Frequent nasal administrations of recombinant cholera toxin B subunit (rCTB)-containing tetanus and diphtheria toxoid vaccines induced antigen-specific serum and mucosal immune responses in the presence of anti-rCTB antibodies

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Abstract

Vaccination via a mucosal route is a very attractive means for immunization, because both local and systemic immune responses are inducible and vaccines can be administered easily and safely from infants to elderly persons. For developing widely applicable mucosal vaccines using recombinant cholera toxin B subunit (rCTB) as a safe adjuvant, we examined whether frequent nasal administrations of rCTB-containing same and different vaccines could induce antigen-specific immune responses without induction of systemic tolerance and suppression by pre-existing anti-rCTB immunity. Ten repetitive nasal administrations to mice of tetanus toxoid (TT)+rCTB or diphtheria toxoid (DT)+rCTB raised and maintained high levels of antigen- and rCTB-specific serum IgG including high levels of tetanus/diphtheria antitoxin titres and raised nasal, salivary, lung, vaginal and fecal secreted IgA, suggesting that the regimen did not induce systemic tolerance to TT/DT and rCTB. Mice successively received repetitive five doses of TT as the first antigen and subsequent five doses of DT as the second antigen, and vice versa, raised serum IgG to the second antigen at various levels including low but sufficient protective levels of antitoxin titres and induced mucosal IgA in the lungs, the vaginas and feces, but hardly in the nasal secretions and salivas. After an interval of 22 weeks between the dosage of the first and second antigens, mice induced serum IgG to the second antigen at high levels and mucosal IgA in all sites. In conclusion, anti-TT and -DT serum and mucosal antibody responses induced by repeated intranasal immunization using rCTB adjuvant lasted for a long period, and for improving the effectivity of vaccination, different rCTB-containing vaccines should be administered at appropriate intervals.

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1. Introduction

Vaccination via a mucosal route is rational and a natural means for immunization because initial defense at the mucosal sites is available against most microbial pathogens which invade and/or colonize mucosally. Both local and systemic immune responses are inducible by mucosal immunization, while only systemic responses are raised by parenteral vaccination. The mucosal route has an advantage over intramuscular and subcutaneous injections in easy and safe administration without much invasiveness, and thus in willing good compliance from infants to elderly persons. However, poor immunogenicity of the mucosally administered antigens and development of mucosal tolerance have been thought to be potential limitations of mucosal vaccines. Elson and Ealding reported first that cholera toxin (CT) as an adjuvant abrogated mucosal tolerance and potentiated systemic and mucosal immune responses to an unrelated protein antigen [1,2].

For developing safe, stable and effective mucosal vaccines applicable for preventing various infectious diseases:
The Th1 type response was additionally observed [6–9]. More-
genens, and in case of HBs vaccine, a significant level of the
biased towards the Th2 type response to these vaccine anti-
gens to determine whether frequent nasal administrations of
the rCTB-containing vaccines are applicable without induction of systemic tolerance and without suppression of antigens-specific immune responses by pre-existing anti-rCTB antibodies.

2. Materials and methods

2.1. Antigen and adjuvant preparations

Tetanus toxoid (TT) containing 2667 Lf units mg\(^{-1}\) protein and diphtheria toxoid (DT) containing 2985 Lf units mg\(^{-1}\) protein, which were not aluminium-adsorbed, were provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). Recombinant CTB was purified from the culture supernatant of \(B.\) \textit{brevis} (pNU212-CTB) [3] by galactose–agarose affinity chromatography [4]. The rCTB preparation mainly contains stable pentamers and is able to bind to GM1 ganglioside like a native CTB preparation (Sigma, St. Louis, USA) [4]. Moreover, it has no ability to stimulate cyclic AMP formation in macrophages [12], and has been evaluated histopathologically as a safe adjuvant in the nasal cavity, the small intestine and the muscle of experimental animals [5].

