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Direct Effects on Antigen-Presenting Cells and T Lymphocytes Explain the Adjuvanticity of a Nontoxic Cholera Toxin Mutant[1]

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The present study has elucidated two distinct mechanisms that may explain how a mutant of cholera toxin (mCT), E112K, retains adjuvant effects though it lacks ADP-ribosyltransferase activity and associated toxicity. In the first mechanism, we show that mCT E112K, like native cholera toxin (nCT), enhances B7-2 expression, but, to some extent, also enhances B7-1 on Peyer's patch B cells and macrophages. Cocultivation of $CD4^+$ T cells with E112K- or nCT-treated B cells and macrophages in the presence of anti-CD3 stimulation resulted in the induction of T cell-proliferative responses. Further, the responses were blocked by mAbs to B7-1 and/or B7-2; however, the effect of anti-B7-1 was minimal. In the second mechanism, addition of mCT E112K or nCT to anti-CD3 mAb-stimulated Peyer's patch $CD4^+$ T cells inhibited proliferative responses, while recombinant CT-B subunit (rCT-B) did not. Analysis of cytokine responses showed that both mCT E112K and nCT preferentially inhibited IFN-γ production. Interestingly, however, nCT, but not mCT E112K, induced apoptosis in $CD4^+$ T cells activated via the TCR-CD3 complex. These results indicate that CT uses at least two pathways for inhibition of Th1 responses and that, while nCT induces cAMP accumulation that in turn leads to apoptosis in Th1-type cells, mCT E112K, which lacks ADP-ribosyltransferase activity, inhibits IFN-γ synthesis by a separate mechanism. Thus, mCT E112K, like nCT, induces adjuvant responses via up-regulation of mainly B7-2 on APCs and through preferential inhibition of Th1-type $CD4^+$ T cell responses in the absence of ADP-ribosyltransferase activity. ***The Journal of Immunology*****, 1999, 162: 7015–7021.**

Cholera toxin (CT)[3] produced by *Vibrio cholerae* is a potent immunogen and induces Ag-specific secretory-IgA (S-IgA) and serum Ab responses (1). Furthermore, CT can act as an adjuvant for the enhancement of mucosal and serum Ab responses to coadministered Ag after oral or nasal presentation (1–5). Earlier studies had suggested that mucosal adjuvanticity of CT and the related heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* were closely associated with ADP-ribosyltransferase activity (6). Therefore, these adjuvants had been thought to be unsuitable for use in humans, since ADP-ribosylation of the G protein Gsα, with subsequent cAMP accumulation in intestinal epithelial cells, causes severe diarrhea (7, 8).

Recently, several groups have reported that single amino acid substitution mutants of LT (R7K, S63K, and R192G) lack ADP-ribosyltransferase activity, yet retain their adjuvant properties (9–11). Our 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 nontoxic; however, these mutants of CT (mCTs) still supported Ag-specific immune responses when administered parenterally (12). Further, we showed that mCT S61F acts as a mucosal adjuvant by inducing $CD4^+$ Th2 cells secreting IL-4, IL-5, IL-6, and IL-10, which provided effective help for Ag-specific mucosal S-IgA, as well as serum IgG1, IgE, and IgA Ab responses (5, 13). These studies provided evidence that ADP-ribosyltransferase activity is not required for CT-induced enhancement of immune responses to coadministered proteins. However, the mechanisms for induction of Ag-specific $CD4^+$ Th2 cells and regulation of mucosal IgA Ab responses by mCT or native CT (nCT) have not been elucidated.

Signal transduction through CD28 induces important costimulation for initial T cell responses when triggered via B7-1 and B7-2 on APCs (14–20). Past studies have shown that both nCT and a fusion protein with the intact A1 subunit that retained ADP-ribosyltransferase enzyme activity enhanced B7-1 (CD80) and B7-2 (CD86) expression on B cells (21). In contrast, other studies have shown that nCT enhances B7-2, but not B7-1, expression by macrophages, and, when treated with nCT, these APCs enhance T cell-proliferative responses (22). Interestingly, administration of anti-B7-2 mAbs inhibited keyhole limpet hemocyanin (KLH)-specific serum IgG and mucosal IgA Ab responses in mice given KLH

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[3] Abbreviations used in this paper: CT, cholera toxin; LT, *Escherichia coli* heat-labile toxin; mCT, mutant CT; nCT, native CT; rCT-B, recombinant CT B subunit; CT-A, CT A subunit; S-IgA, secretory-IgA; KLH, keyhole limpet hemocyanin.

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plus CT orally (22). These studies raise the possibility that costimulation via B7-1 and B7-2 plays a critical role in adjuvanticity induced by CT.

In this study, we have examined the direct effects of mCT E112K on APCs and $CD4^+$ T cells from mucosal inductive tissues, e.g., Peyer's patches. The novel results obtained by this study show that mCT E112K, like nCT, induces mainly B7-2 expression on APCs that enhanced $CD4^+$ T cell-proliferative responses when activated via the TCR-CD3 receptor complex. Further, the nontoxic CT mutant directly signaled $CD4^+$ T cells to preferentially down-regulate Th1-type cytokine responses. It is this ability of mCT to affect two cell types that may account for its ability to act as a mucosal adjuvant in the absence of ADP-ribosyltransferase activity.

