Recombinant cholera toxin B subunit (rCTB) as a mucosal adjuvant enhances induction of diphtheria and tetanus antitoxin antibodies in mice by intranasal administration with diphtheria–pertussis–tetanus (DPT) combination vaccine

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Abstract
Recombinant cholera toxin B subunit (rCTB) which is produced by Bacillus brevis carrying pNU212-CTB acts as a mucosal adjuvant capable of enhancing host immune responses specific to unrelated, mucosally co-administered vaccine antigens. When mice were administered intranasally with diphtheria–pertussis–tetanus (DPT) combination vaccine consisting of diphtheria toxoid (DTd), tetanus toxoid (TTd), pertussis toxoid (PTd), and formalin-treated filamentous hemagglutinin (fFHA), the presence of rCTB elevated constantly high values of DTd- and TTd-specific serum ELISA IgG antibody titres, and protective levels of diphtheria and tetanus toxin-neutralizing antibodies but the absence of rCTB did not. Moreover, the addition of rCTB protected all mice against tetanic symptoms and deaths. DPT combination vaccine raised high levels of serum anti-PT IgG antibody titres regardless of rCTB and protected mice from Bordetella pertussis challenge. These results suggest that co-administration of rCTB as an adjuvant is necessary for induction of diphtheria and tetanus antitoxin antibodies on the occasion of intranasal administration of DPT combination vaccine.

Keywords: DPT combination vaccine; Recombinant cholera toxin B subunit (rCTB); Intranasal administration; Diphtheria and tetanus antitoxin antibodies

1. Introduction
Cholera toxin (CT), a major enterotoxin produced by Vibrio cholerae, is composed of two subunits—a toxigenic A subunit (CTA) which activates ADP-ribosylation activity and a pentameric B subunit (CTB) which is responsible for CT binding to the cell membrane GM1 gangliosides. The basic chemical structure of heat-labile toxin (LT) which is produced by enterotoxigenic Escherichia coli is almost the same as that of CT. Both CT and LT act as a mucosal immunogen and a mucosal adjuvant, and induce antigen-specific systemic IgG and secretory IgA antibody responses at various mucosal sites to unrelated vaccine antigens when administered along with them via the nasal or oral route [1–5]. Only an A subunit of LT (LTA) and various mutant LTs and CTs lacking ADP-ribosylation activity also retain the mucosal adjuvant properties of wild CT and LT [5–11]. Moreover, it has been shown that recombinant CTB (rCTB) and B subunit of LT (rLTB) also have potent adjuvant activity towards intranasally or orally administered antigens [12–14]. We demonstrated that rCTB, which is produced by Bacillus brevis HPD31 carrying pNU212-CTB [15] and purified with affinity chromatography using d-galactose immobilized agarose [16], effectively delivers tetanus toxoid (TTd) and diphtheria toxoid (DTd) to mucosal inductive sites and elicits antigen-specific serum IgG and mucosal IgA antibody responses when given intranasally along
with the toxoids [17,18]. With intranasal co-administration of an acellular pertussis vaccine the adjuvant activity of rCTB was vague because of the mucosal adjuvanticity of pertussis toxoid (PTd) [19]. No distinct local histopathological reactions were observed in the muscle, the nasal cavity and the small intestinal loop given rCTB [20], and frequent nasal administrations of rCTB-containing same and different vaccine-induced antigen-specific immune responses without induction of systemic tolerance and suppression by pre-existing anti-rCTB immunity [21]. In this study we investigated the adjuvant effect of rCTB on intranasal administration of a 30 μl (15 μl per nare) of a diphtheria–pertussis–tetanus (DPT) combination vaccine consisting of DTd, TTd, PTd, and formalin-treated filamentous hemagglutinin (FHA) and found that rCTB has an important role in the induction of diphtheria and tetanus antitoxin antibodies.

