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Immunology

A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity

(cholera toxin mutant/Th1 and Th2 subsets/mucosal immunity)

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ABSTRACT **We have characterized a nontoxic mutant of cholera toxin (CT) as a mucosal adjuvant in mice. The mutant CT was made by substitution of serine with phenylalanine at position 61 of the A subunit (S61F), which resulted in loss of ADP ribosyltransferase activity and toxicity. Mice were intranasally immunized with ovalbumin, tetanus toxoid, or influenza virus either alone or together with mutant CT S61F, native CT, or recombinant CT-B. Mice immunized with these proteins plus S61F showed high serum titers of protein-specific IgG and IgA antibodies that were comparable to those induced by native CT. Further, high protein-specific IgA antibody responses were observed in nasal and vaginal washes, saliva, and fecal extracts as well as increased numbers of IgG and IgA antibody forming cells in cervical lymph nodes and lung tissues of mice intranasally immunized with these proteins and S61F or native CT, but not with recombinant CT-B or protein alone. Both S61F and native CT enhanced the induction of ovalbumin-specific $CD4^+$ T cells in lung and splenic tissues, and these T cells produced a Th2-type cytokine pattern of interleukin 4 (IL-4), IL-5, IL-6, and IL-10 as determined by analysis of secreted proteins and by quantitation of cytokine-specific mRNA. These results have shown that mutant CT S61F is an effective mucosal adjuvant when administrated intranasally and induces mucosal and systemic antibody responses which are mediated by $CD4^+$ Th2-type cells.**

Both cholera toxin (CT) produced by *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxin (LT) have been widely used in experimental systems, since both induce significant antibody (Ab) responses to themselves and are also potent mucosal adjuvants for coadministrated, unrelated antigens, especially when given by the oral route (1–4). Previous studies have shown that CT elicits adjuvant responses by inducing antigen-specific $CD4^+$ T cells secreting interleukin 4 (IL-4), IL-5, IL-6, and IL-10 that correlated directly with serum IgG1 and IgG2b subclass, IgE, and mucosal IgA responses (5–7). However, it is well known that both CT and LT account for the clinical manifestations of cholera and enterotoxigenic *E. coli* enteritis, and thus neither are suitable for use as mucosal adjuvants in humans. Both CT and LT are multisubunit macromolecules composed of two structurally, functionally, and immunologically separate A and B subunits (8–10). The B subunit of each toxin consists of five identical peptides, but differ functionally from each other in that the B subunit of CT (CT-B) is the ligand for GM1 ganglioside (11), while the B subunit of LT (LT-B) binds GM1 as well as asialo GM1 and GM2 (12). Following binding of the B subunit to GM1 or GM2 expressed by epithelial cells, the A subunit reaches the cytosol and binds to NAD and catalyzes ADP ribosylation of the G protein, $G_s\alpha$ (13). This GTP-binding protein activates adenylate cyclase, resulting in elevation of intracellular cAMP levels, which in epithelial cells causes secretion of water and chloride ions into the small intestine (14).

Earlier studies have attempted to dissociate diarrhoeagenicity and adjuvanticity of these molecules; however, it was shown that a mutant LT E112K (15), which involved a single amino acid substitution in the ADP ribosyltransferase active center (16), was nontoxic and also lacked adjuvanticity (17). Recently, two groups have reported that single amino acid substitution mutants of LT R7K (18) and R192G (19) were nontoxic but retained adjuvant properties when given by intranasal or oral routes, respectively. However, one of these mutants has the substitution outside of the ADP ribosyltransferase cleft and displayed low ADP ribosyltransferase activity which potentially could cause diarrhea in humans when administrated orally. In addition, another LT mutant designated S63K (20) was shown to be without toxicity, but exhibited poor mucosal adjuvant properties when administrated intranasally (21).

We have shown that two mutants of CT (mCTs), S61F and E112K, which harbor single amino acid substitutions in the ADP ribosyltransferase active center, completely lack ADP ribosyltransferase activity and diarrhoeagenicity (22). Both mutants supported antigen-specific responses which were comparable to native CT (nCT) when given parenterally even though mCT S61F exhibited slightly higher adjuvanticity than mCT E112K (22). In this study, we addressed the important issue of whether a mCT S61F could be given by a mucosal route for induction of both mucosal and systemic immune responses. A mechanism for adjuvant responses induced by mCT when compared with toxic nCT is presented based upon analyses of B cell and Ab isotype responses and of $CD4^+$ Th1- and Th2-cytokine patterns.

MATERIALS AND METHODS

Purification of mCT and Recombinant CT-B (rCT-B). mCT (22) and rCT-B (23) were purified using a D-galactose immo-

Abbreviations: Ab, antibody; AFC, Ab-forming cells; CLN, cervical lymph nodes; CT, cholera toxin; LT, *Escherichia coli* heat-labile toxin; mCT, mutant CT; nCT, native CT; rCT-B, recombinant B subunit of CT; OVA, ovalbumin; TT, tetanus toxoid; IL, interleukin; RT-PCR, reverse transcriptase–PCR.

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bilized column (Pierce) from a cell suspension prepared by sonication of the bacteria as described (24). When ADP ribosyltransferase activity was examined in the presence of 5 μg of ADP ribosylation factor (ARF) in a total volume of 300 μl as described (22, 25), no ADP ribosylation was observed with 4 μg of mCT S61F (111 ± 14.5 cpm per 50 μl of reaction mixture) or with PBS (113 ± 6.3 cpm), while the activity of 4 μg of nCT (List Biological Laboratories, Campbell, CA) was significantly enhanced with ARF (5101 ± 380 cpm) when compared with nCT only (1735 ± 69 cpm).

