The mucosal adjuvanticity of two nontoxic mutants of *Escherichia coli* heat-labile enterotoxin varies with immunization routes

Eun Jeong Park¹, Ji Hoon Chang¹, Jang Seong Kim¹, Jung Sun Yum^{1,2} and Soo Il Chung¹

¹Mogam Biotechnology Research Institute, 341 Pojungri, Koosungmyun, Yongsin, Kyonggido 449-910, Korea

²Corresponding author: Tel, +82-331-260-9835; Fax, +82-331-260-9808; E-mail, jsyum@greencross.com

Accepted 25 May 2000

Abbreviations: LT, *Escherichia coli* heat-labile enterotoxin; CT, cholera toxin; WCL, whole cell lysate; ELISA, enzyme-linked immunosorbent assay; IG, intragastric; IN, intranasal

Abstract

Escherichia coli heat-labile enterotoxin (LT), which causes a characteristic diarrhea in humans and animals, is a strong mucosal immunogen and has powerful mucosal adjuvant activity towards coadministered unrelated antigens. Here we report the different mucosal adjuvanticity of nontoxic LT derivatives, LTS63Y and LTΔ110/112, generated by immunizing through two different mucosal routes. Intragastric (IG) immunization with *Helicobacter pylori* urease alone resulted in poor systemic IgG and IgA responses and no detectable local secretory IgA, but IG co-immunization with urease and LTΔ110/112 induced high titers of urease-specific local secretory IgA and systemic IgG and IgA, comparable to those induced by wild-type LT. LTS63Y showed far lower adjuvant activity towards urease than LTΔ110/112 in IG immunization, but was more active than LTΔ110/112 in inducing immune responses to urease by intranasal (IN) immunization. LTΔ110/112 predominantly enhanced the induction of urease-specific IgG1 levels following IG immunization, whereas LTS63Y induced high levels of IgG1, IgG2a and IgG2b following IN immunization. In addition, quantitative *H. pylori* culture of stomach tissue following challenge with *H. pylori* demonstrated a 90-95% reduction ($p < 0.0002$) in bacterial burden in mice immunized intranasally with urease using either mutant LT as an adjuvant. These results indicate that the mechanism(s) underlying the adjuvant activities of mutant LTs towards coadministered *H. pylori* urease may differ between the IN and IG mucosal immunization routes.

Keywords: LTS63Y, LTΔ110/112, secretory IgA, mucosal adjuvant

Introduction

Heat-labile enterotoxin (LT) produced by enterotoxigenic *Escherichia coli* (ETEC) and cholera toxin (CT) produced by *Vibrio cholerae* show 80% homology at the primary amino acid sequence level and have similar 3-D structures (Mekalanos *et al.*, 1983; Sixma *et al.*, 1991; Spangler, 1992). Both toxins are composed of two functionally distinct domains, the enzymatically active A subunit with ADP-ribosylating activity (Noda *et al.*, 1989; Lycke *et al.*, 1992; Dickinson *et al.*, 1995), and the pentameric B subunit that contains a G_{M1} (monosialoganglioside) receptor-binding site (Tsuji *et al.*, 1985; Spiegel, 1990; Bäckström *et al.*, 1997). The A subunit intoxicates eucaryotic cells by activating Gs, a GTP-binding protein that regulates the levels of second messenger cAMP (Guerrant *et al.*, 1974). Enhancement of cAMP levels alters ion transport, inducing secretion of water and chloride ions into the small intestine, resulting in traveler's diarrhea or cholera (Spangler, 1992).

Both CT and LT are strong immunogens by oral and other mucosal routes, via which most antigens are unable to induce immune responses. CT and LT are also known to act as potent mucosal adjuvants and induce immune responses against coadministered non-respondent antigens (Spangler, 1992; Jackson *et al.*, 1993). However, the highly-toxic nature of these proteins has precluded their clinical use in humans. Attempts have been made to overcome this problem with LT (Lobet *et al.*, 1991; Douce *et al.*, 1995; Di Tommaso *et al.*, 1996; Marchetti *et al.*, 1998) and with CT (Douce *et al.*, 1997; Yamamoto *et al.*, 1997a; Yamamoto *et al.*, 1997b), using mutagenesis aimed at neutralization of toxicity without compromising immunogenicity. Mutation of amino acids in the NAD binding sites (residues 58-76) or active sites (residues 7, 44, 61, 110 and 112) of the A subunit of cholera toxin resulted in inactivation of the ADP-ribosyltransferase activity and loss of toxicity in Y1 cells (Fontana *et al.*, 1995). Similar point mutations in the A subunit of *E. coli* enterotoxin (LTK7, LTK63, LTR72, LTE110D, LTE112D, LTE112K and LTR192G) have produced inactive forms of LT (Tsuji *et al.*, 1990; Lobet *et al.*, 1991; Tsuji *et al.*, 1991; Pizza *et al.*, 1993; Grant *et al.*, 1994; Dickinson *et al.*, 1995; Douce *et al.*, 1995; Giuliani *et al.*, 1998). However, substitution of Ala to His

