A two-codon mutant of cholera toxin lacking ADP-ribosylating activity functions as an effective adjuvant for eliciting mucosal and systemic cellular immune responses to peptide antigens

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Abstract

Vaccination with peptide antigens is an effective strategy against mucosal viral infections. We tested a two-codon mutant of cholera toxin (CT-2*) lacking ADP-ribosylating activity and toxicity as a mucosal adjuvant for T cell epitope peptides for intranasal immunization of mice. Efficient induction of helper and cytotoxic T lymphocyte responses associated with TH1 cytokine production were observed in the systemic and mucosal compartments including nasal, gut, and vaginal associated lymphoid tissues. Single or multiple dosing with the peptide antigen and CT-2* induced strong memory immunity without tolerance. These results demonstrate CT-2* as a suitable mucosal adjuvant for priming antigen-specific cellular immune responses.

Keywords: Intranasal immunization; Mucosal tissues; Native and mutant cholera toxin; Cellular immune responses

1. Introduction

Vaccination against intracellular pathogens like viruses requires induction of strong antigen-specific cellular immune responses mediated by helper T cells and cytotoxic T lymphocytes (TH and CTL, respectively) for protection. Since T cell immunity depends on the recognition of antigenic peptides on infected cells, immunization with viral peptides could offer a safer and economical strategy for vaccination against viruses. Many viruses infect the host by attacking the mucosal membranes. Therefore, vaccination strategies directly targeting the mucosal surfaces would be beneficial for protection against viral infections. The major concerns for inducing the immune response through the mucosal route include poor availability and/or absorption of the antigen at the mucosal sites, and in some cases the development of tolerance in the host. Therefore, it is important to use adjuvants compatible with the mucosal surfaces for enhancing the immunogenicity of the co-administered antigens [1,2]. A number of bacterial toxins have been tested as mucosal adjuvants [3,4], and prominent among them have been cholera toxin (CT), an enterotoxin produced by Vibrio cholerae [4,5], and the heat labile enterotoxin (LT) derived from Escherichia coli [6,7]. Even though potential localization of these toxin adjuvants in the central nervous system was shown not to cause any pathological changes in brain tissue after nasal administration [8,9], these native toxins are not suitable for human use because of their role in inducing secretory diarrhea [10]. Therefore, alternative approaches have been pursued to develop genetically modified mutant toxins that completely lack toxicity but retain adjuvanticity [11]. Here, we tested the mucosal adjuvanticity of CT-2*, a cholera toxin mutant derived by introducing two-codon

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substitutions (Arg7-Lys and Glu112-Gln) into the CT-A subunit [12]. Compared to native CT, the CT-2* lost its ability to ADP-ribosylate the regulatory protein (G_s/H9251) and failed to induce any detectable fluid secretion in the rabbit ligated ileal loops [12,13]. We obtained data demonstrating that CT-2* is an efficient mucosal adjuvant for priming antigen-specific cellular immune responses at the mucosal and systemic compartments to peptide antigens administered by the intranasal route.

2. Materials and methods

2.1. Animals

Female Balb/c (H-2^d) and B6C3F1 (H-2^kxb) mice of 6–8 weeks age were purchased from Charles River Laboratories (Wilmington, MA). All procedures with the animals were performed in accordance with institutionally approved protocols.

2.2. Cell lines and cell cultures

The cell lines, P815 (DBA/2, H-2^d, Mastocytoma), and EL-4 (C57BL/6, H-2^b, Thymoma), were maintained in DMEM and RPMI, respectively. Both the media were supplemented with 10% heat inactivated FBS, 50 U/ml of penicillin, 50 μg/ml streptomycin and 50 μg/ml gentamicin.

2.3. Peptides

Synthetic peptides corresponding to known TH and CTL epitopes [4,14–19] were used in the study for priming immune responses in association with CT-2* for intranasal immunization of mice. Amino acid sequences and the origin of various peptides employed in different experiments are listed in Table 1. All the peptides were synthesized by the solid-phase method of Merrifield [20] either on a modified Vega250 automatic peptide synthesizer (Vega Biochemicals, Tucson, Arizona) or by the ‘Bag’ method as described by Houghten [21]. The peptides were >90% pure as determined by high-pressure liquid chromatography (HPLC) and mass-spectrometry analyses.

