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Enterotoxigenic *Escherichia coli* heat-stable toxin and heat-labile toxin toxoid fusion 3xSTa_{N12S}-dmLT induces neutralizing anti-STa antibodies in subcutaneously immunized mice

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*Corresponding author: Department of Diagnostic Medicine/Pathobiology, Kansas State University College of Veterinary Medicine, 1800 Denison Ave., Manhattan, KS 66506. Tel: +1-785-532-0831; Fax +1-785-532-4039; E-mail: wpzhang@vet.k-state.edu[†]Authors equally contributed to the study described in this paper.**One sentence summary:** Toxoid fusion antigen 3xSTa_{N12S}-dmLT of enterotoxigenic *Escherichia coli* is a desirable antigen for vaccines against children's diarrhea and travelers' diarrhea.**Editor:** Alejandra Bravo

ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) bacteria producing heat-stable toxin (STa) and/or heat-labile toxin (LT) are among top causes of children's diarrhea and travelers' diarrhea. Currently no vaccines are available for ETEC associated diarrhea. A major challenge in developing ETEC vaccines is the inability to stimulate protective antibodies against the key STa toxin that is potentially toxic and also poorly immunogenic. A recent study suggested toxoid fusion 3xSTa_{N12S}-dmLT, which consists of a monomer LT toxoid (LT_{R192G/L211A}) and three copies of STa toxoid STa_{N12S}, may represent an optimal immunogen inducing neutralizing antibodies against STa toxin [IAI 2014, 82(5):1823-32]. In this study, we immunized mice with this fusion protein following a different parenteral route and using different adjuvants to further characterize immunogenicity of this toxoid fusion. Data from this study showed that 3xSTa_{N12S}-dmLT toxoid fusion induced neutralizing anti-STa antibodies in the mice following subcutaneous immunization, as effectively as in the mice under intraperitoneal route. Data also indicated that double mutant LT (dmLT) can be an effective adjuvant for this toxoid fusion in mice subcutaneous immunization. Results from this study affirmed that toxoid fusion 3xSTa_{N12S}-dmLT induces neutralizing antibodies against STa toxin, suggesting this toxoid fusion is potentially a promising immunogen for ETEC vaccine development.

Keywords: diarrhea; ETEC (enterotoxigenic *E. coli*); toxoid fusion; neutralizing antibodies; vaccine

INTRODUCTION

Escherichia coli bacteria that produce heat-labile toxin (LT) and/or heat-stable toxin (STa), known as enterotoxigenic *E. coli* (ETEC), are the leading cause of moderate-to-severe diarrhea to children in developing countries and to children and adults from

developed countries traveling to ETEC endemic regions (Sanders et al. 2005; WHO 2006; Sack et al. 2007; Black et al. 2010; Kotloff et al. 2012). ETEC fimbrial or non-fimbrial adhesins and enterotoxins LT and STa are virulence determinants in diarrheal disease. Adhesins initiate adherence of ETEC bacteria to host cell receptors and promote bacteria colonization in small

Table 1. Doses of immunogen 3xSTa_{N12S}-dmLT and adjuvants used in mouse SC and IP immunization studies.

Routes Doses	SC	IP
Antigen	3xSTa _{N12S} -dmLT (40 µg in 50 µl)	3xSTa _{N12S} -dmLT (200 µg in 200 µl)
Adjuvant	(1) No adjuvant (2) ISA51 (50 µl) (3) dmLT (2 µg in 50 µl)	(1) FIA (200 µl) (2) dmLT (0.2 µg in 200 µl) (3) dmLT (2 µg in 200 µl)

intestines. Enterotoxins enter host small intestinal epithelial cells and enzymatically disrupt homeostasis, resulting in elevation of intracellular cyclic AMP (by LT) and cyclic GMP (by STa) levels, fluid and electrolyte hypersecretion and watery diarrhea (Nataro and Kaper 1998; Svennerholm and Tobias 2008). Vaccination is regarded the most effective and the most practical preventive approach against ETEC diarrhea (Svennerholm 2011; Zhang 2012). Unfortunately, there are no vaccines available for ETEC-associated diarrhea.

