Intranasal or subcutaneous co-administration of recombinant cholera toxin B subunit stimulates only a slight or no level of the specific IgE response in mice to tetanus toxoid

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Abstract

Whether recombinant cholera toxin B subunit (rCTB) co-administered intranasally or subcutaneously with aluminium-non-adsorbed tetanus toxoid (nTT) can induce the production of tetanus toxoid (TT)-specific IgE antibodies in mice was investigated compared with aluminium-adsorbed tetanus toxoid (aTT) intranasally or subcutaneously. Mice immunized intranasally or subcutaneously with nTT together with rCTB showed a high level of TT-specific serum IgG antibody response and no or a slight level of TT-specific serum IgE antibody response. On the other hand, in mice vaccinated intranasally or subcutaneously with aTT alone, higher levels of TT-specific IgG and IgE antibodies were induced in comparison with intranasal or subcutaneous inoculation of nTT together with rCTB. These results suggest that intranasal or subcutaneous co-administration of rCTB with nTT is better than intranasal or subcutaneous administration of aTT to avoid IgE-mediated allergic reactions.

Keywords: Recombinant cholera toxin B subunit (rCTB); Aluminium-non-adsorbed tetanus toxoid; Aluminium-adsorbed tetanus toxoid; Immunoglobulin E; Adverse effects

1. Introduction

Aluminium compounds are the only widely used adjuvants in vaccines for humans at present to stimulate an antigen-specific IgG antibody response. However, they have been reported to occasionally produce subcutaneous nodules [1, 2], alum granulomas [3–7] and sterile abscesses [1], and they may enhance IgE antibody formation in experimental animals [8–10]. High IgE responses to aluminium-adsorbed tetanus-diphtheria vaccines have also been reported in humans [11–13].

We reported previously [14] that recombinant cholera toxin B subunit (rCTB) acts as an adjuvant for the systemic and mucosal responses of mice to intranasally co-administered aluminium-non-adsorbed tetanus toxoid (nTT), that elevated TT-specific serum IgG antibodies can provide complete protection against tetanus toxin challenge in the same manner as that to subcutaneously injected aluminium-adsorbed tetanus toxoid (aTT), and that a small amount of aluminium compound included in aTT may also act as a strong adjuvant on intranasal immunizations.

In this study, we examined whether intranasal or subcutaneous immunization with nTT in the presence of rCTB induced TT-specific serum IgE antibody responses compared with intranasal administration or subcutaneous injection of aTT alone.

2. Materials and methods

2.1. Animals and immunogens

Female BALB/c mice aged 7 weeks and aluminium-adsorbed tetanus toxoid (aTT) containing 10Lf units
were purchased from SLC (Shizuoka, Japan) and The Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan), respectively. Aluminium-non-adsorbed tetanus toxoids (nTT) containing 400 Lf units ml$^{-1}$ and 920 Lf units ml$^{-1}$ were kindly provided by Research Institute for Microbial Diseases, Osaka University (Osaka, Japan) and The Chemo-Sero-Therapeutic Research Institute, respectively. Recombinant cholera toxin B subunit (rCTB) was prepared by cultivating *Bacillus brevis* bearing pNU212-CTB and purifying from the culture supernatant, as we have previously described [15].

### 2.2. Immunization of mice

Mice were vaccinated in four ways as shown in Fig. 1; (A) intranasal administration of nTT with rCTB, (B) subcutaneous injection of nTT with rCTB, (C) intranasal administration of aTT alone and (D) subcutaneous injection of aTT alone. Two experimental groups were used for each way except (B). In case of intranasal inoculation, mice were immunized under light ether anaesthesia with 30 µl of solution containing tetanus toxoid (TT) with and without rCTB. Group one of nasal administration with nTT together with 10 µg of rCTB consisted of mice receiving 0.5 Lf nTT on days 0 and 14, followed by immunization with 5 Lf nTT on days 21, 28 and 35, and blood was obtained on day 42 (Fig. 1A). Mice belonging to group two were intranasally inoculated with 5 Lf nTT with 10 µg of rCTB on days 0, 14, 21 and 28, and collection of blood sample was performed on day 35 (Fig. 1A); this experiment was done twice. Subcutaneous injection of 5 Lf nTT with 10 µg of rCTB was carried out on days 0, 14, 21, 28 and 35, followed by blood collection on day 42 (Fig. 1B). Intranasal inoculation of 0.5 Lf aTT alone was performed on days 0, 14 and 21 (group 1) and on days 0, 14, 21 and 28 (group 2), and serum was obtained after 1 week from the last booster, respectively (Fig. 1C). In case of subcutaneous injection of aTT alone, mice belonging to group one were immunized with 0.5 Lf aTT on days 0 and 28, followed by blood collection on day 42, and mice belonging to group two were immunized with 0.5 Lf aTT on days 0, 14, 21 and 28, and blood collection was carried out on day 35 (Fig. 1D).

