Induction of systemic and mucosal antibody responses in mice immunized intranasally with aluminium-non-adsorbed diphtheria toxoid together with recombinant cholera toxin B subunit as an adjuvant

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Abstract

Nasal mucosal immunization is very attractive for vaccination to prevent various bacterial and viral infectious diseases because of induction of systemic and mucosal immune responses. The aim of the present study was to investigate the possibility of changing the immunization procedure of diphtheria toxoid (DT) from intramuscular or subcutaneous injection to intranasal administration. Intranasal immunization with aluminium-non-adsorbed diphtheria toxoid (nDT) together with recombinant cholera toxin B subunit (rCTB, 10 μg) induced, at a concentration of 5 Lf, high levels of serum DT-specific IgG antibody responses and high or moderate levels of the specific IgA antibody responses in all mice and only a slight level of the specific IgE antibody responses in some mice. Furthermore, sufficiently high diphtheria antitoxin titres more than 0.1 international units (IU) ml^{-1} were obtained from mice which showed high levels of serum DT-specific IgG antibody responses. Under the same experimental conditions, induction of significant levels of mucosal DT-specific IgA antibody responses occurred in the nasal cavity, the lung, the saliva and vaginal secretions and the small and large intestines of all mice, although there were different titres between individual mice. Similar results were also obtained with rCTB-specific serum IgG and IgA and mucosal IgA antibody responses; serum rCTB-specific IgE antibody titres were not detected. These results show that intranasal administration of nDT with rCTB must be a very useful means for vaccination against diphtheria. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Many infections in humans and animals are caused by bacterial and viral pathogens attaching initially to the mucous membranes, remarkably in the respiratory, gastrointestinal and urogenital tracts. Accordingly, these mucosal membranes represent the first defensive line and are natural sites for immunization. Based on the concept of the common mucosal immune system which allows protective immune responses induced at one mucosal site to be expressed at other mucosal sites and glandular tissues [1], the oral and nasal mucous membranes are very attractive and potential sites for

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vaccination. Moreover, oral or nasal mucosal immunization has the potential to stimulate both mucosal and systemic immunity. If we are forced to say, the intranasal route may be better than the oral route because of more effective delivery of small amounts of antigen to the mucosal immune system, simple mixture of an antigen and a mucosal adjuvant and a considerably lower exposure of an immunogen to adverse conditions such as low pH and the proteolytic enzymes [2–5].

Diphtheria is a bacterial disease whose clinical manifestations are caused by the toxin produced by *Corynebacterium diphtheriae*. The presence of toxin-neutralizing antibodies induced by immunization with diphtheria toxoid (DT) is very important for preventing this disease. The administration method of DT which has been in practice for a long time is a subcutaneous or intramuscular injection but oral administration of toxoid incorporated into lozenges and inhalation of aerosolized toxoid had been tried a long time ago [6,7]. However, the latter two methods had need of very high amounts of antigen or induced various allergic reactions. Recently, intranasal administration of fluid DT with non-ionic excipients in mice [8] or with an enhancer mixture of caprylic/capric glycerides and polysorbate in humans [9] has been reported.

Cholera toxin (CT) composed of two subunits, a toxigenic A subunit (CTA) which activates the adenylate cyclase system and a pentameric B subunit (CTB) which is responsible for CT binding to the cell membrane GM1 gangliosides, is a potent immunogen for the induction of an antigen-specific serum and mucosal IgA antibody responses. Moreover, CT is an adjuvant and enhances serum and mucosal antibody responses to unrelated antigens when given orally or intranasally [10–15]. The same statements are true for recombinant CTB (rCTB) produced by *Bacillus brevis* carrying pNU212-CTB or rCTB-expressing *Escherichia coli,* and it has been shown that the highly purified rCTB has an adjuvant activity when administered intranasally with bovine serum albumin (BSA), aluminium-non-adsorbed tetanus toxoid (nTT) or *Streptococcus mutans* surface protein AgI/II [4,5,16,17].