2.4. Cholera toxin (CT) and mutant cholera toxin (CT-2*)

Native CT was purchased from List Biological Laboratory (Campbell, CA). The CT-2* protein from the culture filtrates of a vaccine strain of V. cholerae was purified to homogeneity by sodium hexametaphosphate precipitation, affinity purification on a galactose column, and Sephadex G75 gel filtration chromatography [13,22–24]. The purified toxin was dissolved in pyrogen-free water. The Limulus amebocyte lysate assay (QCL-1000 kit, BioWhitaker, Walkerville, MD) was used to determine the level of lipopolysaccharide (LPS) in the purified CT-2*. The amount of LPS detected in 1 μg of the CT-2* injected into mice was 0.5 pg, which did not stimulate production of any cytokines in the mouse ligated ileal loops.

2.5. Immunizations

Mice were immunized by the intranasal route once or twice at 5-day intervals with the test peptide (100 μg/mouse) along with or without CT-2* (1 μg/mouse) in phosphate buffered saline (PBS). Native CT (1 μg/mouse) was used as a positive control adjuvant for comparative analysis in some experiments because it was shown to be effective as an adjuvant for priming peptide-specific CTL responses by the intranasal route [25,26]. Mice were anaesthetized by intra peritoneal injection with ketamine–xylazine mixture, and 10 μl of a mixture containing the antigen and the adjuvant in PBS was introduced into each nostril. Five days after the last immunization cell suspensions were prepared from the nasal associated lymphoid tissue (NALT), cervical lymphnodes (CLN), mesentric lymphnodes (MLN), Peyer’s patches (PP), vaginal associated lymphoid tissue (VALT), and spleen by homogenization [27] or by enzymatic dissociation using collagenase type IV (Sigma).

2.6. CTL assay

The CTL assay was carried out as described previously [14] using cells isolated from the various organs after restimulation for five days with the cognate peptide used for immunizing the mice. The effector cells from Balb/c and B6C3F1

Table 1 Synthetic peptides employed as antigens and/or immunogens for determining the cellular immune responses

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Region</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>R15K</td>
<td>V3 loop of gp 120 of HIV1 IIIB</td>
<td>RQGPRGRAFVTIGK</td>
<td>[14]</td>
</tr>
<tr>
<td>Ova 237-264</td>
<td>Chicken ovalbumin</td>
<td>SINEFELK</td>
<td>[18]</td>
</tr>
<tr>
<td>R10I</td>
<td>V3 loop of gp 120 of HIV1 IIIB</td>
<td>RQGPRGRAVTI</td>
<td>[18]</td>
</tr>
<tr>
<td>NP147-158 (R-)</td>
<td>Nucleoprotein of influenza virus</td>
<td>TYQRTAAYTV</td>
<td>[17]</td>
</tr>
<tr>
<td>64</td>
<td>Fifth conserved region in gp 160 of HIV 1</td>
<td>FLGGFAAAGTMAASLTVQARC</td>
<td>[18]</td>
</tr>
<tr>
<td>104</td>
<td>First conserved region in gp 160 of HIV 1</td>
<td>VVYGVVPWKEA</td>
<td>[19]</td>
</tr>
<tr>
<td>111</td>
<td>First conserved region in gp 160 of HIV 1</td>
<td>LWQQLKPCVKLT</td>
<td>[18]</td>
</tr>
</tbody>
</table>
mice were tested for cytolytic activity against 51Cr-labeled P815 and EL4 target cells, respectively, after pre-incubation with the cognate peptide. The percentage (%) of specific lysis was calculated by the following formula: % specific lysis = (experimental release − spontaneous release)/maximum release − spontaneous release) × 100, where spontaneous release represents the radioactivity obtained when the target cells were incubated in culture medium without effectors and maximum release represents the radioactivity obtained when the target cells were lysed with 1% Triton X-100.

