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Short communication

Comparative analysis of the production of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) from macrophages exposed to high virulent and low virulent strains of *Edwardsiella tarda*

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ABSTRACT

We previously reported that high virulent strain (NUF251) of *Edwardsiella tarda* has an ability to prevent the production of reactive oxygen species by macrophages, and is even capable of surviving and multiplying within Japanese flounder (*Paralichthys olivaceus*) peritoneal macrophages, whereas the low virulent strain (NUF194) has no such ability. In this study, we found that NUF251 and NUF194 induced NO and TNF-α production from Japanese flounder peritoneal macrophages, and NUF251 caused faster induction of NO release and much higher level of TNF-α production than NUF194. In addition, similar differences between two strains in terms of the induction of NO and TNF-α production were also observed in mouse macrophage cell line RAW264.7 cells. Our results suggest that the potent ability to induce the production of NO and TNF-α from macrophages may be one of the factors responsible for the virulence of *E. tarda*.

Edwardsiella tarda, a Gram-negative bacterium, is one of the major bacterial pathogens in freshwater and marine fish, and natural *E. tarda* infection has been recorded predominantly in Japanese eel, Japanese flounder (*Paralichthys olivaceus*), channel catfish (*Ictalurus punctatus*) [1,2], and in many other fish species [3]. Some mammals are also known to be infected with *E. tarda* [4]. Edwardsiellosis in Japanese flounder shows suppurative inflammation and hernia as main symptoms [5], and infectious disease caused by *E. tarda* is one of the most serious problems in aquaculture industry especially to Japanese flounder. In spite of the serious threat of *E. tarda* as a fish pathogen and the increasing frequency of the disease, little is known about the exact pathogenesis of *E. tarda* infection. Dermatotoxin [6], hemolysis [7], or ability to invade epithelial cells [8] have been suggested as potential virulence factors of this bacterium. In our previous study, we found that the responses of peritoneal macrophages of Japanese flounder (*P. olivaceus*) against high virulent strain (NUF251) and low virulent strain (NUF194) of *E. tarda* were different [9], and our results suggested that NUF251 may have an ability to prevent ROS generation by macrophages and survive inside macrophages.

Macrophages play pivotal roles in the defense mechanism against invading bacteria by producing bactericidal agents such as NO and ROS, and proinflammatory cytokines (e.g. TNF-α). NO and TNF-α are known to play not only important roles in immune defense system but also to injure host cells and tissues [10–13]. Similar to mammalian immune systems, previous studies have demonstrated that several bacterial infections in fish are also accompanied with the induction of various inflammatory mediators. For instance, *Aeromonas hydrophila* induced iNOS-mediated NO production and secretion of TNF-α and other cytokines from kidney cells of zebrafish (*Danio rerio*) [14]. NO release was observed in carp (*Cyprinus carpio* L.) kidney leukocytes during *in vivo* bacterial infection [15]. It has been reported that production of TNF-α like factor by rainbow trout head kidney macrophages was detected by L929 cell killing assay during *in vitro* bacterial infection model [16]. However, the exact roles of inflammatory factor such as

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NO and TNF-α in bacterial infection in fish are unclear. Therefore, in this study, we investigated the responses of Japanese flounder peritoneal macrophages and mouse macrophage cell line RAW 264.7 cells after exposure to high and low virulent strains of *E. tarda* in terms of NO and TNF-α production.

The culture and maintenance of two strains of *E. tarda* (NUF251 and NUF194), the details of handling of Japanese flounder used for the preparation of peritoneal macrophages, and the culture of the macrophages were executed by the same methods as described previously [9]. To detect the production of NO, we employed Griess method that is an assay for spectrophotometric quantification of nitrite, a stable reaction product of nitric oxide with molecular oxygen, using Griess reagent (3 mM sulfanilic acid, 30 μM N-1-naphtyl-ethlenediamine dihydrochloride, 25% glacial acetic acid). The freshly isolated peritoneal macrophage population from Japanese flounder were cultured in 96-well plates (2×10^4 cells/well) in RPMI 1640 medium containing 10% FBS for 24 h at 25 °C, and then *E. tarda* cells (final 2×10^4 CFU/well) were added and incubated at 25 °C. After the indicated periods of time, the culture medium was collected to determine nitrite concentration. In the experiment, we used a macrophage population from one fish, but when the amount of cells was insufficient, pooled cells from two fish were used. No significant differences between pooled and non-pooled cells were observed. For the analysis, we repeated three separate experiments and conducted triplicate measurements for each experiment. As shown in Fig. 1, both strains of *E. tarda* induced NO production from Japanese flounder peritoneal macrophages in a time-dependent manner, and NUF251 induced NO production with faster kinetics than that of NUF194. After 3 h incubation, the NO level in the culture medium reached the maximum when exposed to virulent NUF251, whereas only half of the maximum level of NO was detected in the case of low virulent strain NUF194. No significant level of NO was detected in the culture supernatant of Japanese flounder peritoneal macrophages without the addition of these bacterial strains (data not shown). To ascertain whether or not Japanese flounder peritoneal macrophages can produce TNF-α into the culture medium responding to *Edwardsiella tarda*, we employed sandwich enzyme-linked immunosorbent assay (ELISA) with monoclonal and polyclonal anti-mouse TNF-α antibodies (R&D Systems, Minneapolis, MN) to two different epitopes on mouse TNF-α molecule. The ELISA procedure was performed according to the manufacturer's protocol. The TNF-α concentration was estimated from a reference to a standard curve for serial twofold dilution of recombinant mouse TNF-α (R&D Systems, Minneapolis, MN). As shown in Fig. 2, low but significant level of TNF-α was detected in the culture medium of Japanese flounder macrophages exposed to NUF251, whereas no significant level of TNF-α was induced by NUF194 even after 48 h incubation. Although these results suggest that NUF251 has a greater activity to induce TNF-α secretion from Japanese flounder macrophages than NUF194, it is uncertain if the exact level of fish TNF-α could be detected by our ELISA system in which anti-mouse TNF-α antibodies were used instead of the antibodies against actual Japanese flounder TNF-α. To confirm the different responses of macrophages against NUF251 and NUF194, we carried out the same analysis regarding NO and TNF-α production in mouse macrophage cell line RAW264.7.