Materials and Methods

Mice

C57BL/6 mice, 8–12 wks of age, were purchased from The Jackson Laboratory Animal Resources Center (Bar Harbor, ME) and were maintained in this facility under pathogen-free conditions in microisolator cages.

Native, nontoxic, and recombinant B subunit of CT

E. coli strains containing the plasmids for the mCT E112K or the rCT-B were grown in Luria-Bertani medium (10 mg/ml NaCl, 5 mg/ml yeast extract, 10 mg/ml tryptone) with 100 μg/ml of ampicillin. The mCT E112K and rCT-B were purified using a D-galactose-immobilized column (Pierce, Rockford, IL) from a cell suspension prepared by sonication of the bacteria, as described previously (12, 23). The purity of mCT E112K and rCT-B was assessed by SDS-PAGE, and no contaminating protein bands were noted. The nCT was purchased from List Biologic Laboratories (Campbell, CA).

Purification of $CD4^+$ T cells and T cell depletion

Peyer's patch $CD4^+$ T cells were purified by use of the magnetic cell sorter system (Miltenyi Biotec, Sunnyvale, CA). Briefly, cells were incubated in a nylon wool column (Polysciences, Warrington, PA) at 37°C for 1 h, and the effluent containing T cells was eluted with complete medium (RPMI 1640; Cellgro Mediatech, Washington, DC) containing 10% FCS, 50 μM 2 ME, 10 mM HEPES buffer, 1% L-glutamime, 10 U/ml penicillin, and 100 μg/ml streptomycin. The T cell populations were incubated with biotinylated anti-CD8 (53-6.7), anti-Mac-1 (M1/70), and anti-B220 (RA3-6A2) mAbs (PharMingen, San Diego, CA), followed by streptavidin-conjugated microbeads and then passed through the magnetic column. Two cycles of the above procedure yielded enriched T cell preparations that were >98% $CD4^+$ T cells. To obtain T cell-depleted Peyer's patch cells, T cells were removed by incubation with anti-Thy 1.2 (30-H12), followed by infant rabbit complement (Pel-Freez Biologicals, Rogers, AR). The cells were then incubated with biotinylated anti-CD4 (GK1.5) and with anti-CD8 (53-6.7) mAbs, followed by streptavidin-conjugated microbeads before passage through the magnetic column. This procedure routinely resulted in cell preparations with <1% $CD3^+$ T cells and of >98% viability.

Culture conditions

To examine the effect of mCT E112K on expression of costimulatory molecules by B cells and macrophages, 1 μg/ml of mCT E112K, nCT, or rCT-B was added to T cell-depleted Peyer's patch cells (1×10^6 cells/ml) and incubated for 20 h. In some experiments, these molecules were incubated with GM1-ganglioside (Sigma, St. Louis, MO) at a 1:100 (mole/mole) ratio for 1 h at 37°C, and GM1-treated mCT E112K, nCT, or rCT-B was then added to T cell-depleted Peyer's patch cell cultures. After incubation, cells were removed, washed extensively, and stained first with either FITC-conjugated anti-B220 or anti-Mac-1 mAb and then with either biotinylated anti-B7-1 (1G10) or anti-B7-2 (GL1; PharMingen), followed by streptavidin-PE (PharMingen). Labeled cells were analyzed by FACScan (Becton Dickinson, Sunnyvale, CA). To assess the costimulatory effect of B7-1 and B7-2 on $CD4^+$ T cells in vitro, T cell-depleted Peyer's patch cells were pretreated with mCT E112K or nCT. After a 24-h incubation, cells were washed with PBS and fixed with 0.5% paraformaldehyde. Peyer's patch $CD4^+$ T cells (2×10^6 cells/ml) were incubated with T cell-depleted Peyer's patch cells treated with mCT E112K or nCT for 48 h at 37°C in 5% CO_2 in the presence of a suboptimal dose (100 ng/ml) of immobilized anti-CD3 mAb. In some experiments, mAbs to B7-1 (10 ng/ml) and/or B7-2 (10 ng/ml) were added to the cultures.

To assess the direct effect of mCT E112K and nCT on $CD4^+$ T cells in vitro, Peyer's patch $CD4^+$ T cells (2×10^6/ml) were incubated with several concentrations of mCT E112K, nCT, or rCT-B in the presence of a suboptimal dose (1 μg/ml) of immobilized anti-CD3 mAb for 48 or 72 h. To measure cell proliferation, 1.0 μCi of [^{3}H]thymidine (DuPont New England Nuclear Products, Boston, MA) was added to individual culture wells 15 h before termination, and the uptake of cpm was determined by scintillation counting.

Quantitative analysis of apoptosis by flow cytometry

For the quantitative analysis of apoptosis, emergence of hypodiploid DNA was measured (24). Briefly, cells (1×10^6) were fixed with 100% ethanol. After fixation, the cells were resuspended in propidium iodide solution containing 50 μg/ml of propidium iodide and 40 μg/ml of RNase A for 30 min in the dark. Labeled cells were analyzed by FACScan (Becton Dickinson).

