Original

Nasally administered cholera toxin A-subunit acts as a mucosal adjuvant

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Abstract: It is well established that cholera toxin (CT) produced by *Vibrio cholerae* acts as a potent mucosal adjuvant; however, the native form of this molecule causes severe diarrhea. Furthermore, both native CT and its B-subunit derivative bind to monosialogangliosides (GM1) in membrane raft microdomains on neural tissues and are thus unsuitable for use in humans. In this study, we evaluated the adjuvanticity of the CT A-subunit (CT-A) administered with ovalbumin (OVA) by the nasal route. We found that nasal administration of OVA plus CT-A elicited both mucosal and systemic antibody (Ab) responses. Immunization of mice with OVA plus CT-A resulted in the induction of OVA-specific IgA Abs in saliva and nasal secretions. Furthermore, significant OVA-specific serum immunoglobulin (Ig) G and IgA Ab responses were induced. Antibody-forming cell (AFC) analysis confirmed the Ab titer findings by revealing significant numbers of OVA-specific IgA AFCs in submandibular glands. In addition, splenic lymphocytes restimulated with OVA *in vitro* exhibited significant proliferative responses. Thus, CT-A might be a candidate for an effective adjuvant for inducing antigen (Ag)-specific Ab responses in human systemic and mucosal compartments, such as the oral cavity. (J. Oral Sci. 45, 25-31, 2003)

Key words: choleratoxin A; mucosal adjuvant

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Introduction

Cholera toxin (CT) consists of an enzymatically active A-subunit that catalyzes adenosine diphosphate (ADP)-ribosylation of the Gsα protein. The B-subunits of CT (CT-B) bind to monosialoganglioside (GM1) (1) (Fig. 1). The binding of CT to intestinal epithelial cells induces a cascade of events that ultimately results in secretion of chloride and water with subsequent diarrhea (1,2). CT is a mucosal adjuvant and when given with protein vaccines by the oral or nasal route it induces mucosal and systemic immunity (3-8). Furthermore, mutants of CT (9,10) have been shown to be devoid of toxicity but to retain full mucosal adjuvanticity when given by the nasal route.

The adjuvant activity of CT was found to involve up-regulation of co-stimulatory molecule expression by
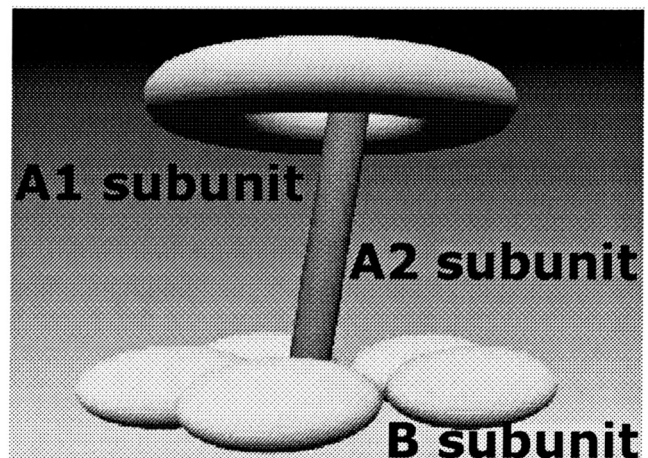

Fig. 1 Cholera toxin (CT) consists of an enzymatically active A-subunit that catalyzes the ADP-ribosylation of the Gsαi protein. The B-subunits of CT (CT-B) bind to GM1.

antigen-presenting cells (APCs) (11-13). CT-B, however, failed to stimulate APC co-stimulatory molecule expression (8). In addition, studies have shown that CT-B does not enhance immune responses to mucosally co-administered protein antigens (Ags) (10,13,14). However, some workers observed that CT-B enhanced soluble peptide presentation by peritoneal macrophages *in vitro* (15) and CT-B-pretreated B-cell hybridomas enhanced interleukin (IL)-4 production by Th2 cell lines after stimulation (16).

