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Highly purified mutant E112K of cholera toxin elicits protective lung mucosal immunity to diphtheria toxin

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Abstract

We demonstrated that the mutant of cholera toxin (mCT) E112K which was LPS-free supported the induction of protective immunity in mucosal (e.g. lung lavage) and systemic (e.g. serum) compartments when given nasally with vaccine-grade diphtheria toxoid (DT) to mice. Significant DT-specific mucosal IgA antibody (Ab) and serum IgG, IgA and IgM Ab responses were induced when LPS-depleted mCT E112K or native CT (nCT) was co-administered nasally with DT. The analysis of DT-specific Ab-forming cell (AFC) responses supported the Ab titers and significant numbers of DT-specific IgA AFC were present in the lungs, nasal passages and submandibular glands. Furthermore, DT-specific IgG AFC in cervical lymph nodes (CLN) and the spleen were induced in mice administered with DT nasally with either mCT or nCT. The analysis of antigen-specific T cell responses revealed that increased DT-specific $CD4^+$ T cell proliferative and Th2-type cytokine responses were induced in mice nasally-immunized with DT and the LPS-free form of mCT. The neutralization of diphtheria toxin by Abs showed that DT-specific IgG Ab responses in serum and lung lavages of mice immunized with DT and mCT were protective. Furthermore, it was shown that an IgA-enriched fraction of lung lavages possessed diphtheria toxin-specific neutralizing activity. These results are the first demonstration that nasally co-administered mCT E112K can induce DT-specific protective Ab responses in mucosal compartments (e.g. lung lavages and the lungs).

Keywords: Cholera toxin; Diphtheria toxin; Mucosal adjuvant; Mucosal immunity; Mutant toxin

1. Introduction

Cholera toxin (CT) is a bacterial protein enterotoxin produced by *Vibrio cholerae* and is similar to the heat labile toxin (LT) secreted by enterotoxigenic *Escherichia coli* [1]. These toxins are known to be strong adjuvants when given by the oral, nasal or parenteral routes [2–5]. Moreover, both enterotoxins are capable of inducing long-term memory to co-administered protein as well as to themselves [6,7]. However, both holotoxins cause watery diarrhea in humans which is mediated by the A subunit of the molecule. This region is responsible for catalysis of ADP-ribosylation of the Gsα, G protein [1]. Therefore, a large number of studies have focused on altering the A subunit of the molecule to eliminate Gsα ADP-ribosyltransferase activity to determine if the molecule retains adjuvanticity [8–13].

The first report of a mutant of the LT holotoxin suggested that neither the recombinant CT B subunit (CT-B) nor a nontoxic mutant of LT E112K (e.g. a glutamic acid to lysine substitution at position 112 in the A subunit) exhibited adjuvanticity when given orally [14]. The authors concluded that ADP-ribosyltransferase activity was necessary for adjuvanticity by either CT or LT [14]. However, later work showed that mutant LT (mLT) R7K [8], mLT S63K [9] and mLT A72R [10] retained their adjuvanticity when given nasally, suggesting that ADP-ribosyltransferase activity is not necessary for their adjuvant activity. Furthermore, the effectiveness of mLT R192G as a mucosal adjuvant has been shown by a combination of oral and parenteral

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immunization [15]. The mLT G33D, which lacks the ability to bind to the receptor Gm1 was found to be an effective nasal adjuvant [16], although it induced neither IgG nor IgA directed to co-administered antigen when given orally [17].