Immunization and Sample Collection. C57BL/6 mice were obtained from the Charles River Laboratories and were used at 8–12 weeks of age. Mice were intranasally immunized with a 20 μl aliquot (10 μl per nostril) containing 100 μg of ovalbumin (OVA; Sigma) alone or together with 0.1, 1 or 5 μg of mCT, 0.1 or 0.5 μg of nCT, or 5 μg of rCT-B on days 0, 7, and 14. Further, 25 μg of tetanus toxoid (TT) (kindly provided by Y. Mutai, Osaka University, Biken Foundation, Osaka) or formalin-treated influenza virus (B/Yamagata) (2 μg of hemagglutinin equivalent) (kindly provided by R. B. Couch and I. N. Mbawuike, Baylor College of Medicine, Houston) were immunized intranasally together with 5 μg of mCT S61F, 0.5 μg of nCT, or 5 μg of rCT-B using the same protocol as employed for OVA. Nasal and vaginal washes were collected by gently flushing the nasal passage or vaginal canal with 20 μl or 50 μl of sterile PBS, respectively. Saliva was obtained following intraperitoneal injection of mice with 100 μl of 1 mg/ml pilocarpine (Sigma). Fecal extract samples were obtained by adding weighed pellets to PBS containing 0.1% sodium azide (1 ml/100 mg fecal sample). The pellet was vortex mixed and centrifuged, and the supernatants were collected for assay.

Cell Isolation. Cervical lymph nodes (CLN) and spleen were aseptically removed and single cell suspensions were obtained as described (26). The nasal passage, submandibular gland, lung, or vaginal tissues were carefully excised, teased apart, and dissociated using collagenase type IV (Sigma) in Joklik-modified medium (Life Technologies, Gaithersburg, MD). After removal of Peyer's patches, the small intestine was stirred in PBS containing 1 mM EDTA at 37°C for 30 min and the lamina propria lymphocytes were subsequently isolated using collagenase type IV. The mononuclear cells were obtained at the interface of the 40% and 75% layers of a discontinuous percoll gradient (Pharmacia) (26, 27).

Detection of Antigen-Specific Ab Titers by ELISA and Ab-Forming Cells (AFC) by ELISPOT Assay. Ab titers in serum and mucosal secretions were determined by ELISA as described (6, 7). 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. Enumeration of antigen-specific AFC from various tissues was performed by ELISPOT assay as described (5, 7).

Detection of Total and Antigen-Specific IgE in Serum. Total IgE levels were determined by ELISA (6). Antigen-specific serum IgE was detected by a modified IgE-capture luminometric assay as described (22). Light development was carried out in a Dynatech ML-3000 luminometer by injection of Ca^{2+} buffer (22). Endpoint titers were determined as the dilution of each sample showing a 2-fold higher level of luminometric units above background.

OVA- and CT-B-Specific $CD4^+$ T Cell Responses. Single cell suspensions were isolated from lung tissues and spleen in complete medium (6). Isolated cells were fractionated on a nylon wool column 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 GK1.5) and streptavidin microbeads (MACS; Miltenyi Biotec, Sunnyvale, CA) (22). Purified splenic $CD4^+$ T cells (>98% purity) were cultured at a density of 2×10^6 cells/ml with OVA (1 mg/ml) or with CT-B-coated beads (10^7 beads/ml) with T cell-depleted, irradiated (3,000 rads) splenic feeder cells (2.5×10^6 cells/ml) and IL-2 (10 units/ml) (PharMingen) in complete medium (5–7). To measure antigen-specific $CD4^+$ T cell proliferative responses, [methyl-^{3}H]thymidine was added into the culture 18 h before termination. Cells were harvested after 96 h of culture for measurement of radioactivity (22). To determine cytokine production by antigen-specific $CD4^+$ T cells, the cells were harvested after 48 h of culture for quantitative reverse transcriptase–PCR (RT-PCR) analysis of cytokine-specific mRNA. Supernatants were collected after 96 h for evaluation of cytokines by ELISA. For IL-2 analysis by ELISA, supernatants from 48-h cultures were used (22).

Detection of Cytokines by ELISA. Cytokines in culture supernatants were determined by ELISA (6, 7). Briefly, 96-well plates were coated with of 2.5 μg/ml of rat anti-mouse interferon γ, IL-2, IL-4, IL-5, IL-6, and IL-10 mAb (PharMingen). Serial dilutions of culture supernatants or standard cytokines (PharMingen) were added in duplicate. For secondary Ab and detection enzymes, 0.2 μg/ml of biotinylated rat anti-mouse cytokine mAb (PharMingen) and 1:4000 diluted horseradish peroxidase-labeled anti-biotin (Vector Laboratories) were employed as described (6, 7). The ELISA assays were capable of detecting 20 pg/ml for interferon γ, 0.1 unit/ml for IL-2, 10 pg/ml for IL-4, 2 units/ml for IL-5, 1 ng/ml for IL-6, and 0.5 ng/ml for IL-10.

Quantitative RT-PCR Analysis of Cytokine-Specific mRNA. Total RNA isolated from $CD4^+$ T cells was subjected to standard RT-PCR. RT products with a series of diluted recombinant DNA internal standards (27) were amplified by PCR (28), and quantitative analysis of RT-PCR products was conducted by capillary electrophoresis with a laser-induced fluorescence detection system (LIF-P/ACE; Beckman) (28).