CT binds to GM1 on epithelial cells via CT-B and requires endocytosis followed by transport across the epithelial cell to reach the basolateral membrane where it induces water and chloride secretion resulting in the characteristic cholera-type diarrhea (1). GM1 is also expressed abundantly in the central nervous system (CNS) and, after administration to mice by the intranasal route, both CT and CT-B have been shown to accumulate in the main olfactory nerves and epithelium (ON/E) and olfactory bulbs (OBs) (18). Furthermore, GM1-mediated transport is responsible for this accumulation of CT-B and CT in the OBs. Thus, following intranasal delivery, CT or CT-B targets the ON/E and OBs, which raises the important possibility of neuronal damage with the intranasal use of these proteins in humans.

In order to eliminate the binding of CT-B to GM1 with subsequent neuronal damage when CT is given nasally, we have evaluated the adjuvant activity of nasally administered CT A-subunit (CT-A). The results obtained in this study suggest that nasal administration of CT-A would be an effective adjuvant for the induction of Ag-specific mucosal and serum antibody (Ab) responses to co-administered proteins.

Materials and Methods

Mice

BALB/c mice were purchased from Charles River Japan and maintained under pathogen-free conditions in the experimental room of Nihon University School of Dentistry at Matsudo. All mice were provided with sterile food and water *ad libitum* and were used in this study when they were 8-12 weeks of age.

Antigen and adjuvants

Chicken egg ovalbumin (OVA; grade V) was purchased from Sigma Chemical Co. (St. Louis, MO). CT-A and CT were purchased from List Biologic Laboratories (Campbell, CA).

Immunization and sample collection

Mice were immunized nasally on days 0, 7, and 14 with a 10-μl aliquot (5 μl per nostril) of phosphate-buffered saline (PBS) containing 100 μg OVA alone or combined with 500 ng of CT-A or CT. Serum, saliva and nasal wash samples were collected from each group, as described elsewhere (2-5,8), on day 21 to examine Ag-specific Ab responses.

Detection of Ag-specific Ab isotype responses

The serum, saliva and nasal wash sample Ab titers were determined by enzyme-kinked immunosorbent assay (ELISA) as described elsewhere (2-5,8). The assay was conducted using Falcon Microtest III assay plates (Becton Dickinson, Oxford, CA), which were coated with OVA (1 mg/ml), incubated overnight at 4°C in a humid atmosphere, washed with PBS and blocked with PBS containing 1% w/v bovine serum albumin. After washing with PBS, serially diluted serum, saliva or nasal wash samples were added, in duplicate, to the wells. Following 4 hours of incubation at room temperature, the plates were washed with PBS containing 0.05% v/v Tween 20 and horseradish peroxidase-labeled goat anti-mouse μ, γ or α heavy chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added to the appropriate wells. The plates were incubated at room temperature for 4 hours, washed with PBS and developed with 1.1 mM of 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) in 0.1 M citrate-phosphate buffer containing 0.01% v/v H_2O_2 (Moss, Inc., Pasadena, MA) for 15 minutes at room temperature. IgG subclass analysis was carried out using horseradish peroxidase-labeled goat anti-mouse γ1, γ2a, γ2b, or γ3 subclass specific Abs (Southern Biotechnology Associates) exactly as described above for the anti-heavy chain-specific analysis. End-point titers were expressed as the reciprocal $\log_2$ of the highest dilution, which gave an optical density in a plate reader at 414 nm (model Biolumin 960; Molecular Dynamics) that was 0.1 units greater than the background level after incubation for 15 min.

ELISPOT for assessment of the numbers of Ab-forming cells (AFCs)

Single cell suspensions were obtained from the spleens (SPs) and salivary glands (SGs) of nasally-immunized mice, as described previously (2-5). The mononuclear cells were obtained at the interface of the 40% and 75% layers of a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Piscataway, NJ). To determine the numbers of Ag-specific AFCs, an ELISPOT assay was performed, as described previously (2-5). Briefly, 96-well nitrocellulose plates (Millititer HA; Millipore Corp., Bedford, MA) were coated with OVA (1 mg/ml), incubated for 20 h at 4°C, washed thoroughly with PBS and blocked with RPMI 1640 (Gibco BRL) containing 10% v/v fetal calf serum.