The first evidences for mCTs as adjuvants, e.g. mCT S61F and E112K, were provided by studies showing comparable enhancing activity as native CT (nCT) when given via either the parenteral or nasal routes [12,13]. Neither mutant exhibited ADP-ribosyltransferase activity with an artificial substrate, agmantine, nor enterotoxicity employing an in vivo ileal loop assay or in vitro with Chinese hamster ovary cells. Two mCTs, S61F and E112K, both elicit $CD4^+$ Th2-type responses via IL-4 production which enhances serum IgG1 and IgG2b Ab subclasses and mucosal IgA Ab responses when given nasally [12]. Adjuvanticity of mCT E112K was induced via up-regulation of B7-2 on APCs and through preferential inhibition of $CD4^+$ Th1-type cytokine responses [18]. Moreover, nasal immunization of mice with pneumococcal surface protein A (PspA) plus mCT S61F-induced protection against a fatal systemic challenge with *Streptococcus pneumoniae* [19]. Others have also shown that mutants of CT exhibit mucosal adjuvant activity [11]. However, we still do not know whether nasally administered vaccine antigen and mCT (e.g. S61F or E112K) can induce protective immunity in the mucosal compartment (e.g. the respiratory tract).

In our previous adjuvant studies purified mCT preparations E112K or S61F contained LPS levels ranging from 2.5 to 12 ng of LPS per 10 μg of mCT based upon the limulus assay. Although this is approximately 1000-fold lower than the levels of lipid A-derivatives required to elicit oral or nasal adjuvant responses in mice [20,21], it was important to eliminate any potential biological effects of LPS which could act together with mCT to induce mucosal adjuvant responses. Furthermore, studies in humans will mandate the use of GMP products which have reduced LPS levels when used as mucosal adjuvants. Thus, the purpose of the present study was to elucidate whether nasally-administered high purified mCT can induce DT-specific protective immunity in the mucosal compartment (e.g. the lungs) as well as in the systemic compartment. We assessed mCT as a nasal adjuvant for diphtheria toxoid (DT), where LPS was depleted by solid-phase chromatography.

2. Materials and methods

2.1. Mice

C57BL/6 mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD) and SLC (Shizuoka, Japan). Mice were maintained in horizontal laminar flow cabinets and were free of microbial pathogens, as determined by Ab screening and tissue histopathology. All mice received sterile food and water ad libitum, and were between 8 and 16 week of age when used in these experiments.

2.2. Preparation of LPS-free grade of mCT

An *E. coli* strain containing the plasmid for mCT E112K was grown in Luria–Bertani medium with 100 μg/ml of ampicillin. The mCT E112K was prepared using a D-galactose-immobilized column (Pierce Chemical Co., Rockford, IL) from cell suspension prepared by sonication of the bacteria as previously reported [13].

For the preparation of the LPS-free form of mCT E112K, Detoxi-Gel^{TM} endotoxin binding columns (1 ml) were used (Pierce Chemical Co.). The column was washed with 1% deoxycholate, Milli-Q water followed by sterile PBS. A 500 μg aliquot of mCT E112K in 50 mM Tris and 1 mM EDTA (pH 8.3) was applied to the column and then eluted with PBS (pH 7.4). A total of 110 μg of mCT per millimeter was recovered. Possible residual endotoxin was measured in the preparation with an LAL pyrochrome kit (Associates of Cape Cod Inc., Woods Hole, MA). The post-column sample contained 2.9 ng of endotoxin (107.3 EU per total) or 0.26 ng of LPS per 10 μg of mCT used as nasal adjuvant. To increase the recovery of mCT, elution and a second purification was carried out with 50 mM Tris, 1 mM EDTA, 0.5 M NaCl (pH 8.3).

The protein content of the LPS-free mCT was assayed by use of the BioRad protein assay system (Hercules, CA) based on the Bradford method (where BSA was used as standard). Total protein recovered was 494 μg of that applied (442.4 EU of LPS per total). Furthermore, two additional preparations with similar recoveries resulted in approximately 3 mg of endotoxin-depleted mCT E112K. The fractions containing mCT E112K were concentrated and sterilized by passage through a 0.45 μm filter. This preparation contained 1.9 mg of mCT protein/1.7 ml and 10.4 EU or ∼1 ng of LPS/10 μg mCT. The levels of LPS in nCT purchased from List Biologic Laboratories Inc. (Campbell, CA) was 4.8 EU or ∼0.5 ng LPS/10 μg of protein.