2.2. Immunization of mice

Female BALB/c mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Mice 6 weeks of age were lightly anesthetized with ethyl ether and vaccinated intranasally using a micropipette with 5 Lf of TT or 5 Lf of DT plus 10\(\mu\)g of rCTB in total volume of 30\(\mu\)l of phosphate buffered saline (PBS). Mice received totally 10 doses of vaccine on weeks 0, 2, 3, 4, 5, 6, 7, 8, 9 and 10 (schedule 1), or on weeks 0, 2, 3, 4, 5, 27, 29, 30, 31 and 32 (schedule 2). Groups A (six mice) and B (five mice) were vaccinated with TT plus rCTB and with DT plus rCTB, respectively, throughout the experiments. Groups C (six mice) and E (five mice) were given totally five doses of TT plus rCTB followed by five doses of DT plus rCTB and groups D (five mice) and F (five mice) five doses of DT plus rCTB followed by five doses of TT plus rCTB, respectively; groups A–D were carried out in schedule 1 and groups E and F in schedule 2. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Nagoya City University Medical School.

2.3. Collection of specimens for antibody analyses

During the immunization period blood and fecal samples were collected: the former was taken from the retro-orbital plexus of ether-anesthetized mice. After 5.6 weeks of the final boost in schedule 1 and after 2 weeks of the final boost in schedule 2, mice were anesthetized by intraperitoneal administration of Avertin (1.8% 2,2,2-tribromoethanol: 1.25% isooamyl alcohol; 0.4 ml per mouse) and saliva, nasal wash, lung wash, vaginal fluid and blood samples were collected as previously described [7]. Total volumes of the collected nasal, lung and small-intestinal washes were 0.4, 1 and 3 ml, respectively. Specimen dilution of saliva, after salivation by intraperitoneal injection of pilocarpin, vaginal fluid and fecal extract was 10\(\times\)v/w. Diluents contained protease inhibitors, bovine serum albumin and NaN\(_3\) as previously described [7].
enzyme-linked immunosorbent assay (ELISA) according to the methods previously described [9]. Briefly, individual serum and mucosal specimens were added to microtiter plates (MaxiSorp U16; Nunc, Roskilde, Denmark) pre-coated with either DT, TT or rCTB antigen (each, 1 μg ml⁻¹ in PBS). Binding of serum IgG antibody was detected with biotinylated goat anti-mouse IgG antibody, peroxidase-conjugated streptavidin (Southern Biotechnology, AL, USA), and color development with tetramethylbenzidine-H₂O₂ (Bio-Rad Laboratories, CA, USA) at A₄₅₀ after stopping the reaction with H₂SO₄ was measured. Binding of mucosal IgA antibody was detected with biotinylated goat anti-mouse IgA antibody, β-galactosidase-conjugated streptavidin (Southern Biotechnology), and color development with o-nitrophenyl-β-galactoside (Southern Biotechnology) at A₄₀₅ after stopping the reaction with EDTA was measured. The end point titer was estimated as the maximum dilution of the specimen giving an absorbance greater than three-fold S.D. + mean value of the unimmunized specimen.

2.5. Analyses of serum antitoxin titres

Serum anti-tetanus and anti-diphtheria toxin titres were determined by particle agglutination methods (KPA methods [13]) using Tetanus Antibody Assay Kit KAKETSUKEN and Diphtheria Antibody Assay Kit KAKETSUKEN (Chemo-Sero-Therapeutic Research Institute) and expressed as international unit (IU) ml⁻¹. There is a significant correlation [13] between the level of anti-tetanus KPA titres and the hemagglutination of tetanus antitoxin titres [14], and between the level of anti-diphtheria KPA titres and the micro cell culture method of diphtheria antitoxin titres using Vero cells [15].