The blocking solution was discarded, lymphoid cell suspensions at various dilutions (10^6/100 μl/well, 2×10^5/100 μl/well and 4×10^4/100 μl/well) were added to the wells and the plates were incubated for 4 hours at 37°C in 5% CO_2 in moist air. The AFCs were detected using horseradish peroxidase-conjugated goat anti-mouse α or γ heavy chain-specific Abs. Following overnight incubation at 4°C with the required Ab, the plates were washed with PBS and developed by adding 3 μg/ml of 3-amino-9-ethylcarbazole dissolved in 100 mM sodium acetate buffer containing 0.01% H_2O_2 (Moss) to each well. The plates were incubated at room temperature for 15-20 min, washed with water and the AFCs were counted with the aid of a stereomicroscope.

Stimulation of OVA-specific splenocytes

Splenocytes (5×10^6 cells/ml) isolated from immunized mice were cultured with 1 mg of OVA in 1ml of complete RPMI 1640 (Gibco BRL) for 4 days at 37°C in 5% v/v CO_2 in air. One μCi of [^{3}H] Thymidine was added to each culture 18 h before the end of the culture period when the OVA-specific lymphocyte proliferative responses were measured.

Statistics

The group data are expressed as means ± standard deviation (SD) and compared using the unpaired Mann Whitney U-test. statistical significance ($P < 0.05$) was analyzed using the Statview II statistical program (Abacus Concepts, Inc., Berkeley, CA).

Results

Analysis of Ag-specific systemic and mucosal Abs after nasal administration of OVA and CT-A

In the initial experiment, we examined whether nasal administration of OVA plus CT-A as a mucosal adjuvant could induce OVA-specific Ab responses. Nasal immunization of mice with OVA plus CT induced significant OVA-specific serum IgG, IgA and IgM Ab titers when compared with mice given OVA alone. Further, those serum Ab responses were comparable to those induced by nasally administered OVA plus CT (Fig. 2). As expected, administration of CT-A or CT alone did not induce OVA-specific Ab responses that were above the dilution cut-off level (Log_2 of 7). Analysis of the IgG subclass responses of mice given OVA plus CT-A or CT revealed that the major subclasses were IgG1 and IgG2b (Fig. 3).

As CT given nasally has been shown to be an effective adjuvant for the induction of Ag-specific mucosal IgA Abs against co-administered proteins (3-5,8), it was important to determine whether OVA-specific IgA Ab responses were induced in mucosal secretions when CT-A was given with OVA. Our results showed that nasal administration of OVA plus CT-A induced significant levels of OVA-specific mucosal IgA Ab responses in saliva and nasal wash samples when compared with those induced by OVA alone (Fig. 4A saliva, and 4B nasal secretions). As expected, nasal delivery of CT-A or CT alone failed to induce OVA-specific Ab titers greater than log_2 of 3 (data not shown).
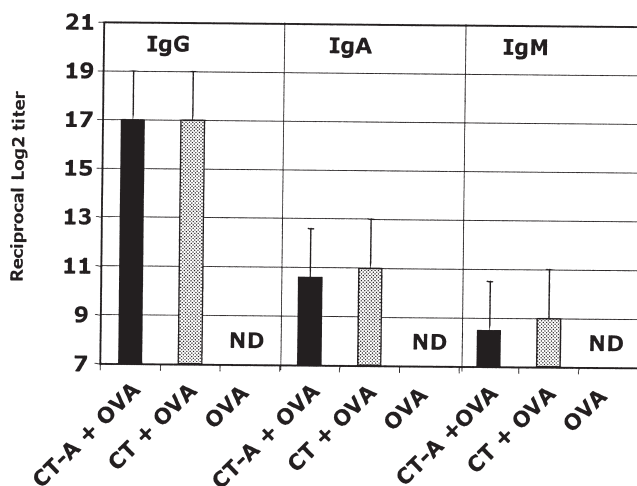