2.10. Proliferation assay

Proliferative responses of cells isolated from spleen and cervical lymph nodes (CLN) of immunized mice were determined using the standard 3H thymidine incorporation assay as described earlier [18]. Briefly, a 100 μl aliquot of the cell suspension (1 × 10^6 cells in complete RPMI) was dispensed into each well of a round-bottom 96 well microtitre plate and incubated with the peptide antigen (2 μg/well). In all experiments, an unrelated TH epitope peptide derived from the human papillomavirus (HPV) was used as a negative control (2 μg/well), while Con A (1 μg/well) served as positive control. All treatments were done in triplicate wells. The cultures were incubated at 37 °C in 5% CO₂ for 96 h and during the final 16–18 h 1 μCi of [3H] thymidine was added to each well. The proliferative response was calculated as fold increase in radioactivity with the test antigen over that for cells cultured in the medium alone, and is represented as stimulation index (SI). The responses to peptides are considered positive when the SI values are ≥2.0, and also ≥2.0 above the values for negative control peptide used in the same experiment.

2.11. Measurement of IFN-γ production by ELISPOT assay

Cells isolated from NALT, PP, MLN, VALT, and CLN, and spleens of immunized mice were subjected to ELISPOT analysis for determining the antigen specific IFN-γ producing cells as reported earlier [30] using antibodies purchased from Pharmingen. The spots, representing individual IFN-γ-producing cells as spot forming cells (SFC), on the membranes were enumerated by an independent agency (Zellnet Consulting Inc., New York, NY) using the KS-ELISPOT automatic system (Carl Zeiss Inc., Thornwood, NY). Responses were considered positive as per the criterion described in the literature [31], which is a minimum of 5 SFC/well and at least double the number that obtained in cells cultured with the negative control peptide.

2.12. Cytokine analysis by cytokine bead array (CBA)

Aliquots (1 × 10^6/ml) of cells from NALT, MLN, PP, CLN, and spleen of mice immunized with peptide antigen and CT2+ were cultured with the peptide antigen (10 μg/ml) for 36–48 h at 37 °C, and the culture supernatants were analyzed for TNF-α, IFN-γ, and IL-2 (TH-1 cytokines) or IL-4 and IL-5 (TH-2 cytokines) by the cytoketric bead array (CBA) kit (BD Biosciences, San Diego, CA) according to manufacturer’s instructions. Peptide-specific cytokine production was measured as the cytokine concentration
in culture supernatant of cells stimulated with the peptide minus that without the peptide.

2.13. Statistical analysis

The data were analyzed for statistical significance using the student’s t-test for paired samples, and a P-value of < 0.05 was considered significant.

3. Results

3.1. Mutant cholera toxin, CT-2* is an effective adjuvant for priming antigen specific CTL responses by intranasal administration of peptide antigens

The ability of CT-2* as a mucosal adjuvant was evaluated in comparison to native CT for the induction of antigen specific CTL responses in Balb/c mice immunized by the intranasal route with R15K, an H-2d-restricted HIV CTL epitope peptide. Strong induction of peptide-specific CTL response was observed in the draining cervical lymph nodes (CLN) and spleens of mice receiving either CT-2* or CT as adjuvant (Fig. 1A and B, respectively). The specific lysis values obtained at an E:T ratio of 100:1 were 36 and 34% in the CLN, and 72 and 44.5% in the spleen with CT-2* and CT, respectively indicating that CT-2* is similar or slightly better compared to CT as a mucosal adjuvant for priming antigen-specific CTL responses. Control experiments involving intranasal administration of either the peptide alone or the adjuvant (CT or CT-2*) by itself to different groups of mice did not elicit peptide-specific CTL responses (the percent specific lysis values ranged between 0 and 5).

Similar to results obtained with the HIV peptide R15K, potent antigen-specific CTL responses were also observed in B6C3F1 mice immunized with CT-2* and peptides Ova 257-264 or NP 321-336, corresponding to H-2b-restricted CTL epitopes in the ovalbumin and the nucleoprotein of sendai virus, respectively (Fig. 1C). Together, these results demonstrate the effectiveness of CT-2* as a potent mucosal adjuvant for inducing antigen-specific CTL responses with a variety of peptide antigens representing CTL epitopes in the context of different MHC haplotypes.

3.2. CTL response induced with CT-2* as adjuvant is mediated by CD8+ T cells

In order to determine whether the observed CTL activity generated after intranasal immunization with the peptide antigen and CT-2* was mediated by CD8+ T cells, splenocytes from immunized mice were subjected to positive selection using the Dynal beads. The isolated CD8+ T cells, along with the CD8-depleted population and the unseparated splenocytes were assayed for antigen-specific CTL activity against P815 target cells pulsed with the cognate peptide (Fig. 2A).

