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Safety evaluation of recombinant cholera toxin B subunit produced by *Bacillus brevis* as a mucosal adjuvant

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Abstract

Mucosal immune responses are known to play important roles in the establishment of protective immunity to microbial infections through mucosa. We examined the toxic effects of recombinant cholera toxin B subunit (rCTB) secreted by Gram-positive bacterium *Bacillus brevis* as a mucosal adjuvant. Incubation of guinea-pig peritoneal macrophages with cholera toxin (CT) or aluminium hydroxide gel (Al-gel) released a significantly higher activity of lactate dehydrogenase than did commercial natural CTB (CTB) or rCTB. Intraintestinal or intramuscular administration of CT, CTB or Al-gel caused severe histopathological reactions. CT also caused infiltration of neutrophils and irregular arrangement or partial loss of the respiratory epithelium. In addition, CT and CTB elicited vascular permeability-increasing effects. rCTB elicited no toxic effects to macrophages and no vascular permeability-increasing effects. Moreover, it is noticeable that no distinct local histopathological reactions were observed in the nasal cavity, the small-intestinal loop or the muscle given rCTB. These results suggest that, from a safety standpoint, rCTB is a useful candidate as mucosal vaccine adjuvant.

Keywords: Recombinant cholera toxin B subunit (rCTB); Intranasal administration; Mucosal adjuvant; Safety

1. Introduction

The induction of mucosal immunity plays an important role in prophylaxis of respiratory, gastrointestinal and genitourinary tract diseases. The nasal cavity and gastrointestinal tract offer adequate conditions for induction of mucosal immunity. Cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin are structurally related with adenosine diphosphate-ribosylating toxins that have a powerful mucosal adjuvant activity and may be useful for development of mucosal vaccines [1–5]. Because of the toxicity of A subunit (CTA), however, researchers have analysed for the potential of B subunit (CTB) for use as a mucosal adjuvant. Its adjuvant activity is low and requires addition of a small amount of CTA [6]. To solve these problems, we have purified a large amount of recombinant CTB (rCTB) secreted by Gram-positive, nonpathogenic bacterium *Bacillus brevis* carrying pNU 212-CTB for use as an adjuvant, investigated the mucosal and systemic immunoadjuvant activities of rCTB on the occasion of coadministration with highly purified bovine serum albumin (BSA) [7] or tetanus toxoid (TT) [8], and shown that rCTB coadministered intra-

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nasally with BSA or TT can induce a high level of antigen-specific serum IgG antibody responses and moderate or slight levels of antigen-specific IgA antibody responses in the nasal and pulmonary lavages. All mice intranasally immunized with TT and rCTB were exempted from onset of tetanus symptoms.

The purpose of this study was to evaluate the safety of rCTB as a mucosal adjuvant for vaccines. In the present study, we examined whether rCTB as an adjuvant for the toxic effects compared with the other related substances.

2. Materials and methods

2.1. Animals

Female guinea-pigs of the Hartley strain, weighing approximately 300 g, and female BALB/c mice, aged 8 weeks (Japan SLC Co., Shizuoka, Japan), were used for toxicological tests. Female Japanese white rabbits, weighing approximately 3 kg (Japan Laboratory Animals Inc., Tokyo, Japan), were used for vascular permeability tests.

2.2. Samples

Recombinant CTB (rCTB): rCTB was prepared by cultivating *B. brevis* bearing pNU 212-CTB at 30°C for 5 days [9] and purified from the culture supernatant by affinity chromatography on D-galactose immobilized agarose [10]. It contained little or no leucocytosis-promoting factor, as described before [7].

Cholera toxin (CT) and cholera toxin B subunit (CTB): CT was purchased from List Biological Laboratories Inc. (LBL, Campbell, CA). CTBs were purchased from LBL, Research Biochemicals Inc. (RBI, Natick, MA) and Sigma Chemical Co. (SIG, St. Louis, MO).

Aluminium hydroxide gel (Al-gel): Al-gel was prepared by mixing an aluminium chloride solution (Wako Pure Chemical Industries, Osaka) and a 1 M sodium hydroxide solution (Wako) and neutralizing the mixture with 1 M HCl. Al-gel, 3 mg in 1 ml of physiological saline corresponding to about 1 mg Al ml^{-1}, was prepared for the test [11].