Developing effective ETEC vaccines is proven difficult. One major challenge is the inability to identify safe antigens to induce protective antibodies against the key STa toxin (Svennerholm 2011; Zhang 2012). As potent enterotoxins, both native STa and LT cannot be used directly as vaccine components. However, unlike LT, of which non-toxic LT B subunit (LT_B) or detoxified LT mutants can be used as safe antigens to elicit protective antibodies against LT, safe STa antigens to induce neutralizing antibodies against STa toxin had not been identified until very recently. In addition, the poorly immunogenic 19-amino acid STa toxin itself cannot induce host anti-STa antibody response. Recent studies demonstrated that STa with mutations of a single amino acid became less or not toxic (Zhang et al. 2010, 2013; Liu et al. 2011). These low or non-toxic STa molecules or toxoids were found safe STa antigens and also became immunogenic when they were genetically fused to a LT toxoid carrier, demonstrated by that derived STa-LT toxoid fusions induced antibodies neutralizing STa and LT toxins (Zhang et al. 2010, 2013; Liu et al. 2011). A more recent study revealed that LT-STa toxoid fusions possessing different STa toxoids exhibited anti-STa antigenicity variously. Among the LT-STa toxoid fusions we examined, 3xSTa_{N12S}-dmLT, a peptide consisting of three STa toxoid STa_{N12S}, one LT A subunit (LT_A) and one (not five) LT B subunit (LT_B), induced stronger neutralizing anti-STa antibodies in the intraperitoneally (IP) immunized mice (Ruan et al. 2014). This suggests that 3xSTa_{N12S}-dmLT may represent an optimal toxoid fusion for inducing neutralizing anti-STa antibodies and potentially an immunogen for ETEC vaccines.

To verify anti-STa antigenicity of this 3xSTa_{N12S}-dmLT toxoid fusion, we carried out mouse subcutaneous (SC) immunization and also mouse IP immunization with different adjuvant, and examined the antigen for induction of anti-STa antibody response. In addition, we evaluated antibodies derived from the SC and IP immunized mice for *in vitro* neutralization activity against STa toxin to assess potential of this toxoid fusion in ETEC vaccine development.

MATERIALS AND METHODS

Antigens and adjuvants used in mouse SC and IP immunization

Toxoid fusion 3xSTa_{N12S}-dmLT was purified from recombinant *Escherichia coli* strain 9331 (Ruan et al. 2014) as the immunogen.

This fusion protein has three copies of STa toxoid STa_{N12S} fused to a monomeric LT mutant (one LT_A subunit fused to one LT_B subunit) as a single peptide. Montanide ISA51 (kindly provided by SEPPIC, Fairfield, NJ, USA), Freund's incomplete adjuvant (FIA; Sigma, ST. Louis, MO, USA) and holotoxin-structured double mutant LT (dmLT; provided by Walter Reed Army Institute of Research, Silver Spring, MD, USA) were used as adjuvants in mouse SC and IP immunization (Table 1).

Mouse SC or IP immunization

A total of seven groups of 8-week-old female BALB/c mice (Charles River Laboratories International, Inc., Wilmington, MA, USA) were included in immunization studies: three SC immunization groups, three IP immunization groups and one negative control group.

For SC immunization, five mice in a group were each administered with 40 µg 3xSTa_{N12S}-dmLT toxoid fusion protein without adjuvant, with adjuvant ISA51 (SEPPIC) or with 2 µg dmLT adjuvant. For IP immunization, a group of five mice was administered with 200 µg toxoid fusion protein (in 200 µl) and 200 µl FIA, a group of six mice was administered with 200 µg toxoid fusion protein and 0.2 µg dmLT adjuvant (in 200 µl) and a third group of six mice was administered with 200 µg toxoid fusion protein and 2 µg dmLT adjuvant (in 200 µl). Each immunized mouse received two boosters with the same route and the same dose of the primary, at the interval of 2 weeks. Five mice in the control group received no IP or SC administration. Mouse serum samples were collected before the primary administration and 10 days after the final booster and stored at -80°C.

Mouse immunization complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (Revision 2015; <http://grants.nih.gov/grants/olaw/references/phspol.htm>). All mouse studies were approved by the Kansas State University IACUC and supervised by a university staff veterinarian.

Mouse serum IgG antibody titration

Mouse serum anti-STa and anti-LT IgG antibody responses were measured in ELISAs as previously described (Zhang et al. 2010, 2013; Ruan et al. 2014).