Statistical Analysis. Results are reported as mean ± one standard error (SE). Statistical significance ($P < 0.05$) was determined by Student's *t* test and by the Mann–Whitney *U* test of unpaired samples.

RESULTS

Mucosal Adjuvant Properties of mCT S61F. In the initial studies, we assessed systemic and mucosal Ab responses of mice given OVA and various doses of CT derivatives by the intranasal route. Neither OVA alone nor an admixture of OVA with 5 μg of rCT-B elicited detectable serum anti-OVA IgM or IgA Ab responses, although low IgG Ab responses were detected (Fig. 1*A*). In preliminary dose-response studies, admixture of 0.1–1 μg of mCT S61F or 0.1 μg of nCT induced OVA-specific IgG Abs in serum and elevated numbers of OVA-specific IgG and IgA AFC in lung tissues and spleen; however, these doses of adjuvants did not elicit optimal OVA-specific IgA Ab responses in saliva, fecal extracts, or vaginal washes (data not shown).

On the other hand, mice intranasally immunized with OVA plus 5 μg of mCT S61F or 0.5 μg of nCT showed significantly high serum Ab titers of OVA-specific IgG, IgA, and IgM isotypes (Fig. 1*A*) and elevated IgG1 and IgG2b subclass responses (Fig. 1*B*). Assessment of AFC responses also revealed significant numbers of splenic OVA-specific IgG AFC in mice given OVA with mCT or nCT as mucosal adjuvants, whereas only low numbers of AFC were observed in mice given OVA alone or OVA together with rCT-B (Fig. 2*A*). Significant OVA-specific IgG and IgA AFC responses were also observed in CLN and in lung tissue cell isolates of mice immunized with OVA and mCT or nCT as mucosal adjuvants. Only low AFC responses occurred in mice given OVA alone or OVA together with rCT-B (Fig. 2). It should be emphasized that significant OVA-specific IgA Ab responses were seen in multiple mucosal secretions of mice given OVA and mCT as adjuvant. For example, anti-OVA IgA Abs were seen in nasal and vaginal

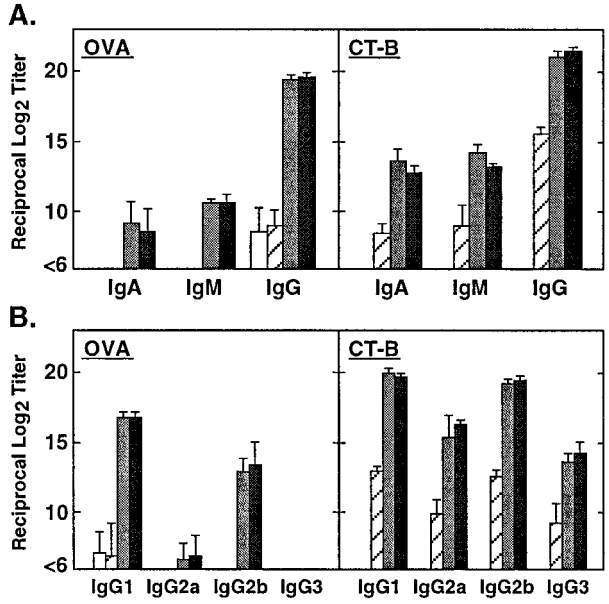

FIG. 1. Serum OVA- and CT-B-specific IgA, IgM, and IgG (*A*) and IgG subclass (*B*) responses on day 21 following intranasal immunization with OVA combined with mCT S61F or nCT as adjuvants were determined by endpoint ELISA. Groups of C57BL/6 mice were immunized with 100 μg of OVA alone (□) or together with 5 μg of rCT-B (▨), 0.5 μg of nCT (▤), or 5 μg of mCT S61F (■) on days 0, 7, and 14. Serum samples were collected 1 week after the last immunization. Bars represent the mean Ab titer ± 1 SE in each group. Each group consisted of five mice, and the data are representative of three separate experiments.

washes, saliva, and fecal extracts of mice immunized with OVA and mCT or nCT as adjuvants, while OVA alone or OVA plus rCT-B failed to elicit detectable IgA anti-OVA responses in

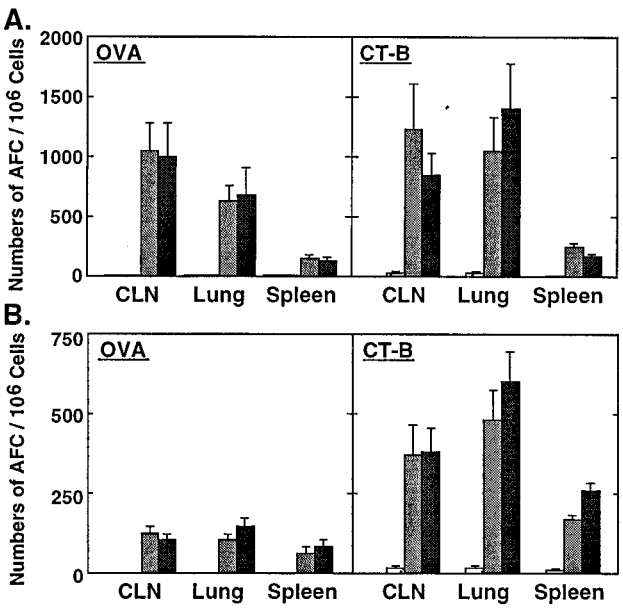

FIG. 2. Numbers of OVA- and CT-B-specific IgG (*A*) and IgA (*B*) AFC in CLN, lung tissues and spleen following intranasal immunization with OVA combined with mCT S61F or nCT as mucosal adjuvants were determined by ELISPOT assay. Groups of C57BL/6 mice were immunized with 100 μg of OVA alone (□) or together with 5 μg of rCT-B (▨), 0.5 μg of nCT (▤), or 5 μg of mCT S61F (■) on days 0, 7, and 14. Samples were collected 1 week after the last immunization. Bars represent the mean numbers of AFC ± 1 SE and each group contained five mice. The data are representative of three separate experiments.