In this study, for the purpose of changing the immunization procedure of DT from intramuscular or subcutaneous injection to intranasal administration, we examined anti-DT-specific serum IgG, IgA and IgE and mucosal IgA antibody responses to aluminium-non-adsorbed DT (nDT) given intranasally with rCTB as an adjuvant in mice. Furthermore, we investigated whether intranasal immunization with nDT in the presence of rCTB provided mice with protective immunity against diphtheria toxin.

2. Materials and methods

2.1. Animals

Female BALB/c mice (SLC, Shizuoka, Japan) aged 7 weeks were used in this study. Each group consisted of five or six mice.

2.2. Immunogens

Aluminium-non-adsorbed DT containing 200 Lf units ml^{-1} (67 μg protein nitrogen ml^{-1}, purity; 2,985 Lf mg^{-1} protein nitrogen) was provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). Recombinant CTB was prepared by cultivating *Bacillus brevis* bearing pNU212-CTB at 30°C for 5 days [18] and purified from the culture supernatant with affinity chromatography using D-galactose immobilized agarose [19] and contained little amount of leukocytosis promoting factor, as described before [4].

2.3. Immunization of mice

Mice were immunized intranasally under light ether anaesthesia with 30 or 130 μl of solution containing nDT and rCTB on days 0, 14, 21 and 28 and sacrificed on day 35. In case of 1 and 5 Lf nDT in the presence of 10 μg of rCTB, mice received 30 μl of the solution once a day and in case of 25 Lf nDT with and without 10 μg of rCTB, mice received 32.5 μl of the solution four times at 30 min intervals a day.

2.4. Sample collection

Collection of lung and nasal cavity lavages, small and large intestinal washes, saliva and vaginal secretions and blood was carried out as described before [16]. Fecal pellets were collected from each immunized mouse and solution II [4,16] was added to them at a ratio of 10 μl/mg of pellets, homogenized and then centrifuged at 16,000 g for 60 min at 4°C. The supernatant fluids were stored at −20°C until use for ELISA.

2.5. Measurement of antibody levels by ELISA

Diphtheria toxoid- and rCTB-specific IgG or IgA antibody titres in serum, washing samples of lung, nasal cavity and small and large intestines, saliva and vaginal secretions and feces were estimated using ELISA as described previously [4,16], except that nDT (1 μg ml^{-1}) was used instead of nTT.

The mean and standard deviation (S.D.) of values at 450 nm, which were read on an automatic microplate reader (Bio-Rad Laboratories, Richmond, CA) after
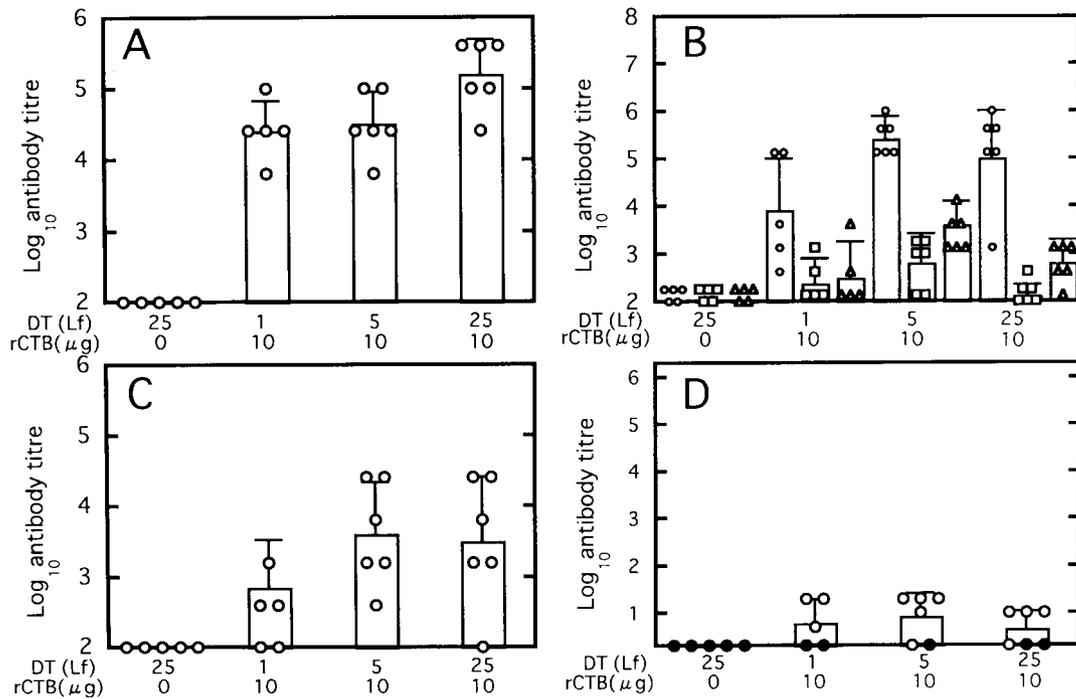
Fig. 1. Serum antibody responses to nDт co-administered intranasally with and without rCTB. (A) IgG. (B) IgG subclass: (○) IgG1; (□) IgG2a; (△) IgG2b. (C) IgA. (D) IgE: (○) positive; (●) negative. Each symbol represents a mouse in all the figures.