Quantitative analysis of cytokine-specific mRNA

For evaluation of cytokine-specific mRNA levels, a quantitative RT-PCR was employed. Total RNA was isolated by the acid guanidinium thiocyanate phenol chloroform extraction procedure. Aliquots of total RNA were subjected to standard RT, and RT products with a series of diluted rDNA internal standards were amplified by PCR (12, 25). For quantification, capillary electrophoresis with the laser-induced fluorescence detection system (LIF-P/ACE; Beckman Instruments, Fullerton, CA) was applied as described previously (26). The fluorescence content of each cytokine-specific RT-PCR product was expressed as the peak area of relative fluorescent light units. Plotting peak areas vs the serial dilutions of rDNA internal standard or sample RNA resulted in a linear relationship.

Analysis of secreted cytokines

Cytokine levels in culture supernatants were determined by cytokine-specific ELISA (27, 28). Nunc (Naperville, IL) MaxiSorp immunoplates were coated with monoclonal anti-IFN-γ (R4-6A2) and anti-IL-4 (BVD4-1D11) (PharMingen). After blocking, samples and serial 2-fold dilutions of standards were added to duplicate wells and incubated overnight at 4°C. The wells were washed and incubated with biotinylated monoclonal anti-IFN-γ (XMG 1.2) and anti-IL-4 (BVD6-24G2) (PharMingen). After incubation, peroxidase-labeled anti-biotin Ab (Vector Laboratories, Burlingame, CA) was added and developed with ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) containing H_2O_2 (Moss, Pasadena, MD). Standard curves were generated using mouse rIFN-γ and rIL-4 (Endogen, Woburn, MA).

Statistics

The data are compared using an unpaired Mann-Whitney *U* test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Berkeley, CA) for Macintosh computers and were considered to be statistically significant if p values were <0.05.

Results

The mCT E112K, like nCT, induces B7-1 and B7-2 expression on APCs

Since past studies have shown that CT induces B7 expression on APCs that provide a costimulatory function for T cell activation (21, 22), we initially determined whether mCT E112K also exhibited this property. Addition of mCT E112K or nCT to T cell-depleted Peyer's patch cells resulted in significant up-regulation of B7-2 expression on B cells ($B220^+$) and macrophages ($Mac\text{-}1^+$). Further, nCT and its nontoxic derivative enhanced expression of B7-1 on B cells and macrophages; however, the levels were significantly lower than those of B7-2. In contrast, rCT-B failed to induce either B7-1 or B7-2 expression on B cells or macrophages (Fig. 1).

To verify that the enhancement of B7-1 and B7-2 expression occurred after CT-B binding to GM1, mCT E112K, nCT, or rCT-B were treated with GM1 before addition to B cell or macrophage cultures. Pretreatment of mCT E112K or nCT with GM1 blocked B7-1 and B7-2 expression on both B cells and macrophages (Table I). These results showed that initial binding of CT-B to APCs

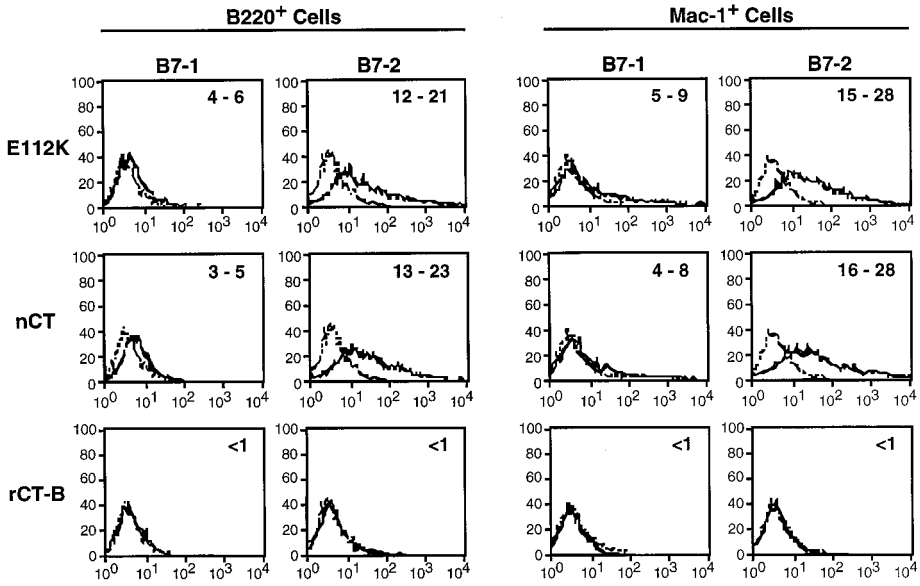

FIGURE 1. Expression of B7-1 and B7-2 by $B220^+$ and Mac-1^+ cells isolated from mouse Peyer's patches. T cell-depleted Peyer's patch cells (1×10^6/ml) were stimulated with 1 μg/ml of nCT, mCT E112K, or rCT-B. After 20 h of incubation, cells were removed, washed, and then stained first with either FITC-conjugated anti-B220 or anti-Mac-1 and then with biotinylated anti-B7-1 or anti-B7-2 followed by streptavidin-PE. The data were electronically gated for either $B220^+$ cells or Mac-1^+ cells. The solid and dotted lines represent stimulated and nonstimulated samples, respectively. The numbers express the range of percent differences between stimulated and nonstimulated cells relative to isotype matched controls after electronic gating for $B220^+$ or Mac-1^+ cells. The data were similar and are representative of four separate experiments.

occurs via GM1 and results in the induction of B7-1 and B7-2 costimulatory molecules.