While CD8-depleted fraction showed only background lysis, the purified CD8+ T cells showed strong antigen-specific CTL activity (48% specific lysis at an E:T ratio of 20:1), that was comparable to unseparated splenocyte population (45% specific lysis at an E:T ratio of 100:1). Further, flowcytometric analysis of splenocytes isolated from Balb/c mice immunized with R15K and CT-2* revealed higher levels of intracellular IFN-γ production by the CD8+ T cells in response to stimulation with the peptide as compared to non-stimulated cells (Fig. 2B). These results indicate the CD8+ phenotype of the CTL generated by intranasal immunization with the peptide antigen using CT-2* as the mucosal adjuvant.

3.3. CTL generated using CT-2* as adjuvant exhibit antigen and MHC specificity

Splenocytes isolated from Balb/c mice immunized with R15K and CT-2* by the intranasal route were analyzed...
Fig. 2. (A) The CTL response induced by intranasal immunization of mice with R15K along with CT-2* is mediated by CD8+ T cells. Balb/c mice were immunized on days 0 and 5 with the HIV CTL epitope peptide R15K (100 μg) and CT-2* (1 μg), and were sacrificed 5 days after the last immunization. Splenocytes isolated from mice were tested either directly or separated into CD8+ and CD8− population using magnetic beads and tested for CTL activity by the standard chromium release assay. Target cells (P815) were pulsed with R15K and the specific lysis values shown were adjusted by subtracting lysis values with P815 cells incubated in medium alone; (B) the CD8+ cells from mice immunized with the CTL epitope peptide and CT-2* exhibit antigen-specific intracellular IFN-γ production. Balb/c mice were immunized on days 0 and 5 with the HIV CTL epitope peptide R15K (100 μg) and CT-2* (1 μg), and were sacrificed 5 days after the last immunization. Splenocytes isolated from mice were stimulated with the cognate peptide, Con A (positive control) or medium alone (negative control) for 6 h in the presence of brefeldin A. The IFN-γ produced was measured in the CD8+ lymphocytes by flow cytometry as described in Section 2.

for CTL activity against MHC-matched (H-2d) and MHC-mismatched (H-2b) target cells that were pulsed with either the cognate peptide or an irrelevant peptide (Fig. 3). Strong CTL activity was observed against the MHC-matched P815 cells pulsed with the cognate peptide R15K, but not the control peptide NP 147-158 (R) from the influenza nucleoprotein, even though this control peptide is also a known H2d-restricted CTL epitope. Also, we did not observe lysis of MHC mismatched EL-4 cells (H-2b) pulsed with the R15K peptide at any E:T ratios tested. Splenocytes from mice immunized with R15K + CT-2* exhibited CTL activity against P815 cells pulsed with R10I, a smaller peptide within R15K sequence that is known to be the optimal H2d-restricted CTL epitope sequence [18]. In repeat experiments, we did not see a significant difference between these two peptides with respect to specific lysis by CTL from mice immunized with the R15K peptide. Importantly, we observed CTL activity against target cells infected with recombinant vaccinia virus for expressing the HIV-envelope protein gp160 (vPE16), but not a control vaccinia virus (vSC8) demonstrating that the CTL were able to recognize and lyse target cells that processed the antigenic protein and presented the proper epitope in the context of the appropriate MHC allele (Fig. 3). These results were similar to those we reported earlier with cells from mice immunized in the footpad with the R15K peptide emulsified in complete Freund’s adjuvant [14]. Together, these results demonstrated that mice immunized with peptide antigens along with CT-2* by the intranasal route generated CTL with MHC and
Fig. 3. CTL responses induced by intranasal immunization of mice with a peptide antigen along with CT-2* as mucosal adjuvant are antigen-specific, and MHC-restricted. Balb/c mice were immunized on days 0 and 5 with the HBV CTL epitope peptide R15K (100 μg) and CT-2* (1 μg), and were sacrificed 5 days after the last immunization. Splenocytes isolated from the mice were restimulated with the cognate peptide for 5 days. The CTL activity was assayed against target cells (P815) that were either pulsed with the cognate peptide (R15K), a peptide of 10 amino acids in length that corresponds to the optimal epitope (R10I) or a control unrelated peptide (NP 147-158 (R-)) corresponding to the nucleoprotein of influenza virus. The CTL activity of the restimulated splenocytes was also assayed against target cells infected with control (vSC8) or recombinant vaccinia virus for the expression of HIV envelope protein gp160 (vPE16). Additional controls included P815 cells pulsed with CT-2* (1 μg) and EL4 cells pulsed with the R15K peptide (MHC-mismatched target cells). The values shown were obtained after subtracting the background lysis of control target cells (P815/EL4) from that of peptide-pulsed target cells. The data presented is the mean value obtained from three different experiments.