Fig. 2 OVA-specific serum Ab titers of mice immunized nasally with OVA alone and plus CT-A or CT as an adjuvant. Groups of 8 mice were immunized nasally with 100 μg OVA alone and plus 500 ng CT-A or CT on days 0, 7, and 14. Serum Ab titers were assessed on day 21. The results are expressed as means ± SD.
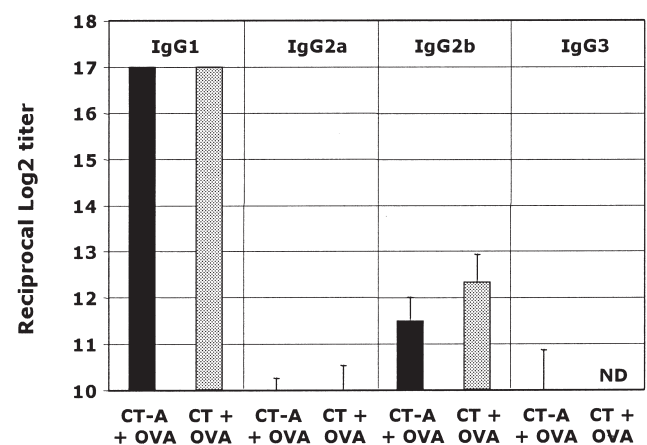

Fig. 3 Anti-OVA serum IgG subclass Ab responses of mice immunized nasally with OVA alone and plus CT-A or CT as an adjuvant. Groups of 8 mice were immunized nasally with OVA alone and plus CT-A or CT as described in the legend to Figure 2. Serum IgG subclass Ab titers were assessed on day 21. The results are expressed as the means ± SD.

Nasal delivery of CT-A induced OVA-Specific IgA AFCs

Analysis of AFC responses confirmed the Ab titer results and revealed the presence of OVA-specific IgA AFCs in SGs following nasal immunization with OVA plus CT-A or CT (Table 1). Furthermore, analysis of B cells producing OVA-specific Abs supported the presence of increased titers of serum Abs. Thus, significant numbers of OVA-specific IgG and IgA AFCs were found in the SPs of mice immunized nasally with OVA plus CT-A or CT when compared with mice given OVA alone (Table 1). These findings further demonstrated that CT-A acted as an adjuvant for the induction of Ag-specific Ab responses in both mucosal and systemic compartments.

Analysis of OVA-specific T cell responses

As nasal immunization with OVA plus CT-A induced OVA-specific Ab responses, it was important to examine the Ag-specific T-cell responses. When splenic mononuclear cells from SP of mice immunized with OVA plus CT-A were restimulated with OVA *in vitro*, the levels of the proliferative responses induced were identical to those seen with splenocytes from mice immunized with OVA plus CT (Fig. 5). Splenocytes from mice given OVA alone showed essentially no increased proliferation (Fig. 5). These results indicate that CT-A, like CT, was an effective adjuvant for the induction of Ag-specific T-cell proliferative responses to a co-administered protein Ag.

Discussion

In the present study, we assessed the potential of CT-A to induce a mucosal IgA response to a co-administered protein Ag. Our results showed that nasal administration of OVA plus CT-A as a mucosal adjuvant induced significant OVA-specific IgG, IgA and IgM Ab responses in serum and IgA Ab responses in mucosal secretions. Furthermore, splenocytes from mice given OVA plus CT-A exhibited significant OVA-specific proliferative responses. These findings indicate that CT-A is an effective adjuvant for the induction of Ag-specific Ab responses in both systemic and mucosal compartments when given by a mucosal route, such as the intranasal one.