### 2.3. Sample collection and assays for IgG and IgE antibodies

Blood was collected from the retro-orbital plexus of ether-anesthetized mice by using capillary tubes and an indirect ELISA was used to determine the TT-specific IgG antibody concentrations in the serum, as described before [14]. The mean and standard deviation (SD) of values at 450 nm on an automatic microplate reader (Tosoh Co., Tokyo) were calculated with sera of five non-immunized mice, and antibody-positive cut-off values were set as the mean + 3-fold SD. ELISA anti-TT antibody titers were expressed as the highest endpoint dilution of each sample giving the positive reaction, and shown as the geometric mean (GM) ± SD. Tetanus toxoid-specific serum IgE antibodies were determined by passive cutaneous anaphylaxis (PCA) tests in Sprague–Dawley rats, using a modification of the method previously described [16]. Briefly, 0.1 ml of each dilution of serum was injected into the shaved dorsal skin of rats. Two days later, 1 ml of phosphate-buffered saline, pH 7.2, (PBS) containing 200 µg of nTT and 2.5 mg of Evans blue was injected intravenously into the dorsal penile vein. After 30 min, the rat was killed, the skin on the back was removed, and the diameters of blue spots on the internal surface of the skin were measured. Sera were titrated in serial 2-fold dilutions in PBS, starting from a dilution of 1:5 with all mice immunized; 0.1 ml of non-diluted serum was also injected into the dorsal skin of rats according to circumstances. Five or six mice were used for each test. Anti-TT IgE PCA titers were expressed as the reciprocal of the highest dilution of each sample giving a reaction of 5 mm or more, and shown as GM ± SD.
2.4. Statistics

The differences among four experimental ways were evaluated by Fisher’s protected least significant difference (Fisher’s PLSD).

3. Results

Generally speaking, mice vaccinated intranasally and subcutaneously with nTT together with rCTB showed little or no production of TT-specific serum IgE antibody, whereas TT-specific IgE antibodies were detected in mice immunized intranasally and subcutaneously with aTT. Tetanus toxoid-specific serum IgG and IgE antibodies of all mice examined were summarized in Figs. 2–5. In mice immunized intranasally with 0.5 and 5 Lf nTT in the presence of rCTB (group 1), TT-specific serum IgG antibodies reached titres of 1:25,600, but TT-specific IgE antibodies showed titres of 1:1 in sera of only two of five mice; the remaining three mice did not induce TT-specific serum IgE at all (Fig. 2A). Intranasal administration of 5 Lf nTT with rCTB (group 2) induced significant levels (titres between 1:25,600 and 1:409,600) of TT-specific serum IgG antibody and very low levels (titres between 1:5 and 1:10) of TT-specific serum IgE antibody in all mice examined (Fig. 2B, 1st experiment). The second experiment of the same immunization schedule also showed high serum IgG antibody titres (1:25,600 to 1:102,400) to TT but TT-specific serum IgE antibody was detected in only one of five mice (titre of 1:10) (Fig. 2B, 2nd experiment). Tetanus toxoid-specific IgG antibody titres of mice which received subcutaneous injection of nTT and rCTB reached 1:819,200 and over, but TT-specific IgE antibody was not detected in all mice examined (Fig. 3). These results show that intranasal and subcutaneous immunization with nTT in the presence of rCTB may induce only a slight or no level of TT-specific serum IgE antibody.