or Glu at position 72 resulted in a protein with a toxicity indistinguishable from that of wild-type LT, whereas Ala to Arg substitution at the same position greatly reduced toxicity (Giuliani *et al.*, 1998). These observations suggest that mutations at key positions in the active site may affect ribosyltransferase activity, and that the specific amino acid used to replace wild-type residues may affect binding affinities for NAD or substrates. We constructed two novel LT mutants by point mutation of serine at amino acid position 63 to tyrosine (LTS63Y), and double deletion of glutamic acid at positions 110 and 112 (LT Δ 110/112) (Park *et al.*, 1999). Modification of these residues, which are located at the putative ADP-ribosyltransferase active center, has been shown to inactivate ADP-ribosyltransferase activity and enterotoxicity. Here we investigated the mucosal adjuvant properties of the mutant LTs, using *H. pylori* urease as a coadministered antigen.

Materials and Methods

Mice and immunization

Six-week-old female BALB/c mice were purchased from Charles River (Japan). To test the mucosal adjuvanticity of mutant LTs, mice were immunized either intragastrically with 125 μ g of *H. pylori* urease together with 25 μ g of toxin on days 0, 7, 14, and 21, or intranasally with 20 μ g of the same antigen coadministered with 2 μ g of toxin on days 0, 7, and 14 (Yamamoto *et al.*, 1997a). For nasal immunization, small volume of antigen suspension was dropped using micropipets. For intragastric immunization, antigen suspension was fed using blunt-ended needle. Fecal extracts and serum samples were collected and monitored for immune responses.

ELISA

Urease-specific antibodies were measured in an enzyme-linked immunosorbent assay (ELISA) as described previously (Di Tommaso *et al.*, 1996; Guidry *et al.*, 1997). Briefly, plates were coated with 1 μ g of urease per well, incubated at 37°C for 1 h, washed three times and blocked for 1 h at 37°C with 2.5% skim milk in PBS containing 0.05% Tween 20 (PBST). Sera and fecal samples from each mouse were tested using two-fold serial dilutions for the estimation of urease-specific secretory IgA and serum IgG and IgA. Serum or fecal sample (100 μ l) was added to each well and incubated for 2 h at 37°C. After washing six times with PBST, the plates were incubated for 1 h at 37°C with horseradish peroxidase (HRP)-conjugated anti-mouse antibodies specific for mouse immunoglobulin G (IgG) (1 : 5000), IgA (1 : 2000) (KPL) or IgG1, IgG2a or IgG2b (1 : 2000) (Biosource). After washing six times with PBST, bound antibody was visualized by addition of 3,3',5,5'-tetra-

methylbenzidine (TMB). The absorbance at 450 nm was determined and ELISA titers were recorded as the highest dilution of serum which gave an absorbance value above the level measured in preimmune samples.

Bacterial challenge and assessment of colonization

Helicobacter pylori SS1, a mouse-adapted strain originally isolated from a peptic ulcer patient, was provided by Dr. A. Lee (University of New South Wales, Australia) and was used as the bacterial challenge inoculum (Lee *et al.*, 1997). The cells were grown as previously described (Kim *et al.*, 1999).

Two weeks after the last IN immunization, mice were fed 0.35 M NaHCO₃ to neutralize gastric acidity and then challenged intragastrically with 5×10^7 colony-forming units (CFUs) of *H. pylori* SS1 twice over a two-day period. At day 42 all mice were sacrificed by cervical dislocation. The stomachs were excised, cut along the greater curvature, and rinsed in PBS to remove the stomach contents. The stomach tissues were homogenized with a pellet pestle (Kontes Scientific Glassware) in a 1.5 ml microfuge tube in 0.5 ml of BHI broth. Serial dilutions of the resulting suspension were plated onto blood agar plates and protection levels were assessed by viable counting of the recovered bacteria.