2.3. Preparation and culture of macrophages

Guinea-pig peritoneal macrophages (Mø) were collected in Dulbecco's phosphate-buffered saline (D-PBS) 4 days after intraperitoneal injection of 20 ml of sterilized 10% proteose peptone. The ascites was centrifuged at 500 × *g* for 10 min, and the cells were washed twice with D-PBS at 4°C and finally suspended in Dulbecco's minimum essential medium (DMEM, Nissui Pharmaceutical Co. Ltd., Tokyo). The suspended cells were allowed to adhere to a 24-well plate for 2 h at 37°C. The nonadherent cells were washed off with D-PBS warmed at 37°C. The Mø monolayers were covered with 3 ml of DMEM containing 5% heat-inactivated fetal calf serum (FCS-DMEM) and incubated overnight. The final count of the adherent cells was approximately 3×10^5 per well.

To eliminate influence of adsorption of LDH, Al-gel was preincubated with FCS-DMEM for several hours at 37°C. Each material at 200 μg ml^{-1} was added to the well containing 1 ml of FCS-DMEM. After incubation for 48 h at 37°C, the 24-well plates were centrifuged at 500 × *g* for 10 min to sediment the detached cells. The supernatant was assayed for the lactate dehydrogenase (LDH) activity. The cells treated in the same manner were added to the well containing 1 ml of 0.1% Triton X-100 and disrupted by repeated pipetting. The lysate was used as a positive control for LDH assay.

2.4. Assay for LDH

The LDH activity released from Mø was measured with a commercial LDH assay kit (MTX "LDH") produced by Kyokuto Seiyaku, Tokyo.

2.5. Vascular permeability test

Vascular permeability at the site of intracutaneous injection was assayed in rabbits by a minor modification of the method described previously [12]. The rabbit was injected intravenously into *V. auricularis* with 10 ml of 1% Evans' blue in a physiological saline solution. Immediately after the injection, 30 μg of each material in 0.1 ml was injected intracutaneously into the previously clipped flanks of a rabbit. Al-gel in a dose of 0.1 ml containing 0.1 mg Al was given in the same way. Five hours later, each blue spot arising at the injection site was excised from the skin, minced, and extracted for about 24 h with a mixture of acetone and a sodium sulfate solution. The extract was centrifuged, and the supernatant was measured spectrophotometrically at 620 nm to estimate the Evans' blue extracted. The mean of three spots was expressed as the vascular permeability reaction for each test material.

2.6. Administration of test materials

2.6.1. Nasal cavities

Guinea-pigs and mice were each given with a micropipette under light ether anaesthesia into both the nasal cavities in a 50-μl dose containing 15 μg (guinea-pig) or a 30-μl dose containing 9 μg (mouse) of each material. Al-gel was administered intranasally in a 50-

μl dose containing 50 μg Al (guinea-pig) or a 30-μl dose containing 30 μg Al (mouse). The nasal cavities were excised 6 h after the administration of each sample.

2.6.2. Small-intestinal loop

Guinea-pigs and mice under ether anaesthesia were given into the small-intestinal loop in a 0.1-ml dose containing 30 μg of each material.

Al-gel was injected in a 0.1-ml dose containing 0.1 mg Al. The intestinal loop was excised 3 h after the inoculation of each sample.

2.6.3. Muscle of the hind leg

Guinea-pigs and mice were inoculated intramuscularly into the femur of the hind leg in a 0.5-ml dose containing 150 or 15 μg (guinea-pig) or a 0.1-ml dose containing 3 μg (mouse) of each material. Al-gel was injected in a 0.5-ml dose containing 0.5 mg Al (guinea-pig) or a 0.1-ml dose containing 0.1 mg Al (mouse). The tissue was excised 48 or 72 h after the injection of each sample.

The same amount of physiological saline was administered to the control group in all tests.

2.7. Histopathological examination

The tissues excised from the sites of administration were fixed in 10% neutral-buffered formalin and embedded in paraffin by the ordinary methods. Sections were stained with hematoxylin and eosin (H–E).

3. Results

3.1. Release of LDH activity from guinea-pig peritoneal Mø cultured with each test material

Irreversible cell death was measured by the release of LDH activity. As shown in Fig. 1, incubation of Mø with CT or Al-gel released a significantly higher activity of LDH than did rCTB or CTB.