the enzyme reaction of peroxidase was stopped by adding H_2SO_4, were calculated with sera and each washing sample of five nonimmunized mice and antibody-positive cut-off values were set as the mean + threefold SD. ELISA anti-Dт and anti-rCTB antibody titres were expressed as the highest endpoint dilution of each sample giving the positive reaction and shown as geometric mean (GM) ± SD.

2.6. Assay of IgE antibody

Diphtheria toxoid-specific and rCTB-specific serum IgE antibody titres were measured by passive cutaneous anaphylaxis (PCA) tests in Sprague-Dawley rats, as described previously by Isaka et al. [17] and according to the method described by Marinaro et al. [20], respectively. Anti-Dт and anti-rCTB IgE PCA titres 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.

2.7. Determination of diphtheria antitoxin titres

The titration of diphtheria antitoxin of mouse serum was carried out by micro cell culture method using VERO cells as described by Miyamura et al. [21] and diphtheria antitoxin titres were expressed as international units (IU) ml^{-1}.

3. Results

3.1. Effects of dose of nDт on serum antibody responses to intranasally administered nDт with and without rCTB

We determined nDт concentrations necessary for sufficient induction of serum anti-Dт-specific antibody responses with a constant amount of rCTB (10 µg). Intranasal administration of nDт in the absence of rCTB did not cause any Dт-specific serum IgG antibody response even at a concentration of 25 Lf (Fig. 1A). On the other hand, in the presence of rCTB, the dose of 25 Lf nDт showed the highest antigen-specific serum IgG antibody titres and intranasal immunization with 1 or 5 Lf nDт also induced considerably high Dт-specific serum IgG antibody responses in all mice examined (Fig. 1A). The major subclass of serum IgG responses to Dт was IgG1 followed by IgG2b and significant IgG2a antibody responses were detected in two, four and only one of five or six mice immunized with 1, 5 and 25 Lf nDт, respectively (Fig. 1B). Diphtheria toxoid-specific serum IgA antibody was induced in all mice that received 5 Lf nDт and rCTB, in three of five mice given 1 Lf nDт with rCTB and in five of six mice administered 25 Lf nDт in the presence of rCTB (Fig. 1C); in case of nTT, tetanus toxoid (TT)-specific serum IgA antibody was not induced at all even in the presence of rCTB [16]. Very low levels (titres between 1:1 and 1:20) of Dт-specific serum IgE
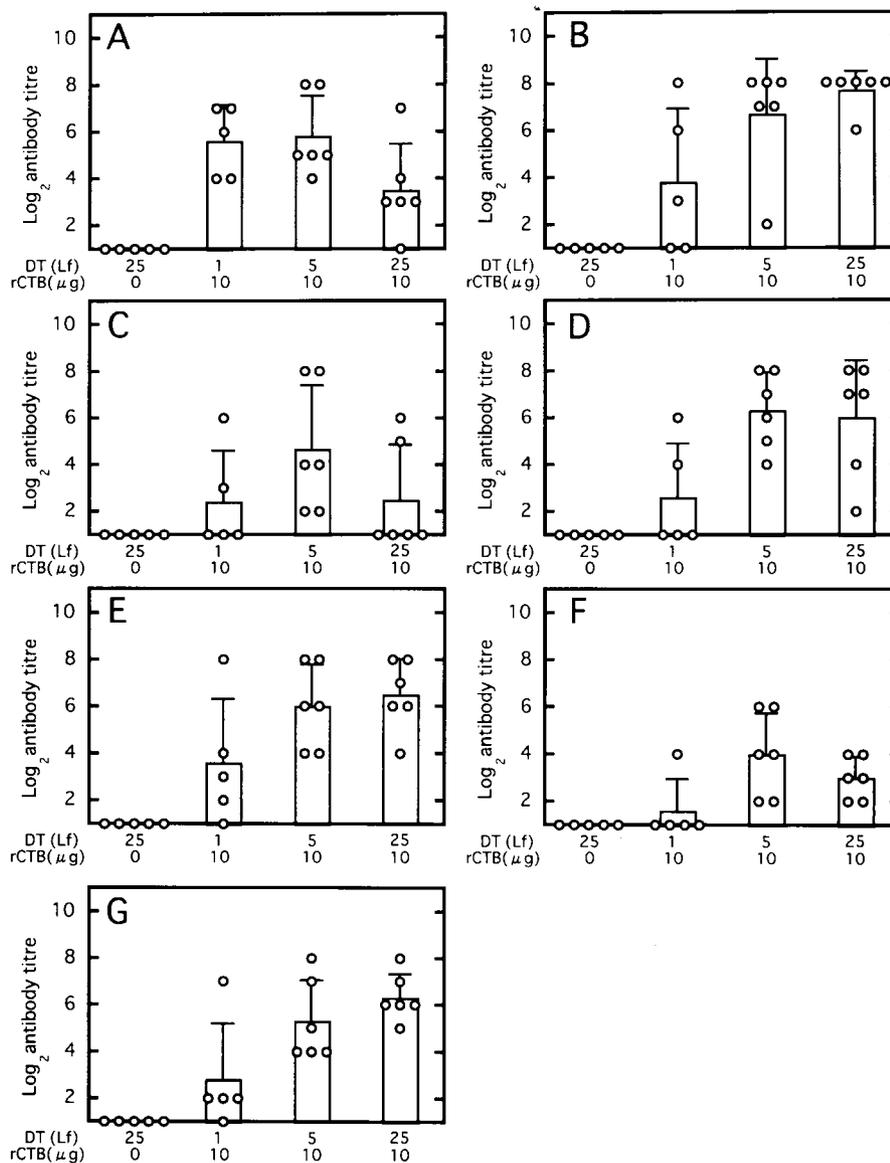
Fig. 2. Mucosal IgA antibody responses to nDT co-administered intranasally with and without rCTB. (A) nasal cavity. (B) lung. (C) saliva. (D) vagina. (E) small intestine. (F) large intestine. (G) feces.

antibody were detected in three to five of five or six mice regardless of nDT concentrations (Fig. 1D).

3.2. Effects of dose of nDT on mucosal immune responses to intranasally administered nDT with and without rCTB

Intranasal administration of nDT without rCTB did not induce mucosal IgA responses to DT at all mucosal sites examined of mice (Fig. 2A–G). Mice given 1 Lf nDT with rCTB exhibited generally lower levels of DT-specific mucosal IgA antibody titres than those given 5 or 25 Lf nDT with rCTB, both of which gave rise to similar mucosal IgA responses to DT at all

mucosal sites (Fig. 2A–G). If examined in detail, in case of 5 Lf nDT administration, DT-specific mucosal IgA antibody titres of 16-fold dilution and over were detected in the nasal cavities, the vaginal secretions, the small intestines and the feces of all mice (Fig. 2A,D,E and G), in the lungs of five mice (Fig. 2B) and in the saliva secretions and the large intestines of four mice (Fig. 2C and F). The same levels of DT-specific mucosal IgA antibody responses were observed in the lungs, the small intestines and the feces of all mice that received 25 Lf nDT with rCTB (Fig. 2B,E and G), in the vaginal secretions of five mice (Fig. 2D) and in the nasal cavities, the saliva secretions and the large intestines of two mice (Fig. 2A,C, and F).