antigen specificity, and also relevant for vaccination because they recognized target cells expressing naturally processed epitope from cognate antigen.

3.4. CT-2* is a potent mucosal adjuvant at very low concentration and a single immunization is effective in inducing antigen-specific CTL responses

To determine the lowest concentration of CT-2* effective as an adjuvant for eliciting antigen-specific CTL response, groups of Balb/c mice were immunized by the intranasal route with the R15K peptide using one of four different concentrations of CT-2* (50, 100, 500 or 1000 ng). Strong CTL activity (47% specific lysis at an E:T ratio of 100:1) was observed in the splenocytes from mice immunized with the R15K peptide in the presence of 50 ng, the lowest concentration of CT-2* used (Fig. 4A). This level of CTL activity was similar to that observed at the 100 and 500 ng of CT-2*, and was about 65% of that with 1000 ng of CT-2*. Thus, despite lowering the dose of CT-2* from 1000 to 50 ng (95% reduction) strong CTL activity was observed, demonstrating that CT-2* is a potent adjuvant at very low concentrations for priming antigen-specific CTL responses by intranasal immunization.

To further realize the potency of CT-2* as an adjuvant, we analyzed for antigen-specific CTL responses in mice after a single intranasal immunization. As shown in Fig. 4B, both CLN and spleen cells exhibited strong peptide-specific CTL activity (45 and 81% specific lysis, respectively at an E:T ratio of 100:1). This level of CTL activity was comparable to that seen in mice after two or three immunizations (Fig. 1 and data not shown). Antigen-specific CTL activity was not generated in mice immunized either once or up to three times with the R15K peptide in the absence of CT-2* (the percent specific lysis values ranged between 0 and 5). These results demonstrated that a single immunization of mice with the peptide antigen in the presence of CT-2* as an adjuvant was sufficient to generate antigen-specific CTL responses in both the local and systemic compartments (CLN and spleen, respectively).
Repeated intranasal immunizations using peptide antigen along with CT-2* as mucosal adjuvant prime efficient CTL response without tolerance induction. Balb/c mice were immunized on days 0 and 5 with the HIV CTL epitope peptide R15K (100 µg) and CT-2* (1 µg), and CLNs and splenocytes were isolated either 40 days (A) after the last immunization or boosted twice (day 40 and 45) after the last immunization (B) were assayed for memory CTL activity. Target cells (P815) were pulsed with R15K and the specific lysis values shown were adjusted by subtracting lysis values with P815 cells incubated in medium alone. The data presented is the mean value obtained from three different experiments.

We also tested whether repeated dosing, sometimes required for sustained immunity, would be a potential problem for mucosal immunization with the antigen and adjuvant in terms of inducing tolerance to the antigen. Antigen-specific CTL responses were determined in spleens of mice immunized with the peptide antigen (R15K) along with CT-2* by the intranasal route twice at 0 and 5 days followed by two additional doses at days 40 and 45. Strong antigen-specific memory CTL activity was observed in the spleens, but not CLN of mice prior to boosting at day 40 (Fig. 5A). However, after the two booster immunizations, strong peptide-specific CTL responses were observed in both the spleen and CLN from all five mice tested (Fig. 5B). These results suggest that multiple dosing with the peptide antigen and the mucosal adjuvant CT-2* do not induce tolerance.