3. Results

In the first experiment, each TT and DT vaccine was repeatedly co-administered intranasally with rCTB adjuvant to BALB/c mice. The time course for serum IgG and fecal IgA antibody responses is shown in Figs. 1 and 2. Mice (group A) immunized with repetitive ten doses of TT + rCTB over a period of 10 weeks raised serum anti-TT and anti-rCTB
IgG antibodies gradually and maintained their titers at high levels during the experimental period (A1, A3), and also raised fecal anti-TT and anti-rCTB IgA antibodies (A2, A4). Similar serum and fecal antibody responses were observed in group B mice administered with repetitive 10 doses of DT + rCTB (B1–B4). Mucosal responses after 15.6 weeks at the other sites showed that anti-TT/anti-DT and anti-rCTB IgA antibodies were induced in the nasal wash, the saliva (a part of mice with TT), the lung wash and the vaginal wash in groups A and B mice (Fig. 3). Thus, rCTB acted as an immunogen as well as a mucosal adjuvant for co-administered toxoids, and serum and mucosal anti-rCTB antibodies did not seem to suppress both systemic and mucosal antibody responses to these antigens.

In the second experiments, repeated five administrations of TT/DT + rCTB were followed by five doses of DT/TT + rCTB after a short interval (1 week; group C/D mice). As shown in Fig. 4, all mice induced serum IgG antibodies to TT administered first and the levels of the titres were maintained for 10 weeks in four mice but declined in two mice (C1). Fecal IgA antibody responses to TT were induced in four mice but hardly in two mice whose serum IgG responses decreased (C2). Serum IgG antibodies to DT given secondly were also induced but after the third administration their titres dispersed and declined in two mice (C3). Fecal IgA antibody responses to DT administered secondly were observed in some mice (C4). Serum and fecal anti-rCTB antibody responses showed almost the same profiles as groups A and B mice (C5, C6; Fig. 1: A3, A4; Fig. 2: B3, B4). In replacement of TT with DT (Fig. 5), all mice induced high levels of anti-DT serum IgG antibody (D1) and maintained the levels and induced fecal IgA responses (D2). Serum IgG...
Fig. 5. Time course of serum IgG and fecal IgA antibody responses to DT, TT and rCTB in group D mice intranasally administered first DT (5 Lf) + rCTB (10^7 H9262 g) five times (open arrows) and second TT (5 Lf) + rCTB (10^7 H9262 g) five times (closed arrows). Interval between the former and the latter was 1 week. The same symbols belong to the same animals through D1 to D6.

Fig. 6. Mucosal IgA antibody responses to TT, DT and rCTB after 15.6 weeks of groups C and D mice shown in Figs. 4 and 5, respectively. Nasal, saliva, lung and vaginal IgA antibody titres (mean + S.D.) of TT (open column), DT (gray column) and rCTB (hatched column) are shown. The exception that in each group one mouse whose serum IgG response to the second antigen decreased much (Fig. 4: C3; Fig. 5: D3) and also did not induce lung and vaginal IgA responses. Anti-rCTB IgA antibodies were induced at almost all the mucosal sites (Fig. 6).

In order to investigate a cause of the dispersion of serum IgG antibody titres and partly low mucosal IgA antibody titres of the second administered antigen, repeated administration of the second antigen (DT/TT) + rCTB were started 22 weeks after the final administrations of the first antigen (TT/DT) + rCTB (the third experiment, groups E, F mice). As shown in Figs. 7 and 8, high levels of serum IgG antibodies to TT/DT administered first were maintained at least 34 weeks (E1, F1) and serum IgG antibody responses to the second antigen (DT/TT) reached high levels without delay and dispersion (E3, F3); high anti-rCTB serum IgG antibody titres continued until 34 weeks (E5, F5). With the first administered antigen, fecal anti-TT/anti-DT IgA antibody responses of some mice increased during the first several weeks and then gradually decreased, but retained detectable levels even over 30 weeks (E2, F2); a mouse showing the lowest serum anti-TT IgG titre did not induce detectable levels of fecal anti-TT IgA (E1, E2). Anti-rCTB IgA antibodies
were detected after 34 weeks (E6, F6). On the other hand, with the second administered antigen, both fecal anti-TT and anti-DT IgA antibodies were observed in only a few mice (E4, F4). However, as shown in Fig. 9, mucosal anti-DT and anti-TT antibody titres were higher than or comparable to those obtained from a short interval (1 week) at all the mucosal sites (E, F; Fig. 6; C, D). Considerably high levels of mucosal anti-CTB IgA antibody titres were detected at all sites (E, F). Out of figures, in the small-intestinal washes, anti-TT, anti-DT and anti-CTB IgA antibody titres (log2) were 1.9 ± 0.9, 3.6 ± 1.4 and 4.8 ± 1.2 with group E mice and ≤ 1.0 ± 0, 4.2 ± 0.8 and 6.5 ± 1.4 with group F mice, respectively.

