

Protective Effect of *Clostridium septicum* Alpha-Toxoid Vaccine against Challenge with Spores in Guinea Pigs

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ABSTRACT. The protective effect of an alpha-toxoid vaccine of *Clostridium septicum* purified alpha-toxin was investigated in guinea pigs. Purified alpha-toxin was treated with formalin to make toxoid, and alpha-toxoid vaccine was prepared by mixing alpha-toxoid (4 to 64 μ g/dose) with an aluminum phosphate gel as adjuvant. Guinea pigs were immunized twice with different doses of alpha-toxoid vaccine, and challenged with spores of *C. septicum*. The guinea pigs surviving after challenge had been immunized with 8 μ g/dose or more of alpha-toxoid. All these animals produced titers of 20 units or higher of antitoxin at the challenge. The results suggest that *C. septicum* alpha-toxin plays an important role in protection against challenge with spores in guinea pigs.

KEY WORDS: alpha-toxin, *Clostridium septicum*, toxoid.

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Clostridium septicum is one of the agents causing malignant edema with high mortality in many kinds of animals [5]. *C. septicum* produces four extracellular toxins, alpha-, beta-, gamma- and delta-toxin [2, 11]. It is still unclear whether these toxins play a role in the pathogenesis of the disease. Alpha-toxin has high lethal activity [2, 6, 8], and may be closely associated with the disease. At present, two types of commercial *C. septicum* vaccines, toxoid or bacterin/toxoid, are available for preventing the disease [5]. It is believed that anti-alpha-toxin is efficacious against the disease [5, 8]. However, the protective effect of alpha-toxoid in *C. septicum* vaccine has not been proved to satisfactory. Ballard *et al.* [2] examined the protective effect of highly purified alpha-toxin against challenge with *C. septicum* cells in mice, but their preparation was not inactivated, and they did not check the antitoxin.

In this study, we examined the effect of purified *C. septicum* alpha-toxin on protection against lethal challenge with *C. septicum* spores in guinea pigs by measurement of anti-toxin titers of guinea pig sera.

C. septicum strain No.44 was cultured in brain-heart infusion broth (Difco Laboratories, MI) containing 0.3% glucose and 0.05% L-cysteine hydrochloride, pH 7.4, at 37°C for 18 hr. Alpha-toxin was purified by the method described by Ballard *et al.* [2] with slight modifications. Briefly, the filtered culture supernatant was fractionated by high performance liquid chromatography using a SP-5PW cation exchange column (Tosoh, Tokyo, Japan) and a G3000SW gel filtration column (Tosho). The fractions with alpha-toxin were monitored for cytotoxicity. The cytotoxic positive fractions, which were purified alpha-toxin, were pooled, then concentrated to a protein concentration of 200 μ g/ml by ultrafiltration (10 kDa cut off).

SDS-PAGE of the purified alpha-toxin was performed by the method of Laemmli [9]. It was detected as a nearly single band with a molecular weight of 45 kDa (Fig. 1). The

lethal activity of purified alpha-toxin was measured by intravenously injecting 4 ddY male mice weighing about 20 g. The activity was expressed as the 50% lethal dose (LD₅₀) per kg of body weight for mice. The cytotoxic activity of alpha-toxin was determined as described previously [1]. Briefly, Vero cells were inoculated with a sample and after 24 hr, the surviving cells were detected by MTT assay [10]. The number of cytotoxic units (CU) was determined as the reciprocal of the highest dilution of toxin killing 50% or more of cells as compared to the control. The lethal and cytotoxic activities of the purified alpha-toxin were 14 μ g/kg of body weight for mice and 5,800 CU/ μ g in Vero cells, respectively. Ballard *et al.* [3] reported that the molecular weight was estimated as 46.450 kDa from the result of DNA sequencing, and that 1 LD₅₀ of purified alpha-toxin was

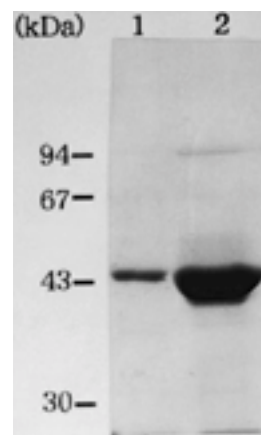


Fig. 1. SDS-PAGE (10% running gel) profiles of purified alpha-toxin. The gel was stained with CBB R-250. Lane 1, purified alpha-toxin (110 μ g/ml); lane 2, purified alpha-toxin concentrated by ultrafiltration (1/20 of the original volume).

approximately 10 µg/kg of body weight for mice [2, 4]. Our results are consistent with these numbers.