Neither rCTB nor CTB released LDH on incubation with Mø even when the concentration of rCTB or RBI-CTB was increased to 200 μg ml^{-1}.

3.2. Effects of the test materials on vascular permeability

The method (see Materials and methods) used to estimate the vascular permeability was also used to assess the degree of oedema arising in the skin. The amount of Evans' blue extracted from the skin site inoculated with CT was much greater than that with any other material. Although CTB also elicited vascular permeability-increasing effect, the intensity of vascular permeability evoked differed among the manufacturers or lots of CTB. rCTB induced no vascular permeability-increasing reaction (Fig. 2).

3.3. Histopathological reactions

Nasal cavity: CT caused infiltration of polymorphonuclear neutrophilic leucocytes and oedema around the capillaries in the lamina propria (Fig. 3A) and irregular arrangement or partial loss in the respiratory epi-

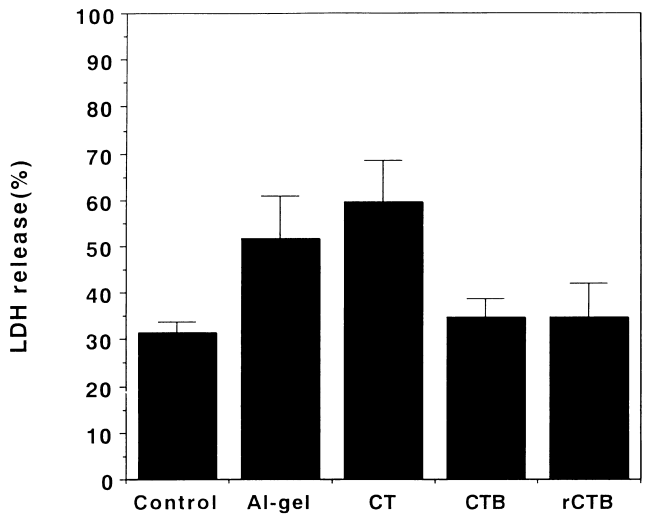

Fig. 1. LDH release from macrophages cultured for 48 h with a test material at 200 μg ml^{-1}. Control: FCS-DMEM. Approximately 3×10^5 cells per well were cultured in 1 ml of FCS-DMEM. Each point indicates the mean of triplicate tests and each vertical bar the standard deviation of the mean.

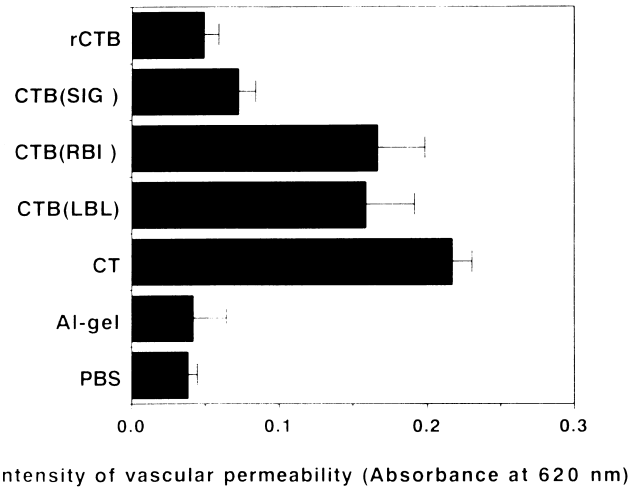

Fig. 2. Effects of test materials on vascular permeability. Rabbits, previously given Evans' blue, were injected intracutaneously with 0.1 ml of test material. Each point indicates the mean absorbance of the sum of three spots in rabbit and each horizontal bar the standard deviation of the mean. Sigma Chemical Co.: SIG; Research Biochemicals Inc.: RBI; List Biological Laboratories Inc.: LBL.

thelium (Fig. 3B). LBL-CTB induced slight infiltration of neutrophils and oedema around the capillaries in the lamina propria (Fig. 3C). Al-gel occasionally induced irregular arrangement in the epithelium of mice, whereas intranasal administration of rCTB (Fig. 3D and E) or CTB (SIG and RBI) caused no distinct local histopathological reaction.

