FULL PAPER *Bacteriology*

Protective Effects of *Clostridium sordellii* LT and HT Toxoids against Challenge with Spores in Guinea Pigs

Katsuhiko AMIMOTO[1)], Eiji OISHI[1)], Hisao YASUHARA[1)], Osamu SASAKI[1)], Shigeji KATAYAMA[1)], Takashi KITAJIMA[1)], Akihiro IZUMIDA[1)] and Tadashi HIRAHARA[1)]

[1)]*Division of Veterinary Microbiology, Kyoto Biken Laboratories, 24–16 Makishima-cho, Uji, Kyoto 611–0041, Japan*

(Received 8 November 2000/Accepted 17 April 2001)

ABSTRACT. The protective effects of *Clostridium sordellii* lethal toxin (LT) and hemorrhagic toxin (HT) toxoids against challenge with spores in guinea pigs were investigated. Purified LT and partially purified HT were obtained from the culture supernatant of *C. sordellii* strain 3703, and then were treated with formalin to make toxoids. LT, HT and combined LT and HT (LT / HT) toxoid vaccines were prepared by mixing each toxoid with an aluminum phosphate gel as adjuvant. Guinea pigs immunized twice with the respective toxoid vaccines were challenged with spores of strains 3703 or KZ1047. The latter strain does not produce HT. LT toxoid vaccine conferred protection against challenge with strain KZ1047, but not strain 3703, in guinea pigs. All guinea pigs immunized with HT toxoid vaccine died after challenge with spores of either strain. LT/HT toxoid vaccine gave complete protection against challenge with spores of strains 3703 and KZ1047 to guinea pigs. These results suggest that not only LT toxoid, but also HT toxoid, are essential protective antigens of *C. sordellii*.

KEY WORDS: *Clostridium sordellii*, HT, LT, toxoid.

J. Vet. Med. Sci. 63(8): 879–883, 2001

Clostridium sordellii causes gas gangrene in humans [19] and animals [7, 18], and it has also been known as an agent of enteritis in cattle and sheep [2–4, 7]. The diseases associated with *C. sordellii* have been controlled by toxoids and bacterins in animals [7]. The pathogenic *C. sordellii* strains produce lethal toxin (LT) alone or both LT and hemorrhagic toxin (HT) [15, 24]. LT has edematizing and cytotoxic activities in addition to its lethal toxicity [5, 17]. HT has much weaker lethal and cytotoxic activities than LT [5, 12, 17]. Consequently, LT masks the action of HT in culture supernatants, and the differentiation between pathogenic and nonpathogenic *C. sordellii* strains has been conventionally based on LT production [8]. A consequence of this is that *C. sordellii* vaccines have been produced on the basis of inducing LT-specific antitoxins alone, however, the lethal activity of HT is not as low as the activities of other clostridial lethal toxins [6, 20, 21].

The toxins of *C. sordellii* have often been compared with toxin A and toxin B produced by *C. difficile* [5, 12, 15, 17]. The biological and antigenic properties of toxin A and toxin B are very similar to those of HT and LT [5, 12, 17, 23, 24]. Both toxoid A and toxoid B are essential for complete protection in hamsters with a *C. difficile* vaccine [10].

The necessity of HT toxoid for protection against bacterial infections has not fully been investigated. In this study, we examined the protective effects of toxoids against lethal challenge with *C. sordellii* spores in guinea pigs.

MATERIALS AND METHODS

Bacterial strains: *C. sordellii* strain 3703 (LT-positive, HT-positive) [14–16, 23, 24] and strain KZ1047 (LT-positive, HT-negative) [14, 16, 23] were obtained from the Department of Bacteriology at the School of Medicine of Kanazawa University, Kanazawa, Japan.

Preparations of purified LT and partially purified HT toxoids: Strain 3703 was incubated and the culture supernatant was separated into two cytotoxically positive fractions by DEAE column chromatography described by Yamakawa *et al.* [23]. Fractions of the first peak were pooled, and named partially purified HT. Fractions of the second peak were pooled, dialyzed against 50 mM Tris-HCl buffer, pH 6.5, and applied to a G5000SW high performance liquid chromatography gel filtration column (7.5 mm I.D. $\times$ 60 cm: Tosho, Tokyo, Japan), which was washed with the same buffer at a flow rate of 5 m*l*/min. Fractions containing LT were monitored for cytotoxicity in Vero cells and measured for molecular weight using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). LT fractions were pooled and designated purified LT. Protein concentration was estimated by the method of Lowry *et al.* [11].