2.3. Immunization protocol

A standard nasal immunization protocol was used in this study [12]. C57BL/6 mice were nasally immunized to both nostrils on days 0, 7 and 14 with 25 μg of diphtheria toxoid (DT) (1050 Lfu/mg) kindly provided by the Biken Foundation (Osaka University) either alone or combined with 5 μg of the LPS-free form of mCT or 0.5 μg of nCT (List Biologic Laboratories Inc.) as mucosal adjuvants.

2.4. DT-specific ELISA and ELISPOT assays

We assessed DT-specific Ab titers in serum and mucosal secretions (e.g. lung lavages) by ELISA as described previously [22,23]. Endpoint titers were expressed as the

reciprocal $\log_2$ of the last dilution giving an optical density at 450 nm (OD_{450}) of >0.1 above negative controls. The ELISPOT assay was employed for the evaluation of antigen-specific Ab-forming cells (AFCs) from various mucosal and systemic tissues such as those of the nasal passages, lungs, SMG, spleen and cervical lymph nodes (CLN) [5,23,24]. The submandibular glands (SMG) and lung tissue were carefully excised, teased apart, and dissociated using collagenase-type IV. Mononuclear cells were obtained at the interface of the 40 and 75% layers of a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Buckinghamshire, UK). Mononuclear cells from the spleen and CLN were mechanically dissociated.

2.5. DT-specific $CD4^+$ T cell responses

The spleen and CLN were removed from immunized and control mice and single-cell suspensions were prepared as described previously [24]. The isolated cells were initially applied to a Nylon wool column (Polysciences, Warrington, PA) and incubated at 37 °C for 1 h to remove adherent cells. The $CD4^+$ T cell subset was then obtained by positive sorting using a magnetic bead separation system consisting of biotinylated anti-CD4 mAb (clone GK 1.5) and streptavidin microbeads (MACS; Miltenyi Biotech, Sunnyvale, CA). Purified $CD4^+$ T cells (>98% purity) were cultured at a density of 4×10^6 cells/ml with DT (10 μg/ml) and T cell-depleted, irradiated (3000 rad) splenic feeder cells (8×10^6 cells/ml) and rIL-2 (10 U/ml) (PharMingen, San Diego, CA) in complete medium. The $CD4^+$ T cell cultures were incubated for 4 days at 37 °C in 5% CO_2 in air. To measure DT-specific T cell proliferation, 0.5 μCi of [^{3}H] thymidine (Du Pont/New England Nuclear Products, Boston, MA) was added to individual cultures 18 h before termination, and the uptake of [^{3}H] thymidine in counts per minute (cpm) was determined by scintillation counting [13].

2.6. Cytokine analysis by ELISA

The levels of Th1 and Th2 cytokines in culture supernatants from in vitro DT stimulated $CD4^+$ T cells were determined by a cytokine-specific ELISA as described previously [23,25]. For coating and detection, the following mAbs were used: for anti-IFN-γ: R46A2 and XMG 1.2 mAbs; for anti-IL-2: JES6-1A12 and JES6-5H4 mAbs; for anti-IL-4: BVD4-1D11 and BVD6-24G2 mAbs; for anti-IL-5: TRFK-5 and TRFK-4 mAbs; for anti-IL-6: MP5-20F3 and MP5-32C11 mAbs; and for anti-IL-10: JES5-2A5 and JES5-16E3 mAbs (PharMingen). The levels of Ag-induced cytokine production were calculated by subtracting the results of control cultures (e.g. without Ag stimulation) from those of Ag-stimulated cultures. This ELISA was capable of detecting 0.2 ng/ml of IFN-γ; 0.01 U/ml of IL-2; 10 pg/ml of IL-4; 0.4 U/ml of IL-5; 400 ng/ml of IL-6; and 20 pg/ml of IL-10.