Small-intestinal loop: Intraintestinal administration of CT, CTB or Al-gel caused severe erosion of the intestinal mucosa consisting of epithelial necrosis and epithelial defect with hemorrhage. Most villi of the surface mucosa in the ligated small intestines disappeared (Fig. 4A, B and D). There were, however, a great deal of differences in the evoked lesions of intestinal mucosa among the manufacturers of CTB. Administration of SIG-CTB caused relatively mild injury (Fig. 4C). rCTB induced no distinct histopathological reaction in either animal except for some congestion provoked by the ligature (Fig. 4E)

Muscle: marked exudative inflammatory reactions were induced by CT or Al-gel. CT-induced cellular reactions consisting mainly of severe infiltration of neutrophils, eosinophilic exudate and hemorrhage were seen at the injection sites (Fig. 5A). In the animals injected with Al-gel, a large amount of adjuvant mass with severe infiltration of polymorphonuclear leucocytes (PMN) and oedema in the interstitial connective tissues between degenerated muscles were seen (Fig. 5D). LBL-CTB also elicited active inflammatory reactions consisting of infiltration of PMN, oedematous reaction and hyperemia (Fig. 5B). However, SIG-CTB caused a slight inflammatory reaction (Fig. 5C), while rCTB induced no histopathological reaction at the injection site (Fig. 5E).

4. Discussion

In the present study, we investigated the toxic effects of rCTB to Mø and the administration sites. The toxi-

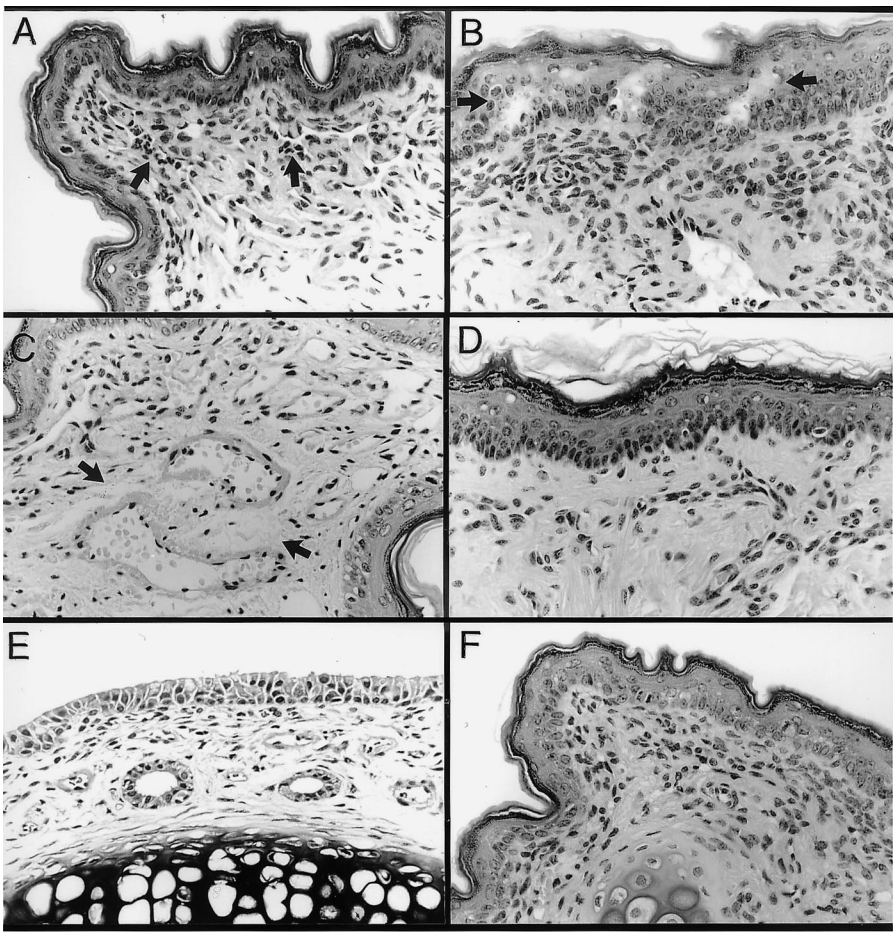

Fig. 3. Guinea-pig. Intranasal (i.n.) administration site of each material (15 μg/50 μl) after 6 h. (A) CT: Infiltration of polymorphonuclear leucocytes in the lamina propria (arrows). (B) CT: Partial disarrangement and loss of epithelial cells in the epithelium (arrows). (C) LBL-CTB: Perivascular oedema in the lamina propria (arrows). (D) rCTB: The epithelial layer and lamina propria have no sign of damage. (E) Mouse, rCTB (9 μg/30 μl): No change is observed in the respiratory epithelium and lamina propria. (F) Physiological saline (50 μl): No change is observed. H–E, (original magnification: ×100).