The mCT E112K- or nCT-treated APCs enhance $CD4^+$ T cell responses

Since mCT E112K, as well as nCT, induced B7-1 and B7-2 expression on Peyer's patch B cells and macrophages, it was important to assess the effect of costimulatory signals induced by these molecules on $CD4^+$ T cell responses. Thus, Peyer's patch $CD4^+$ T cells were incubated with mCT E112K- or nCT-treated APCs in the presence of a suboptimal dose (100 ng/ml) of anti-CD3 mAb. Cocultivation of $CD4^+$ T cells with mCT E112K- or nCT-treated APCs revealed significantly higher proliferative responses than were induced by anti-CD3 mAb stimulation alone. Further, the addition of mAb to B7-2 resulted in a marked reduction in T cell-proliferative responses. In contrast, anti-B7-1 or a combination of anti-B7-1 and anti-B7-2 mAbs only slightly inhibited the response when compared with isotype-matched control or anti-B7-2 alone, respectively (Fig. 2). These results showed that B7-2, but not B7-1, expression, enhanced by mCT E112K or nCT, supported $CD4^+$ T cell-proliferative responses when activated via the TCR-CD3 receptor complex.

mCT, like nCT, exert direct effects on $CD4^+$ T cells

We next investigated whether mCT E112K and nCT directly influence T cell responses when activated via the TCR-CD3 receptor complex. When 10 ng/ml of mCT E112K was added to $CD4^+$ T cells stimulated by anti-CD3 mAb, a small but significant down-regulation of proliferative responses was seen. Further, a 100 ng/ml dose gave a strong inhibitory effect (Fig. 3). Addition of nCT also inhibited $CD4^+$ T cell-proliferative responses. Interestingly, 1 ng/ml of nCT revealed a greater inhibitory effect than did 10 ng/ml of mCT E112K. When 10 ng/ml of nCT was added, the proliferative responses were completely eliminated. In contrast, both 10 and 100 ng/ml doses of rCT-B failed to inhibit $CD4^+$ T cell-proliferative responses (Fig. 3).

Analysis of cytokine synthesis by $CD4^+$ T cells showed that 100 ng/ml of mCT E112K significantly down-regulated IFN-γ production at both the mRNA and protein levels. Further, the synthesis of this cytokine was at a marginal level when $CD4^+$ T cells were treated with 10 ng/ml of mCT E112K (Fig. 4). In contrast, the lower dose (10 ng/ml) of mCT E112K did not inhibit IL-4 production. Further, significant IL-4 responses were maintained when 100 ng/ml of mCT E112K was added to cultures, even though T

Table I. *Enhanced expression of B7-1 and B7-2 by Peyer's patch B cells and macrophages treated with mCT E112K, nCT, or rCT-B*[a]

				GM1-Treated		
Increase (%)[b]	E112K	nCT	rCT-B	E112K	nCT	rCT-B
$B220^+$ cells						
B7-1	4	4	<1	<1	<1	<1
B7-2	18	21	<1	<1	<1	<1
Mac-1^+ cells						
B7-1	5	4	<1	<1	<1	<1
B7-2	22	23	<1	<1	<1	<1

[a] T cell-depleted Peyer's patch cells (1×10^6/ml) were incubated with 1 μg/ml of nCT, mCT E112K, or rCT-B for 20 h. In some experiments, mCT E112K, nCT, or rCT-B were incubated with GM1-ganglioside at a molar ratio of 1:100 for 1 h at 37°C, and GM1-treated nCT, mCT, or rCT-B was then incubated with T cell-depleted Peyer's patch cells. After incubation, cells were removed, washed, and then costained with FITC-conjugated anti-B220 or anti-Mac-1 and biotinylated anti-B7-1 or anti-B7-2 mAbs followed by streptavidin-PE.

[b] Increases in the frequency of B7 expression were shown as the difference between treated and untreated cells when compared with isotype-matched controls after electronic gating for $B220^+$ or Mac-1^+ cells.