CT has been widely used as an adjuvant for mucosal immunization (3,5, 19-24). In this study, we also found that nasal administration of CT acted as an adjuvant to OVA and induced OVA-specific mucosal IgA, as well as serum IgG, IgA and IgM, Ab responses. However, despite these beneficial attributes, CT is unsuitable for use in humans because the ADP-ribosyltransferase activity of CT-A causes severe diarrhea (1,25). Thus, a number of groups have assessed the adjuvant effects of CT-B and found that CT-B enhances soluble peptide presentation by peritoneal macrophages *in vitro* (16). Furthermore, CT-B-pretreated B-cell hybridomas enhanced IL-4 production by Th2 cell lines after stimulation with either an Ag or an anti-CD3 monoclonal Ab (15), suggesting that CT-B may have some adjuvant properties. However, CT-B does not appear to be as effective as CT for induction of immune responses (17). These studies suggest that CT-B acts mainly as a

Table 1 OVA-specific Ab producing cells in salivary glands of mice nasally immunized with OVA plus CT-A, OVA plus CT or OVA alone

Tissue	Vaccine	Antibody forming cells/10^6 cells	
		IgG	IgA
Spleen	OVA + CT-A	224.6 ± 35.9	164.6 ±161.7
	OVA +CT	101.1±21.3	96±9.8
	OVA only	0	0
SG	OVA + CT-A	0	301.8 ± 99.8
	OVA +CT	0	300.0 ± 212.1
	OVA only	0	0

Groups of mice were nasally immunized with OVA alone or together with CT-A or CT on days 0, 7 and 14; and AFCs were assessed on day 21.

targeting protein for GM1 and following binding of CT, the CT-A component actually enhances immune responses.

In addition, both CT and CT-B given by the intranasal route have been demonstrated to accumulate in the ON/E and OBs of mice (18) and preincubating either molecule with its ligand GM1 before intranasal application inhibited accumulation of CT-B and CT in the OB. In mammals, the sense of smell is controlled by the ON/E, which is directly connected to the OBs as the first neural integrative center and to the olfactory cortex as the second integrative center (18,26,27). The neuronal connections between the ON/E and the OBs are used by pathogens to enter the CNS. These findings imply that following intranasal application, CT-B targets the ON/E and OB, which raises the important possibility of neuronal damage with the intranasal use of these proteins in humans. Thus, in this study, in order to eliminate the binding of CT-B to the GM1 receptor, CT-A was administered nasally with a protein Ag. The results presented above demonstrate that nasal administration of OVA plus CT-A as an adjuvant elicited OVA-specific Ab responses in both the serum and mucosal secretions. However, it is interesting to note that OVA-specific salivary IgA Ab titers in mice given OVA plus CT-A were lower than those seen in mice given OVA plus CT, despite the numbers of anti-OVA AFCs in the salivary glands of both groups of mice being identical. A possible explanation is that the amounts of Abs secreted by each AFC in the salivary glands of mice given OVA plus CT-A may have been lower than those in mice given OVA plus CT. Thus, the adjuvant activity of CT-A in mucosal lymphoid tissues may vary and certain mucosal tissues, such as salivary glands, may be less sensitive to CT-A than to CT. Studies are underway to elucidate the reason(s) for this difference. Taken together, these results suggest that CT-A alone is an effective adjuvant for nasal immunization and that binding of CT-B to GM1 is not required for the adjuvant effect of CT. However, it should be noted that the CT-A used in this study was purified from *Vibrio cholerae* and therefore, may have been contaminated with a trace amount of CT-B. If it was, then the residual CT-B may have contributed to the adjuvanticity of CT-A. We are currently generating recombinant CT-A to eliminate CT-B contamination for use in a future immunization study.

