



NOTE

Public Health

Immunogenicity and protective efficacy of *Vibrio vulnificus* flagellin protein FlaB in a wound infection model

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ABSTRACT. *Vibrio vulnificus* is known as an opportunistic bacterial pathogen that causes primary septicemia and wound infection in humans. Recently, the incidence of wound infection by *V. vulnificus* is increasing in warm countries. In this study, we examined a vaccine antigen against *V. vulnificus* in mice. FlaB, a component protein of the *V. vulnificus* flagellum, was expressed as a recombinant protein, named rFlaB. After immunization of mice with rFlaB, the mice were challenged by subcutaneous inoculation with *V. vulnificus*. Bacterial burdens in muscular tissue at the infection site in rFlaB-immunized mice were significantly decreased compared with those of control mice. We found that rFlaB immunization can partially suppress proliferation of *V. vulnificus* at the local infection site.

KEY WORDS: epsis, vaccine, *Vibrio vulnificus*, wound infection

Vibrio vulnificus, a gram-negative estuarine bacterium, causes primary septicemia and wound infections in humans [7, 8, 15, 18, 21, 23]. Primary septicemia is caused by the ingestion of contaminated seafood, and wound infections can result from exposure to seawater or through handling marine products [8, 15, 18]. Septicemia caused by *V. vulnificus* is associated clinically with liver cirrhosis, immune compromised condition, hemochromatosis, and alcoholism. Over 50% of patients with septicemia die within the first 48 hr of admission. Furthermore, Oliver reported recently that over 80% of infected people with *V. vulnificus* wound infection has no underlying diseases, and that the incidence of wound infection per year has more than doubled in the last decade [21]. Thus, prevention of *V. vulnificus* infection, especially wound infection, is urgently needed.

It is well known that bacterial flagella contribute to the pathogenesis of many bacterial pathogens through adhesion, invasion, and chemotaxis in a host [1, 2, 4, 5, 9, 17, 20, 22]. Flagella are composed of multiple proteins and subunits and are large motility organelles for bacterial cells [9, 25]. Flagellin proteins, the main component of bacterial flagellum, have become target antigens for vaccine development in various bacterial infections due to recognition by Toll-like receptor 5 (TLR5) [6, 11, 12, 19, 24]. *V. vulnificus* possesses six flagellin genes, *flaA*, *-B*, *-C*, *-D*, *-E* and *-F* in two loci, and at least 5 flagellin proteins, FlaA, *-B*, *-C*, *-D* and *-F* were expressed *in vitro* [9]. Within these flagellin proteins, FlaB and *-D* share 99% identity at the amino acid level, and deletion of both genes resulted in a significantly decreased length of flagellum, motility, cytotoxicity and lethality [9]. FlaB of *V. vulnificus* appears to be the most crucial component of the flagellum and is known to have mucosal adjuvant activity to induce protective immunity by activating innate immune responses via the TLR5 signaling pathway [11, 19, 24]. From these facts, FlaB of *V. vulnificus* appears to be a good candidate vaccine antigen to prevent proliferation of *V. vulnificus*. Therefore, we investigated whether or not immunization with purified FlaB can prevent wound infection by *V. vulnificus*. Full length of FlaB encoding gene, *flaB*, was amplified from *V. vulnificus* CMCP6 genome, and cloned into pET29b (Novagen, MA, U.S.A.). Recombinant FlaB protein (rFlaB) fused to a His-tag at the C-terminus was purified using Ni-NTA affinity column (Merck Biosciences, Darmstadt, Germany). Female 5-week-old C57BL/6 mice (Charles River Laboratories Japan, Yokohama, Japan) were immunized by subcutaneously (s.c.) injection with 50 μ g rFlaB, and boosted with 50 μ g rFlaB at 14 and 35 days after 1st immunization. Mice in the control group were s.c. injected with sterile phosphate buffered saline (PBS) without rFlaB in the same procedures. All experiments involving mice were carried out in accordance with the Kitasato University guidelines for animal treatment (Approval No.16-059). At 2 weeks after last immunization, sera were collected from the control mice and the rFlaB-immunized mice. The rFlaB and sonicated *V. vulnificus* were applied to SDS-PAGE at 0.5 μ g/lane and 50 μ g/lane respectively. The rFlaB-immunized mice sera were used as primary antibody. Primary antibodies were detected using horseradish peroxidase (HRP)-conjugated