Mouse serum antibody neutralization assays

Anti-STa and anti-LT antibody neutralization activities were examined from mouse serum samples pooled from each immunization group or the control group (in triplicates) using T-84 cells and a cAMP EIA kit or a cGMP EIA kit (Enzo Life, Farmingdale, NY, USA), as described previously (Zhang et al. 2010, 2013; Liu et al. 2011; Ruan et al. 2014).

Statistical analysis

Mouse antibody titration and antibody neutralization data were analyzed with SAS program (SAS Institute, Cary, NC, USA) and

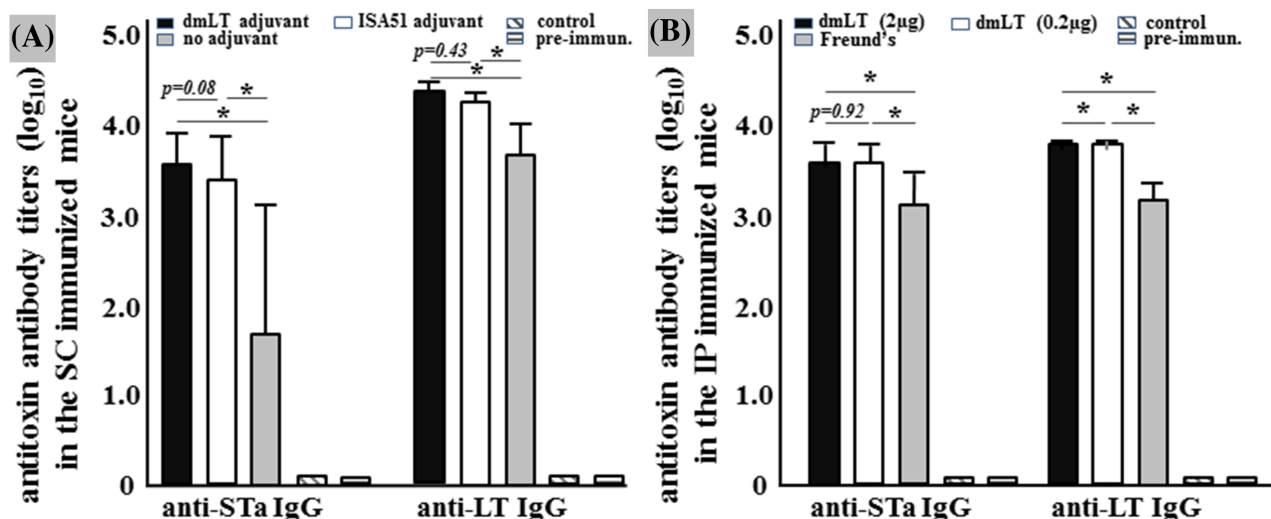


Figure 1. Mouse serum anti-STa and anti-LT IgG antibody titers (in log₁₀) detected from the immunized groups and the control group. (A) Anti-STa and anti-LT IgG antibody titers from the serum samples of mice SC immunized with toxoid fusion 3xSTaN_{12S}-dmLT without adjuvant, or with dmLT (2 μg) or Montanide ISA51 as the adjuvant. (B) Anti-STa and anti-LT IgG antibody titers from the serum samples of mice IP immunized with toxoid fusion 3xSTaN_{12S}-dmLT with 0.2 μg dmLT, 2 μg dmLT or FIA as the adjuvant. Serum samples of the control mice and serum samples collected from mice prior to immunization were included for anti-STa and anti-LT IgG antibody titration. P values were calculated from Student's t-test. Boxes and bars represented means and standard deviations of antibody titers, respectively.

presented in means ± standard deviations. Different treatment groups were pairwise compared using two-tailed distribution and two-sample equal or unequal variance. Student's t-test P value of <0.05 refers statistically significant difference.

RESULTS

Mice SC immunized with toxoid fusion 3xSTaN_{12S}-dmLT developed antibodies specific to STa and LT toxins

Mice developed antibody responses specific to STa and LT following SC administration of 3xSTaN_{12S}-dmLT (Fig. 1A). Mice SC administered with the toxoid fusion and adjuvant dmLT and mice SC with the toxoid fusion and adjuvant ISA51 had similar anti-STa IgG titers (3.62 ± 0.42 and 3.42 ± 0.59 ; in log₁₀) and anti-LT IgG titers (4.40 ± 0.15 and 4.30 ± 0.09) detected in their serum samples. No IgG antibody responses to STa or LT were detected from the control mouse serum samples or the serum samples collected prior to immunization (Fig. 1A).