2.7. Detection of neutralizing Abs by use of diphtheria toxin-specific Vero cell assay

The cytotoxicity induced by the diphtheria toxin (List Biological Laboratories Inc.) in Vero cell cultures was used to measure the toxin-specific neutralizing titers of the Abs induced by the nasal vaccine. The neutralizing assay was basically the method described by others [26,27]. Vero cells (1.25×10^4 per well) were added to 96-well microtiter plates and then incubated with 20 pg per well of diphtheria toxin. The cultured cells were assessed after 7 days of incubation. For toxin neutralization studies, diphtheria toxin was pre-incubated with serially-diluted lung lavages or serum for 1 h at 25 °C. In addition, these Ab samples were reacted with protein G in order to deplete the IgG fraction for the enrichment of IgA antibodies. The absorption of IgG from sera and lung lavages was performed with high performance protein G sepharose (BioRad).

2.8. Statistical analysis

The results are reported as the mean ± 1 standard error (S.E.). Statistical significance ($P < 0.05$) was determined by Student's *t*-test and by the Mann–Whitney *U*-test of unpaired samples.

3. Results

3.1. Induction of antigen-specific mucosal and systemic Abs by nasal immunization of DT and LPS-free grade of mCT

In the initial study, we determined whether nasal co-administration of DT plus LPS-free grade of mCT E112K as a mucosal adjuvant could induce DT-specific Ab responses. Mice nasally immunized with DT plus mCT showed significant levels of serum DT-specific IgG, IgA and IgM Ab responses (Fig. 1A). The DT-specific Ab responses were slightly less than those induced by nasal delivery of Ag plus nCT as mucosal adjuvant. This difference, however, was not statistically significant. In contrast, DT-specific Ab responses were low but detectable after nasal immunization with DT alone (Fig. 1A). With regard to the induction of DT-specific mucosal IgA Abs by co-administered protein, our results showed that nasal delivery of DT and LPS-free mCT induced DT-specific mucosal IgA Ab responses in nasal washes and saliva. Furthermore, both DT-specific mucosal IgA and IgG Ab responses were induced in lung lavages of nasally immunized mice (Fig. 1B). The magnitude of these DT-specific mucosal Ab responses was less than those induced by co-administered nCT as adjuvant. Again, however, the difference was not statistically significant. DT-specific mucosal IgM Ab responses were not detected in lung lavages of nasally immunized mice (data not shown). In control group, essentially no mucosal

DT-specific Abs were detected in mice that received nasal vaccine antigen alone (Fig. 1B).

3.2. Nasally delivery of LPS-free grade of mCT-induced DT-specific IgA and IgG AFC

Analysis of AFCs confirmed the above ELISA results and revealed the presence of DT-specific IgA AFCs in nasal passages and lungs following nasal immunization with DT plus mCT or nCT (Fig. 2A). Furthermore, DT-specific IgG AFCs were induced in lungs following nasal delivery of DT plus mCT or nCT (Fig. 2B). However, DT-specific IgM AFCs were not detected in the lungs of mice immunized with DT plus mCT or nCT (data not shown). The DT-specific AFC responses were not seen in these respiratory tissues of mice given DT alone. Increased numbers of DT-specific IgA AFCs were also detected in mononuclear cells isolated from the SMG of mice nasally immunized with DT and either mCT or nCT as mucosal adjuvants (Fig. 2A). Furthermore, analysis of B cells producing DT-specific Abs supported the presence of increased titers of antigen-specific serum Abs. Thus, significant numbers of DT-specific IgG AFCs were found in the spleen, the lung and the CLN of mice nasally immunized with DT plus mCT or nCT as adjuvant (Fig. 2B). These findings further demonstrated that the LPS-free form of mCT provided mucosal adjuvant activity for the induction of Ag-specific Ab responses in both mucosal and systemic compartments.