2. Materials and methods

2.1. Animals, immunogens, immunization, and sample collection

All procedures on animals were conducted according to the Guidelines for the Care and Use of Laboratory Animals of the Nagoya City University Medical School under protocols approved by the Institutional Animal Care and Use Committee at the Nagoya City University Medical School. Female BALB/c mice (SLC, Shizuoka, Japan) aged 7 weeks were used in this study. Each group consisted of 5, 10, 15 or 16 mice. TTd containing 200 Lf units ml⁻¹ (66 μg protein nitrogen (PN) ml⁻¹ and purity: 3025 Lf mg⁻¹ PN), DTd containing 200 Lf units ml⁻¹ (67 μg PN ml⁻¹ and purity: 2985 Lf mg⁻¹ PN), PTd containing 16 μg PN ml⁻¹, pertussis toxin (PT) containing 30 μg PN ml⁻¹, formalin-non-treated filamentous hemagglutinin (FHA) containing 32 μg PN ml⁻¹ and FHA containing 75 μg PN ml⁻¹ were provided by The Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). PT and FHA were used for an enzyme-linked immunosorbent assay (ELISA) and TTd, DTd, and PTd were not adsorbed to aluminum salt. Mice were administered and boosted intranasally with 30 μl of solution consisting of 5 Lf DTd, 5 Lf TTd, 0.5 μg PTd, and 5 μg FHA (DPT combination vaccine) with and without 10 μg rCTB under light ether anesthesia. Intranasal administrations were performed on days 0, 14, 21, and 28. Mice were sacrificed on day 35 and blood was collected. Almost the same experiments were repeated four times and designated as Experiments 1–4. Experiment 4 was mainly carried out for a challenge test of tetanus toxin (TT). In Experiment 1 collection of lung and nasal cavity lavages, small and large intestinal washes, saliva and vaginal secretions, and feces was done for measurement of mucosal IgA titres as described before [12,17]. These samples were stored at −20°C until use.

2.2. Measurement of antigen-specific IgG, IgA, and IgE antibody levels

Antigen-specific serum IgG and IgA, and mucosal IgA antibody titres were measured using ELISA (in-house ELISA) as described before [17,22] and antigen-specific serum IgE antibody was detected by fluorometric capture ELISA as described by Sakaguchi et al. [23] except the use of 5-[5-(N-succinimidylsulfonyl) pentylamido]hexyl n-biotinamide (Dojindo Laboratories, Kumamoto, Japan) in place of α-hydroxy succinimidobiotin to conjugate antigen with biotin; serum and mucosal IgA, and serum IgE antibody titres were determined only in Experiment 1. Calibration of the mean and standard deviation (S.D.) of values at 450 and 405 nm with sera and each washing sample of five non-immunized mice, setting of antibody-positive cut off values and expression of antigen-specific antibody ELISA titres and the geometric mean (G.M.) ± S.D. were carried out according to the previous description [19].

2.3. Cell culture method (CCM) for measurement of diphtheria antitoxin titres

In Experiments 1–4, the titration of diphtheria antitoxin of mouse serum was performed by CCM using Vero cells as described by Miyamura et al. [24] and diphtheria antitoxin titres were expressed as international neutralizing antibody units (IU) ml⁻¹.

2.4. Determination of anti-tetanus toxin antibody

The TT neutralization (TTN) assay was carried out in mice as described in “Minimum Requirement for Biological Products [25]”. Briefly, six-fold dilution (in Experiments 2 and 3) and two-fold dilution (in Experiment 4) of pooled sera were performed and assayed for TT level of L+/1000. Tetanus antitoxin titres were calculated relatively for the standard tetanus antitoxin [26,27] and expressed in IU ml⁻¹.

2.5. TT direct challenge test

Five days after final collection of blood on day 35, mice were challenged by subcutaneous injection of approximately 100 LD₉₀ of TT and was observed throughout their life and death after 7 days.

