrapment of microbes and the bactericidal functions of their histone components, NETs also signals danger forming a part of the "danger associated molecular patterns" (DAMPs) [2].

ETs formation was later on discovered likewise in the invertebrates, which solely depend on innate immunity, establishing that this phenomenon is thus an ancient immune response [5,6]. Similar to that of the mammalian NETs, in the oyster *Crassostrea gigas*, DNA traps were armed with antimicrobial histones [6].

Recently, ETs formation was also reported in hemocytes of shrimp *Litopenaeus vannamei* [7] where its DNA fibers were shown to have antimicrobial activity against *Escherichia coli* [7,8]. Although ETs in *L. vannamei* was likewise found to be armed with histone proteins, whether it is also directly involved in the ETs antimicrobial mechanism together with the DNA fibers, is yet to be established. In the case of three other invertebrate species: crab *Carcinus maenas*, mussel *Mytulis edulis*, and sea anemone *Actinia equina*, AMPs were also discussed to aid in the antimicrobial activity of ETs in a study on ETs coupled antimicrobial histones [5]. Although clear evidence of the interaction of these AMPs in invertebrate ETs has not been well established.

In penaeid shrimp, several types of AMPs were identified including penaeidin, crustin, anti-lipopolysaccharide factor, histone and lysozyme [9,10]. C-type lysozyme in particular was found to be a component of mammalian NETs [11]. Lysozymes are characterized into 6 types: chicken- or conventional- lysozyme (c-type), goose-lysozyme (g-type), plant lysozyme, T4-phage lysozyme (phage-type), and invertebrate- lysozyme (i-type) [10]. In, kuruma shrimp, a c-type lysozyme was identified and found antimicrobial activity against both Gram-positive and Gram-negative bacteria and that the deficiency of c-type lysozyme reduces circulating hemocyte number [12,13].

However, little is known regarding the relationship between the direct involvements of these particular AMPs to ETs formation in penaeid shrimp. Here we investigate ETs formation by hemocytes of kuruma shrimp (*Marsupenaeus japonicus*) and its relationship with antimicrobial peptides. This study provides additional information on both shrimp ETs formation and c-type lysozyme.

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http://dx.doi.org/10.1016/j.fsi.2016.03.039
1050-4648/

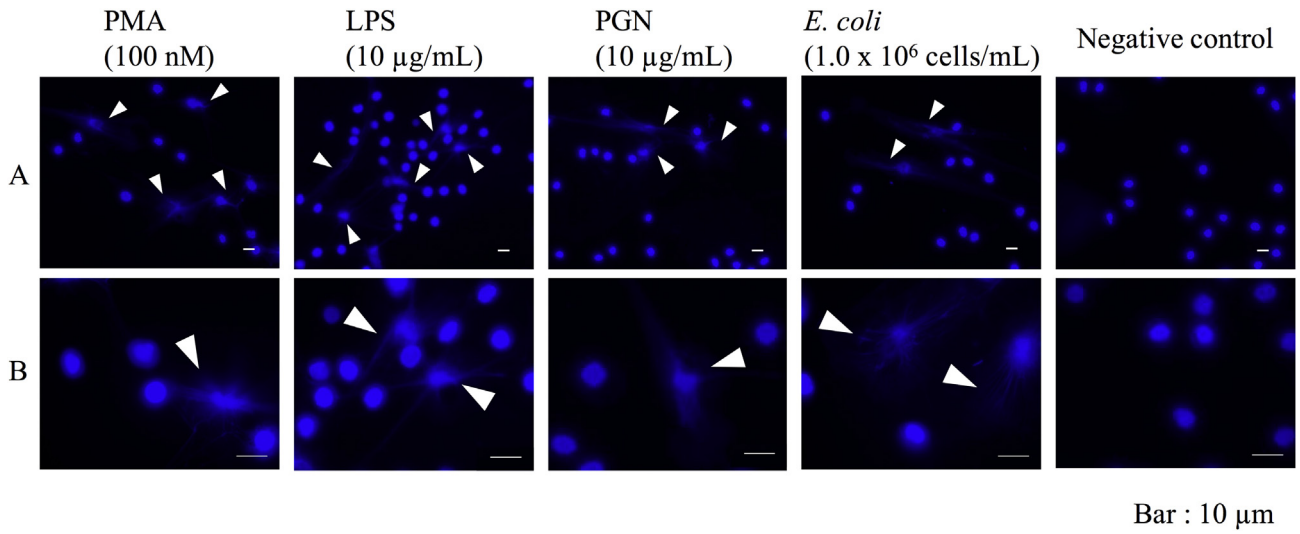

Fig. 1. Kuruma shrimp hemocytes formed ETs after stimulation. Fluorescence microscopy images of kuruma shrimp hemocytes stimulated with 100 nM PMA, 10 μg/mL LPS, 10 μg/mL PGN and 1.0×10^6 cells/mL of *E. coli*. ET fibers and DNA (blue) were formed in several hemocytes as indicated by white arrows. Rows A and B show different magnifications. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