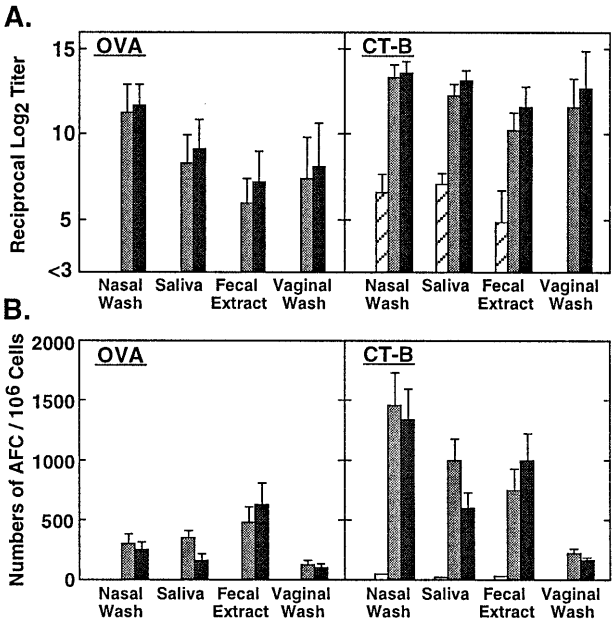

FIG. 3. OVA- and CT-B-specific IgA Ab responses in mucosal secretions were determined by ELISA (*A*) and numbers of IgA AFC in mucosal tissues by ELISPOT assay (*B*) following intranasal immunization with OVA combined with mCT S61F or nCT as mucosal adjuvants. Groups of C57BL/6 mice were immunized with 100 μg of OVA alone (□) or together with 5 μg of rCT-B (▨), 0.5 μg of nCT (▤), or 5 μg of mCT S61F (■) on days 0, 7, and 14. Tissue samples and external secretions were taken 1 week after the last immunization. Bars represent the mean Ab titer or numbers of AFC ± 1 SE in each group. Each group contained five mice and the data are representative of three separate experiments.

any mucosal secretion (Fig. 3*A*). These results were consistent with AFC analyses which demonstrated significant numbers of OVA-specific IgA AFC in these mucosal effector tissues from mice given OVA and mCT or nCT (Fig. 3*B*).

It was important to ensure that mCT S61F is indeed an adjuvant for more conventional vaccines. Groups of mice were also immunized intranasally with TT or with influenza virus mixed with mCT, nCT, or rCT-B. In these studies, the mCT as well as nCT enhanced Ab responses to TT and to influenza virus. Protein-specific IgG, IgA, and IgM Ab responses in serum and protein-specific IgA Ab responses in mucosal secretions were significantly enhanced in mice given mCT or nCT as adjuvants (Table 1). Furthermore, single cell analyses showed significant numbers of protein-specific IgG and IgA AFC in spleen, CLN, and lung tissues and protein-specific IgA AFC in mucosal sites of mice immunized with mCT or nCT as adjuvants (data not shown). On the other hand, rCT-B failed to act as adjuvant for coadministrated TT or influenza virus (Table 1).

Immunogenicity of mCT S61F. Mice immunized with OVA together with mCT or nCT also showed high CT-B-specific IgG, IgA, and IgM Ab responses (Fig. 1*A*), and IgG1 and IgG2b subclass anti-CT-B Ab responses in serum (Fig. 1*B*). Anti-CT-B Ab responses were also seen in mice given rCT-B, but were lower in magnitude when compared with mCT or nCT. Single cell analyses revealed significant numbers of CT-B-specific IgG and IgA AFC in spleen, CLN, and lung tissues from mice coimmunized with OVA plus mCT or nCT, with lower numbers of AFC in mice given rCT-B (Fig. 2). CT-B-specific IgA Ab responses were also elevated in mucosal secretions (i.e., nasal and vaginal washes), saliva, and fecal extracts of mice given mCT or nCT (Fig. 3).

IgE Ab Responses. Serum IgE levels peaked 2 weeks after the initial immunization. Differences in total and OVA-

Table 1. Ab responses in serum and in mucosal secretions following intranasal immunization of mice with TT or influenza virus and mCT S61F or nCT as mucosal adjuvants

Antigen used*	Adjuvant*	Serum isotypes,† reciprocal $\log_2$ titer			Mucosal IgA,† reciprocal $\log_2$ titer			
		IgA	IgM	IgG	Nasal wash	Saliva	Fecal extract	Vaginal wash
TT	None	7	9	10	3	4	<3	<3
	rCT-B	7	9	11	4	4	<3	<3
	nCT	10	14	17	8	7	7	6
	S61F	11	13	18	9	8	8	5
Influenza virus	None	9	10	13	8	9	7	<3
	rCT-B	10	10	13	8	9	7	<3
	nCT	13	12	18	13	10	10	7
	S61F	12	13	19	14	11	10	7

*C57BL/6 mice were immunized by the intranasal route as described. Each group comprised five mice and the results are representative of two separate experiments.
†Ag-specific Ab titers in serum and in mucosal secretions on day 21 were determined by endpoint ELISA.

specific IgE levels were noted between the groups given mCT and nCT as adjuvants, but the differences were not significant. CT-B-specific IgE levels in mice given mCT were significantly lower than seen with nCT (Table 2).

