한국어병학회지 제31권 제2호 (2018)
J. Fish Pathol., 31(2) : 109~113

www.ksfp.org
pISSN 1226-0819, eISSN 2233-5412
http://dx.doi.org/10.7847/jfp.2018.31.2.109

Olive flounder (*Paralichthys olivaceus*) leukocytes stimulated with poly (I:C) could kill *Miamiensis avidus* (Ciliophora: Scuticociliatia) only when ciliates were immobilized by antiserum

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The purpose of the present study was to determine whether the scuticocidal activity of olive flounder (*Paralichthys olivaceus*) head-kidney leucocytes can be enhanced by stimulation with polyinosine-polycytosine [poly (I:C)]. The growth of *Miamiensis avidus* was not affected by exposure to unstimulated or poly (I:C)-stimulated leucocytes alone, heat-inactivated immune serum alone, or unstimulated leucocytes plus heat-inactivated immune serum. However, leucocytes stimulated with poly (I:C) showed clearly high scuticocidal activity against *M. avidus* in the presence of heat-inactivated immune serum. Furthermore, numerous poly (I:C)-stimulated leucocytes occupied the surface of scuticociliates in the presence of the heat-inactivated immune serum, which led to lysis of scuticociliates. These results suggest that both of the stimulation of leukocytes and the immobilization of scuticociliates are necessary to kill scuticociliates by leukocytes.

Key words: *Miamiensis avidus, Paralichthys olivaceus,* Scuticocidal activity of leucocytes, Poly (I:C)-stimulation, Immobilization

Scuticociliatosis caused by several species of histophagous scuticociliates has been one of the most devastating diseases in farm-reared fish, especially in flat fishes such as turbot *Scophthalmus maximus* (Sterud et al., 2000; Iglesias et al., 2001; Alvarez-Pellitero et al., 2004; Puig et al., 2007; Ramos et al., 2007) and olive flounder *Paralichthys olivaceus* (Yoshinaga & Nakazoe, 1993; Jee et al., 2001; Kim et al., 2004; Jung et al., 2007). In Korea, *Miamiensis avidus* (=*Philasterides dicentrarchi*) has been a notorious culprit that leads to mass mortality in cultured olive flounder (Kim et al., 2004; Jung et al., 2007).

Previous studies have demonstrated that complement activated through the classical pathway plays an important role in defense against scuticociliates in vaccinated fish (Iglesias et al., 2003; Lee and Kim, 2008a,b; Leiro et al., 2008; Sitjà-Bobadilla et al., 2008; Piazzon et al., 2011). However, researches on the role of leucocytes against scuticociliates have been poorly conducted. Although the scuticocidal activity of nitric oxide and oxygen radicals produced from activated leucocytes was demonstrated through *in vitro* exposure experiments (Lee et al., 2004; Piazzon et al., 2011), most studies showed that excretory-secretory components including proteases from scuticociliates affected on the immune responses of leucocytes (Kwon et al., 2002; Parama et al., 2007a,b). Recently, Piazzon et al. (2011) reported that turbot leucocytes were not helpful to kill *P. dicen-*

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trarchi in antibody-dependent complement-mediated killing assay.

The aim of the present study was to examine the role of leucocytes of olive flounder in killing of *M. avidus*, and we found that leucocytes stimulated with polyinosine-polycytosine [poly (I:C)] could actively kill *M. avidus* in the presence of heat-inactivated immune serum.

Materials and methods

Ciliates

Miamiensis avidus was isolated from the abdominal cavity of infected olive flounder (*Paralichthys olivaceus*) that were obtained from a fish farm in South Korea. Polymerase chain reaction was performed to confirm *M .avidus* using 18S rRNA gene-specific primers (Kim et al., 2004). The isolated ciliates were cultured in chinook salmon embryonic (CHSE)-214 cells supplemented with 10% fetal bovine serum (FBS, Sigma). The ciliates for experiments were harvested at the logarithmic phase by centrifugation at 1000 g for 5 min.

Immunization of fish to obtain immune serum and serum agglutination assay

Experiments for obtaining immune sera of olive flounder and for serum agglutination assay were conducted according to the methods described in Lee and Kim (2008b).