Purified LT was inactivated by adding formalin at a final concentration of 0.4% (v/v) at 37°C for 2 days. Partially purified HT was inactivated with formalin at a final concentration of 0.2% (v/v) at 37°C for 24 hr. These toxoids did not show any cytotoxicity in Vero cells.

Preparation of crude toxoid: Strains 3703 and KZ1047 were cultured in cooked meat medium (Difco Laboratories, Michigan, U.S.A.). The actively growing cells were transplanted to trypticase soy broth (Becton Dickinson, U.S.A.) containing 0.3% glucose and 0.1 M HEPES (Dojin Laboratories, Kumamoto, Japan) and cultured at 37°C for 20 hr. The culture fluids containing toxins and bacterial cells were added with formalin at a final concentration of 0.4% (v/v) and cultured at 37°C for 3 days. The formalin-treated culture fluids were centrifuged at 10,000 $\times$ g for 1 hr to remove

the bacterial cells. The supernatants were concentrated to approximately 1/10 of the original volume by ultrafiltration using a Labo module model ACP-1010 (13 kDa cut off: Asahi Kasei Corporation, Tokyo, Japan). They were used as crude toxoid.

Vaccine preparation: The purified LT toxoid was diluted 1/10 with 0.15 M phosphate buffered saline (PBS). The partially purified HT toxoid was also diluted 1/20 with PBS. The toxoids indicated in Tables 2 and 3 were mixed to a final concentration of 20 % (v/v) with aluminum phosphate gel as adjuvant.

Production of antiserum: Purified LT and partially purified HT toxoids were emulsified with equal volumes of Freund's incomplete adjuvant (Sigma, St. Louis, U.S.A.). Male rabbits and guinea pigs were injected intramuscularly with the respective emulsions twice at 4 weeks intervals. Two weeks after the final injection, blood samples were collected from there. Sera were separated and stored at –20°C.

Protection test: Five female Hartley guinea pigs (4 weeks old) were immunized intramuscularly twice with 0.5 m*l* of each toxoid vaccine at 2-week intervals. Ten days after the final immunization, all guinea pigs were challenged with strains 3703 or KZ1047 spores adjusted to 100 LD_{50} per 0.5 m*l* of 3% $CaCl_2 \cdot 2H_2O$ solution. Protective effects of the toxoid vaccines were judged by the survival rate at 7 days after challenge. Blood samples were collected from the heart before the challenge to measure antitoxin titers.

Determination of cytotoxic activities and measurement of antitoxin titers: Cytotoxic activities of LT and HT were determined as described previously [1] except that LT and HT samples were neutralized with anti-HT and anti-LT sera at 37°C for 1 hr, respectively. The samples were inoculated on Vero cells. After 48 hr, the surviving cells were detected by MTT assay [13]. The reciprocal of the highest dilution of toxin showing under 50% of live cells against control cells was considered as the number of cytotoxic units (CU).

The antitoxin titers were measured by neutralization tests using Vero cells as described previously [1]. Two-fold serial dilutions of serum samples neutralized with 10 CU of partially purified LT or HT were inoculated on Vero cells, and then the cells were incubated for 48 hr. The units (U) of antitoxin were expressed as the reciprocal of highest dilution showing cells of over 50% of surviving cells detected by MTT assay.

Cross-neutralization test: Anti-LT, anti-HT and normal rabbit sera were diluted 1/128. Then, two-fold serial dilutions of each partially purified toxin were mixed with an equal volume of the diluted sera, and incubated at 37°C for 1 hr. The mixtures were inoculated onto Vero cells. Two days after inoculation, the CU of each toxin in the presence of the diluted serum was measured. Antiserum titers were expressed as neutralization indices as follows: antiserum titer=$\log_2$ (CU of toxin in the presence of diluted normal rabbit serum) — $\log_2$ (CU of toxin in the presence of diluted antitoxin serum).

Analytical gel electrophoresis: SDS-PAGE was performed by the method of Laemmli [9]. Samples were mixed with an equal volume of 2 × sample buffer containing 10% β-mercaptoethanol. The samples were heated in boiling water for 1 min prior to SDS-PAGE in a 6% running gel and 4% stacking gel. The gel was stained with Coomassie brilliant blue (CBB) R-250.