cities after a single administration of rCTB were compared with those of CT, CTB and Al-gel in mice, guinea-pigs and rabbits.

LDH release assay using guinea-pig peritoneal Mø was made to measure the in vitro cytotoxicity. The method, which detected simultaneously the LDH activity of injured and surviving cells, is widely recognized to be sensitive [13–15]. Furthermore, to investigate the cell damage due to rCTB, the growth rate and morphological change of the human hepatoma cells were observed. rCTB at the concentrations of 2, 10 or 50 μg ml^{-1} did not show any effect on the growth rate nor morphological change of the human hepatoma cells, HuH-7, maintained in a chemically defined medium, ISE-RPMI 1540 for 48 h (unpublished data). The toxicity of rCTB was also tested for survival, mobility and growth of *Tetrahymena pyriformis* (protozoa) maintained in a medium supplemented with proteose peptone and tryptone. No abnormality due to rCTB was found in any morphological testing using *Tetrahymena* even after the administration of 200 μg ml^{-1} of rCTB (unpublished data).

Nasal cavity mucous membranes are composed of various epithelia such as squamous epithelium, transitional epithelium, respiratory epithelium and olfactory epithelium. Among these epithelia, transitional epithelium, respiratory epithelium and olfactory epithelium are sensitive to various chemical substances [16]. Desquamation, regeneration, hyperplasia, irregular arrangement and squamous metaplasia in the respiratory epithelium have been reported as lesions caused by glutaraldehyde, dibasic esters, formaldehyde [17–19]. There have been a few reports concerning lesions in the nasal cavity mucous membranes caused by CT or CTB [20].

Although there are many defence mechanisms such as dilution and ciliary movement in the nasal cavity, we found some histopathological changes in the epi-