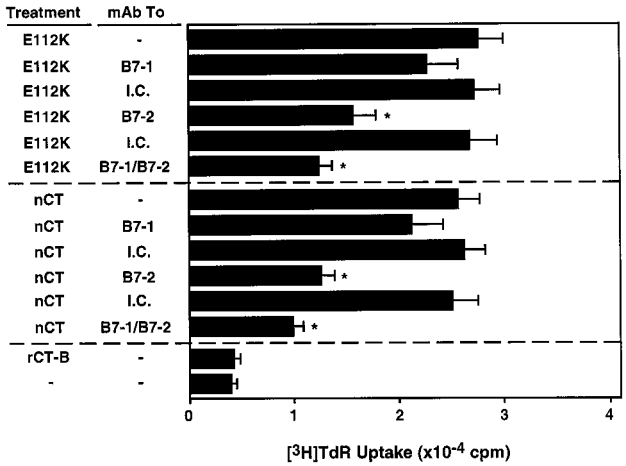

FIGURE 2. CT-treated Peyer's patch APCs result in enhanced $CD4^+$ T cell-proliferative responses. T cell-depleted cells were pretreated with mCT E112K or nCT, as described in Fig. 1. After treatment, the cells were fixed with 0.5% paraformaldehyde and added to $CD4^+$ T cells in the presence of anti-CD3 (100 ng/ml) stimulation. In some experiments, anti-B7-1 (10 ng/ml) and/or anti-B7-2 (10 ng/ml) were added to the cultures. The results were expressed as the mean ± SEM. The data were similar and are representative of three separate experiments. I.C., isotype-matched Ab control. *, $p < 0.05$ when compared with cultures using I.C.

cell-proliferative responses were clearly reduced (Figs. 3 and 4). In the case of nCT, 1 ng/ml of nCT strongly inhibited IFN-γ synthesis by $CD4^+$ T cells activated via the TCR-CD3 receptor complex, whereas IL-4 responses were only slightly reduced. Addition of 10 ng/ml of nCT essentially inhibited both IFN-γ and IL-4 synthesis. These results showed that Th1- and Th2-type cells possessed different sensitivities to the inhibitory signals provided by mCT E112K and nCT, since both mCT E112K and nCT preferentially

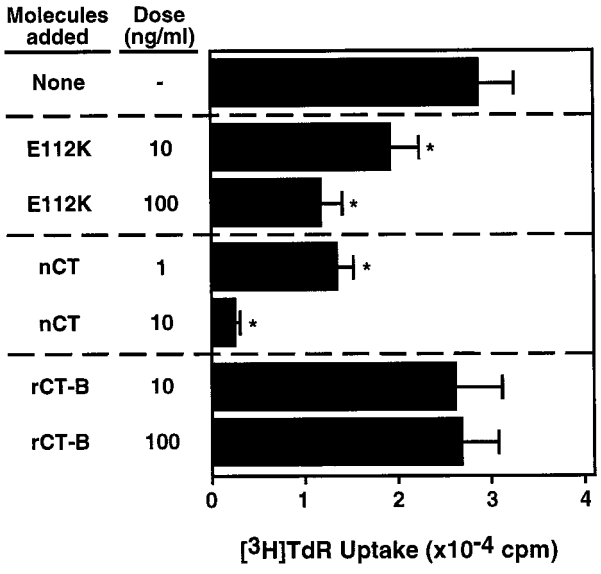

FIGURE 3. The effect of mCT E112K on proliferative responses of Peyer's patch $CD4^+$ T cells activated via the TCR-CD3 complex. $CD4^+$ T cells (2×10^6/ml) isolated from Peyer's patches were incubated with mCT E112K, nCT, or rCT-B in the presence of 1 μg/ml of immobilized anti-CD3 mAb. The results were expressed as the mean ± SEM. The data were similar and are representative of three separate experiments. *, $p < 0.05$ when compared with cultures stimulated by anti-CD3 mAb alone.

inhibited Th1-type cytokine responses. As expected, comparable doses of rCT-B did not affect either IFN-γ or IL-4 synthesis (Fig. 4).

mCT and nCT differ in apoptotic signals for $CD4^+$ T cells

Our results showed that both mCT E112K and nCT inhibited proliferation and cytokine synthesis of $CD4^+$ T cells activated via the TCR-CD3 complex. To determine whether these inhibitory effects were due to apoptosis, we quantitatively analyzed apoptosis by assessing the emergence of hypodiploid DNA (sub G_0/G_1 phase). Addition of 1 ng/ml of nCT to $CD4^+$ T cells activated via the TCR-CD3 complex resulted in increased apoptosis. Further, the frequency of apoptotic cells was increased when cultures were incubated with 10 ng/ml of nCT (Fig. 5). In contrast, doses as high as 100 ng/ml of mCT E112K failed to induce apoptosis. As expected, rCT-B did not induce apoptosis in $CD4^+$ T cells (Fig. 5). These results indicate that while the inhibitory effect of nCT could be explained by induction of apoptosis in $CD4^+$ T cells, mCT E112K inhibits Th1-type cytokines via other mechanisms.

Discussion

Previous studies have shown that nontoxic mCT S61F is an effective adjuvant and, when administered nasally with Ag, it induces $CD4^+$ T cells to produce Th2-type cytokines that enhance S-IgA Ab responses (5, 12, 13). Further, our most recent study has provided evidence that oral mCT E112K also acts as an effective mucosal adjuvant by inducing $CD4^+$ Th2 cells.[4] However, the mechanisms by which mCT acts as adjuvant for induction of $CD4^+$ Th2 cells in mucosal tissues and regulation of S-IgA Ab responses are unknown. The current study considered two distinct mechanisms to explain the adjuvant effects of mCT E112K. In the first, mCT E112K induced mainly B7-2 (CD86) expression on APCs, which subsequently enhanced proliferative responses in Peyer's patch $CD4^+$ T cells activated via the TCR-CD3 complex. In the second, mCT E112K directly induced Peyer's patch $CD4^+$ T cells to preferentially down-regulate Th1-type cytokine production.