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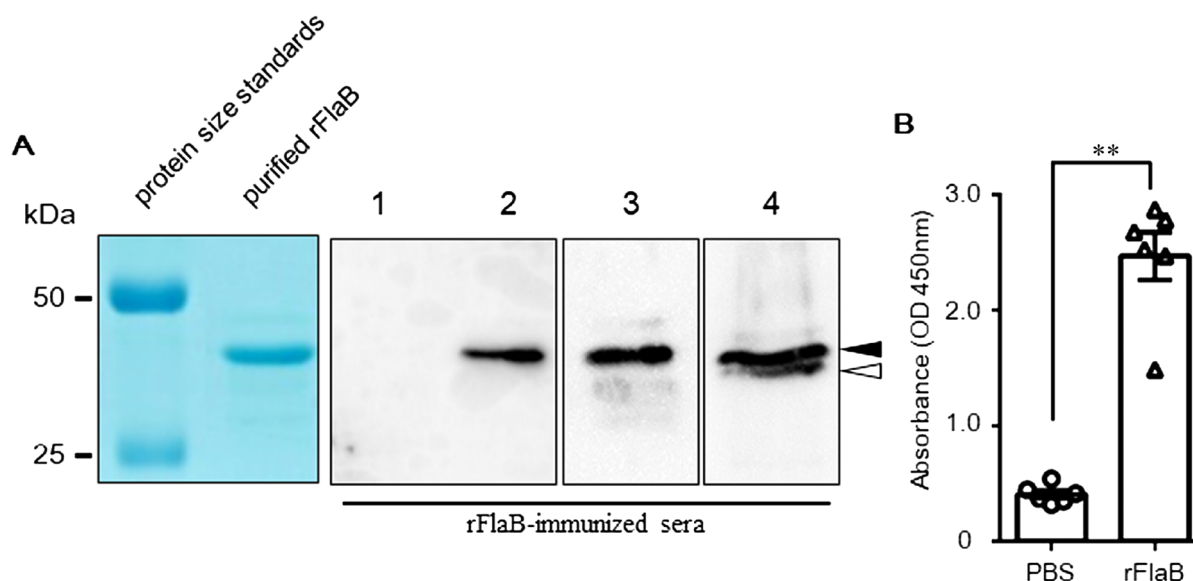


Fig. 1. A. Marker proteins and purified rFlaB applied to SDS-PAGE were shown as protein size standards (left panel). Arrowheads indicate detected bands. The rFlaB-immunized mice sera were analyzed by western blotting for reactivity against sonicated JE109 (DE) with pET29b empty vector (lane 1), sonicated JE109 (DE) with pET29b encoding *flaB* (lane 2), rFlaB (lane 3), and sonicated *V. vulnificus* (lane 4). B. Anti-rFlaB antibody titers, reflected by serum IgG level against rFlaB, were determined by IgG specific ELISA. Each symbol represents an individual mouse. Error bars indicate SEM. ** $P < 0.01$; Mann-Whitney *U*-test. Mice immunized PBS and adjuvant without rFlaB served as the control group.