Serum IgG antibody titers specific to STa and LT in the mice administered with the fusion antigen alone (without adjuvant) were detected at 1.74 ± 1.66 and 3.76 ± 0.43 (log₁₀), which were significantly lower than those from the mice administered with the toxoid fusion and dmLT adjuvant ($P < 0.01$) or ISA51 adjuvant ($P < 0.01$).

Mice IP administered with 3xSTaN_{12S}-dmLT developed antibodies specific to STa and LT

Mice developed anti-STa and anti-LT antibody responses following IP administration of 3xSTaN_{12S}-dmLT (Fig. 1B). Anti-STa IgG titers were 3.62 ± 0.21 , 3.61 ± 0.18 and 3.12 ± 0.36 (log₁₀) in the serum of the IP-administered groups using 2 μg dmLT, 0.2 μg dmLT or FIA as the adjuvant. Anti-LT IgG antibody titers were 3.8 ± 0.02 , 3.73 ± 0.02 and 3.16 ± 0.24 in the serum of the three administered groups with adjuvant 2 μg dmLT, 0.2 μg dmLT or FIA, respectively. The STa and LT IgG titers in the two immunization groups with dmLT adjuvant were significantly greater than

those in the immunization group with FIA adjuvant ($P < 0.01$, $P < 0.01$). No anti-STa or anti-LT IgG titers were detected from the serum samples of the control mice or the pre-immunization serum samples (Fig. 1B).

Anti-STa IgG antibody titers detected from the serum samples of the two IP administered groups using dmLT adjuvant were not different ($P = 0.92$), but IgG titers specific to LT in the group using 2 μg dmLT adjuvant were greater than those in the group using 0.2 μg dmLT adjuvant ($P < 0.01$).

Serum samples of the mice SC or IP administered with 3xSTaN_{12S}-dmLT neutralized STa toxin

Serum samples pooled from mice following SC or IP immunization neutralized STa toxin *in vitro* (Fig. 2). Intracellular cGMP levels of the T-84 cells treated with 2 ng STa and 30 μl pooled serum from the SC immunization group using dmLT and ISA51 adjuvant were 1.06 ± 0.34 and 2.12 ± 0.59 pmole ml⁻¹, respectively (Fig. 2A).

Intracellular cGMP levels of the T-84 cells treated with 2 ng STa and 30 μl pooled serum from the IP immunization groups using 2 μg dmLT adjuvant, 0.2 μg dmLT adjuvant and FIA were 0.81 ± 0.16 , 0.79 ± 0.06 and 0.77 ± 0.05 pmole ml⁻¹, respectively (Fig. 2B).

Baseline intracellular cGMP levels of T-84 cells, in which cells were treated with culture medium only, were 0.67 ± 0.37 pmole ml⁻¹. In contrast, the cGMP levels in the T-84 cells treated with STa alone and STa with the control mouse serum were 11.7 ± 0.47 and 12.3 ± 0.06 pmole ml⁻¹, respectively.

Serum samples of the SC or IP administered mice neutralized CT

Intracellular cAMP levels of the T-84 cells that were treated with 10 ng CT and 30 μl pooled serum sample of the mice SC administered with the toxoid fusion and dmLT adjuvant or ISA51 adjuvant were 5.42 ± 0.08 and 7.54 ± 0.46 pmole ml⁻¹, respectively (Fig. 3A). Intracellular cAMP concentrations of the T-84 cells that