CD4⁺ T Cell Subset Responses. In the present study, $CD4^+$ T cells from lung tissues and spleen, when restimulated with either OVA or CT-B, resulted in significant proliferative responses, clearly indicating the presence of both OVA- and CT-B-specific $CD4^+$ Th cells in mice which had received either mCT or nCT (Fig. 4). Furthermore, OVA-specific $CD4^+$ T cells from lung tissues of mice given OVA and mCT released high levels of Th2-type cytokines (IL-4, IL-5, IL-6, and IL-10) into the culture. These responses were comparable to those obtained when nCT was used as adjuvant (Fig. 5); however, cytokine responses produced by $CD4^+$ T cells from mice given OVA alone or OVA plus rCT-B were detectable but considerably lower than observed with mCT or nCT (Fig. 5). Again, by quantitative RT-PCR, Th2-type cytokine-specific mRNA was readily detected in OVA-specific $CD4^+$ T cells from lung tissues of mice given OVA with mCT or nCT as mucosal adjuvants. Much lower levels of these cytokines were noted in cultures from mice given OVA alone or OVA plus rCT-B (Fig. 5). On the other hand, Th1-type cytokines (interferon γ and IL-2) were detectable at low levels in all samples (Fig. 5). CT-B-specific $CD4^+$ T cells from lung tissues of mice given OVA plus mCT or nCT also exhibited high levels of Th2-type and low levels of Th1-type cytokines (data not shown). Splenic OVA- or CT-B-specific $CD4^+$ T cells from mice given mCT or nCT also showed high levels of Th2-type cytokines by both ELISA and quantitative RT-PCR (data not shown).

DISCUSSION

In the present study, a nontoxic mutant of CT S61F, which was shown to lack ADP ribosyltransferase activity and diarrhoeagenicity (22), was assessed for potential mucosal adjuvant properties. Our recent studies showed that S61F was immunogenic and capable of enhancing Ab responses to a coadministrated protein when given parenterally (22), and in the present study this molecule was tested with several proteins given by the intranasal route. In this study, mCT elicited significant protein-specific IgG, IgA, and IgM Ab responses in serum and IgA Abs in mucosal secretions after intranasal administration, and these responses were comparable to those induced by nCT as mucosal adjuvant. Furthermore, significant enhancement was seen with three different protein components, poorly immunogenic OVA as well as TT and influenza virus. On the other hand, rCT-B failed to enhance anti-protein Ab responses in serum or in the external secretions, indicating that the A subunit of CT is necessary for adjuvant activity.

The mechanisms by which CT acts as a mucosal adjuvant are only partially understood. One study showed that cAMP activates the IL-5 promoter in EL-4, a thymoma T cell line (29). On the other hand, CT has been shown to inhibit mitogen- and anti-CD3-stimulated T cell proliferative responses (30, 31). In another study, CT as well as forskolin were shown to inhibit IL-2 production and proliferation in cloned

Table 2. Serum IgE responses induced by intranasal immunization with OVA and either mCT S61F or nCT as mucosal adjuvants

Treatment* group	Total IgE,† ng/ml	Ag-specific IgE,† reciprocal $\log_2$ titer†	
		OVA	CT-B
OVA alone	186 ± 108	3.99 ± 1.25	<3
OVA + rCT-B	159 ± 83	3.63 ± 0.51	<3
OVA + nCT	1,094 ± 237	9.14 ± 0.79	6.22 ± 0.84
OVA + S61F	768 ± 218	8.10 ± 1.05	4.24 ± 0.88‡

*Mice were immunized as described. Each group contained five mice, and the results are representative of three separate experiments.
†Serum IgE levels reached maximum levels at 2 weeks following the initial immunization and were determined by ELISA (for total IgE) or by IgE-capture luminometric assay (for Ag-specific IgE).
‡Significantly lower when compared with nCT ($P < 0.05$).

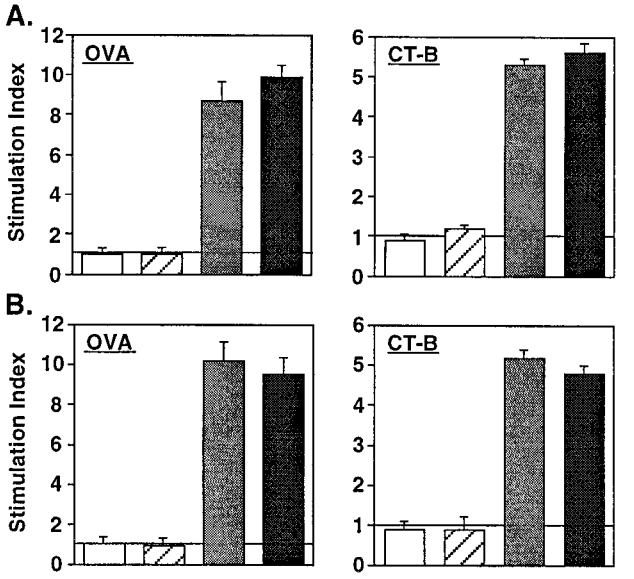

FIG. 4. OVA- and CT-B-specific $CD4^+$ T cell proliferative responses isolated from lung (*A*) and spleen (*B*). Groups of C57BL/6 mice were immunized with 100 μg of OVA alone (□) or together with 10 μg of rCT-B (▨), 0.5 μg of nCT (▤), or 5 μg of mCT S61F (■) on days 0, 7, and 14. Bars represent the mean stimulation index ± 1 SE and each group contained five mice. The data were similar and are representative of four separate experiments.