2.6. Kaketsuken particle agglutination (KPA) method

The particle agglutination test with poly (γ-methyl L-glutamate), which was developed and shortened to KPA by KAKEITSUKEN (The Chemo-Sero-Therapeutic Research Institute) [28], was carried out beforehand for the purpose of reference to measure tetanus antitoxin titres in
Experiments 1–4 and diphtheria antitoxin titres in Experiments 2–4, using Tetanus and Diphtheria Antibody Assay kits, respectively. The procedure was done according to the manufacturer’s instructions. The highest dilution showing positive agglutination was determined and the unitage of serum sample was calculated against the reference serum and expressed as IU ml$^{-1}$. There is a significant correlation [28] between the level of anti-tetanus KPA titres and that of tetanus antitoxin titres by hemagglutination [29] and between the level of anti-diphtheria KPA titres, and that of diphtheria antitoxin titres by CCM [24].

2.7. Measurement of anti-PT and anti-FHA antibody titres by calibration against a reference mouse serum

Serum PT- and FHA-specific IgG antibody titres were also expressed as ELISA units (EU) ml$^{-1}$ on the basis of a comparison of the response curve of the test serum to that of the reference mouse serum provided by the National Institute of Infectious Diseases, Tokyo, Japan (anti-PT IgG antibody: 886 EU ml$^{-1}$ and anti-FHA IgG antibody: 21 900 EU ml$^{-1}$). This was done according to a modified version of the parallel line assay procedure described by Sato and Sato [30].

2.8. Intranasal challenge of Bordetella pertussis

Intranasal infection of B. pertussis 18 323 phase I was performed using mice immunized intranasally with DPT combination vaccine with and without rCTB according to the method described before [19] and three mice per group were used.

2.9. Statistics

Analysis of antibody titres was performed on logarithmically transformed data and the G.M. and S.D. values were calculated. Mann-Whitney’s U-test was used to compare mean values of different groups with serum and mucosal antibody titres. Statistical significance was designated as $P < 0.01$ or $P < 0.05$.

3. Results

3.1. DTd-specific serum IgG antibody and diphtheria antitoxin responses: comparison among in-house ELISA, CCM, and KPA titres

We assessed the immune responses to DTd induced by intranasally delivered DPT combination vaccine. Mice immunized with DPT plus rCTB showed much higher DTd-specific serum IgG antibody titres by in-house ELISA than those without rCTB except Experiment 1, indicating the statistically significant adjuvant effect of rCTB in Experiments 2 and 3 (Fig. 1A).

Mice immunized with DPT plus rCTB showed the mean log$_{10}$ diphtheria antitoxin titres $\pm$ S.D. values of 0.47 $\pm$ 0.36, 0.80 $\pm$ 0.37, and $−0.21 \pm 0.65$ IU ml$^{-1}$ in Experiments 1–3, respectively, which were measured by CCM (Fig. 1B). These titres were sufficiently greater than 0.01 (log$_{10}$ $−2$) IU ml$^{-1}$ of serum and significantly higher than those obtained from mice treated with DPT alone, clearly showing that rCTB specifically induces diphtheria antitoxin antibody even if in-house ELISA antibody titres are almost the same irrespective of rCTB (Fig. 1A, Experiment 1). Diphtheria antitoxin titres measured by KPA in Experiments 2 and 3 also showed significant differences between mice immunized with DPT in the presence and absence of rCTB (Fig. 1C).

3.2. TTd-specific serum IgG antibody and tetanus antitoxin responses: comparison among in-house ELISA, KPA, and TTN titres

The addition of rCTB to DPT combination vaccine induced sufficiently high serum anti-TTd IgG antibody titres, which were significantly high only in Experiment 3 when compared with no addition of rCTB (Fig. 2A).