Results

Mucosal adjuvanticity of mutant LTs in IG immunization

We previously showed that both mutant LTs were immunogenic following either IG or IN administration, and that antibody titers induced by IN immunization were slightly higher than those induced by IG immunization (Park *et al.*, 1999). In the present study, the abilities of mutant LTs to act as mucosal adjuvants were assessed by IG immunization in mice. Groups of mice were immunized intragastrically with 125 μ g of *H. pylori* urease alone or together with 25 μ g of wild-type LT, LTS63Y or LT Δ 110/112. As shown in Figure 1, mice immunized with *H. pylori* urease alone showed no significant production of urease-specific antibodies. Mice immunized by coadministration of urease and LT Δ 110/112 produced strong anti-urease antibody responses, comparable to those observed in mice co-immunized with wild-type LT. In contrast, mice immunized by coadministration of the antigen and LTS63Y showed no significant sero-conversion. Altering the dose of LT Δ 110/112 to 10 or 125 μ g did not cause any change in the immune response to urease. LTS63Y did not show any significant adjuvanticity, even when 125 μ g was coadministered with the urease (data not shown). To test the adjuvanticity of LT Δ 110/112 with other antigens, whole cell lysate (WCL) of *H. pylori* was used as an antigen and it was observed

Figure 1. Anti-urease secretory IgA and serum IgG and IgA antibody responses following IG immunization: IgA in fecal extracts and IgG and IgA in sera of mice immunized 4 times. 1, PBS; 2, urease 125 µg; 3, urease 125 µg plus LT 25 µg; 4, urease 125 µg plus LTS63Y 25 µg; 5, urease 125 µg plus LTΔ110/112 25 µg. Results are shown as mean titers and error bars indicate standard deviations from the mean.

that LTΔ110/112 also induced antibody responses to WCL (data not shown). These data suggest that LTΔ110/112 effectively functions as a mucosal adjuvant in IG immunization by inducing strong mucosal and systemic antibody responses to coadministered antigens such as *H. pylori* WCL or urease.

To further assess the immunologic response induced by LTΔ110/112 following IG immunization, IgG subclass responses were determined by ELISA. Urease antibody responses enhanced by LTΔ110/112 were largely restricted to IgG1 rather than IgG2a or IgG2b subclass in sera, and similar antibody patterns were observed using wild-type LT as an adjuvant (Figure 3A). This pattern was not consistent with the immune responses induced by wild-type LT in previous reports, but rather resembled the responses induced by activation of CD4⁺ Th2-type cells by CT (Munoz *et al.*, 1990; Marinaro *et al.*, 1995; Takahashi *et al.*, 1996; Yamamoto *et al.*, 1997a).

Mucosal adjuvanticity of mutant LTs in IN immunization

The ability of mutant LTs to function as a mucosal adjuvant was also assessed by IN immunization in mice. IN administration of wild-type LT or LTS63Y demonstrated the capacity of this immunization route to generate powerful mucosal adjuvant responses, as shown in Figure 2. Groups of mice were immunized intranasally with 20 µg of *H. pylori* urease alone or coadministered with 2 µg of wild-type LT, LTS63Y or LTΔ110/112. Mice immunized intranasally with urease and LTS63Y showed high levels of mucosal and systemic anti-urease responses, including urease-specific secretory IgA, serum IgG and IgA antibodies, which were equivalent to the responses observed when wild-type LT was used as the adjuvant. However, mice immunized by coadministration of urease and LTΔ110/112 showed lower levels of antibodies to urease, similar to the levels induced by

Figure 2. Anti-urease secretory IgA and serum IgG and IgA antibody responses following IN immunization: IgA in fecal extracts and IgG and IgA in sera of mice immunized 3 times. 1, PBS; 2, urease 20 µg; 3, urease 20 µg plus LT 2 µg; 4, urease 20 µg plus LTS63Y 2 µg; 5, urease 20 µg plus LTΔ110/112 2 µg. Results are shown as mean titers and error bars indicate standard deviations from the mean.

Figure 3. Anti-urease serum IgG subclass antibody responses following IG (A) and IN (B) immunization. IgG1 (black bars), IgG2a (light gray bars) and IgG2b (dark gray bars) of mice immunized 4 (IG) or 3 (IN) times. 1, PBS; 2, urease (125 µg / 20 µg); 3, urease plus wild-type LT (125 µg + 25 µg / 20 µg + 2 µg); 4, urease plus LTS63Y (125 µg + 25 µg / 20 µg + 2 µg); 5, urease plus LTΔ110/112 (125 µg + 25 µg / 20 µg + 2 µg). The amounts of protein used in each immunization are shown in parenthesis (IG dose / IN dose). Results are shown as mean titer and error bars indicate standard deviations from the mean.

urease alone. When 0.2 µg of LTS63Y was used, antibody responses to urease in both fecal extracts and sera were lower by a factor of approximately 10, but when 6 µg of LTS63Y was used, no increase in antibody responses to urease was induced. Thus, a 2 µg dose of LTS63Y appeared to be adequate for eliciting adjuvanticity *via* IN administration.