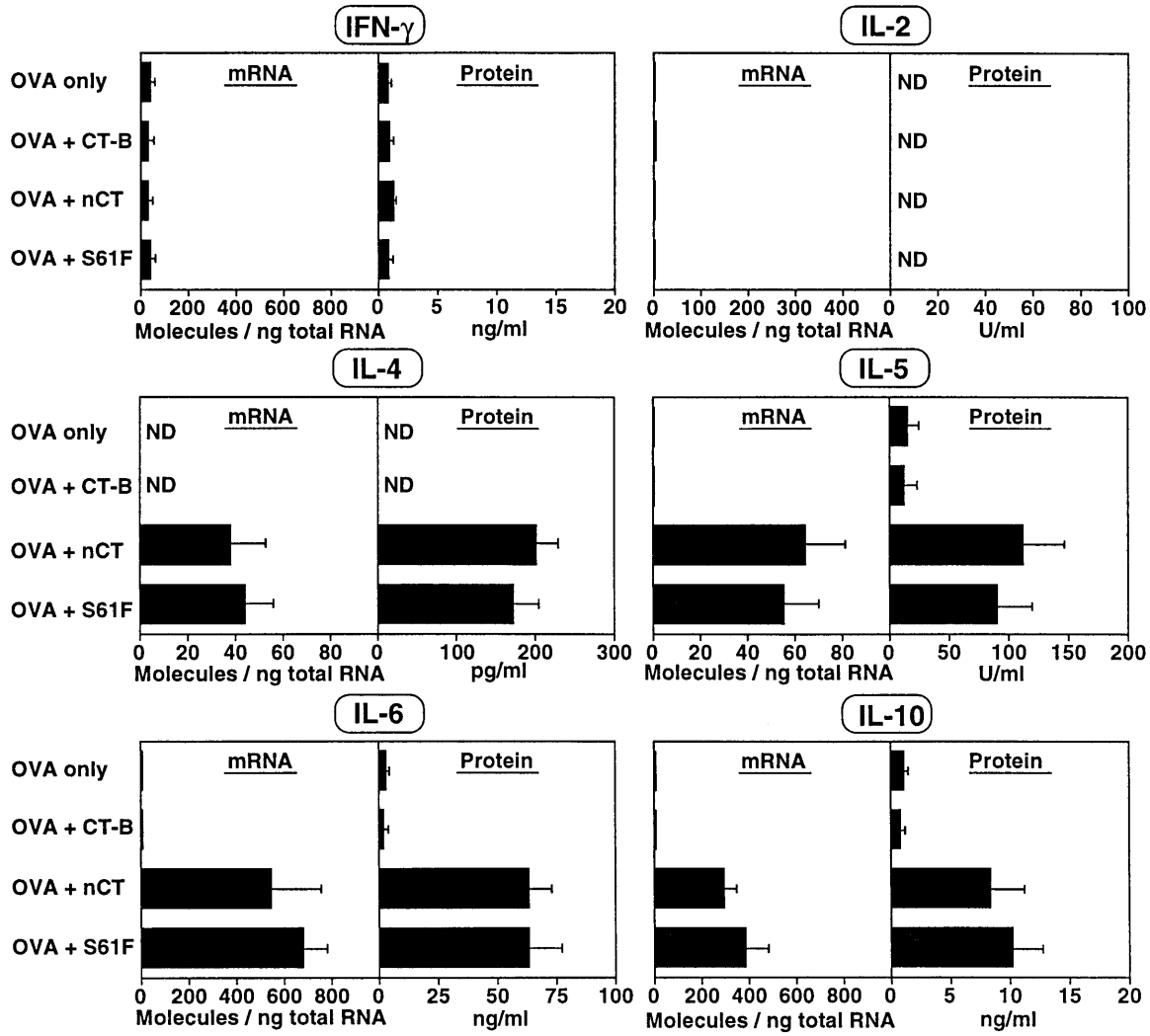

FIG. 5. Cytokine production from OVA-specific $CD4^+$ T cells isolated from lung tissues. Molecules of cytokine-specific mRNA were determined by quantitative RT-PCR. Cytokine protein production was determined by ELISA. The scale of each figure corresponds to mRNA molecules and protein levels produced by nonimmunized $CD4^+$ T cells stimulated with anti-CD3 mAb. ND, not detected; IFN-γ, interferon γ. Bars represent the mean cytokine profile ± 1 SE in each group. The data are representative of four separate experiments.

Th1 cells but not IL-4 production and proliferation in a clone of Th2 cells, indicating that Th1 and Th2 cells differ in their sensitivity to an increase in cAMP (32). However, our results showed that mCT, despite lack of ADP ribosyltransferase activity with resultant cAMP induction, elicited serum IgG1 and IgG2b subclass and mucosal IgA Ab responses. Further, OVA-specific $CD4^+$ T cells from mice given mCT as adjuvant yielded a clear pattern of Th2-type responses which were identical to those induced when nCT was used as adjuvant. Thus, our findings indicate that the $CD4^+$ Th2-type T cell responses induced by CT are elicited via a pathway separate from the adenyl cyclase system. Taken together, CT may possess the property of up-regulation of a yet to be characterized pathway that induces $CD4^+$ T cells into a Th2-type subset, and this mechanism has not been elucidated by previous *in vitro* studies. Such adjuvant properties of CT appear to be associated with the A subunit, since rCT-B did not enhance protein-specific Ab responses.