The mean log$_{10}$ tetanus antitoxin titres $\pm$ S.D. values measured by KPA were 0.65 (pool sera), $−1.75 \pm 0.69$ and $−1.47 \pm 0.96$ in Experiments 1–3, respectively, in the absence of rCTB and 2.53 (pool sera), 0.89 $\pm$ 0.05 and 0.75 $\pm$ 0.31 in Experiments 1–3, respectively, in the presence of...
rCTB (Fig. 2B). These tetanus antitoxin titres were significantly different between the presence and absence of rCTB unlike serum anti-TTd IgG in-house ELISA antibody titres. Subsequently, the toxin neutralization tests were carried out by six-fold dilution using the pool sera of mice obtained from Experiments 2 and 3. In Experiment 2 the TTN titres of pool sera from each five mice were less than 0.01 IU ml\(^{-1}\) without rCTB and 0.36–2.16 IU ml\(^{-1}\) with rCTB (Table 1). In Experiment 3, 15 or 16 mice were divided into two groups by KPA titres, i.e. into a higher and lower one. In mice immunized with DPT in the presence of rCTB both higher group (5 mice) showing KPA titres less than 0.0025 unit ml\(^{-1}\), and 11 mice showing KPA titres from 0.01 to 1.0 units ml\(^{-1}\) were less than 0.01 IU ml\(^{-1}\) (Table 1). On the other hand, in mice immunized with DPT in the presence of rCTB both higher group (5 mice) showing KPA titres between 8 and 16 unit ml\(^{-1}\) and lower one (10 mice) showing KPA titres between 2 and 4 unit ml\(^{-1}\) demonstrated TTN titres from 1.80 to 10.8 IU ml\(^{-1}\) (Table 1). Judging from these results, the addition of rCTB is essential to elevate tetanus antitoxin titres unlike anti-TTd antibody in-house ELISA titres.

Table 1

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>rCTB</th>
<th>Number of mice</th>
<th>KPA titres (units ml(^{-1}))</th>
<th>TTN titres (IU ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 2</td>
<td>0.5</td>
<td>5</td>
<td>0.0025–0.16</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5</td>
<td>6.4–8.0</td>
<td>0.36–2.16</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>0.5</td>
<td>5</td>
<td>&lt;0.0025</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10</td>
<td>2.0–4.0</td>
<td>1.8–10.8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5</td>
<td>8.0–16.0</td>
<td>1.8–10.8</td>
</tr>
</tbody>
</table>

*See Fig. 3A.

Pooled sera used and TTN tests were carried out by six-fold dilution.

3.3. Correlation between TTd-specific KPA and TTN titres

In Experiment 4, the correlation between TTd-specific KPA and TTN titres was investigated in detail in combination with DTd + TTd + PTd + fFHA + rCTB. As shown in Fig. 3, tetanus antibody titres measured by the KPA method with rCTB were significantly higher in comparison with those in the absence of rCTB irrespective of fFHA. Two of the 10 mice immunized with the mixture of DTd, TTd, PTd, and fFHA without rCTB showed very low anti-tetanus KPA titres, and one of them died on day 1 and the other one was paralyzed (Fig. 3A). Only one of the 10 mice immunized with DTd, TTd, and PTd in the absence of both fFHA and rCTB showed very low anti-tetanus KPA titre and died on day 1 (Fig. 3B). All mice inoculated with phosphate buffer saline died on day 1 (Fig. 3C). However, the addition of rCTB to DPT combination vaccine surely protected all mice against tetanic symptoms and deaths regardless of fFHA (Fig. 3A and B). TTN titres obtained from pooled sera of each ten mice immunized without rCTB were 2.77 IU ml\(^{-1}\) in the presence of fFHA and 0.36 IU ml\(^{-1}\) in the absence of fFHA. The addition of rCTB showed high TTN titres of 11.60 and 10.30 in the presence and absence of fFHA, respectively. Diphtheria antitoxin titres measured by CCM and KPA in the same samples were significantly higher in the presence of rCTB like Experiments 1–3 (data not shown).