To further characterize the adjuvant properties of LTS63Y in IN immunization, IgG subclass responses were also analyzed. As shown in Figure 3B, LTS63Y induced IgG1, IgG2a and IgG2b subclass antibody responses to *H. pylori* urease, which is consistent with a previous report suggesting that antibody response profiles induced by LT are regulated by both CD4⁺ Th1 and Th2 cell types (Takahashi *et al.*, 1996). These results were quite different from the IgG subclass response (predominantly IgG1) induced by LTΔ110/112 *via* the IG route (Figure 3A).

Colonization of the mouse stomach

The adjuvant activities of LTS63Y and LTΔ110/112 were further examined by assessing the protection achieved using *H. pylori* urease as a vaccine immunogen (Figure 4). The results indicate that *H. pylori* could not colonize the stomachs of mice immunized intranasally with *H. pylori* urease coadministered with either mutant or wild-type LT, although a small number of *H. pylori* cells were detected. The protection against *H. pylori* challenge was not consistent with level of IgA titers induced by IN immunization with either of mutant LT. It appears that the antibody response to *H. pylori* urease may not be the only requirement for protection against bacterial challenge, since IN immunization with urease plus LTΔ110/112 as an adjuvant elicited weak antibody responses comparable to those induced by urease alone. This observation is consistent with the demonstration by

Figure 4. Quantitative *H. pylori* culture of gastric tissue from mice immunized intranasally with urease alone (U) or together with wild-type LT (L), LTS63Y (S) or LTΔ110/112 (D). Colony counts are presented as CFU per gram of gastric tissue of each animal. Colonization levels of *H. pylori* were significantly reduced ($\geq 90\%$) in mice immunized with mutant or wild-type LT as an adjuvant.

Ermak *et al.* (1998) that protection of mice against *H. pylori* infection by immunization with urease is dependent on MHC class II-restricted, cell-mediated mechanisms (Ermak *et al.*, 1998).

Discussion

The ability of LT to function as a strong mucosal adjuvant and immunogen has prompted many studies of the mechanisms by which LT induces mucosal immunity, and numerous attempts to eliminate toxicity without impairing adjuvanticity by site-directed mutagenesis of LT. There remains some dispute over the correlation between ADP-ribosyltransferase activity and adjuvanticity. A nontoxic, enzymatically inactive mutant LT (LTE-

112K) was shown to lack adjuvanticity in oral immunization in mice, suggesting that ADP-ribosyltransferase activity may be required for adjuvanticity (Lycke *et al.*, 1992). However, when delivered *via* the IN route, the same mutant LTE112K has been shown to exert adjuvant activity (Verweij *et al.*, 1998). On the other hand, Giuliani *et al.* (1998) indicated that enzymatic activity is required for full mucosal adjuvanticity by demonstrating that LTR72, which had only 0.6% of wild-type LT enzymatic activity, exhibited a greater mucosal adjuvanticity than the nontoxic, enzymatically inactive mutant LTK63 (Giuliani *et al.*, 1998). Thus, ADP-ribosyltransferase activity may contribute to the adjuvanticity of LT.

In this study, the nontoxic mutants LTS63Y and LT Δ 110/112 were administered alone or together with *H. pylori* urease *via* two immunization routes to assess mucosal adjuvanticity. These mutant LTs were shown to be immunogenic in a previous study and induce high titers of LT-specific antibodies following IG and IN immunization (Park *et al.*, 1999). In the present study, the mutant LTs showed no ADP-ribosyltransferase activity, but retained adjuvant activity. LTS63Y was an effective mucosal adjuvant when administered intranasally and induced mucosal and systemic antibody responses, while LT Δ 110/112 was also an effective adjuvant when administered *via* the IG route. These observations suggest that these mutant LTs enhance the immunogenicity of a coadministered, unrelated protein by a mechanism independent of the ADP-ribosyltransferase activity of wild-type LT. Our results appear to contradict a previous report suggesting that ADP-ribosyltransferase activity is required for adjuvanticity (Lycke *et al.*, 1992). However, this study involved different LT mutants from ours, and the adjuvant activity of CT or LT may be mediated by two or more different mechanisms, one of which may include intracellular cAMP accumulation following ADP-ribosylation (Yamamoto *et al.*, 1997a).