3.3. Nasally co-administered LPS-free mCT-induced DT-specific T cell responses

Since nasal immunization with DT plus the LPS-free mCT induced Ag-specific Ab responses at both mucosal and systemic sites, it was important to investigate the nature of the $CD4^+$ T cell help for DT-specific B cell and Ab

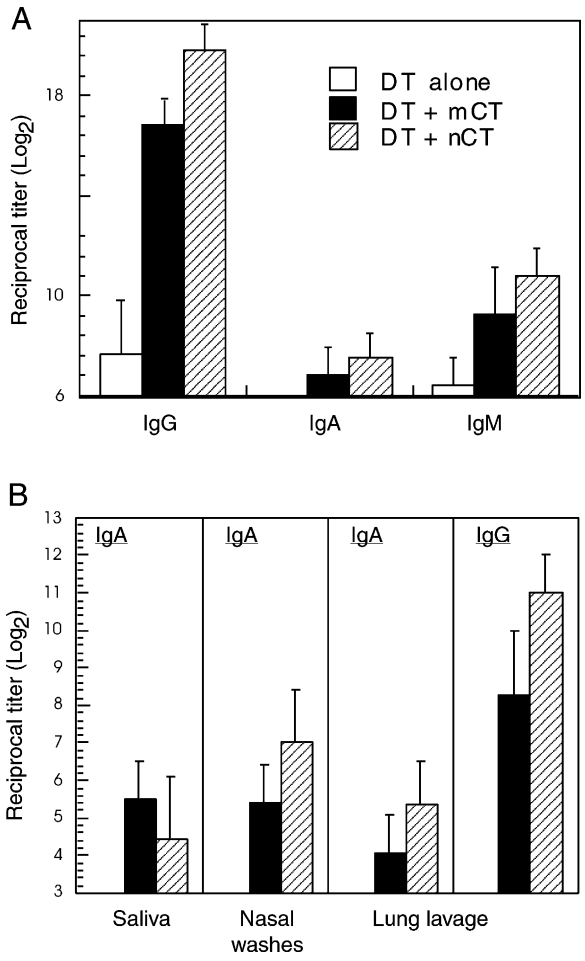

Fig. 1. Induction of DT-specific Ab responses in serum (A) and mucosal secretions (B) by nasal immunization with DT and LPS-free mCT E112K. Groups of five C57BL/6 mice were nasally immunized with 25 μg of DT plus 5 μg of the LPS-free form of mCT (■); 0.5 μg of nCT (▨), DT alone (□) on days 0, 7 and 14. Serum and mucosal Ab titers were assessed on day 21. The results are expressed as the mean ± S.E.M. obtained from three separate experiments.

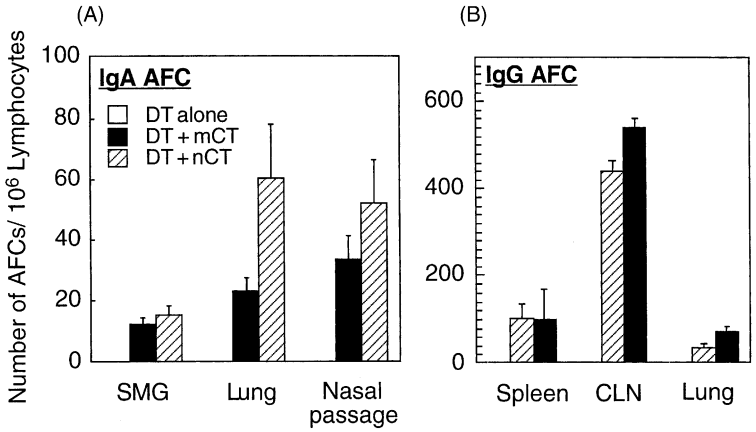

Fig. 2. Induction of DT-specific Ab-forming cells (AFCs) in the lungs, nasal passages, SMG and CLN and spleen of mice nasally immunized with DT and LPS-depleted mCT E112K. Groups of C57BL/6 mice were immunized with DT plus mCT (■); nCT (▨); DT alone (□) as described in Fig. 1. Mononuclear cells were isolated from the lungs, nasal passages, SMG, spleen and CLN of nasally immunized mice at day 21 and then examined by DT and isotype-specific ELISPOT assay. The results are expressed as the mean ± S.E.M. obtained from three separate experiments with five mice per group.