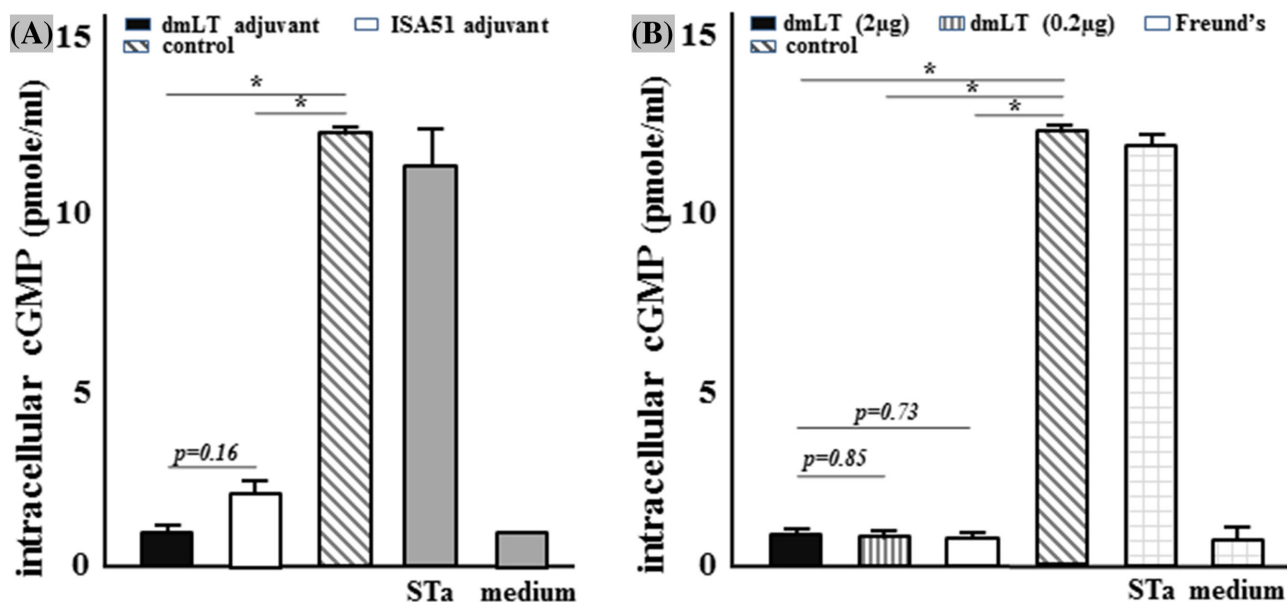


Figure 2. Mouse serum antibodies against STa toxin *in vitro* antibody neutralization assay with T-84 cells and EIA cGMP kit. Pooled serum samples (30 μ l) from each immunization group or the control group were used to incubate STa (2 ng). The serum/toxin mixture samples were added to T-84 cells. After 1 h incubation, intracellular cGMP levels (pmole ml^{-1}) in T-84 cells were measured. (A) Intracellular cGMP levels in T-84 cells incubated with STa toxin and pooled serum from the SC immunized group using 2 μ g dmLT or ISA51 adjuvant, or from the control group. (B) Intracellular cGMP levels in T-84 cells incubated with STa toxin and pooled serum from the IP immunized groups using 2 μ g dmLT, 0.2 μ g dmLT or FIA as the adjuvant, or from the control group. Baseline cGMP levels (T-84 cells incubated with cell culture medium) and cGMP levels in T-84 cells incubated with 2 ng STa (as positive reference) were included. P values were calculated with Student's t-test. Boxes and bars represented means and standard deviations of cGMP levels, respectively.

were treated with 10 ng CT and 30 μ l pooled serum from the mice IP administered groups using 2 μ g dmLT adjuvant, 0.2 μ g dmLT adjuvant or FIA were 2.3 ± 0.37 , 2.7 ± 0.06 and 13.3 ± 1.2 pmole ml^{-1} , respectively (Fig. 3B).

Intracellular cAMP levels of the T-84 cells treated with 10 ng CT and the control mouse serum were 53.7 ± 1.3 pmole ml^{-1} . The baseline cAMP levels in T-84 cells that were cultured with cell medium were 2.5 ± 0.03 pmole ml^{-1} .

DISCUSSION

Results from this study affirmed that toxoid fusion 3xSTa_{N12S}-dmLT induces antibodies neutralizing STa toxin and CT toxin that is structurally and functionally homologous to LT. Mice SC or IP administered with 3xSTa_{N12S}-dmLT, with different adjuvants, develop strong antibody responses specific to STa and LT. More importantly, induced mouse antibodies were shown to neutralize STa and CT. These results suggest that toxoid fusion 3xSTa_{N12S}-dmLT is potentially a desirable antigen for ETEC vaccines. With a safe toxoid fusion antigen that induces neutralizing anti-STa antibodies, we potentially overcome a key challenge and are able to accelerate ETEC vaccine development.