CT-2* is an effective adjuvant for priming antigen-specific T helper (TH) responses after intranasal immunization

We tested whether in addition to antigen-specific CTL responses, CT-2* would also be effective as a mucosal adjuvant for eliciting antigen-specific TH responses to peptide antigens administered by the intranasal route. Cells isolated from spleen and CLN of mice immunized with CT-2* as adjuvant along with one of three separate peptides representing TH epitopes from the HIV envelope protein were tested for peptide-specific proliferative responses (Fig. 6). Significant levels of proliferation responses specific to each of the three peptides were observed in both spleen and CLN cells from multiple mice tested. On the other hand, cells from mice immunized with the peptide in the absence of CT-2* showed no specific proliferative responses (the SI values ranged between 1 and 1.5). These results together with those showing efficient CTL responses in mice immunized with CTL epitope peptides and CT-2* demonstrate the efficiency of CT-2* to serve as a mucosal adjuvant for priming antigen-specific cellular immune responses.

CT-2* is efficient in priming strong antigen-specific cellular immune responses in several mucosal compartments after intranasal immunization with peptide antigens

Mice immunized by the intranasal route with CTL and TH epitope peptides using CT-2* as an adjuvant were evaluated for cellular immune responses in terms of IFN-γ production in response to peptide-stimulation by cells isolated from various mucosal tissues and lymph nodes (Fig. 7). In case of mice immunized with the CTL epitope peptide R15K, strong immune responses scored as IFN-γ spot forming cells (SFC) were observed with cells from CLN and spleen as well as nasal associated lymphoid tissue (NALT) that is proximal to the immunization site by the intranasal route and also distant sites like the gut- and vaginal-associated lymphoid tissues (GALT and VALT, respectively). Also, strong responses were observed with cells from the Peyer’s patches (PP). Control mice immunized with the R15K peptide alone...
Fig. 7. ELISPOT analysis of peptide-specific IFN-γ production by cells from mucosal and systemic compartments of mice immunized with a CTL epitope peptide R15K and CT-2*. Balb/c mice were immunized on days 0 and 5 with the HIV CTL epitope peptide R15K (100 ng) and CT-2* (1 µg), and were sacrificed 5 days after the last immunization. Cells isolated from NALT, PP, MLN, VALT, CLN, and spleen were stimulated with or without the cognate peptide, and analyzed for IFN-γ spot forming cells (SFC) as described in Section 2. The results shown were obtained after subtracting SFC with the cells incubated in medium alone and represented as IFN-γ SFC per 1 × 10^6 cells, and represent an average of three different experiments.

showed only background levels of responses (<10 IFN-γ SFC/10^6 cells). Further analyses, in terms of cytokine production in culture supernatants of NALT, PP, MLN, CLN, and spleen cells revealed high levels of TNF-α and IFN-γ (TH1-type cytokines), and low to undetectable levels of IL-5 and IL-4 (TH2-type cytokines) in all cases (Fig. 8A). Similarly, mice immunized by the intranasal route with a TH epitope and CT-2* showed production of IL-2, TNF-α, IFN-γ, and IL-5 but not IL-4 in NALT as well as spleen and CLN cells (Fig. 8B). These results strongly support CT-2* as a potent mucosal adjuvant for priming antigen-specific cellular immune responses in multiple mucosal compartments.

4. Discussion

The present investigation demonstrated the ability of CT-2*, a two-codon mutant of CT lacking ADP-ribosylating activity [12], as a mucosal adjuvant for a variety of peptide antigens to elicit T cell responses in terms of antigen-specific proliferation and cytotoxic activities (TH and CTL, respectively). Further, CT-2* induced TH1-type cytokine response in mucosal and systemic compartments but not tolerance after repeated dosing at shorter and longer time intervals.

In comparison to the earlier described mutants of CT and LT with single point mutations substituting either arginine at position 7 or glutamic acid at position 112, the CT-2* was derived with substitutions at both these positions, and CT-2* exhibited no enzymatic activity and toxicity [12,13]. While the genotypic and some phenotypic properties of CT-2* were described earlier, this study is the first to elaborate the potential of CT-2* as a mucosal adjuvant, particularly for synthetic peptide antigens representing known TH and CTL epitopes.