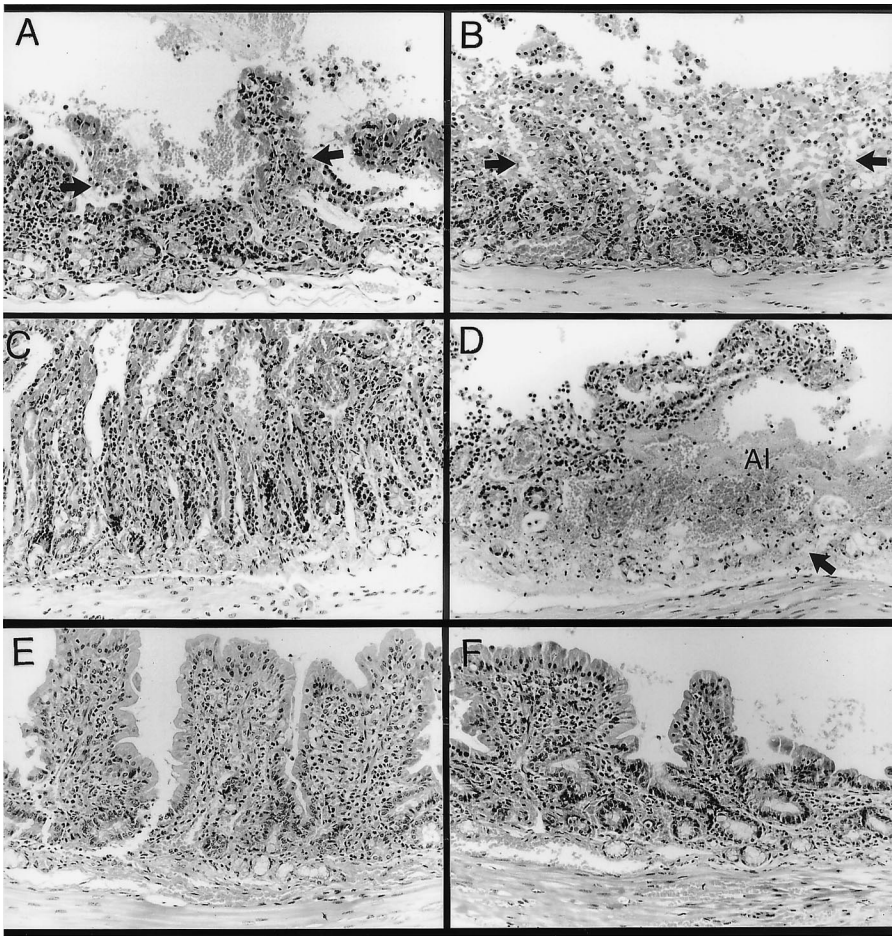

Fig. 4. Guinea-pig. Intraintestinal (i.i.) administration site of each material (30 μg/0.1 ml) after 3 h. (A) CT: Severe erosion of small intestinal mucosa due to epithelial destruction with hemorrhage (arrows). (B) RBI-CTB: Villi of the small intestinal mucosa have disappeared (arrows). These changes may occur to the intestinal mucosa with a small amount of CT holotoxin frequently present in commercial CTB. (C) SIG-CTB: Epithelial lesions are extremely slight compared with those of RBI-CTB. (D) Al-gel (0.1 mg Al/0.1 ml): The severest villous lesions are seen (arrow). Most intestinal mucosa has been destroyed by Al-gel (Al). (E) rCTB: No histopathological change is observed in the intestinal mucosa. (F) Physiological saline (0.1 ml): No change is observed. H–E, (original magnification: ×50).

thelium and/or lamina propria even after a single administration. On the other hand, Al-gel used as a depot-forming-type adjuvant induced no distinct histopathological reaction in the nasal cavity of guinea-pigs. The antigen (protein)-adsorption ability plays a very important role in adjuvanticity of such depot-forming-type adjuvants as aluminium and calcium gels. Since Al-gel adsorbs nonspecifically protein, it adsorbs nasal fluid in the nasal cavity. Thus, no Al-gel-induced lesions in the nasal cavity mucous membranes might be attributable to insufficient exposure to Al-gel.

Intraintestinal administration of CT, CTB or Al-gel caused severe erosion with epithelial defect of intestinal mucosa. Such severe histopathological reactions were also observed in muscle of the hind leg. We have reported very severe histopathological reactions following intramuscular injection of Al-gel in mice [21,22] and guinea-pigs [23].

On the other hand, rCTB induced no distinct histopathological reaction in the ligated intestinal loop of guinea-pigs or mice as well as the nasal cavity, since it does not contain any CT holotoxin regardless of its ability to bind to the cell membrane GM1 gangliosides of the intestinal epithelium, which has been shown to be important for stimulating mucosal immunity [10,24]. It is well known that CT holotoxin, even in its highly purified form, strongly increases the capillary permeability [25,26]. Gizurarson et al. [20] have reported that major histopathological changes were seen in the tissues exposed to the CT holotoxin, causing cell damage and cell loss.

The use of adjuvants should reduce the amount of purified antigen required for successful vaccination,