Our results showed that CT-A was an effective adjuvant and, when given nasally, it elicited mucosal and systemic Ab responses to a co-administered protein Ag. Importantly, however, it is well established that CT-A accounts for the clinical manifestations of cholera, and is thus unsuitable for use in humans. CT-A binds to nicotinamide adenosine diphosphate and catalyzes ADP-ribosylation of Gsα. This guanosine triphosphate (GTP)-binding protein activates
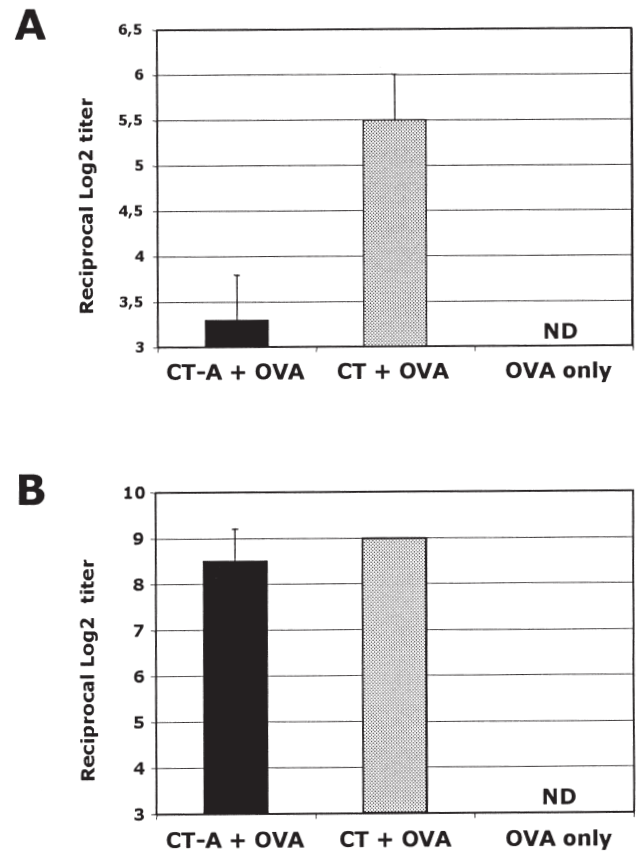

Fig. 4 OVA-specific IgA Ab titers in saliva (A) and nasal wash samples (B) of mice immunized nasally with OVA alone and plus CT-A or CT as an adjuvant. Groups of 8 mice were immunized nasally with OVA alone and plus CT-A or CT, as described in the legend to Figure 2. Saliva and nasal wash samples were assessed for IgA Ab titers on day 21. The results are expressed as means ± SD.
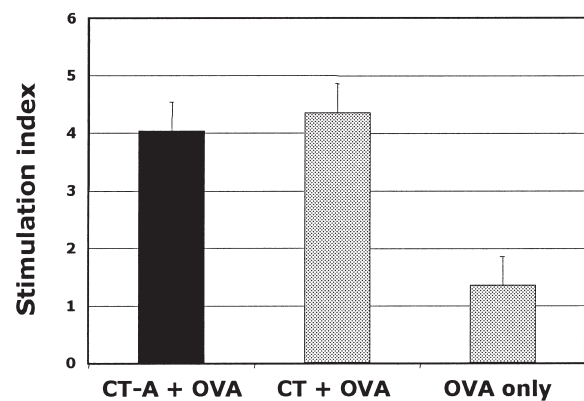

Fig. 5 OVA-specific splenocyte proliferative responses. Groups of 8 mice were immunized nasally with OVA alone and plus CT-A or CT, as described in the legend to Figure 2. Splenocytes were isolated on day 21 and cultured with 1 mg/ml OVA. The results are expressed as the means ± SD.

adenyl cyclase with subsequent elevation of cyclic adenosine monophosphate (cAMP) levels, which in epithelial cells results in secretion of water and chloride ions into the small intestine (1). Thus, in order to develop an adjuvant suitable for use in humans, the ADP-ribosyltransferase activity of CT-A must be eliminated.

Several groups have reported that single amino acid substitution mutants of the heat-labile enterotoxin of enterotoxigenic *Escherichia coli* (R7K, S63K and R192G) lack ADP-ribosyltransferase activity yet retain their adjuvant properties (29-31). Other studies have also shown that mutating CT by substituting a single amino acid in the ADP-ribosyltransferase active center rendered two mutants of CT (S61F and E112K) enzymatically inactive and thus non-toxic; however, these mutant CTs still supported Ag-specific immune responses when administered parenterally (3,9,10). These studies provide evidence that ADP-ribosyltransferase activity is not required for adjuvant activity of CT and, together with our findings, suggest that nasal administration of genetically manipulated non-toxic mutant CT-A as a mucosal adjuvant could be a practical regimen for use in humans.

Acknowledgments

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