Serum anti-tetanus KPA mean titres of the last sampling day of groups A, C, D, E and F mice were 53.3 (range, 64–32), 25.2 (64–1), 4.1 (8–0.5), 14.6 (32–1) and 16.0 (16–16) IU ml\(^{-1}\), respectively. Anti-diphtheria KPA mean titres of groups B, C, D, E and F mice were 89.6 (range, 128–64), 4.9 (16–0.25), 13.0 (32–1), 13.6 (32–8) and 32 (32–32) IU ml\(^{-1}\), respectively. Both KPA titres showed the highest level with 10 repetitive administrations of the same antigen (groups A and B) and the lowest level to the second antigen with the short interval experiments (group D for tetanus and group C for diphtheria); however, each KPA titre was greater than 0.1 IU ml\(^{-1}\), an estimated protective level of tetanus and diphtheria antitoxins of human serum [13].
In relation to protective activity, we have previously demonstrated that mice immunized intranasally four times with TT + rCTB were protected from tetanus toxin challenge [7] and those immunized with DT + rCTB raised sufficient serum diphtheria antitoxin titres higher than protective levels determined by micro cell culture method using Vero cells [8].

4. Discussion

Oral administration of antigens, depending on antigen dose and frequency of administration, results in the induction of a state of systemic immunological tolerance, i.e. oral tolerance [16,17], which is an important physiological mechanism to avoid developing inflammatory immune reactions to many ingested food proteins. The same phenomenon is also observed with nasally inhaled antigens [18]. CT [1], CTB-containing a trace amount of CT [19,20] and rCTB [6–9,21] abrogate the mucosally induced immunological tolerance and induce systemic and mucosal immune responses against co-administered antigens. Coupling of certain antigens to CTB enhances mucosal antibody responses [22,23] and/or systemic tolerance against conjugated antigens [24,25]. Both adjuvanticity and tolerogenicity are involved in complex immunomodulating activities of the CTB molecule, and thus clinical applications...
Fig. 9. Mucosal IgA antibody responses to TT, DT and rCTB after 34 weeks of groups E and F mice shown in Figs. 7 and 8, respectively. Nasal, saliva, lung and vaginal IgA antibody titres (mean ± S.D.) of TT (open column), DT (gray column) and rCTB (hatched column) are shown. Four nasal washes and two salivas, lung washes and vaginal washes of group F mice failed.

of CTB to mucosal vaccine development and prevention of autoimmune diseases and allergy are now of great interest [26,27].

As induction of systemic tolerance is not desirable for anti-infectious mucosal vaccines, it was investigated in this study whether the frequent mucosal administrations of the same or different antigen together with rCTB affected the development of antigen-specific systemic and mucosal responses in mice. As shown in the first experiment, 10 repetitive nasal inoculations of TT/DT + rCTB developed and maintained high levels of systemic and mucosal antibody responses, but systemic tolerance did not occur. Moreover, over 0.5 year-long persistence of not only systemic and mucosal antibody responses to TT, DT and rCTB but also tetanus and diphtheria antitoxin immunity were observed after five administrations of rCTB-containing vaccines, as shown in the third experiment. Such strong and persisting systemic and mucosal immunological responses would be one of the advantages of using CTB adjuvant for mucosal vaccine development. Additionally, presence of anti-rCTB IgA antibody in the intestines may contribute partly to protection against cholera [28]; in fact the washes of small intestines of groups E and F mice contained significant levels of anti-rCTB IgA antibodies.