Surprisingly, our mutant LTs exhibited different abilities to act as mucosal adjuvants depending on the route of administration. Although the mutant LTs resembled the holotoxin in terms of assembly and cell binding activity, the mutations in the A subunit may influence receptor-binding properties and hence exert different effects on lymphocytes of the mucosal immune system. In contrast to CT, which binds to only G_{M1}, LT exhibits a broad range of binding affinities including G_{M1}, G_{M2}, and asialo-G_{M1} (Tsuji *et al.*, 1985; Spiegel, 1992; Bäckström *et al.*, 1997). This suggests that different mutant forms of LT may require different immunization routes for adequate adjuvanticity, and that specific immunization route may elicit specific mechanisms of up-regulation of immune responses, independent of cAMP levels. Thus, selection of the route of immunization may be critical for determining the mucosal adjuvant activity of mutant LTs. Furthermore, in this study, different patterns of antigen-specific IgG subclass responses were induced by dif-

ferent immunization routes, regardless of cAMP levels. LTS63Y as well as wild-type LT induced antigen-specific IgG1, IgG2a and IgG2b responses by IN immunization, indicating that LTS63Y may promote both Th1- and Th2-type responses. This result is consistent with the general pattern of T-cell help induced by LT described by other workers who have studied the cytokines such as INF- γ and IL-5, released by T cells taken from mice following immunization with LT as a mucosal adjuvant (Takahashi *et al.*, 1996). In contrast, LT Δ 110/112 as well as wild-type LT induced predominant IgG1 responses following IG immunization, which more closely resembled the Th2-type responses induced by CT (Snapper *et al.*, 1993; Marinaro *et al.*, 1995; Douce *et al.*, 1997). Kleanthous *et al.* (1998) showed different IgG1/IgG2a responses following IN, peroral or rectal immunization with recombinant *H. pylori* urease using wild-type LT as an adjuvant in a Swiss-Webster mouse model. CT has been reported to elicit adjuvanticity by promoting antigen-specific CD4⁺ Th2-type responses associated with high levels of IL-4 and IL-5 production and provision of help for IgG1 subclass responses (Hörnquist *et al.*, 1993; Yamamoto *et al.*, 1997a). The mechanism(s) by which these molecules stimulate the mucosal immune responses in different ways has remained largely obscure to date. Different subsets of T helper lymphocytes may be activated by different mucosal immunization routes.

As described above, the choice of administration route may be very important for effective induction of mucosal immune responses. Mucosal vaccines delivered into the nasal tract provide several advantages. For example, lower doses of antigen and adjuvant are required to induce effective antibody responses when compared to IG immunization, which can decrease the cost of vaccination (Partidos *et al.*, 1996; Yamamoto *et al.*, 1997a). IN immunization effectively induces not only systemic IgG but also secretory IgA responses in mucosal effector tissues. In this study, IN immunization induced higher levels of secretory IgA and systemic IgG and IgA antibodies than IG immunization, despite requiring only 15% of the *H. pylori* urease and 6% of the adjuvant. In addition, IN immunization may involve less risk of anaphylactic reactions, since the doses used in IN immunization induce lower total and antigen-specific IgE level in serum (Marinaro *et al.*, 1995; Yamamoto *et al.*, 1997a). The results of our *H. pylori* challenge experiments demonstrated that immunization of mice with urease together with either mutant LT significantly suppressed *H. pylori* infection (Figure 4). Although IN immunization with *H. pylori* urease and LTS63Y or LT Δ 110/112 did not entirely prevent *H. pylori* colonization of the stomachs of the immunized mice after intragastric challenge, it significantly diminished the colonization levels. Similar protection results were also reported by Kleanthous *et al.* (1998) with wild-type LT as an adjuvant. Thus, intranasal

vaccination using nontoxic mutant LT as an adjuvant may be useful in human disease prevention, although it remains to be determined whether the antibody responses elicited in humans will be sufficient to confer protection.

In conclusion, the mutant LTs in this study retained the high adjuvanticity of native LT but lacked ADP-ribosyltransferase activity and would therefore not be expected to induce diarrhea in humans. These mutant LTs would be suitable for use in any vaccine formulation designed to elicit immunity *via* mucosal delivery; in particular, LTS63Y functioned as an effective adjuvant when given intranasally, whereas LT Δ 110/112 showed high adjuvanticity by IG immunization. It is possible that depending on immunization routes, LT, LTS63Y, or LT Δ 110/112 may activate either Th2-type or balanced Th1/Th2-type responses *via* induction of specific cytokine profiles. The mutant LTs in this study may retain different biological properties and may be useful tools with which to examine the mechanisms of induction of mucosal adjuvanticity.

Acknowledgement

The authors are grateful to Dr. A. Lee for providing *H. pylori* SS1. We thank J. H. Lee for excellent technical assistance in animal experiments and J. T. Oh for helpful discussions. This work was supported by the Korea Green Cross Corporation.

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