The purified alpha-toxin was inactivated by adding formalin at a final concentration of 0.4% (v/v) and maintaining the mix at 37°C for 4 days. The purified alpha-toxoid was diluted to final concentrations of 4, 8, 16, 32 and 64 µg/0.32 ml with 0.15 M phosphate-buffered saline, and mixed with aluminum phosphate gel at a ratio of 8:2. Twenty-five female Hartley guinea pigs (4 weeks old), five for each vaccine dose, were immunized intramuscularly twice with 0.4 ml of toxoid vaccine at 2-week intervals. Ten days after the final immunization, all guinea pigs were challenged with 100LD₅₀ (1.0×10^2 colony forming units) of strain No. 44 spores suspended in 0.5 ml of 3% CaCl₂-2H₂O solution. The survival of the animals was recorded for 7 days after the challenge. Blood samples were collected from the heart before the challenge to measure antitoxin titers. Antitoxin titers were measured as follows. Two-fold serially diluted serum samples were mixed with 10 CU of *C. septicum* culture filtrate. The mixtures were inoculated on Vero cells, and then the cells were incubated for 24 hr. The units (U) of antitoxin were expressed as the reciprocal of the highest dilution at which over 50% of cells survived as detected by MTT assay. One IU of standard *C. septicum* antitoxin from the National Institute of Infectious diseases (Tokyo, Japan) was 40 U by this measurement method. The protective effects of purified alpha-toxoid vaccines with various protein concentrations were examined in guinea pigs (Fig. 2). The guinea pigs immunized with purified alpha-toxoid vaccine containing 8 µg or more of protein per dose exhibited 80% survival after challenge with *C. septicum* spores. All guinea pigs immunized with the vaccine at 4 µg / dose died. The guinea pigs with an antitoxin titer of 10 U or lower did not survive after the challenge, whereas the animals with 160 U or higher were completely protected. Three of 14 guinea pigs with intermediate levels of antitoxin died after the challenge. Guinea pigs immunized with purified alpha-toxoid vaccines required at least 20 U of antitoxin to survive against the challenge. Although 100% protection was observed in guinea pigs with 160 U or more, two of 8 guinea pigs with 80 U of antitoxin died. Immunoblotting analysis was performed with guinea pig sera (Fig. 3). The guinea pigs were immunized with purified alpha-toxoid vaccine. Culture supernatant of *C. septicum* was electrophoresed by SDS-PAGE, and transferred to a membrane. The membrane was incubated with 1:200 diluted guinea pig sera, which had antitoxin of 20 and 80 U respectively from guinea pigs surviving and dying after challenge. After washing, the membranes were incubated with 1:200 diluted horseradish peroxidase-conjugated anti-guinea pig Ig-G (Cappel, U.S.A.). The reactivity was visualized with 50 mM Tris-HCl buffer, pH 8.0 containing 0.05% 3-3'-diaminobenzidine (Dojin Laboratories, Kumamoto, Japan) and 0.02% H₂O₂. The sera with antitoxin of 20 U from the surviving guinea pig and guinea pig which died after challenge showed profiles similar to a protein of 45 kDa. Moreover, 2 sera with antitoxin of 80 U showed a similar profile. So, we

