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Short communication

Edwardsiella tarda evades serum killing by preventing complement activation via the alternative pathway

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ABSTRACT

Edwardsiella tarda is a Gram-negative bacterium with a broad host range that includes a wide variety of farmed fish as well as humans. *E. tarda* has long been known to be able to survive in host serum, but the relevant mechanism is unclear. In this study, we investigated the fundamental question, i.e. whether *E. tarda* activated serum complement or not. We found that (i) when incubated with flounder serum, *E. tarda* exhibited a high survival rate (87.6%), which was slightly but significantly reduced in the presence of Mg^{2+}; (ii) *E. tarda*-incubated serum possessed strong hemolytic activity and bactericidal activity, (iii) compared to the serum incubated with a complement-sensitive laboratory *Escherichia coli* strain, *E. tarda*-incubated serum exhibited much less chemotactic activity, (iv) in contrast to the serum incubated with live *E. tarda*, the serum incubated with heat-inactivated *E. tarda* exhibited no apparent hemolytic capacity. Taken together, these results indicate for the first time that *E. tarda* circumvents serum attack by preventing, to a large extent, complement activation via the alternative pathway, and that heat-labile surface structures likely play an essential role in the complement evasion of *E. tarda*.

1. Introduction

The complement system is a vital part of innate immune defense against foreign organisms including bacteria, virus, and parasite. The complement system can be activated through three pathways, i.e. the classical pathway, the alternative pathway, and the lectin pathway [1–3]. The three pathways converge at the cleavage of C3 to C3a and C3b, which lead to a series of events involving cleavage of C5 and assembly of C5b, C6, C7, C8, and C9 to generate the membrane attack complex (MAC) that induces osmotic lysis of the target cells [4,5]. In the alternative pathway, C3b derived from spontaneous C3 hydrolysis covalently binds to various activating surface antigens, such as those on bacterial cells, and the bound C3b forms a complex with Factor B. The Factor D present in the serum cleaves Factor B to release Ba and yields the alternative C3 convertase C3bBb. This convertase cleaves the surrounding C3 into C3a and C3b, the latter is able to bind to an activating microbial surface. Such surface bound C3b can then generate additional stable C3 convertase in the presence of Mg^{2+} and Factor B, resulting in the amplification of C3 cleavage and a rapid massive deposition of C3b onto the activating surface. Binding of newly formed C3b to an existing C3 convertase (C3bBb) will lead eventually to the formation of the alternative C5 convertase C3bBb3b and the formation of MAC (C5b-C9) [4,5].

Edwardsiella tarda is a Gram-negative bacterium of the family Enterobacteriaceae. It is recognized as a severe fish pathogen with a broad host range including many species of economically important fish [6]. Fish infected with *E. tarda* develop a systematic disease called edwardsiellosis that can cause heavy economic losses. In addition to fish, *E. tarda* is also a human pathogen and is associated with various clinical diseases in humans. Accumulating evidences have indicated that *E. tarda* is an intracellular pathogen with the capacity to evade host immune defense, which is reflected, in one aspect, in the observation that *E. tarda* can survive in host serum [7,8]. However, the relevant mechanism is unclear. In this study, we aimed to examine the effect of *E. tarda* on serum complement activity and to investigate whether *E. tarda* was able to prevent activation of the alternative pathway.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

The fish bacterial pathogen *E. tarda* TX1 was reported previously [9]. *Escherichia coli* DH5α was purchased from Tiangen (Beijing, China). Both strains were cultured in Luria–Bertani broth (LB) medium at 28 °C (for *E. tarda*) or 37 °C (for *E. coli*).

2.2. Fish

Japanese flounder (*Paralichthys olivaceus*) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. Before experiment, fish were acclimatized in the laboratory for two weeks and randomly sampled for the examination of the presence of bacteria in blood, liver, kidney, and spleen as reported previously [9]. No bacteria were detected in the examined fish. Enzyme-linked immunosorbent assay detected no antibodies against *E. tarda* TX1 or *E. coli* DH5α in the sera of sampled fish. Before blood collection, fish were anaesthetized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA).

2.3. Serum survival assay

Blood was withdrawn from the caudal vein of flounder and placed on ice immediately. The blood was allowed for clotting for 2 h. Serum was then collected after centrifugation and stored in aliquots at −80 °C until use. For serum survival assay, the serum was treated with or without heating at 56 °C for 30 min. *E. tarda* TX1 and *E. coli* DH5α were cultured in LB medium to an OD_{600} of 0.8 in different tubes. The cells were washed and resuspended to 2×10^6 CFU/ml in Hank's Balanced Salt Solution (HBSS) (Solarbio, Beijing, China) that contains no Mg^{2+} or Ca^{2+}. The bacterial suspension was mixed with an equal volume of treated or untreated serum in the presence or absence of 0.04 M ethylenediaminetetraacetic acid (EDTA) disodium salt or 0.02 M $MgCl_2$. The control cells were mixed with HBSS. After incubation at 28 °C for 1 h, the mixture was serially diluted and plated in triplicate on LB agar plates. The plates were incubated at 28 °C for 48 h, and the colonies that appeared on the plates were enumerated. The genetic nature of the colonies was verified by PCR with primers specific to *E. tarda* TX1 and *E. coli* DH5α. The survival rate was calculated as follows: (number of cells that survived serum treatment/number of cells that survived control treatment) × 100%. The assay was performed three times.