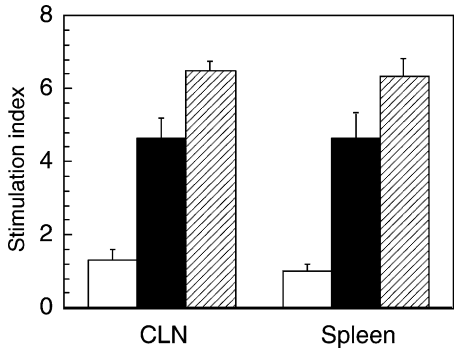

Fig. 3. Induction of DT-specific CD4$^+$ Th2-type responses by a nasal vaccine containing DT and an LPS-free mCT E112K. Groups of C57BL/6 mice were immunized with DT plus mCT (■); nCT (▨); DT (□) alone as described in Fig. 1. CD4$^+$ T cells were isolated from CLN and the spleen of nasally-immunized mice on day 21 and were cultured with 10 μg/ml of DT in the presence of irradiated, T cell-depleted spleen feeder cells and rIL-2 (10 U/ml). The results are representative of one experiment (five mice per group) taken from three separate experiments.

responses. When CD4$^+$ T cells from CLN or the spleen of mice nasally immunized with DT plus mCT were restimulated with DT in vitro, high T cell proliferative responses were induced (Fig. 3). Essentially no antigen-specific CD4$^+$ T cell responses were detected in CLN or the spleen of mice given nasal DT alone. These results indicate that the LPS-free grade of mCT, like nCT, is an effective mucosal adjuvant for the induction of DT-specific CD4$^+$ T cells. Analysis of Th1 and Th2 cytokine production by Ag-stimulated T cells revealed that DT-specific CD4$^+$ T cells from the CLN of mice given DT plus the LPS-free grade of mCT or nCT produced high levels of Th2-type cytokines including IL-4, IL-5, IL-6 and IL-10 with low Th1-type cytokines IFN-γ and IL-2 (Table 1). As expected, CD4$^+$ T cells from CLN of mice given DT alone produced low to undetectable levels of all cytokines measured. These studies show that nasal administration of DT plus the LPS-free form of mCT as an adjuvant preferentially induces Th2-type cytokine responses.

Table 2
Induction of diphtheria toxin-neutralizing Abs in lung lavages and serum of mice nasally immunized with DT and LPS-free form of mCT

Mice immunized with	Neutralizing titer[a]			
	Serum		Lung lavages	
	Without absorption	With absorption[b]	Without absorption	With absorption[b]
DT alone	0[c]	0	0	0
DT + mCT	1/256	0	1/4	1/2.3
DT + nCT	1/512	0	1/5.5	1/4.5

[a] Neutralizing titers are expressed as the highest serum or lung lavage dilution demonstrating 100% inhibition of the toxic effect on Vero cells induced by diphtheria toxin.
[b] An aliquot of samples was absorbed by protein G.
[c] 0: Undetectable.

3.4. Induction of toxin neutralizing mucosal Abs after nasal vaccination with DT and the LPS-free mCT E112K

Since the nasally co-administered LPS-free mCT E112K supported the induction of DT-specific serum Ab responses, we next assessed the ability of the anti-DT Abs to neutralize active diphtheria toxin in Vero cell cultures. The sera from mice nasally immunized with DT and mCT E112K possessed a toxin neutralizing titer of 1/256, which was comparable to that in sera of mice nasally immunized with DT and nCT (1/512) (Table 2). After removal of IgG Abs by passage through a protein G column, the neutralizing activity of serum antitoxin of mice nasally immunized with DT and either mCT or nCT was ablated. These results show that the major neutralizing activity of nasally induced serum Abs reside in the IgG fraction.