On the other hand, mice given 0.5 Lf aTT three times (group 1) and four times (group 2) by the intranasal route exhibited TT-specific serum IgG titres between 1:16,000 and 1:32,000, and between 1:25,600 and 1:1,638,400, respectively, and TT-specific serum IgE titres between 1:10 and 1:160, and between 1:20 and 1:320, respectively (Fig. 4A and B). Tetanus tox-
iod-specific IgG antibody titres reached 1:1,638,400 and TT-specific IgE antibody responses rose to titres between 1:40 and 1:320 in the sera of mice that received subcutaneous injection of aTT twice (group 1) or four times (group 2) (Fig. 5A and B). When each number of mice vaccinated in four experimental ways was summed, there was a significant difference between intranasal (n = 16) or subcutaneous (n = 5) administration of nTT with rCTB and intranasal (n = 10) or subcutaneous (n = 10) administration of aTT for TT-specific IgE antibody titres (P < 0.0001).

4. Discussion

In this experiment, it became clear that fluid nTT administered intranasally or subcutaneously together with free rCTB stimulated only a slight or no level of TT-specific serum IgE antibody response with individual differences among mice, as well as stimulation of a high level of TT-specific serum IgG antibody response (Fig. 2); TT-specific mucosal IgA antibody responses were also induced, as reported before [14]. These results are not coincident with those reported by Tamura et al. about the adjuvant effects of CTB for ovalbumin-specific IgE responses after oral or intranasal administration to BALB/c mice [17]. This discrepancy seems to be due to the presence of 0.1% CT in their study. Marinaro et al. showed that oral immunization with TT and CT induces TT-specific serum IgG (IgG1 and IgG2b subclasses), IgA and IgE responses and TT-specific secretory IgA responses in the gastrointestinal tract, and that CT acts as a mucosal adjuvant to enhance Th2-type cell responses and in particular, IL-4 production [18]. When intranasally immunized with bovine serum albumin (BSA) or nTT, co-administered rCTB seemed to cause predominant activation of Th2 cells, resulting in induction of BSA- or TT-specific serum IgG1 and IgG2b responses and BSA- or TT-specific mucosal IgA responses [14, 19]. In vitro studies have indicated that CTB has the same IgG-, IgE- and IgA-potentiating function as CT, but to a lesser degree [20, 21]. Accordingly, it may be natural that intranasal administration of nTT with rCTB induced TT-specific serum IgE antibody. On the other hand, there is a report that CTB does not potentiate allergic sensitivity to mice for a protein antigen co-administered orally, and the presence of the A subunit of CT is necessary, both for a strong antibody response including mucosal IgA and to sensitize mice [22]. Judging from only a slight induction of TT-specific serum IgE response, rCTB may have a great potential for the separation of TT-specific IgG inducible and IgE inhibitory activities by its direct or indirect effect on Th2 cells producing cytokines that control activation and differentiation of B cells into antibody-secreting cells.

On the other hand, it is said that when administered orally or nasally, antigen alone or antigen coupled to CTB or LTB can induce systemic immunological tolerance, but the oral or nasal administration of antigen coupled to or together with CT or LT, resulting in the induction of IgG, IgA and IgE antibody responses to the antigen [17, 23–27]. As the rCTB preparation which we obtained from B. brevis carrying pNU212-CTB does not contain CT at all [28], a slight induction of TT-specific IgE antibody response is not due to the effect of the holotoxin.

When the adjuvant activity of aluminium phosphate (AlPO₄) and calcium phosphate (CaHPO₄) for TT were compared to nTT in mice and guinea-pigs injected subcutaneously, AlPO₄-adsorbed TT preparations induced not only higher neutralizing and IgG antibodies but also higher IgE antibodies than CaHPO₄-adsorbed TT preparations [8, 10]. In our experiment, aTT alone inoculated intranasally as well as subcutaneously also showed significantly high TT-specific serum IgE antibody response compared to nTT with rCTB inoculated intranasally and subcutaneously (P < 0.0001). Thus aluminium adjuvants may promote IgE-mediated allergic reactions regardless of inoculation method. When administered intranasally, recombinant CTB adjuvant generally induced a little lower TT-specific serum IgG antibody than aluminium adjuvants (Figs. 2 and 4) but protected all mice from challenge of tetanus toxin [14]. Accordingly, intranasal administration of nTT with rCTB seems to be safer and more excellent method to prevent tetanus than intramuscular or subcutaneous injection of aTT, which has been in practice for a long time.

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