Data from this study also suggest dmLT an effective adjuvant in SC immunization. ADP-ribosylating bacterial toxins including CT of *Vibrio cholerae* and LT of ETEC have been explored as vaccine adjuvants (Lycke and Holmgren 1986; Clements, Hartzog and Lyon 1988; Freytag and Clements 2005; Lycke 2005). Unfortunately, CT or LT potent toxicity results in adverse effects, limiting application as a safe adjuvant (Levine et al. 1984; Gagliardi and De Magistris 2003; Lewis et al. 2009). Detoxified CT and LT derivatives were produced as the second generation of adjuvants (Chong, Friberg and Clements 1998; Yamamoto et al. 2001; Lycke 2004). However, remaining enterotoxicity in some LT toxoids is linked to diarrhea following oral administration or Bell's

palsy in volunteers following intranasal immunization (Kotloff et al. 2001; Gagliardi and De Magistris 2003; Mutsch et al. 2004; van Ginkel et al. 2005). Double-mutant holotoxin-structured LT toxoid (dmLT; LT_{R192G/L211A}, one LT_A subunit and five LT_B subunits forming LT holotoxin), which had toxicity further reduced but retained LT adjuvanticity (Norton et al. 2012), was shown to positively immunoregulate antigen-specific mucosal immunity following oral, intragastric, sublingual and intranasal immunizations (Summerton et al. 2010; Leach et al. 2012; Holmgren et al. 2013; Martinez-Becerra et al. 2013; Sjobqvist Ottso et al. 2013). However, dmLT adjuvanticity in parenteral immunizations has not been characterized, nor has its potential application as an adjuvant for ETEC subunit vaccines been explored.

Results from SC immunization study showed that dmLT was an effective adjuvant to immunoregulate toxoid fusion 3xSTa_{N12S}-dmLT in inducing anti-STa antibodies. Anti-STa and anti-LT IgG titers in the serum of mice immunized with toxoid fusion and ISA51 adjuvant or dmLT adjuvant were significantly greater than the anti-STa ($P < 0.01$) and anti-LT ($P < 0.01$) IgG titers in the serum of the mice SC immunized with the toxoid fusion alone (without adjuvant). This suggests that dmLT is an adjuvant equally effective as ISA51 adjuvant in enhancing 3xSTa_{N12S}-dmLT for induction of IgG antibodies to STa toxin.

The enhanced antibody response to LT in SC administered mice using dmLT adjuvant can be resulted from adjuvant dmLT, since this dmLT adjuvant likely also plays a role as an antigen to induce anti-LT antibodies. Enhanced anti-LT antibody response nevertheless is desired for ETEC vaccines since LT is also a key virulence determinant in ETEC-associated children's diarrhea and travelers' diarrhea.

Mice IP administered with the toxoid fusion and dmLT (2 or 0.2 μ g) developed greater antibody responses to STa and LT compared to the mice IP administered with the same antigen but FIA adjuvant. Without a treatment group administered with