B7-1 and B7-2 have been shown to be essential costimulatory molecules for initial activation of $CD4^+$ T cells (14, 16–20). A previous study had suggested that a fusion protein consisting of intact CT-A subunit attached to Ig-binding domains of Staphylococcal protein A enhanced B cell expression of both B7-1 and B7-2, while rCT-B was without effect (21). In the present study, our results also showed that mCT E112K, as well as nCT, enhanced these costimulatory molecules on Peyer's patch B cells and macrophages. Further, both E112K- and nCT-treated APCs enhanced proliferative responses in $CD4^+$ T cells when stimulated by anti-CD3 mAb. These results imply that mCT E112K and nCT induce B7-1 and B7-2 expression on APCs that, in turn, lead to costimulation of $CD4^+$ T cells activated via the TCR-CD3 complex. In support of this hypothesis, enhanced proliferative responses were blocked by anti-B7-2 mAb, indicating that B7-2 expression induced by mCT E112K or nCT was the major costimulatory effect for $CD4^+$ T cell responses. On the other hand, anti-B7-1 treatment only slightly inhibited the costimulatory effect. A plausible explanation for these differences would be that since the percent increase in B7-1 induced by nCT or its nontoxic derivative was relatively low, the costimulatory effect of B7-1 was below the threshold required to support T cell responses. In this regard, it was shown that, although CT-A with Ig-binding domains of Staphylococcal protein A, as well as CT, enhance both B7-1 and

[4] M. Yamamoto, S. Yamamoto, M. Ohmura, M. Yamamoto, M. Kweon, J. L. VanCott, K. Fujihashi, M. Noda, Y. Takeda, H. Kiyono, and J. R. McGhee. A nontoxic mutant of cholera toxin enhances immunity to oral vaccines in mice. *Submitted for publication.*

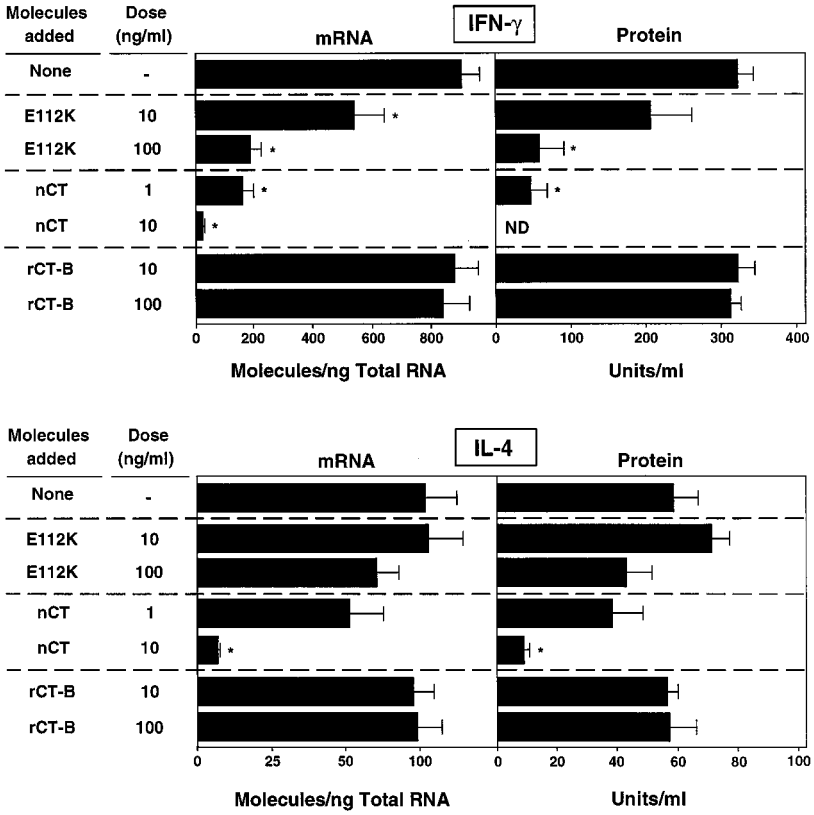

FIGURE 4. The effect of mCT E112K on cytokine synthesis by Peyer's patch $CD4^+$ T cells activated via the TCR-CD3 complex. Peyer's patch $CD4^+$ T cells were incubated with mCT E112K, nCT, or rCT-B, as described in Fig. 3. The number of molecules of Th1- and Th2-type cytokine-specific mRNA was determined by quantitative RT-PCR. After 72 h of incubation, culture supernatants were harvested and analyzed for Th1- (IFN-γ) or Th2-type (IL-4) cytokines by ELISA. The results were expressed as the mean ± SEM. IL-4 was not detected in cells incubated in medium only at both mRNA and protein levels. The mRNA and protein levels for IFN-γ in medium only were 24.5 ± 4.6 and 12.6 ± 3.1, respectively. ∗, $p < 0.05$ when compared with cultures stimulated by anti-CD3 mAb alone. ND, not detected.