On the other hand, several reports have shown that CT could enhance IL-1 or IL-6 production by macrophages or epithelial cells (33, 34) and ADP ribosylating activity by CT appeared to contribute to these effects, since dibutyryl cAMP induced effects similar to those seen with CT (34). In the present study, although mCT showed a potent mucosal adjuvant effect in the absence of ADP ribosyltransferase activity, ≈5- to 10-fold higher doses of mCT were required to elicit comparable adjuvant responses as nCT. These findings suggest that CT may affect a variety of cells including macrophages, epithelial cells, and lymphocytes and its adjuvanticity may be derived from two or more mechanisms, which may include intracellular cAMP accumulation following ADP ribosylation. We are currently investigating the signal transduction pathways induced by mCTs and nCT in lymphoreticular cells—i.e., in $CD4^+$ and $CD8^+$ T cells, B cells, macrophages, as well as dendritic cells.

It is also of interest that even though LT shares a significant homology (≈80% amino acid sequence homology) with CT (8) and is also an effective adjuvant (3), these molecules differ in terms of the nature of $CD4^+$ T cell subsets induced and thus provide help for different Ab isotypes and subclasses. For example, CT effectively promoted $CD4^+$ Th2-type responses with provision of help for IgG1 subclass, IgE, and mucosal IgA responses (5–7), while LT induced both $CD4^+$ Th1- and Th2-type responses with subsequent IgG1, IgG2a, and IgG2b subclass and mucosal IgA Ab responses (35). Further, CT failed to enhance Ab responses to co-administrated proteins in IL-4 gene disrupted ($IL\text{-}4^{-/-}$) mice when used as an oral adjuvant (36, 37), whereas LT was effective as mucosal adjuvant in both normal and $IL\text{-}4^{-/-}$ mice (unpublished data). These differences between CT and LT cannot be ascribed to ADP ribosyltransferase activity since both molecules share this

property. In our studies, mCTs S61F and E112K were shown to exhibit adjuvant properties (22), while a LT mutant, E112K, did not (17). However, the routes of administration by which these adjuvants were assessed should be carefully considered, since we tested the adjuvant properties of mCTs S61F and E112K parenterally in our previous study (22) and mCT S61F by the intranasal route in the present study, while LT mutant E112K was tested by the oral route (17). In this regard, our recent study demonstrated that mCT E112K was also an effective adjuvant when administrated by either the intranasal or oral routes (unpublished data). These observations would again indicate that CT and LT are different in terms of effects on cells of the immune system which result in adjuvanticity.

Intranasal immunization, like other mucosal routes, offers several advantages when compared with parenteral immunization. For example, lower doses of proteins are required to induce Ab responses when compared with other routes, and would decrease the cost for vaccination. To elicit intestinal mucosal IgA responses comparable to those induced by oral immunization, only 5–10% of the quantity of vaccine is required when given intranasally and this dose also effectively induces serum IgG Ab responses when compared with parenteral immunization which required more doses of vaccine (5–7, 22). In addition, the doses used in intranasal immunization in this study induced lower total and antigen-specific IgE levels in serum than protocols used for oral administration (6). This result implies that intranasal immunization may have less risk for anaphylactic reactions. In this regard, mCT has an additional advantage over nCT, since serum total and antigen-specific IgE levels elicited by mCT were lower than induced by nCT, although both molecules induced elevated antigen-specific Ab responses of other isotypes in serum. Further, intranasal immunization effectively induced not only systemic IgG but also mucosal IgA responses in mucosal effector tissues as seen in the present study. Thus, intranasal vaccination using mCT could be useful in humans to prevent systemic, gastrointestinal or respiratory diseases as well as sexually transmitted diseases including HIV infection.

In summary, the present study has shown that mCT S61F, like nCT, is an effective adjuvant by the intranasal route and induces mucosal and systemic Ab responses mediated by $CD4^+$ Th2-type cells elicited via a pathway separate from ADP ribosylation activity, indicating that mCTs should be considered as a candidate for an nontoxic mucosal adjuvant. We are currently concentrating on assessing adjuvanticity and safety of mCTs for use as mucosal adjuvants in humans.