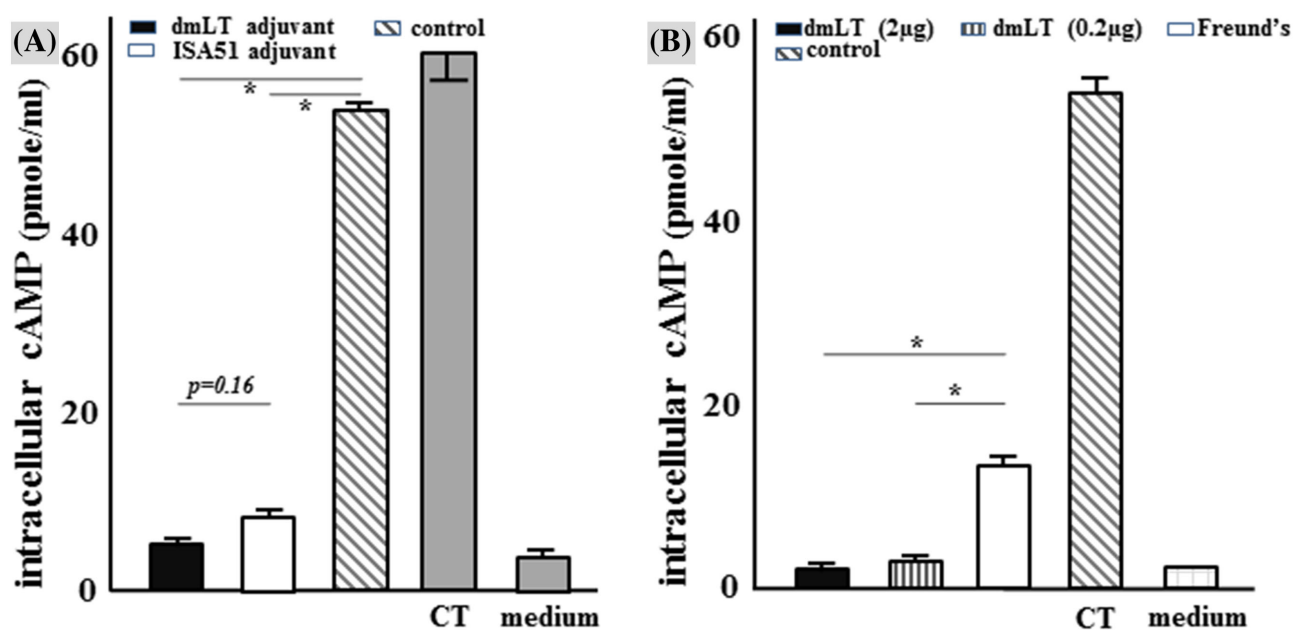


Figure 3. Mouse serum antibodies against CT toxin *in vitro* antibody neutralization assays with T-84 cells and EIA cAMP kit. Pooled serum samples (30 μ l) from each immunization group or the control group were used to incubate CT (10 ng). The serum/toxin mixture samples were added to T-84 cells. After 3 h incubation, intracellular cAMP levels (pmole ml⁻¹) in T-84 cells were measured. (A) Intracellular cAMP levels in T-84 cells incubated with CT toxin and pooled serum from the SC immunized groups using 2 μ g dmLT or ISA51 adjuvant or the control group. (B) Intracellular cAMP levels in T-84 cells incubated with CT toxin and pooled serum from the IP immunized groups using 2 μ g dmLT, 0.2 μ g dmLT or FIA as the adjuvant or the control group. Baseline cAMP levels (T-84 cells incubated with cell culture medium) and cAMP levels in T-84 cells incubated with 10 ng CT (as positive reference) were included as controls. *P* values were calculated with Student's *t*-test. Boxes and bars represented means and standard deviations of cAMP levels, respectively.

the toxoid fusion only (without adjuvant), we were unable to characterize adjuvanticity of dmLT for toxoid fusion 3xSTa_{N125}-dmLT in the IP immunization study. However, serum anti-STa IgG antibody titers in IP immunized group using 2 or 0.2 μ g dmLT were significantly greater than those in mice immunized using FIA adjuvant ($P < 0.01$, $P < 0.01$), suggesting that dmLT could immunoregulate this toxoid fusion equally or more effectively as FIA. Future studies to include a treatment immunized with the toxoid fusion alone and also treatments with various doses of dmLT will help to characterize dmLT adjuvanticity in IP immunization route.

In this study, we used LT as the coating antigen in the anti-LT antibody titration ELISA and CT in the anti-LT antibody neutralization assay. While the structurally homologous LT and CT used as ELISA coating antigens led to same outcomes in anti-LT antibody titration, we preferred CT for anti-LT antibody neutralization assay. CT was commercially available when this assay was developed. Also, we found that CT is more biologically effective in elevating cGMP levels in T-84 cells and that the assay is more reproducible when CT is used. As quality of commercial LT product gets improved, LT can be used in future studies for anti-LT antibody titration as well as *in vitro* antibody neutralization assay.

Antitoxin antibody neutralizing STa and CT was assessed *in vitro* in this study. *In vivo* studies using suckling infant mice can better characterize anti-STa antibody neutralization activity against STa toxin. However, animal challenge studies will be needed to assess protection of antibodies induced by this toxoid fusion. Although data from our preliminary study showed that this toxoid fusion induced neutralizing anti-STa antibodies in intramuscularly administered pigs and passive antibodies were shown to protect suckling piglets against STa⁺ ETEC challenge (data not shown), additional challenge studies using

this pig model or other animal challenge models will be needed to further evaluate induced anti-STa antibodies for protection against STa⁺ ETEC diarrhea.

In conclusion, results from the current study revealed that mice SC or IP administered with toxoid fusion 3xSTa_{N125}-dmLT developed antibodies specific to STa and LT and the induced mouse antibodies neutralized STa and CT toxins. These results provided further evidence that toxoid fusion 3xSTa_{N125}-dmLT can induce neutralizing anti-STa (and anti-LT) antibodies and this toxoid fusion may potentially be a suitable toxin immunogen for ETEC subunit vaccine development.

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Conflict of interest. None declared.

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