Together with the results obtained from Experiments 1–3, these results made it clear that the addition of rCTB to DPT combination vaccine is necessary to protect against tetanus and diphtheria.
Fig. 4. Correlation between serum PT-specific antibody titres by in-house ELISA (A) and ELISA units by calibration against a reference mouse serum (B) with and without rCTB. Mice were administered intranasally with 5 LF DTd + 5 LF TTd + 0.5 µg PTd + 5 µg FHA ± 10 µg rCTB on days 0, 14, 21, and 28 and sacrificed on day 35. The same experiments were repeated three times. Both titres were not measured in Experiment 4.

3.4. Serum PT-specific IgG antibody titres by in-house ELISA and serum anti-PT ELISA units by calibration against a reference mouse serum

High levels of anti-PT serum IgG antibody titres by in-house ELISA were induced irrespective of rCTB through Experiments 1–3 and there were no significant differences in the presence and absence of rCTB (Fig. 4A). Serum anti-PT ELISA units from calibration against a reference mouse serum also increased and the presence and absence of rCTB showed no significant differences (Fig. 4B).

3.5. FHA-specific serum IgG antibody titres by in-house ELISA and serum anti-FHA ELISA units by calibration against a reference mouse serum

Serum FHA-specific IgG antibody titres by in-house ELISA increased substantially irrespective of rCTB and there was a significant difference in the presence and absence of rCTB (Fig. 4A). Anti-FHA ELISA units obtained from calibration against a reference mouse serum were also elevated regardless of the presence or absence of rCTB and a significant increase with rCTB was observed only in Experiment 3 (Fig. 4B).

3.6. Protection from intranasal challenge of B. pertussis in mice intranasally administered with DPT combination vaccine with and without rCTB

The bacteria were not removed from the lungs of non-vaccinated control mice even on day 14 of infection. However, regardless of rCTB, intranasal administration with DPT combination vaccine showed complete clearance of the bacteria from the lungs by 6 days postinfection (data not shown).

3.7. DTd-, TTd-, PT-, and FHA-specific serum IgA and IgE antibody responses

Serum IgA antibodies specific to all components of DPT vaccine were produced independent of rCTB and anti-DTd, TTd, and FHA IgA antibody titres were significantly higher in the presence of rCTB (Fig. 6A). Serum IgE antibody responses to DTd, TTd, and PT were seen in the presence of rCTB and TTd- and PT-specific IgE antibody titres were significantly higher (Fig. 6B).
Mucosal antigen-specific IgA antibody titres were measured only in Experiment 1. Significantly higher levels of mucosal DTd-specific IgA antibody titres were observed in the lungs, small intestines, feces and the saliva and vaginal secretions in the presence of rCTB (data not shown), almost coinciding with the results obtained from intranasal immunization with DTd ± rCTB [18]. Mucosal TTd-specific IgA antibody titres rose significantly in the nasal cavities, lungs, small intestines, feces, and the saliva and vaginal secretions in the presence of rCTB (data not shown), being different from the previous results from intranasal immunization with TTd ± rCTB [17]. The difference between two data may be due to the presence of PTd. High or moderate levels of PT-specific mucosal IgA antibody responses were induced in the lungs and the vaginal secretions regardless of rCTB and mucosal anti-FHA IgA antibody titres in the presence of rCTB increased significantly in the lungs and small intestines but not in the other mucosal sites (data not shown). These results were roughly in agreement with the those from intranasal immunization with PTd ± FHA ± rCTB [19].

4. Discussion

In this study the mucosal adjuvant effect of rCTB on intranasal administration of DPT combination vaccine consisting of DTd, TTd, PTd, and FHA was investigated and it was clearly shown that co-administration of rCTB is essential for induction of diphtheria and tetanus antitoxin antibodies. Moreover, all mice intranasally administered with a mixture of DPT combination vaccine and rCTB were surely protected from challenge of TT and diphtheria. Moreover, there was only one paper reported by Nagai et al. [32]. They used some onjisaponins, from the root of Polygala tenuifolia Willdenow, as a mucosal adjuvant and showed significant increases in serum anti-DTd, -TTd, -PTd IgG, and nasal IgA antibody titres. However, there were no data on diphtheria and tetanus antitoxin antibody and anti-DTd, -TTd, and -PTd serum IgE antibody titres.