The LTK63 and CTK63 were among the widely characterized derivatives of LT and CT as adjuvants since both the mutant-toxins had no detectable enzymatic activity or toxicity [32–35]. While the CTK63 was poorly immunogenic, the LTK63 was shown to be capable of inducing antigen-specific CTL responses but was inferior to wild type LT [36]. Other non-toxic CT mutants like CT7K and CT112K with single amino acid substitutions were shown to be effective in inducing IgA and IgG antibody responses when used as adjuvants with nasal influenza vaccine [37]. However, to our knowledge none of the CT mutants described in the literature were effective as adjuvants for peptide antigens to prime specific cellular immune response in mice, particularly when used at concentrations less than 1 µg. Results from the present
investigation demonstrate CT-2* as an effective mucosal adjuvant for priming both CTL and TH responses to known CTL epitopes and TH epitopes from an HIV envelope peptide cocktail vaccine [38], respectively delivered by intranasal immunization. These results were comparable to those of others who reported that native CT and genetically modified CT induce proliferative responses against protein antigens [27,39]. Although numerous studies have described native CT as a potent inducer of TH2-type immune responses [27,40], our study, like only a few others in the literature [25,41], showed that both native CT and CT-2* are effective adjuvants for priming CTL responses, that are normally associated with strong TH1-type cytokine responses. CTL represent an important component of the cellular immune effector mechanisms for protection against viral agents such as HIV [42].

Our results also showed that CT-2* at concentrations as low as 50 ng was sufficient to induce antigen-specific CTL responses, suggesting high potency of CT-2* as a mucosal adjuvant for intranasal immunization in mice. These results were similar to those of others who reported that as little as 10 ng of native CT had measurable adjuvant activity when administered intranasally with HIV peptides for inducing CTL response [25].

The memory immune responses play a key role in defending the host from secondary infections. In this regard, we observed strong antigen-specific CTL responses in the spleen of mice 45 days after one or two immunizations by the intranasal route with the peptide antigen in the presence of CT-2*. These results were similar to those reported when CT was used as the mucosal adjuvant for intranasal immunization [25]. Furthermore, we observed that additional doses of the peptide antigen along with CT-2* administered by the intranasal route to the mice at 40–45 days post-primary immunization resulted in antigen-specific CTL responses in the draining cervical lymph nodes while maintaining high levels of CTL responses in the spleens of mice. The reasons for the lack of antigen-specific memory CTL in the regional lymph nodes (CLN) are not clear, though it is possible that memory CTL do not reside exclusively at local site (CLN) but can be rapidly generated/recruited to this site (CLN) following re-challenge with the antigen (boosting). Further investigations are required to test this possibility. Also, a single immunization by the intranasal route with a peptide antigen and CT-2* was sufficient to prime strong antigen-specific CTL responses. In contrast, others have previously reported that a single dose of OVA in the presence of CT or LT as adjuvant for intranasal immunization resulted in inconsistent OVA-specific CTL responses while consistent OVA-specific CTL responses were obtained only following two immunizations [4]. These results strongly indicate the potential utility of CT-2* as a mucosal adjuvant for priming strong cellular immune responses without inducing tolerance.

The respiratory, gastrointestinal, and vaginal mucosal surfaces play an important role in preventing and/or protecting from microbial infections [43]. Imbamba et al. [44] showed that nasal vaccination with SIV antigen and cholera toxin induced antigen specific IgA antibodies in the reproductive tissue in rhesus macaques. Intranasal immunization with ova protein and mutant CT (S61F) generated protein specific IgA antibody responses in nasal and vaginal washes [27]. However, our data represents the first report to show priming of cellular immune responses, in particular antigen-specific IFN-γ producing cells at nasal (NALT), gastrointestinal (MLN, PP), and vaginal (VALT) compartments after intranasal immunization with peptide antigen and CT-2*. Induction of cellular immune responses, especially at the reproductive sites by intranasal immunization with peptide antigens may be beneficial for protection against viral infections such as those caused by HIV. Further, TH1-type cytokines are essential for the induction of cellular immune responses [45]. Our results showed that CT-2* enhanced the induction of peptide-specific CTL responses that coincided more with TH1-type cytokines (IFN-γ and TNF-α) than TH2-type cytokines (IL-4 and IL-5) in both the mucosal and systemic compartments. However, further investigation is required to determine whether the nature of the antigen employed or the adjuvanticity of CT-2* is associated with preferential induction of TH1-type immune responses. Data from this investigation strongly indicate the potential utility of CT-2* as a mucosal adjuvant for peptide-based antigens and vaccines, and support further studies in primate models for determining the efficacy and safety of mucosal vaccination with this mutant toxin as adjuvant.

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enteroxin-based adjuvants elicits protective, immunopathogenic, and immunomodulatory effects.


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