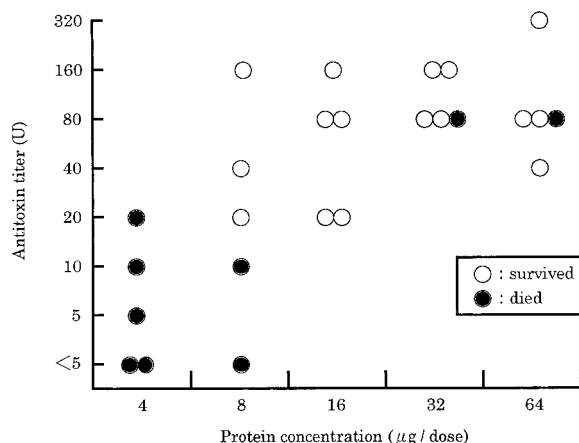


Fig. 2. Protective effect of purified alpha-toxoid vaccine in relation to antitoxin titers before challenge. Guinea pigs were immunized twice with purified alpha-toxoid vaccine at 2-week intervals. Ten days after the final immunization, all guinea pigs were bled to measure antitoxins and then challenged with spores of *C. septicum*. The animals were observed for 7 days after the challenge.

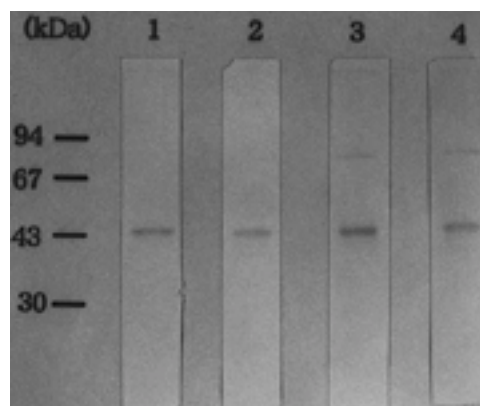


Fig. 3. Immunoblotting analysis of guinea pig sera immunized with purified alpha-toxoid vaccine. Culture supernatant of *C. septicum* was transferred to a membrane. All lanes, incubated with 1:200 diluted guinea pig sera before challenge. Lanes 1 and 2, guinea pig sera with antitoxin titers of 20 U. Lanes 3 and 4, guinea pig sera with antitoxin titers of 80 U. Lanes 1 and 3 incubation with sera from guinea pigs surviving after challenge, and lanes 2 and 4, incubation with sera from guinea pigs which died.

think that antitoxins at 20 to 80 U confer partial protection against challenge with *C. septicum* spores in guinea pigs. The sera with antitoxin of 80 U barely reacted to a protein of close to 94 kDa in immunoblotting. Ballard *et al.* [2] observed the same reaction. Ballard *et al.* [2] suggested that the reaction does not relate to alpha-toxin. We examined the N-terminal amino acid sequence of alpha-toxin and the pro-

tein. Alpha-toxin and the protein of close to 94 kDa were transferred to a membrane and excised and sequenced by Edman degradation analysis on an automatic protein sequencer PSQ-2 (Shimadze, Kyoto, Japan). The N-terminal amino acid sequence of alpha-toxin was L-T-N-F-E-E-G-G-Y-A-, and that of the protein was G-D-N-K-Q-L-E-. We thought that it was antibody to a contaminating protein in purified alpha-toxin as shown in Fig. 1. We determined that the N-terminal amino acid sequence of *C. septicum* alpha-toxin was different from those reported by Ballard *et al.* [3] and Imagawa *et al.* [7] at the fourth amino acid from the N-terminal. But the toxin was certainly *C. septicum* alpha-toxin as judged by the results of the neutralization test with standard *C. septicum* antitoxin and by its biological activities. We thought that the differentiation occurred through a mutation of the strains.

Ballard *et al.* [2] reported that a 0.2 µg/dose of purified alpha-toxin with oil-adjuvant gave 30% protection after challenge with *C. septicum* organisms to mice. However, they used active alpha-toxin at a lethal dose, and they did not measure antitoxin titers of mouse sera. Therefore, it was unclear whether antitoxin and/or some other factor was responsible for the protection. Inactivation of toxin is needed for producing a toxoid vaccine. Our results in guinea pigs showed that *C. septicum* alpha-toxoid is highly effective as

an antigen protecting against challenge with *C. septicum* spores.

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