It is generally known that mucosal IgA plays an important role in protection against infections by enteropathogens and viruses both in human and animal models [33–37]. The inhibition of bacterial adherence by mucosal IgA is considered to be one of the most important defense mechanisms against mucosal bacterial invasion and in vitro, has been shown to limit the attachment of bacteria to epithelial cells isolated from various mucosal sites [38–43]. Moreover, mucosal IgA can also neutralize toxins by blocking their binding to cell receptors [44]. On the other hand, a functional role for serum IgA is still unclear but the IgA, not the IgG, serum fraction, from patients with Clostridium difficile has been reported to neutralize the cytotoxic and enterotoxic properties of the major virulence factor, toxin A, of the bacterium [45]. In this study, rCTB significantly enhanced production of serum IgA antibodies and mucosal ones to DTd in the respiratory tract and the vagina. In our preliminary experiment, IgAs purified from three mouse sera with 13.0, 1.63, and 3.26 CCM IU ml⁻¹, which were intranasally administered with DTd + rCTB, showed 0.102, 0.005, and 0.026 CCM IU ml⁻¹, respectively, and mucosal IgAs purified from the pulmonary lavages of 4 mice intranasally immunized with DTd + rCTB, showed 0.006, 0.102, 0.026, and 0.026 CCM IU ml⁻¹, suggesting the possibility of neutralizing diphtheria toxin of IgA. However, it has been suggested that IgA is not essential for preventing viral infection, reduction in the severity of disease, or both because of no differences between wild-type IgA⁻/⁻ and transgenic IgA⁻/⁻ knockout mice [46]. The role of systemic and mucosal IgA merits further research.

There have been several studies that have tracked the tissue distribution of a marker following intranasal delivery to mice but the results obtained from these studies have been varied [47–51]. Wosiewsaraiah et al. [52] reported in detail that all parameters such as level of anesthesia, position of the animal during and post dye administration, dosing schedule and total volume administered are important for a solution delivered intranasally to be retained in the nasal cavity of a mouse. In case of a total volume of 30 μl, it is important to hold a mouse under heavy anesthesia and in a supine position both during and post dye administration and to administer according to the optimal schedule of administration, i.e. 2 μl per nare at 5 min intervals [52]. However, in our experiment a 15 μl bolus of vaccine per nare (a total volume of 30 μl) was delivered intranasally to moderately anesthetized mice held upright during administration and placed on its stomach in the cage post administration. Accordingly, a part of intranasally administered vaccine may have been swallowed or aspirated into the lungs.

Phase I safety studies conducted with intranasally administered rCTB and LTR92G revealed only minor side effects including self-limiting increased nasal secretions, itching,
runny nose and sneezing within a certain amount of them but not visible effects on the nasal mucosa, systemic adverse events and long-term adverse events [53]. Clinical trials with an inactivated, viro-rome-formulated, LT-adjuvanted, intranasal subunit influenza vaccine showed solicited and unsolicited symptoms such as nasal discomfort, sneezing, nasal pain, stuffy nose, runny nose, shivering, malaise, and runny nose. Moreover, Bell’s Palsy (facial palsy) was observed in a tiny minority [53]. However, a detailed analysis of the observed cases has not revealed a distinct pattern for an influenza vaccine-induced facial paresis. Pre-clinical studies in animal models are needed to evaluate the safety of intranasally administered CT and LT adjuvants, and derivatives prior to the initiation of clinical trials. Intranasal administration of rCTB to BALB/c mice caused no distinct local histopathological reactions in the nasal cavity [20]. If rCTB is confirmed to be completely non-toxic for humans, an intranasal DPT combination vaccine with rCTB could be easily licensed for human use. The needle-free intranasal administration is an attractive alternative procedure, especially in developing countries.

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References