B7-2 on B cells, the extent of increase in the former molecule was much smaller than the latter one (21). Other studies have shown that CT increases costimulatory activity of bone marrow macrophages for anti-CD3-stimulated and allostimulated T cells, an increase that was blocked by anti-B7-2 but not anti-B7-1 mAb (22). These studies, together with our results, suggest that mCT E112K and nCT both induce mainly B7-2 expression on APCs for subsequent costimulatory signaling to $CD4^+$ T cells and, thus, partially explain why both molecules are effective adjuvants.

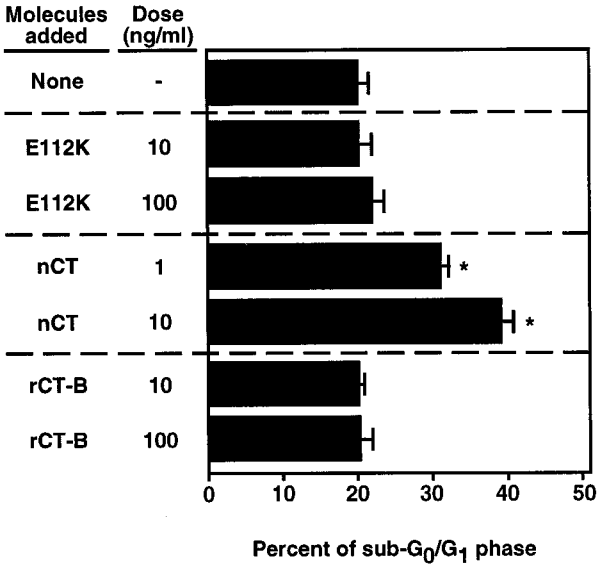

FIGURE 5. The effect of mCT E112K on apoptosis in Peyer's patch $CD4^+$ T cell cultures. Peyer's patch $CD4^+$ T cells were incubated with mCT E112K, nCT, or rCT-B, as described in Fig. 3. The DNA content was analyzed by propidium iodide staining. The results were expressed as the mean ± SEM. The data were similar and are representative of four separate experiments. ∗, $p < 0.05$ when compared with cultures stimulated by anti-CD3 mAb alone.

Past studies have suggested that intact CT-A with ADP-ribosyltransferase enzyme activity is required for B7 expression and adjuvant-induced immune responses (21, 22). However, the results presented here clearly showed that mCT E112K, which lacks ADP-ribosyltransferase activity, also enhanced B7-2 expression on B cells and macrophages that, in turn, led to enhanced proliferative responses by $CD4^+$ T cells activated via the TCR-CD3 complex. These results provide direct evidence that ADP-ribosyltransferase activity is not required for the induction of costimulatory activity in APCs and that mCT E112K retains full adjuvanticity. In fact, our recent results have shown that parenteral (S61F and E112K) or nasal (S61F) administration of mCT induces $CD4^+$ Th2-type cytokines with subsequent IgG1, IgE, and IgA Ab responses specific for the coadministered Ag (5, 12). Collectively, our results suggest that ADP-ribosyltransferase activity is not required for induction of B7 costimulatory molecules and, thus, for the adjuvant effects of CT.

Our results showed that mCT E112K, as well as nCT, induced mainly B7-2, and the mAb anti-B7-2-blocking experiment suggested that this is a major pathway for enhancing $CD4^+$ T cell responses. In this regard, it has been shown that IL-4 produced by T cells is dependent upon B7-2, but not B7-1, costimulation (17, 29, 30). Since CT acts as adjuvant by inducing $CD4^+$ Th2 cells secreting IL-4, IL-5, IL-6, and IL-10, which effectively provide help for Ag-specific S-IgA and serum IgG1, IgE, and IgA Ab responses (2, 3), it is possible that B7-2 induced by CT may contribute to the induction of Th2-type cytokine responses. In support of this, administration of anti-B7-2 mAb inhibited serum IgG and mucosal IgA anti-KLH Ab responses in mice given oral KLH plus CT as adjuvant (22). Thus, Th2 responses induced by CT may be in part mediated through B7-2 costimulatory signals.

rCT-B failed to enhance expression of either B7-1 or B7-2 on B cells or macrophages. It has been shown that rCT-B enhanced presentation of soluble peptide by peritoneal macrophages in vitro (31). Further, both CT- and rCT-B-pretreated B cell hybridomas enhanced IL-4 production by Th2 cell lines after stimulation with either Ag or anti-CD3 mAb (32), suggesting that CT-B may have some adjuvant properties. However, rCT-B does not appear to be as effective as an adjuvant for induction of immune responses (5, 6, 12). Thus, we would conclude that CT-B mainly acts as a targeting protein for GM1 ganglioside, and, following binding of CT, the CT-A component actually enhances maximum B7-2 expression and thus accounts for the adjuvant properties. In support of this, pretreatment of mCT E112K, nCT, or rCT-B with GM1 blocked B7-1 and B7-2 expression on both B cells and macrophages, indicating that initial binding of CT-B to APCs via GM1 was necessary for induction of B7-1 and B7-2 costimulatory molecules.