Table 1
Serum antitoxin titres of mice intranasally immunized with nDT plus rCTB[a]

Mouse no.	Serum DT-specific antibody titre[b]	Diphtheria antitoxin titre (IU ml^{-1})
1	1638400	2.89
2	6400	0.008
3	409600	2.89
4	102400	0.256
5	1638400	1.02

[a] Mice were immunized intranasally with 5 Lf nDT and 10 μg of rCTB on days 0, 14, 21 and 28 and sacrificed on day 35.
[b] Reciprocal of the serum dilution giving an ELISA absorbance value more than antibody-positive cut-off values.

3.3. Induction of diphtheria antitoxin activity by intranasal immunization with nDT in the presence of rCTB

It is generally considered that antitoxin titre of 0.1 IU ml^{-1} is the smallest level necessary to protect man from a challenge of diphtheria toxin. We therefore measured the antitoxin titres for mice which were intranasally immunized with 5Lf nDT plus rCTB. As shown in Table 1, mice which indicated high levels of DT-specific serum IgG antibody titres gave sufficiently high antitoxin titres greater than 0.1 IU ml^{-1} except for No. 2 mouse.

These results clearly demonstrate the phylacagogic effect of intranasal administration of nDT with rCTB.

3.4. Serum anti-rCTB antibody responses

Serum anti-rCTB-specific antibody production was examined in mice given 10 μg of rCTB together with
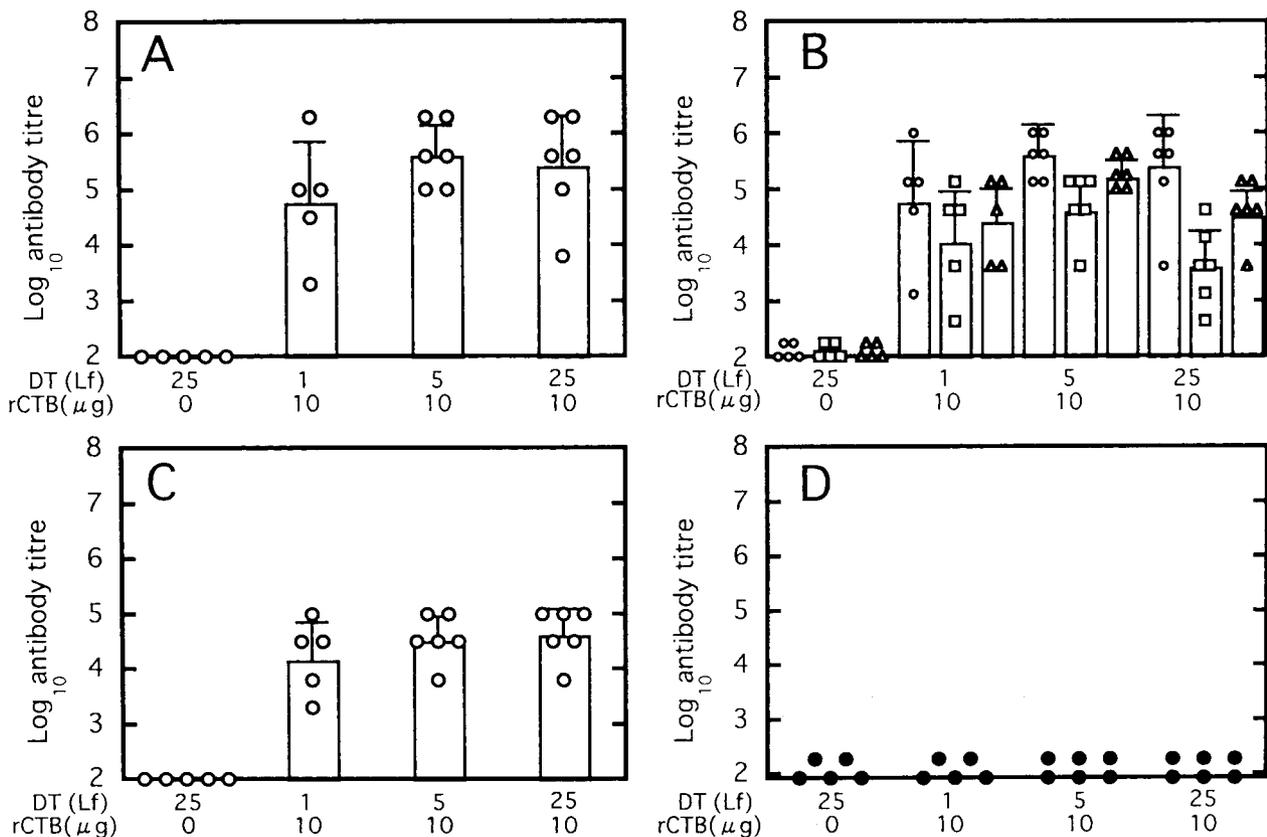
Fig. 3. Serum antibody responses to rCTB co-administered intranasally with nDT. (A) IgG. (B) IgG subclass: (○) IgG1; (□) IgG2a; (△) IgG2b. (C) IgA. (D) IgE: (●) shows negative as well as in Fig. 1D.
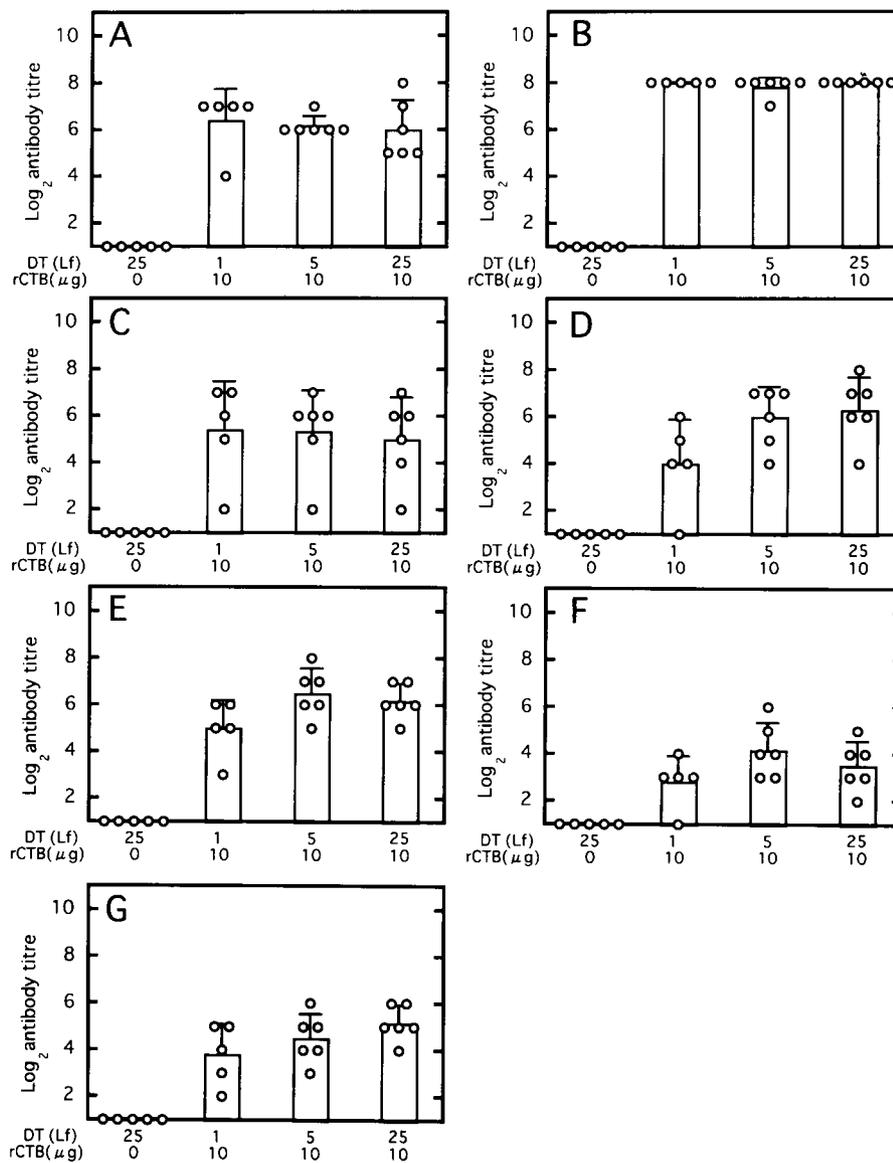
Fig. 4. Mucosal IgA antibody responses to rCTB co-administered intranasally with nDT. (A) nasal cavity. (B) lung. (C) saliva. (D) vagina. (E) small intestine. (F) large intestine. (G) feces.