The lung lavages of mice nasally immunized with DT plus either mCT or nCT expressed toxin neutralizing titers of 1/4 or 1/5.5, respectively (Table 2). No DT-specific IgG Abs were seen in lung lavages after passage through the protein G column (data not shown). However, the toxin-specific neutralizing activity in lung lavages of mice nasally co-administered with mCT or nCT was maintained.

Table 1
Analysis of Th1- (IFN-γ and IL-2) and Th2-type (IL-4, IL-5, IL-6 and IL-10) cytokine synthesis by antigen-specific CD4$^+$ T cells isolated from cervical lymph nodes of mice nasally immunized with DT and LPS-free form of mCT

Mice immunized with	Levels of cytokine produced[a]					
	Th1-type		Th2-type			
	IFN-γ (ng/ml)	IL-2 (U/ml)	IL-4 (pg/ml)	IL-5 (U/ml)	IL-6 (μg/ml)	IL-10 (pg/ml)
DT alone	<0.2[b]	<0.01[b]	7.1 ± 3.7	<0.4[b]	1.3 ± 0.05	<20[b]
DT + mCT	<0.2	<0.01	14 ± 3.3	5.5 ± 1.3	4.5 ± 0.2	820 ± 87
DT + nCT	2.5 ± 0.5	<0.01	32 ± 5.6	7.3 ± 0.8	4.7 ± 0.8	780 ± 35

[a] Culture supernatants were harvested and then analyzed for secreted cytokines using the appropriate cytokine-specific ELISA. The results are expressed as the mean ± S.E.M. from three separate experiments with five mice in each group.
[b] Under limits of detection. The detection limits for the cytokine tested in this study were IFN-γ (0.2 ng/ml), IL-2 (0.01 U/ml), IL-4 (10 pg/ml), IL-5 (0.4 U/ml), IL-6 (0.4 μg/ml) and IL-10 (20 pg/ml).

These results suggest that an IgA-enriched fraction of DT-specific IgA Abs in lung lavages is responsible for this residual neutralizing activity.

4. Discussion

Inasmuch as the mCT was prepared by the use of a recombinant *E. coli* expression system, one must always consider the potential contribution of contaminating LPS to mucosal adjuvant activity of the nontoxic mCT. The results presented here show that the LPS-free form of mCT supported induction of protective immunity against diphtheria toxin. Most importantly, the present study provided the first evidence that a nasally administered LPS-free mCT E112K-induced DT-specific neutralizing Ab responses in the mucosal compartments, including lung secretions. Thus, our findings clearly exclude the possibility that immune responses are due to contamination by LPS in the adjuvant preparation since the LPS-depleted mCT provided mucosal adjuvanticity for the induction of DT-specific, protective humoral immunity in both mucosal and systemic compartments.

The neutralizing Ab activity against diphtheria toxin was removed by absorption of the IgG from serum, indicating that the significant protective Ag-specific serum Abs induced by nasal immunization with DT and LPS-depleted mCT were preferentially of IgG isotype. This finding further suggests that nasal vaccination is an effective immunization regimen to enhance DT-specific systemic immune responses which can neutralize the toxin and protect against the myocardial and neural tissue damage caused by the toxin through blood circulation. The induction of DT-neutralizing Ab responses by nasal vaccine containing DT and LPS-free mCT support the finding of our previous study that nasal immunization with PspA plus mCT elicits protective Ab levels to systemic wild-type *S. pneumoniae* challenge [19].