Cross-immunoblotting analysis: LT and HT of 0.1 μg/lane each were electrophoresed by SDS-PAGE, and were transferred electrophoretically from the gel to an Immobilon membrane (Milipore, Massachusetts, U.S.A.) by the method of Towbin *et al*. [22]. The membrane was incubated with 1:1,600 diluted anti-LT or anti-HT guinea pig sera at room temperature for 30 min. After washing, the membrane was incubated with 1:400 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% H_2O_2.

Determination of lethality: Aliquots, 0.5 m*l*, of two fold serially diluted LT and HT were intravenously injected into 2 ddY male mice (about 19 g) at each dilution. The minimum lethal dose (MLD) was defined as the reciprocal of the highest dilution that killed 100% of the mice within 5 days.

RESULTS

Properties of purified LT and partially purified HT: Purified LT was resolved in a 6% SDS-polyacrylamide-gel under reducing conditions. With CBB-staining, a single band with molecular weight about 250 kDa was observed. The protein concentration of the purified LT was 126 μg/m*l* and its specific activities were 33 CU/*n*g in Vero cells and 250 MLD/μg in mice (1 MLD=4 *n*g). In contrast, partially purified HT was detected as one broad band of about 300 kDa and many slight bands less than 76 kDa in stained gel (Fig. 1). The protein concentration of the partially purified HT was 1.85 mg/m*l* and its specific activities were 89 CU/μg in Vero cells and 17 MLD/μg in mice (1 MLD=59 *n*g). In cross-neutralization tests, anti-LT and anti-HT rabbit sera showed high levels of antitoxin titer against the homologous antigen but did not react with the heterologous antigen (Table 1). In cross-immunoblotting analysis, anti-LT and anti-HT guinea pig sera did not react to HT and LT, respectively (Fig. 2).

Protective effects of purified LT toxoid vaccine and partially purified HT toxoid vaccine: The results of the protection tests are summarized in Table 2. Purified LT toxoid vaccine and partially purified HT toxoid vaccine induced respective antitoxins in guinea pigs. Guinea pigs immunized with mixed toxoid of LT and HT were completely protected from challenge with spores of strain 3703. The guinea pigs had antitoxin titers of 11.5 U against LT and 6.6 U against HT. Neither LT nor HT toxoid vaccine protected guinea pigs against challenge with spores of strain 3703. The antitoxin titers of the guinea pigs immunized with LT toxoid and HT toxoid at the challenge were 23.0 U and <5 U against LT, and <5 U and 10.0 U against HT, respectively. LT toxoid vaccine conferred an antitoxin titer of 11.5 U

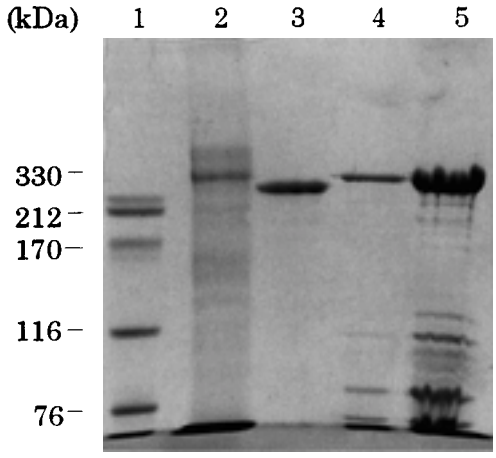

Fig. 1. SDS-PAGE (6.0% gel) profiles of purified LT and partially purified HT. Lane 1, high molecular weight marker (for SDS-PAGE: Pharmacia); Lane 2, high molecular weight marker (for native-PAGE: Pharmacia); lane 3, purified LT; lane 4, partially purified HT diluted 1/20 with PBS; lane 5, partially purified HT.

Table 1. Cross neutralization test between purified LT and partially purified HT with their antisera

Antiserum	Toxin	
	Purified LT	Partially purified HT
Purified LT	11[a)]	0
Partially purified HT	0	6

a) Titers were expressed as neutralization indices as follows: $\log_2$ (CU of toxin in the presence of diluted normal rabbit serum)—$\log_2$ (CU of toxin in the presence of diluted antitoxin serum).

against LT and protection against strain KZ1047 on guinea pigs, while HT toxoid vaccine did not.