2.4. Bacterial surface polarity

Surface polarity was determined by microbial adhesion to solvent (MATS) technique as reported previously [10].

2.5. Hemolytic and bactericidal activity of bacteria-treated serum

To prepare *E. coli*- and *E. tarda*-treated sera, *E. tarda* TX1 and *E. coli* DH5α were cultured in LB medium to an OD_{600} of 0.8, and the bacterial concentration was adjusted to 10^9 CFU/ml in HBSS containing 0.02 M $MgCl_2$ (HBSS-Mg). Flounder serum was diluted two times in HBSS-Mg. The diluted serum was mixed with an equal volume of bacterial suspension or HBSS-Mg. The mixture was incubated at 28 °C for 1 h. After incubation, the mixture was diluted two times in HBSS-Mg and passed through a 0.22 μM filter to remove any bacterial cells. The treated serum thus obtained was diluted serially in HBSS-Mg and used for hemolysis assay and bactericidal activity. For hemolysis assay, rabbit red blood cells (RRBC) (purchased from Guangzhou Future Technology Co., Ltd, Guangzhou, China) were washed and resuspended in HBSS-Mg. Ten microliters of RRBC suspension were mixed with 50 μl different dilutions of the treated serum a in 96-well culture plate. The plate was incubated at 28 °C for 30 min. After incubation, the supernatant was collected by centrifugation and determined for absorbance at 450 nm. Bactericidal assay was performed in HBSS-Mg as described above in "Serum survival assay", and bactericidal activity was defined as {1 - (number of cells that survived serum treatment/number of cells that survived control treatment)} × 100%. The experiments were performed three times.

2.6. Chemotaxis

Flounder peripheral blood leukocytes (PBL) were prepared with 61% Percoll gradient as described previously [11]. *E. coli*- and *E. tarda*-incubated sera were prepared as described above in "Hemolytic and bactericidal activity of bacteria-treated serum". The chemotactic activity of bacteria-incubated serum and normal serum against PBL was determined in Transwell as reported previously [12]. Chemotactic index was presented as fold increase in the number of migrated cells induced by bacteria-treated serum compared to that induced by normal serum. The assay was performed three times. For microscopy, ten microliters of the migrated cells were taken from the Transwell and observed under a microscope (Nikon E800, Japan).

2.7. Statistical analysis

All experiments were performed three times. Statistical analyses were performed using analysis of variance (ANOVA) in SPSS 18.0 package (SPSS Inc., Chicago, IL, USA). Data are presented as means plus or minus standard errors of the means (SEM), and statistical significance was determined with Student's *t*-test. In all cases, significance was defined as $P < 0.05$.

3. Results and discussion

3.1. Survival of E. tarda in fish serum

When *E. tarda* TX1 was incubated with the serum of flounder, a susceptible host of *E. tarda*, the survival rate of the bacteria was 87.6%. In sharp contrast, the survival rate of *E. coli* DH5α, a serum sensitive laboratory strain, incubated under the same condition was 0.8% (Fig. 1). These results confirmed previous observations that *E. tarda* can resist the killing effect of fish serum [13,14]. It is known that divalent metal ions are essential for complement activation [15]. In the alternative pathway, Mg^{2+} is a natural cofactor involved in the formation of stable C3 convertase [16–19]. In our study, we found that in the presence of Mg^{2+}, the serum survival rates of *E. tarda* and *E. coli* were reduced to 67.8% and 0.2% respectively, which were significantly ($P < 0.05$ and $P < 0.01$ respectively) lower than the serum survival rates in the absence of Mg^{2+}. In both the presence and absence of Mg^{2+}, the survival rates of *E. tarda* were significantly higher than those of *E. coli*. When EDTA, which chelates Mg^{2+}, was added to the serum, the survival rates of *E. tarda* and *E. coli* increased to the comparable levels of 97% and 88% respectively. These results indicate an essential role of Mg^{2+} in the bactericidal activity of flounder serum, which implies that the killing is most likely mediated by the complement activated through the alternative pathway. Similar enhancing effects of Mg^{2+} on the alternative pathway of complement activation have been reported previously in other teleost [20,21].