In a second line of investigation, it was shown that nCT directly affects Peyer's patch $CD4^+$ T cells activated via the TCR-CD3 complex and inhibited proliferative responses. These results are consistent with past studies showing that CT reduces T cell activation in vitro (33, 34). Analysis of cytokine responses showed that a lower dose of nCT (1 ng/ml) preferentially inhibited Th1-type cytokine (e.g., IFN-γ) responses. On the other hand, a higher dose of nCT (10 ng/ml) inhibited essentially both IL-4 and IFN-γ responses. In this regard, a previous study had shown that CT inhibits proliferative responses of Th1 clones, but had no effect on Th2 cell clones activated by TCR-mediated signaling (35). In that study, it was concluded that Th1 and Th2 cells differ in their sensitivity to an increase in cAMP, and Th1 cells are more susceptible to cAMP-mediated inhibition. Thus, nCT induces cAMP accumulation, which, in turn, leads to a preferential inhibition of Th1-type cells. Further, it is possible that since cAMP accumulation induced by a higher dose of nCT is above the threshold of sensitivity of Th1- and Th2-type cells, the responses in both Th subsets would be inhibited by nCT. Taken together, these results suggest that nCT preferentially inhibits Th1-type cytokine responses via a cAMP-mediated pathway.

Of interest was the finding that mCT E112K, which lacks ADP-ribosyltransferase activity, also preferentially down-regulated Th1-type cytokine responses. These results imply that E112K may have an alternative mechanism for inhibition of Th1 responses in the absence of ADP-ribosyltransferase activity. To further elucidate the mechanisms for the inhibitory effects of mCT E112K and nCT on $CD4^+$ T cells, we quantitatively analyzed apoptosis. Our results showed that while nCT induced apoptosis in a dose-dependent fashion, the proportion of apoptotic cells did not increase in $CD4^+$ T cells treated with mCT E112K, suggesting that induction of apoptosis by nCT, but not mCT E112K, is probably mediated through increased levels of intracellular cAMP. In this regard, past studies have shown that agents that elevate cAMP stimulate DNA fragmentation and apoptosis in thymocytes (36). However, other studies have reported that cAMP can block apoptosis in other model systems (37, 38). Alternatively, protein kinase C (PKC) antagonists have been shown to induce apoptosis (39). Furthermore, it has been hypothesized that Th1, but not Th2, cells are activated through a PKC-dependent pathway when activated via the TCR-CD3 complex and that the elevated levels of cAMP block the activation of PKC (35). These studies, together with our results, suggest that nCT preferentially induces apoptosis in Th1-type cells via a cAMP pathway. Therefore, mCT E112K, which lacks ADP-ribosyltransferase activity, failed to induce apoptosis in T cells. However, this mutant molecule still inhibited cytokine synthesis by Th1 cells. In this regard, a recent study has shown that CT suppresses production of IL-12 by human monocytes and expression of the IL-12 receptor on T cells (40). In that study, the authors suggest that CT-A-dependent, but not cAMP-dependent, processes could be responsible for the suppression of IL-12 responses. Thus, we would conclude that CT has at least two pathways for selective inhibition of Th1-type responses and that mCT E112K preferentially down-regulates Th1-type responses in the absence of ADP-ribosyltransferase activity, i.e., by an as yet to be characterized cAMP-independent pathway. In this regard, rCT-B failed to down-regulate proliferative responses or to affect cytokine synthesis. Earlier studies have shown that CT-B inhibits T cell-proliferative responses (33, 34). However, the CT-B used in those studies was purified from CT holotoxin and not from recombinant expression. Thus, it is likely that holotoxin contaminated those preparations. In support of this, rCT-B was not inhibitory below 0.1–1 μg/ml, while nCT was inhibitory at doses of 0.1 ng/ml (33). Since rCT-B does not possess the A subunit that is responsible for ADP-ribosylation with subsequent elevation of cAMP levels, the results from studies with rCT-B imply that ADP-ribosyltransferase activity is required for the effects of CT on T cells. However, studies using mCT E112K clearly showed that ADP-ribosyltransferase activity in the CT-A subunit was not necessary for preferential down-regulation of Th1 cells. Thus, CT-A must elicit inhibitory effects on Th1-type cells through different mechanisms, and current studies are focused on elucidating the cAMP-independent inhibitory pathway that emerged from the present work.

In summary, our study has demonstrated that mCT E112K, as well as nCT, enhances mainly B7-2 expression by B cells and macrophages that exert a costimulatory effect on $CD4^+$ T cells. We have further shown that mCT E112K, like nCT, directly affects Peyer's patch $CD4^+$ T cells activated via the TCR-CD3 receptor complex and preferentially inhibits Th1-type cytokine responses. The B subunit of CT acts as a carrier protein to insert CT-A into the cells, but alone did not enhance B7 expression. Thus, from the results presented here, we conclude that mCT E112K elicits adjuvant responses via B7-2 cosignaling and by preferential down-regulation of Th1-type cells. Interestingly, nCT also acts as adjuvant by B7-2 cosignaling and by down-regulation of Th1-type cells; however, this latter effect is explained, in part, by cAMP-mediated apoptosis, a pathway that does not occur when mCT E112K is used as adjuvant.

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