secondary antibodies. Western blotting showed that the rFlaB-immunized mice sera contained antibodies against FlaB and sonicated *V. vulnificus* (Fig. 1A). Only one band appeared against the rFlaB, whereas two bands appeared against sonicated *V. vulnificus* (Fig. 1A). These results indicate that the antibody against rFlaB was able to bind the other flagellin, such as FlaA, -C, -D, -E and -F, that show 51 to 99% sequence identities with FlaB (data not shown). Indeed, it was reported that four flagellin proteins could be detected as the three bands in the isolated flagellin fraction from *V. vulnificus* CMCP6 in western blot analysis using a mixture of six flagellin (FlaA, -B, -C, -D, -E and -F) antibodies [9]. Detailed results of detected flagellin bands in this previous report showed that FlaC was the highest molecular weight, however the FlaC band was recognized as an almost single band together with the next lower single band, which included the FlaB/-D [9]. In addition to these two bands (FlaC and FlaB/-D), FlaA appeared as the lowest band [9]. Based on these previous reports, bands detected by the rFlaB-immunized mice sera in this study would include FlaB/-D, and -C (black arrowhead) and FlaA (white arrowhead) (Fig. 1A). Thus, the rFlaB-immunized mice sera could recognize at least two or three flagellin proteins of *V. vulnificus*.

To determine the antibody response in the rFlaB-immunized mice, anti-rFlaB antibody titers were determined by Enzyme-linked immunosorbent assay (ELISA) using the 3,3',5,5'-tetramethyl benzidine liquid substrate system (Sigma-Aldrich, Tokyo, Japan). We used Mann-Whitney's *U*-test to compare the differences between the two groups, and a *P* value less than 0.05 was considered statistically significant. The specific antibody titer for flagellin protein was significantly higher in the rFlaB-immunized mice sera than in the control mice (Fig. 1B).

To investigate whether or not immunization with purified rFlaB can prevent the wound infection, the control and rFlaB-immunized mice were s.c. inoculated with 1.1×10^7 colony-forming units (CFU)/mouse of *V. vulnificus* CMCP6. The infection mice were monitored for 72 hr, and sacrificed when they displayed critical symptoms that link with death directly by severe infection. Survivors were sacrificed at 72 hr post-infection. Muscle at the infection site and spleen of sacrificed mice were harvested immediately, homogenized in PBS containing 0.1% gelatin, and plated onto LB agar plates containing 50 $\mu\text{g/ml}$ rifampicin and incubated for 12 hr at 37°C. To compare the differences in bacterial burdens of *V. vulnificus* in those tissues between the two groups, we used Mann-Whitney's *U*-test, and a *P* value less than 0.05 was considered statistically significant. In the muscular tissue, the median bacterial burden in the control mice was 3.02×10^8 CFU/g, whereas that in the rFlaB-immunized mice was significantly decreased to 1.78×10^8 CFU/g (Fig. 2A). However, there were no differences in bacterial burdens in the spleen (Fig. 2B). These results indicate that immunization of rFlaB can partly prevent the proliferation of *V. vulnificus* in the muscular tissue. Survival times tended to be longer in the rFlaB-immunized mice compared with that of the control mice, but the two curves finally overlapped at 36 hr after inoculation with *V. vulnificus* (Fig. 3).

Bacterial motility toward their favorable environment is accomplished by regulating the direction of flagellar rotation. Several studies have reported significantly reduced colonization in gastric mucosa by *Helicobacter pylori* mutants lacking flagellar-based motility or chemotaxis [17, 22]. It is also known that *V. vulnificus* are isolated from the necrotic lesion of muscular tissue both in wound infection and after ingestion of contaminated seafood. These facts strongly suggested that *V. vulnificus* has positive