Isolation of head kidney leucocytes and stimulation with polyinosine-polycytosine [poly (I:C)]

Olive flounder were anaesthetized with tricaine methanesulfonate (MS222; Sigma). The head kidney was extracted by ventral incision and transferred to Eagle's minimum essential medium (MEM, Sigma) supplemented with heparin (10 U ml^{-1}, Sigma), penicillin (100 μg ml^{-1}, Gibco) and streptomycin (100 U ml^{-1}, Gibco). To get leucocytes, the cell suspensions obtained by forcing the organ through a nylon mesh were layered over a 51 % Percoll (Sigma). After centrifugation at 600 g for 30 min at 4°C, the leucocytes fraction was removed from the Percoll-medium interface, washed 1 time, stimulated with 100 μg/ml of poly (I:C) (Sigma) for 3 h, and washed 3 times with Hank's buffered salt solution (HBSS).

Analysis of scuticocidal activity of leucocytes

The ciliates were dispensed into wells of flat-bottomed 96-well microplates at a density of $1x10^2$ ciliates/50 μl of MEM supplemented with 10% heat-inactivated FBS (MEM-FBS) per well. The number of leucocytes used for analysis was 1 x 10^7 cells/50 μl of MEM-FBS/well. The experimental groups were as follows: i) ciliates plus 100 μl of MEM-FBS; ii) ciliates plus 50 μl of heat-inactivated (50°C for 30 min) olive flounder immune serum and 50 μl of MEM-FBS; iii) ciliates plus unstimulated leucocytes and 50 μl of MEM-FBS; iv) ciliates plus poly (I:C)-stimulated leucocytes and 50 μl of MEM-FBS; v) ciliates pre-incubated with 50 μl of the heat-inactivated immune serum for 10 min plus unstimulated leucocytes; vi) ciliates pre-incubated with 50 μl of the heat-inactivated immune serum for 10 min plus poly (I:C)-stimulated leucocytes. The micro titration plate was then incubated at 20°C, and counted survived number of ciliates at 3, 6, 12, 24, 36, 48, 60, and 72 h post-inoculation using a light microscope or haemo-cytometer.

Results

Scuticocidal activity of olive flounder head-kidney leucocytes stimulated with poly (I:C) was analyzed (Fig. 1). The results showed that the unstimulated leucocytes enhanced the growth of *M. avidus* in the absence of heat-inactivated immune serum. Whereas leucocytes stimulated with poly I:C showed some inhibitory activity at early period compared to the unstimulated leucocytes, but eventually failed to inhibit growth of the ciliates in the absence of heat-in-

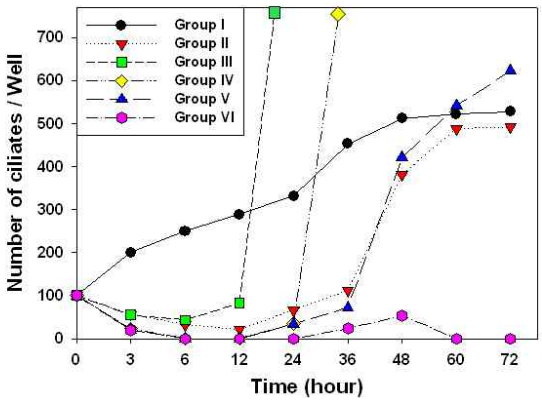

Fig. 1. Effects of polyinosine-polycytosine [poly (I:C)] on scuticocidal activity of olive flounder (*Paralichthys olivaceus*) leucocytes. Ciliates (*Miamiensis avidus*; 1×10^2 cells) were preincubated with heat-inactivated olive flounder immune serum and exposed to 1×10^7 cells of unstimulated leucocytes (Group V) or poly (I:C)-stimulated leucocytes (Group VI). Controls were included MEM supplemented with 10% heat-inactivated FBS (MEM-FBS) alone (Group I), heat-inactivated olive flounder immune serum alone (Group II), unstimulated leucocytes alone (Group III), and poly (I:C)-stimulated leucocytes alone (Group IV). The number of survived ciliates were counted at 3, 6, 12, 24, 36, 48, 60, and 72 h post-inoculation using a light microscope or haemocytometer.

activated immune serum. The growth of *M. avidus* exposed to heat inactivated immune serum alone was retarded at an early period compared to ciliates in culture medium alone. The unstimulated leucocytes in the presence of heat-inactivated immune serum were not effective in *M. avidus* killing, led to a similar growth pattern to that of the ciliates exposed to heat-inactivated immune serum alone. In contrast, leucocytes stimulated with poly (I:C) showed evidently strong scuticocidal activity against *M. avidus* in the presence of heat-inactivated immune serum. Lots of the stimulated leucocytes attached to the ciliates surface in the presence of heat-inactivated immune serum (Fig. 2), and the surrounded ciliates died by lysis in the end.