RAW264.7 cells were from the American Type Culture Collection (Rockville, MD), and were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (100 μg/mL) as described previously [17]. To adherent RAW264.7 cells in 96-well plates (2×10^4 cells/well), *E. tarda* cells (final 2×10^4 CFU/well) were added. After the indicated periods of time, NO levels in the culture medium were determined as described above. As shown in Figs. 3a, similar to Japanese flounder macrophages, the higher level of NO production with faster kinetics from RAW264.7 cells were induced by NUF251 as compared to that induced by NUF194. The presence of an inducible pathway for NO production from L-arginine catalyzed by inducible NO synthase (iNOS) has been well documented in macrophages in response to cytokines and LPS [18,19]. Therefore, we investigated the kinetics of expression level of iNOS mRNA in RAW264.7 cells exposed to two strains of *E. tarda* by RT-PCR. The RT-PCR was performed by the following procedure. Adherent RAW264.7 cells in 12-well plate (2×10^5 cells/well) were exposed to each strain of *E. tarda* (final 2×10^5 CFU/well). After the incubation for the indicated time periods, total RNA was isolated from the cells with Trizol reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed with an oligo dT primer in a 10 μL reaction volume using PrimeScript 1st strand cDNA synthesis Kit (TaKaRa) according to the manufacturer's instructions. PCR was performed with 1 cycle of 70 s at 95 °C, 20 cycles of 55 s at 93 °C, 45 s at 61 °C, 40 s at 72 °C, and 1 cycle of 100 s at 72 °C, in a 25 μL reaction mixture containing 12.5 μL of GoTaq Green

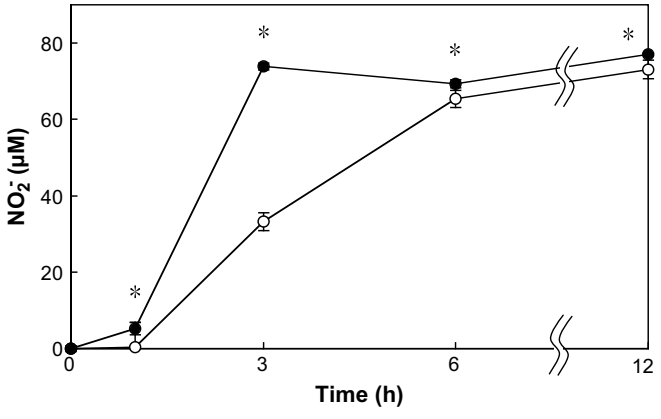

Fig. 1. Time-course analysis of NO production from Japanese flounder peritoneal macrophages after exposure to high virulent strain NUF251 (●) and low virulent strain NUF194 (○) of *E. tarda*. To the adherent macrophages in 96-well plates (2×10^4 cells/well) in RPMI 1640 medium containing 10% FBS, each bacterial cell suspension was added (final 2×10^4 CFU/well). After the indicated periods of time at 25 °C, the culture supernatant was withdrawn from each well, and subjected to the determination of NO level as described in the text. Each point represents an average of triplicate measurements. Each bar represents standard deviation. Asterisks indicate significant differences between two strains ($^*P < 0.05$).

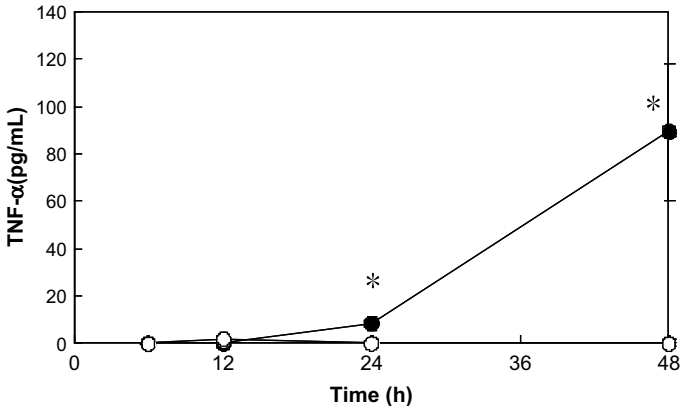

Fig. 2. Time-course analysis of TNF-α production from Japanese flounder peritoneal macrophages after exposure to high virulent strain NUF251 (●) and low virulent strain NUF194 (○) of *E. tarda*. To the adherent macrophages in 96-well plates (2×10^4 cells/well) in RPMI 1640 medium containing 10% FBS, each bacterial cell suspension was added (final 2×10^4 CFU/well). After the indicated periods of time at 25 °C, the culture supernatant was withdrawn from each well, and subjected to the determination of TNF-α level as described in the text. Each point represents an average of triplicate measurements. Each bar represents standard deviation. Asterisks indicate significant differences between two strains ($^*P < 0.05$).