Effect of the crude toxoid vaccines on cross-protection: The guinea pigs immunized with each of the crude toxoid vaccines were challenged with spores of 3703 or KZ1047 strains and their survival rates and antitoxin titers are shown in Table 3. Crude toxoid vaccine prepared from the culture supernatant of strain 3703 gave complete protection against challenge with both 3703 and KZ1047 strains to guinea pigs. Antitoxin titers of the guinea pigs at the challenge were 160 U and 211 U against LT, and 80 U and 139 U against HT. On the other hand, guinea pigs immunized with the crude toxoid vaccine of strain KZ1047 died after challenge with spores of strain 3703, but survived after challenge with spores of strain KZ1047. This vaccine only induced high levels of antibody against LT: the antitoxin titers against LT were 485 U and 422 U, while the antitoxin titers of all the guinea pigs against HT were less than 5 U.

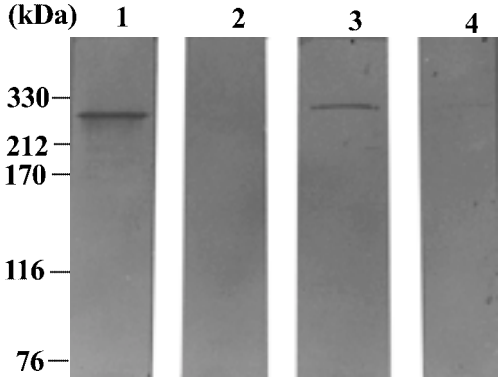

Fig. 2. Cross immunoblotting analysis of purified LT and partially purified HT. Lanes 1 and 2, 0.1 μg of purified LT; Lanes 3 and 4, 0.1 μg of partially purified HT. The membranes were incubated with 1:1,600 diluted anti-LT (lanes 1 and 4) and anti-HT guinea pig sera (lanes 2 and 3).

DISCUSSION

Yamakawa *et al*. [23] first separated cytotoxin I (CT I) from cytotoxin II (CT II) of the culture supernatant of strain 3703 by DEAE anion exchange column chromatography. At present, CT I and CT II are considered to be HT and LT, respectively, because CT I and HT behaved in a similar way in cytotoxic activity, induction of hemorrhagic response in rabbit ileal loop and serological cross reactions to *C. difficile* Toxin A [5, 12, 23, 24]. CT II showed cytotoxic and lethal activities and serological cross reactions to *C. difficile* Toxin B like LT [5, 16, 23, 24]. The molecular weights of LT and HT were estimated to be approximately 240 kDa and 300 kDa by SDS-PAGE, respectively [12, 17]. In our study, the band of purified LT and the major band of partially purified HT were observed at similar positions of about 250 kDa and 300 kDa protein in CBB-stained gel, respectively. We consider that the cytotoxicities of the first and second peaks eluted from the DEAE anion-exchanger must be caused by HT and LT, respectively. That is why the cytotoxicities coincide with the peaks of chromatography (data not shown) as reported by Yamakawa *et al*. [23]. We demonstrated the serological difference between LT and HT preparations by neutralization tests and immunoblotting analysis using polyclonal antibodies, like the CT I and CT II described by Yamakawa *et al*. [23].

Nakamura *et al*. [16] reported that LT of strain 3703 causes rounding of Vero cells without aggregation, while LT of strain KZ1047 aggregates Vero cells. Therefore, it is thought that LT produced by strain 3703 is not completely identical to LT of strain KZ1047. On the other hand, purified LT of strain 3703 gave complete protection to guinea pigs against challenge with spores of strain KZ1047, and besides partially purified LT of strain 3703 was neutralized with the guinea pig antiserum prepared by the crude toxoid

Table 2. Protective effects of purified LT toxoid and partially purified HT toxoid

Immunize toxoid		Antitoxin titer (U)		Challenge strain	Survival rate
LT	HT	LT	HT		(%)
204,800[a)]	4,096	11.5 ± 7.5[b)]	6.6 ± 5.9		100[c)]
204,800	–	23.0 ± 1.3	<5 ± 5	3703	0
–	4,096	<5 ± 5	10.0 ± 6.2		0
204,800	–	11.5 ± 6.5	<5 ± 5	KZ1047	100
–	4,096	<5 ± 5	8.7 ± 7.0		0

a) Cytotoxic activity of the toxin in Vero cells before inactivation. Five guinea pigs were immunized intramuscularly twice with each toxoid vaccine at 2-week intervals. Ten days after the second immunization, the guinea pigs were challenged with spores of 3703 or KZ1047 strains. b) Geometric mean ± standard error of antitoxin titers of five guinea pigs before challenge. c) The guinea pigs were observed for 7 days after challenge. The survival rate was calculated according to the following formula: the number of surviving guinea pigs / the number of the guinea pigs challenged × 100.