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1. Elson, C. O. & Ealding, W. (1984) *J. Immunol.* **132,** 2736–2741.
2. Elson, C. O. & Ealding, W. (1984) *J. Immunol.* **133,** 2892–2897.
3. Lycke, N. & Holmgren, J. (1986) *Immunology* **59,** 301–308.
4. Clements, J. D., Hartzog, N. M. & Lyon, F. L. (1988) *Vaccine* **6,** 269–277.
5. Xu-Amano, J,. Kiyono, H., Jackson, R. J., Staats, H. F., Fujihashi, K., Burrows, P. D., Elson, C. O., Pillai, S. & McGhee, J. R. (1993) *J. Exp. Med.* **178,** 1309–1320.
6. Marinaro, M., Staats, H. F., Hiroi, T., Jackson, R. J., Coste, M., Boyaka, P. N., Okahashi, N., Yamamoto, M., Kiyono, H., Bluethmann, H., Fujihashi, K. & McGhee, J. R. (1995) *J. Immunol.* **155,** 4621–4629.
7. VanCott, J. L., Staats, H. F., Pascual, D. W., Roberts, M., Chatfield, S. N., Yamamoto, M., Coste, M., Carter, P. B., Kiyono, H. & McGhee, J. R. (1996) *J. Immunol.* **156,** 1504–1514.
8. Spangler, B. D. (1992) *Microbiol. Rev.* **56,** 622–647.
9. Gill, D. M. (1976) *Biochemistry* **15,** 1242–1248.
10. Gill, D. M., Clements, J. D., Robertson, D. C. & Finkelstein, R. A. (1981) *Infect. Immun.* **33,** 677–682.
11. van Heyningen, S. (1974) *Science* **183,** 656–657.
12. Fukuta, S., Magnani, J. L., Twiddy, E. M., Holmes, R. K. & Ginsburg, V. (1989) *Infect. Immun.* **56,** 1748–1753.
13. Gill, D. M. & King, C. A. (1975) *J. Biol. Chem.* **250,** 6424–6432.
14. Field, M., Rao, M. C. & Chang, E. B. (1989) *N. Engl. J. Med.* **321,** 800–806.
15. Tsuji, T., Inoue, T., Miyama, A., Okamoto, K., Honda, T. & Miwatani, T. (1990) *J. Biol. Chem.* **265,** 22520–22525.
16. Sixma, T. K., Pronk, S. E., Kalk, K. W., van Zanten, B. A., Berghuis, A. M. & Hol, W. G. (1992) *Nature (London)* **355,** 561–564.
17. Lycke, N., Tsuji, T. & Holmgren, J. (1992) *Eur. J. Immunol.* **22,** 2277–2281.
18. Douce, G., Turcottee, C., Cropley, I., Roberts, M., Pizza, M., Domenghini, M., Rappuoli, R. & Dougan, G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1644–1648.
19. Dickinson, B. L. & Clements, J. D. (1995) *Infect. Immun.* **63,** 1617–1623.
20. Pizza, M., Domenghini, M., Hol, W., Giannelli, V., Fontana, M. R., Giuliani M. M., Magagnoli, C., Peppoloni, S., Manetti, R. & Rappuoli, R. (1994) *Mol. Microbiol.* **14,** 51–60.
21. Di Tommaso, A., Saletti, G., Pizza, M., Rappuoli, R., Dougan, G., Abrignani, S., Douce, G. & DeMagistris, M. T. (1996) *Infect. Immun.* **64,** 974–979.
22. Yamamoto, S., Takeda, Y., Yamamoto, M., Kurazono, H., Imaoka, K., Yamamoto, M., Fujihashi K., Noda, M., Kiyono, H. & McGhee, J. R. (1997) *J. Exp. Med.* **185,** 1203–1210.
23. Dertzbaugh, M. T. & Macrina, F. L. (1989) *Gene* **82,** 335–342.
24. Uesaka, Y., Otsuka, Y., Lin, Z., Yamasaki, S., Yamaoka, J., Kurazono, H. & Takeda, Y. (1994) *Microb. Pathog.* **16,** 71–76.
25. Noda, M., Tsai, S., Adamik, R., Bobak, D. A., Moss, J. & Vaughan, M. (1989) *Biochemistry* **28,** 7936–7940.
26. Fujihashi, K., McGhee, J. R., Kweon, M., Cooper, M. D., Tonegawa, S., Takahashi, I., Hiroi, T., Mestecky, J. & Kiyono, H. (1996) *J. Exp. Med.* **183,** 1929–1935.
27. Hiroi, T., Fujihashi, K., McGhee, J. R. & Kiyono, H. (1995) *Eur. J. Immunol.* **25,** 2743–2751.
28. Yamamoto, M., Kawabata, K., Fujihashi, K., McGhee, J. R., Van Dyke, T. E., Bamberg, T. V., Hiroi, T. & Kiyono, H. (1996) *Am. J. Pathol.* **148,** 331–339.
29. Lee, H. J., Koyano-Nakagawa, N., Naito, Y., Nishida, J., Arai, N., Arai, K. & Yokota, T. (1993) *J. Immunol.* **151,** 6135–6142.
30. Anderson, D. L. & Tsoukas, C. D. (1989) *J. Immunol.* **143,** 3647–3652.
31. Imboden, J. B., Shoback, D. M., Pattison, G. & Stobo, J. D. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5673–5677.
32. Munoz, E., Zubiaga, M., Merrow, M., Sauter, N. P. & Huber, B. T. (1990) *J. Exp. Med.* **172,** 95–103.
33. Bromander, A., Holmgren, J. & Lycke, N. (1991) *J. Immunol.* **146,** 2908–2914.
34. McGee, D. W., Elson, C. O. & McGhee, J. R. (1993) *Infect. Immun.* **61,** 4637–4644.
35. Takahashi, I., Marinaro, M., Kiyono, H., Jackson, R. J., Nakagawa, I., Fujihashi, K., Hamada, S., Clements, J. D., Bost, K. L. & McGhee, J. R. (1996) *J. Infect. Dis.* **173,** 627–635.
36. Vajdy, M., Kosco-Vilbois, M. H., Kopf, M., Kohler, G. & Lycke, N. (1995) *J. Exp. Med.* **181,** 41–53.
37. Okahashi N., Yamamoto M., VanCott, J. L., Chatfield, S. N., Roberts, M., Bluethmann, H., Hiroi, T., Kiyono, H. & McGhee, J. R. (1996) *Infect. Immun.* **64,** 1516–1525.