1, 5 or 25 Lf nDT. Regardless of the nDT concentrations, very high levels of rCTB-specific serum IgG antibody and considerably high levels of rCTB-specific serum IgA antibody were induced by intranasal administration (Fig. 3A and C); little or no serum anti-rCTB IgA antibody was detected in case of intranasal co-administration of rCTB with nTT as reported before [16]. When serum IgG subclass responses to rCTB were assessed, very high IgG1 and IgG2b antibody responses, followed by considerably high IgG2a antibody responses, were observed (Fig. 3B). Serum anti-rCTB IgE antibodies were not detected at all (Fig. 3D).

3.5. Mucosal rCTB-specific IgA antibody responses

Subsequently, mucosal anti-rCTB-specific IgA antibody titres were measured in the same mice. Recombinant CTB-specific mucosal IgA antibody responses were observed in all mucosal sites of almost all mice examined (Fig. 4A–G). The mucosal sites showing anti-rCTB-specific mucosal IgA antibody titres of 16-fold dilution and over in the greater part of mice, regardless of nDT concentrations, were the nasal cavities, the lungs, the saliva and vaginal secretions, the small intestines and feces (Fig. 4A–E and G).

At any rate it is clear that co-administration with nDτ induced mucosal rCTB-specific antibody responses more effectively than that with nTT [16].

4. Discussion

Intranasal immunization of nDτ in the presence of rCTB not only induced high levels of Dτ-specific serum IgG antibody (Fig. 1A) but also provided diphtheria antitoxin titres above a protective level of 0.1 IU ml^{-1} to four of five mice vaccinated (Table 1), clearly showing the effectiveness of this route of immunization in the same manner as nTT plus rCTB [16]. This finding suggests that intranasal administration of nDτ together with rCTB could substitute in humans for the existing protocol in which the vaccine is administered by subcutaneous or intramuscular injection. In fact the effectiveness of intranasal administration in humans has been reported as to nDτ–nTT vaccines in an enhancer mixture of polysorbate and caprylic/capric glycerides [9] and moreover, Waldeyer's ring consisting of tonsils and adenoids in humans is thought to be functionally equivalent to the nasal-associated lymphoid tissue (NALT) in mice [22].

The intranasal immunization with nTT in the presence of rCTB or aTT alone gave a little lower TT-specific serum IgG antibody response than the subcutaneous injection as shown in the previous paper for mice [17] but protected all mice from challenge of tetanus toxin [16]. The same thing could be said of the intranasal administration of nDτ with rCTB because Dτ-specific serum IgG antibody titres obtained at a concentration of 5 Lf (Fig. 1A) were almost similar to TT-specific serum IgG antibody titres acquired under the same conditions [16,17]. The reason for the slightly poorer antibody response following the intranasal immunization may be due to only a small amount of the antigen which is able to pass through the mucosal membrane. Nevertheless intranasal administration seems to be recommended because of definite induction of an antigen-specific serum IgG antibody response, as shown in the experiments concerning mucosal application of a streptococcal surface protein antigen with CTB [3,5] and that of nTT with rCTB or aTT [16], because of a low exposure of a protein antigen to adverse conditions such as low pH and various proteolytic enzymes and because of the nasal mucosa with its large surface and abundant supply of blood vessels [23].