In case of lung lavages, both antigen-specific IgA and IgG antibodies were induced in mice nasally-immunized with DT and mCT or nCT (Fig. 1B). Furthermore, corresponding DT-specific IgA and IgG antibody producing cells were detected in the lungs of those immunized mice (Fig. 2). These findings suggested that these DT-specific IgA and IgG antibodies were locally produced in lungs, although the possibility that a part of these antigen-specific antibodies might result from leakage from the serum can not be excluded. In contrast to serum, the neutralizing activity against the toxin was reduced but retained even by the absorption of the IgG fraction in lung lavages. Furthermore, DT-specific IgM Abs in lung lavages and DT-specific IgM AFCs in the lung of mice nasally administered with DT and mCT or nCT were not detected (data not shown). These findings could suggest that the presence of protective Ag-specific Abs in the lung lavage of mice nasally immunized with DT plus LPS-depleted mCT or nCT was due to not only IgG but also IgA isotype. Therefore, nasal immunization with DT plus LPS-depleted mCT or nCT is an effective vaccination regimen to elicit DT-specific IgA protective immune responses which can inhibit an influx of exotoxin from colonized *Corynebacterium diphtheriae* in the respiratory tract tissue. Thus, DT-specific mucosal IgA Ab responses may lead to the prevention of the development of pathological lesions in the respiratory tract-associated tissues such as lung, tonsils, the throat and subsequent transmission to the myocardium or central nervous system.

When one considers adjuvants for human use, one should always consider the role of contaminating LPS, as long as these preparations are derived from recombinant *E. coli* expression systems [9,13]. In terms of human responses to bacterial endotoxin, there is general agreement that subjective, influenza-like symptoms such as fatigue, headaches, nausea and fever occur in most subjects after injection of high doses (e.g. 3 and 4 ng/kg). A direct relationship was found between the dose of LPS and the fever index where a dose of between 0.1 and 0.5 ng/kg was needed to induce fever. Moreover, endotoxin causes a decrease in myocardial function and coagulation activation such as a rise in plasminogen activator (t-PA) and prothrombin (F1+2) fragments [28]. Common symptoms caused by contaminating LPS include airway irritation, fever, and chest tightness in 17–33% of the subjects receiving endotoxin [28]. Furthermore, the minimum level of endotoxin required to cause pyrogenic activity when infused into humans is approximately 5 EU (∼0.5 ng) per kilogram of body weight [29]. The endotoxin limit for injectable drugs into humans (including biological products), is calculated according to the following formula: 5 EU/kg [(dose × 1.80 m^2)/70 kg] [30]. A guideline for the endotoxin limits in nasal or oral drugs and vaccines has not been formulated to date, and the limits are generally expected to be comparable to that of an injectable drug. The endotoxin level in mCT used in this study was 10 EU/10 μg of protein. Since 350 EU of LPS in mCT can be tolerated in a human adult when the average body weight is considered to be 70 kg, 350 μg of mCT can be co-administered if calculated according to the formula provided for injectable drugs.

The present findings provide a potential application of mCT for clinical trials since the LPS-free form of this adjuvant supported DT-specific immune responses with toxin neutralizing activity. To this end, we have shown that mucosal administration of nasal vaccine containing DT and the LPS-free form of mCT induced antigen-specific IgA Abs in mucosal secretions, as well as serum IgM, IgG and IgA Ab responses. Furthermore, nasally-administered DT with mCT elicited DT-specific $CD4^+$ Th2-type cells in both mucosa-associated and systemic tissues. These findings further support the results of our previous studies which showed that mCT induces significant Th2-type cytokine responses with subsequent serum IgG1, IgG2b and IgA, and mucosal IgA Abs to co-administered protein Ags (e.g. tetanus toxoid, ovalbumin and PspA) when given either nasally or parenterally [12,13]. Moreover, it is important to note that diphtheria toxin-specific Ab responses induced after nasal immunization with DT plus LPS-depleted mCT

E112K provided protective immunity with neutralization of the toxin in both lung lavages and serum. Taken together, our findings indicate that the LPS-free form of mCT E112K could be suitable for use in humans as a mucosal adjuvant.

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