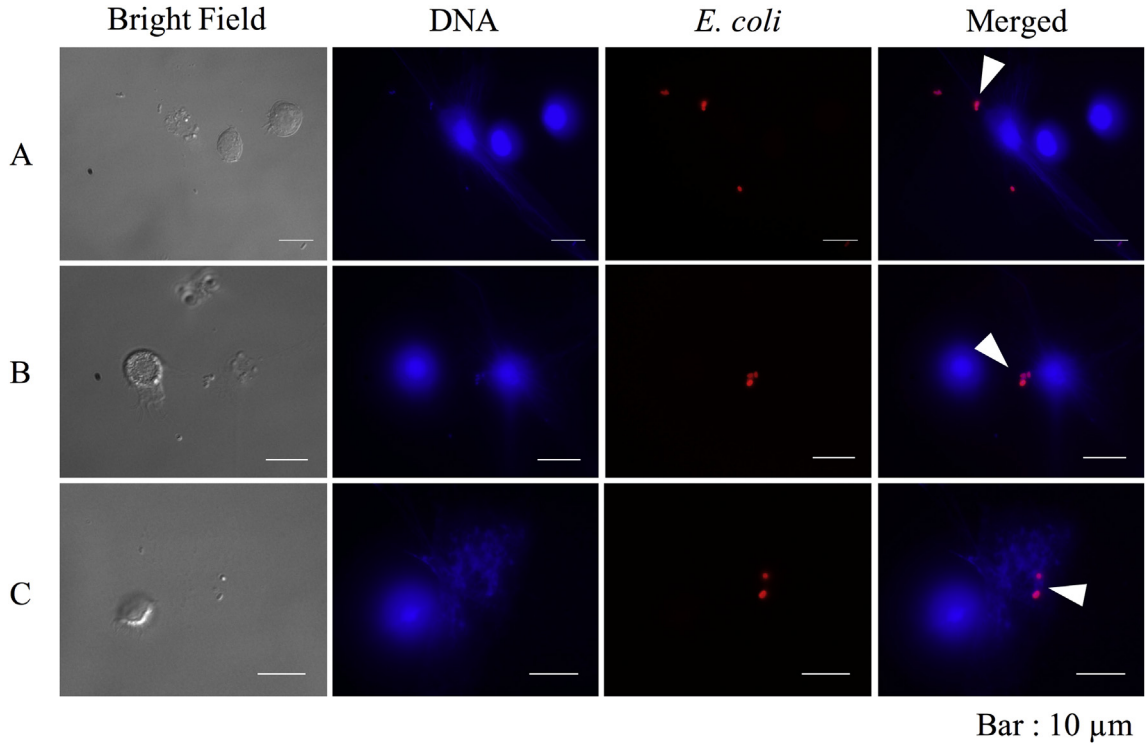

Fig. 2. ETs of kuruma shrimp hemocytes entangle *E. coli* cells. Fluorescence microscopy images showing *E. coli* cells (red) and DNA (blue). Some of the *E. coli* cells (white arrows) are entangled in ET fibers. Rows A, B and C show different fields of view. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Experimental animals

Kuruma shrimp (*Marsupenaeus japonicus)* weighing 10–15 g were purchased from a commercial shrimp farm in Miyazaki, Japan. All shrimps were kept in artificial seawater of 35 ppm at 25 °C and fed with commercial shrimp diet before experimental procedure.

2.2. Bacterial strain and staining of bacterial cells

Escherichia coli JM109 were grown overnight in Luria-Bertani (LB) medium at 37 °C. The *E. coli* cells were fixed with 0.5% formalin solution for 24 h at 4 °C and then stained with PKH26 fluorescent dye (Sigma-Aldrich Co., Japan) following the manufacturer's instructions. The number of stained *E. coli* cells was counted and 1.0×10^6 cells/ml of bacterial cells were used in the succeeding experiments.