The major serum IgG subclass responding to Dτ was IgG1 followed by IgG2b responses (Fig. 1B) in the same manner as serum IgG subclass responses to BSA [4] and nTT [16]; in case of 5 Lf nDτ, significant IgG2a antibody responses were observed in four of six mice, these results being different from those observed

with nTT. It has been shown earlier that the complement binding subclasses IgG2a and IgG2b are more valuable in mice than IgG1 or IgG3 for combating certain infectious agents [24–26]. However, IgG1 and IgG2b seem to play an important role in neutralization of exotoxins produced by *C. diphtheriae* and *Clostridium tetani* according to the patterns of IgG antibody-subclasses (Fig. 1B) [16].

Intranasal administration of 5 Lf nDτ together with rCTB could induce Dτ-specific IgA antibodies at all mucosal sites in all mice examined, although there were differences between individual mice (Fig. 2). On the other hand, in case of intranasal administration of BSA together with rCTB, BSA-specific mucosal IgA antibodies were detected only in the lungs and the nasal cavities of mice [4] and TT-specific mucosal IgA antibody responses occurred at all mucosal sites except the saliva and vaginal secretions upon intranasal immunization with nTT together with rCTB [16]. Moreover, intranasal immunization of rhesus monkeys with *S. mutans* AgI/II mixed with CTB resulted in detectable mucosal IgA antibody responses in the saliva and the nasal and vaginal secretions [3]. Judging from these facts, mucosal effective sites, where antigen-specific mucosal IgA antibodies are produced, seem to vary with different kinds of antigens. Definite production of Dτ-specific IgA antibodies at all mucosal sites examined is very rational because they can bind to exotoxin released by *C. diphtheriae*, which remains on the mucous membrane of the respiratory tract, and prevent the exotoxin from penetrating inside of the mucosal sites; diphtheria toxin is reported to be only neutralized by specific IgG antibodies but neither by IgA nor IgM [27].

The use of CT as a mucosal adjuvant has been reported to induce antigen-specific serum IgE antibody responses in mice and rhesus monkeys [3,20,28,29] when administered orally or intranasally together with some protein antigens, whereas there is a report that CTB does not potentiate allergic sensitivity to mice for a protein antigen co-administered orally [28]. It was shown in the present experiments that rCTB co-administered intranasally with nDτ stimulated a slight level of Dτ-specific serum IgE antibody response in some mice (Fig. 1D) like the intranasal administration of nTT with rCTB [17] but rCTB-specific serum IgE antibodies were not detected at all (Fig. 3D); whether this level of IgE response would cause a potential risk of allergy is unclear. The above discrepancy may depend on the differences in strains of mice and in species of experimental animals.

Intranasal application of rCTB mixed with nDτ evoked higher levels of rCTB-specific serum IgG and IgA responses (Fig. 3A and C) and induced significant levels of rCTB-specific mucosal IgA antibodies at all mucosal sites examined (Fig. 4) unlike that of rCTB

plus nTT [16]. These facts suggest that anti-rCTB antibody levels depend on the antigens that are co-administered intranasally and that the type of antigen is important in considering the composition of cholera vaccine.

It was also shown in this study that rCTB effectively delivers a heterologous antigen to mucosal sites and elicits a protective serum antibody response. Accordingly, intranasal co-administration of rCTB with nDT may be one of the more favorable immunization procedures for diphtheria as well as tetanus. Furthermore, the intranasal inoculation procedure is simple, effective, inexpensive and needle-free and represents an attractive alternative procedure to humans who are afraid of needles.

Acknowledgements

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