Table 3. Cross-protection test between strain 3703 and strain KZ1047

Crude toxoid[a)]	Antitoxin titer (U)		Challenge strain	Survival rate
	LT	HT		(%)
3703	160 ± 6[b)]	80 ± 6	3703	100[c)]
	211 ± 6	139 ± 6	KZ1047	100
KZ1047	485 ± 7	<5 ± 5	3703	0
	422 ± 6	<5 ± 5	KZ1047	100

a) Crude toxoid vaccine of strain 3703 contained 600,000 CU of LT and 1,200 CU of HT per dose. Crude toxoid vaccine of strain KZ1047 contained 600,000 CU of LT per dose. Guinea pigs were immunized with each toxoid vaccine and were challenged with spores of each strain by the same method described in Table 2. b) Geometric mean ± standard error of antitoxin titers of five guinea pigs before challenge. c) The survival rate was calculated by the method used in Table 2.

vaccine of strain KZ1047. Thus, part of the induction of LT protection and neutralization is common to strains 3703 and KZ1047.

Martinez and Wilkins [12] purified HT by affinity column chromatography with a monoclonal antibody against Toxin A. Though we tried to purify HT with ion-exchange (SP-5PW column: Tosho, Tokyo, Japan), gel filtration (G5,000SW column: Tosho), hydrophobic interaction (Phenyl-5PW column: Tosho) and hydroxyapatite (Macro-prep CHT: Japan Bio Rad, Tokyo, Japan) column chromatographies and preparative isoelectric focusing (Prep cell: Japan Bio Rad), we could not purify it without the monoclonal antibody. The DEAE-column chromatography elution pattern of partially purified HT was a clear peak as reported by Yamakawa *et al*. [23]. The results of SDS-PAGE and immunoblotting showed the purity of the partially purified HT. Therefore, we used the partially purified HT in our investigation. We examined the protective effect of LT and HT toxoids against challenge with *C. sordellii* spores. Then, we investigated media components show the high cytotoxicity of LT and HT (data not shown), and examined the protective effect of crude toxoids from the media against *C. sordellii* spores.

In guinea pigs, both LT and HT toxoids played a contributory role in protection against the challenge with spores of strain 3703. The MLDs of LT were reported by Ball *et al*. [5] and Popoff [17] as 5 *n*g and 2.9 *n*g in mice, respectively. On the other hand, the MLDs of HT reported by Ball *et al*. [5] and Martinez and Wilkins [12] were 75 *n*g and 120 *n*g, respectively. In our study, the MLD of LT was 4 *n*g and that of HT was 59 *n*g in mice. LT toxoid is essential for protection against challenge with spores of *C. sordellii*, since LT has very high lethality as indicated in the above studies. Compared with the lethal activity of LT, the lethal activity of HT is significantly lower, and the MLD of HT is equivalent to that of *Clostridium septicum* alpha-toxin [6], *Clostridium perfringens* alpha-toxin [21] and *C. perfringens* iota-toxin [20]. However, HT toxoid may play a considerable role in protection against challenge with HT-positive *C. sordellii* spores in guinea pigs. Although guinea pigs vaccinated with crude toxoid prepared from strain KZ1047 produced high antitoxin titers against LT, all the vaccinated animals died after challenge with spores of strain 3703. Crude toxoid vaccines induced higher antitoxin titers in comparison with purified toxoid vaccines in guinea pigs. Immunogenicity of the toxins varied with the difference of formalin treatment conditions of the toxins and the influence of impurity proteins for formalin. In conclusion, *C. sordellii*

vaccine should be composed of both LT and HT toxoids for perfect protection of animals against *C. sordellii* infection.

ACKNOWLEDGMENT. The authors thank Prof. S. Nakamura of Kanazawa University for providing *C. sordellii* strains 3703 and KZ1047.

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