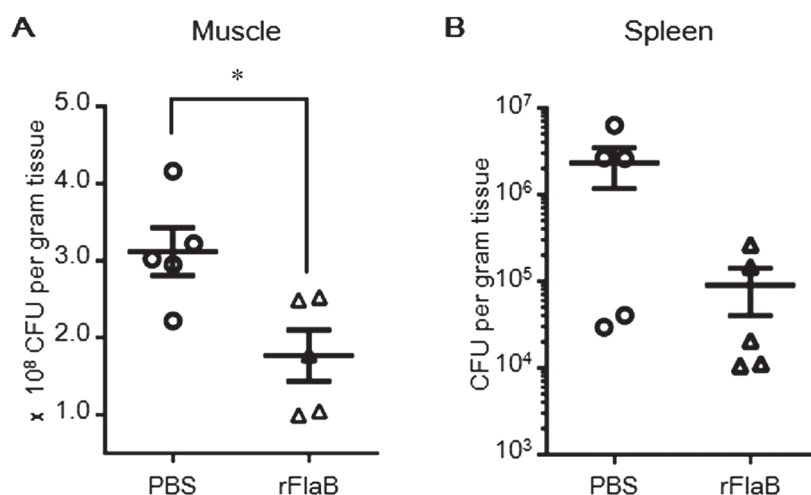


Fig. 2. Bacterial burdens in muscular tissue at the infection site (A) and spleen (B) were calculated as CFU/g in mice s.c.-inoculated with 1.1×10^7 CFU of *V. vulnificus* in the right caudal thigh (n=6/group). Each symbol represents an individual mouse. Error bars indicate SEM. * $P < 0.05$; Mann-Whitney *U*-test. Mice immunized with PBS and adjuvant without rFlaB served as the control group.

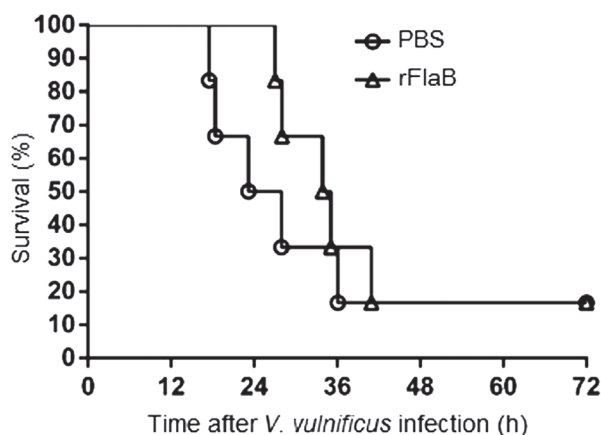


Fig. 3. Kaplan-Meier survival curves for mice (n=6/group) inoculated s.c. with 1.1×10^7 CFU of *V. vulnificus* and monitored for 72 hr. Mice immunized with PBS and adjuvant without rFlaB served as the control group.

chemotaxis attracted toward muscular tissue for colonization and proliferation. Therefore, the rFlaB immunization would inhibit invasion of muscular tissue from subcutaneous tissue by decreasing the motility of *V. vulnificus*. From a different perspective, flagellar-based motility does not seem to be important after the invasion of the systemic circulation. This perspective is in agreement with a report that flagellar-based motility of *Listeria monocytogenes* is not important for the proliferation of this bacterium in systemic circulation [20].

Regardless of the fact that the anti-flagellum antibody titer was elevated by rFlaB immunization, the overall effect of the rFlaB immunization was limited in our study. Improvement of this rFlaB vaccine will be needed in the future. Flagellin has already been found to have high adjuvant activity [19, 24], and therefore should be combined with another additional antigen such as *Vibrio vulnificus* hemolysin VVH, metalloprotease VvpE, and/or RTX toxin [3, 10, 13, 14, 16]. There have been many reports on utilization of these proteins as vaccine antigens, and immunization with these proteins confers effective protection against lethal challenge by *V. vulnificus* on hosts [10]. In addition, immunization of an attenuated strain of *V. vulnificus* with triple deletion in VVH, VvpE, and RTX also provided efficient protection from challenge by a homologous strain [10]. However, in all of these previous reports, routes of infection with *V. vulnificus* after immunization did not assume wound infection such as s.c. inoculation into the thigh of mice. We need to attempt to combine some of the above antigens to develop a vaccine for wound infection caused by *V. vulnificus* in the future.

CONFLICTS OF INTEREST. The authors declare no conflicts of interest.

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