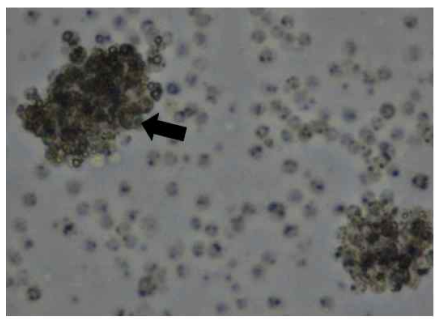

Fig. 2. A micrograph of *Miamiensis avidus* incubated with poly (I:C)-stimulated leucocytes of olive flounder in the presence of heat-inactivated olive flounder immune serum. Lots of leucocytes attached to the surface of the ciliate (arrow). Observation at ×400.

Discussion

Although fish leucocytes such as neutrophils, macrophages, and natural cytotoxic cells (NCC) are one of the first defense lines against pathogens infection, their role in scuticociliates infection is poorly understood. Piazzon et al. (2011) reported that killing of *P. dicentrarchi* by a combination of purified antibodies and normal turbot serum was not changed by addition of turbot leucocytes that were stimulated with various fractions of the ciliates. On the contrary, the leucocytes were fed by the ciliates, leading to increase of the ciliate number. From these results, they concluded that cellular immune responses are not a critical factor for killing of scuticociliates. However, the results of the present study showed that a synthetic Toll-like receptor 3 agonist, poly (I:C), enhanced scuticocidal activity of olive flounder leucocytes in the presence of heat-inactivated immune serum.

Several reasons can be presumed as the cause of difference between the results of Piazzon et al. (2011) and the present study. First, we used poly (I:C) as the stimulant of leucocytes instead of scuticociliates fractions that were used in the study of Piazzon et al. (2011). Poly (I:C) is a synthetic double-stranded RNA analog, and known as the most potent inducer of type I interferons and gamma interferon (IFN-γ)

in vertebrates including fish (Robersten, 2006). Although type I interferons are well-known to elicit strong antiviral immune responses, their protective potential against various protozoan parasites has also been demonstrated in mammals (Bogdan et al., 2004). Furthermore, IFN-γ-mediated signal activates leucocytes, such as macrophages and natural killer cells, and induces expression of inducible nitric oxide synthase (iNOS) gene, which drives production of nitric oxide (Pindado et al., 2007) that is an important factor in host defense against pathogens including extracellular parasites. Thus, the strong scuticocidal activity of leucocytes in the present study suggests that leucocytes stimulated with poly (I:C) might be better able to defeat scuticociliates than those stimulated with the ciliates fractions. Second, the ratio of leucocytes number versus scuticociliates number, 200:1, in Piazzon et al. (2011) was too low when compared with that of the present study (10^5:1). According to our preliminary experimental results, more than 10^4:1 ratio is needed to get results showing evident killing activity of leucocytes.

In the present study, even poly (I:C)-stimulated leucocytes did not show any clear scuticocidal activity when heat-inactivated immune serum was not included. In contrast, numerous poly (I:C)-stimulated leucocytes occupied the surface of scuticociliates in the presence of the heat-inactivated immune serum, which led to lysis of scuticociliates. These results suggest that both of the stimulation of leukocytes and the immobilization of scuticociliates might be needed to kill scuticociliates by leukocytes.

Acknowledgements

This research was a part of the project titled 'Development of Fish Vaccines and Human Resource Training', funded by the Ministry of Oceans and Fisheries, Korea.

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Manuscript Received : Oct 2, 2018
Revised : Nov 19, 2018
Accepted : Nov 19, 2018