When different antigens are given with rCTB sequentially, it seems not to deny the possibility that pre-existing anti-rCTB antibodies suppress the immune responses to the second administered antigen. In the second and third experiments, therefore, we examined the immune responses using TT as the first antigen and DT as the second antigen, and vice versa, and demonstrated that systemic and mucosal antibody responses to both second antigens could be stimulated even in the presence of anti-rCTB antibodies. Although there was no substantial difference in the induction of systemic responses between the short and long administration intervals, variation in antibody levels among mice seemed to be larger and antitoxin titres were lower in the short interval than those in the long interval. Nasal and salivary IgA responses to the second antigen were hardly detectable only in the short interval. Effect of serum anti-rCTB IgG on the differences is unlikely, because high levels of IgG were observed throughout in both cases. Mucosal anti-rCTB IgA levels may be partly responsible for the variation in immune responses. The pre-existing anti-rCTB IgA at the inductive site, the nasal cavity in this study, could trap some of the secondly given rCTB, resulting in a decrease in free CTB molecules necessary for achieving full adjuvanticity. However, mechanisms of the selective influence of the pre-existing rCTB immunity on the serum, nasal and salivary immune responses are unknown. Pre-existing rCTB-specific T cells at the inductive site and in the systemic circulation [29] could play regulatory/stimulatory functions for local and systemic responses. Yet mechanisms of immunomodulating functions of CTB in vivo including immunocytes network have not been fully elucidated. By its remarkable high affinity with cell surface GM1 ganglioside (association constant with GM1: $7.3 \times 10^{-10}$ [30]) of most mammalian cells, CTB facilitates uptake of itself and co-administered antigens from the mucosal surface into the mucosal-associated lymphoid tissues, and CTB further interacts with various immunocytes, such as antigen presenting cells, T and B cells [2,27].

Wu and Russell reported that pre-existing immune responses to CT suppressed the subsequent serum IgG response to Streptococcus mutans antigen, but did not inhibit the mucosal IgA antibody response to the antigen: they achieved intranasal administration of the antigen + CTB after 8 days (i.e. a short interval) of immunization with CTB + CT [33]. Tamura et al. examined the effect of anti-adjuvant immunity by using CTB or Escherichia coli heat labile enterotoxin B subunit (LTB) supplemented with a trace amount of the holotoxin and influenza haemagglutinin (HA) vaccines administered intranasally to mice and reported that the adjuvant-combined vaccines could be
given repeatedly (at least 10 times [34]) without reducing the protective efficacy of the vaccines [34,35]. They also reported that high levels of pre-existing nasal IgA and serum IgG antibodies to LTB, which were provided by repeated pretreatments with adjuvant alone, inhibited the induction of anti-HA antibody responses and reduced the effectiveness of the adjuvant-combined vaccine [34]. Bergquist et al. studied the influence of pre-existing immunity to CTB by using CTB-dextran conjugate + CT and showed that pre-existing immunity to CTB inhibited both lung mucosal response and serum antibody response to dextran, but this effect could be overcome by using a higher dose of conjugate and long intervals (3 months) between immunizations [36]. These results are nearly consistent with our present data using rCTB adjuvant, TT and DT. Taken together, these CTB- or LTB-containing vaccines can be used effectively by consideration to regimens in spite of pre-existing anti-adjuvant immunity.

In summary, we showed here that anti-TT and -DT serum and mucosal antibody responses induced by repeated intranasal immunizations using rCTB adjuvant lasted for a long period and that, for improving the effectiveness of vaccination, different rCTB-containing vaccines should be administered at appropriate intervals.

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