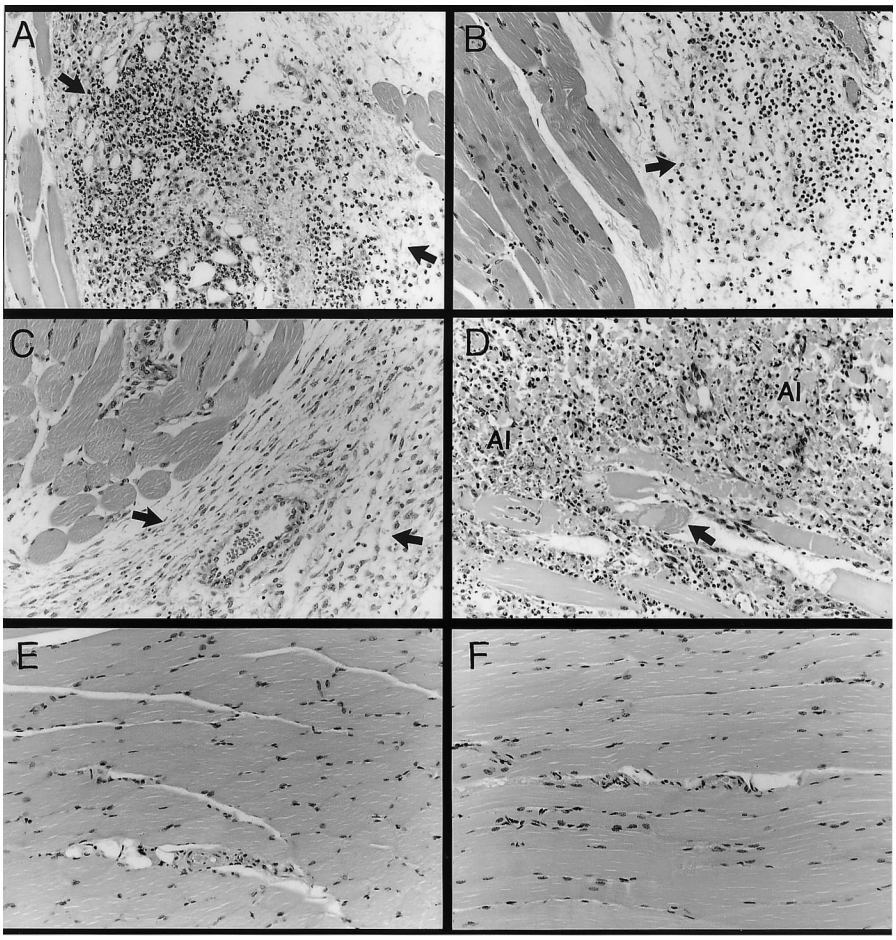

Fig. 5. Guinea-pig. Intramuscular (i.m.) injection site of each material (15 μg/ 0.5 ml) after 48–72 h. (A) CT: Marked infiltration of polymorphonuclear neutrophilic leucocytes and oedematous reaction with eosinophilic exudate and hemorrhage in the interstitial connective tissue of the muscles (arrows). (B) LBL-CTB: Moderate infiltration of neutrophils and oedematous reaction in the interstitial connective tissue of the muscles (arrow). (C) SIG-CTB: Production of fibrocytes and weak oedematous reaction are seen in the interstitial connective tissue between muscle fibers (arrows). Infiltration of neutrophils is rarely seen. (D) Al-gel (0.5 mg Al/0.5 ml): Between the widely separated muscle fibers there is a large amount of Al-gel mass (Al) with severe infiltration of neutrophils having phagocytized Al-gel. Some muscle fibers are intact, others are degenerated and some are necrotic (arrow). (E) rCTB (150 μg/0.5 ml): No inflammatory reaction is seen even when the concentration of rCTB is increased. (F) Physiological saline (0.5 ml): No change is observed. H–E, (original magnification: ×50).

making vaccine production more economical and more feasible. Although the absolute safety of vaccine adjuvants can never be guaranteed, it is noticeable that rCTB induced no in vitro cytotoxicities, local histopathological or vascular permeability-increasing reactions in the experimental animals. We showed also previously that intranasal or subcutaneous coadministration of rCTB with aluminium-nonadsorbed tetanus toxoid was better than intranasal or subcutaneous administration of aluminium-adsorbed tetanus toxoid to avoid IgE-mediated allergic reactions [27]. rCTB, unlike native CT and CTB containing a small amount of holotoxin, show powerful adjuvant activity without increased cyclic AMP formation. This notwithstanding, cytokine responses are similar for CT use (unpublished data: results will be presented in separate reports). Our recombinant system for CTB production in this study has a significant advantage especially for human vaccine use, since the host used was the Gram-positive bacterium *B. brevis* which is nonpathogenic, and the possibility of contamination of endotoxin and other virulence factors was excluded. In addition, rCTB was similar to the native CTB with respect to GM1 binding ability and noticeable stability of the pentamer [10]. These results suggest that, from a safety standpoint, rCTB produced by *B. brevis* is a useful candidate as mucosal vaccine adjuvant, and moreover there is great value in proceeding with this research. Studies on toxicities of combination of rCTB and some vaccines such as tetanus and diphtheria toxoids administered intranasally in a single or multiple doses to the nasal cavity mucous membranes are in progress in this laboratory.

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