Characterization of a Mutant Escherichia coli Heat-Labile Toxin, LT(R192G/L211A), as a Safe and Effective Oral Adjuvant

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Despite the fact that the adjuvant properties of the heat-labile enterotoxins of Escherichia coli (LT) and Vibrio cholerae (CT) have been known for more than 20 years, there are no available oral vaccines containing these molecules as adjuvants, primarily because they are both very potent enterotoxins. A number of attempts with various degrees of success have been made to reduce or eliminate the enterotoxicity of LT and CT so they can safely be used as oral adjuvants or immunogens. In this report we characterize the structural, enzymatic, enterotoxic, and adjuvant properties of a novel mutant of LT, designated LT(R192G/L211A), or dmLT. dmLT was not sensitive to trypsin activation, had reduced enzymatic activity for induction of cyclic AMP in Caco-2 cells, and exhibited no enterotoxicity in the patent mouse assay. Importantly, dmLT retained the ability to function as an oral adjuvant for a coadministered antigen (tetanus toxoid) and to elicit anti-LT antibodies. In vitro and in vivo data suggest that the reduced enterotoxicity of this molecule compared to native LT or the single mutant, LT(R192G), is a consequence of increased sensitivity to proteolysis and rapid intracellular degradation in mammalian cells. In conclusion, dmLT is a safe and powerful detoxified enterotoxin with the potential to function as a mucosal adjuvant for coadministered antigens and to elicit anti-LT antibodies without undesirable side effects.

Bacterially derived enterotoxins, such as the heat-labile enterotoxin (LT) produced by enterotoxigenic Escherichia coli (ETEC) and the closely related chola enterotoxin (CT) produced by Vibrio cholerae, promote secretory diarrhea during microbial infection. Each enterotoxin is composed of an enzymatically active A-subunit noncovalently associated with a pantameric B-subunit. Induction of intestinal fluid secretion occurs after a series of events involving both changes to toxin structure and activation of intracellular signaling pathways (reviewed in reference 14). The B-subunit mediates binding and internalization of the toxin to cells; subsequent proteolytic cleavage and disulfide bond reduction separate the A-subunit into two domains, the enzymatically active A1 subunit and a smaller A2 peptide. Transport of A1 into the cytoplasm results in ADP-ribosylation of Gsα, followed by irreversible activation of adenylate cyclase and increases in intracellular levels of cyclic AMP (cAMP). In intestinal epithelial cells, this induction of cAMP causes a disregulation of cAMP-sensitive ion transport mechanisms, inhibiting intracellular salt absorption, increasing electrolyte transport into the gut lumen, and creating an osmotic gradient favoring intestinal water secretion (12).

An enzymatically active A-subunit is required for intestinal fluid secretion but also enables LT to act as an oral adjuvant, boosting the immune response to coadministered antigens and inducing both humoral and cellular immune responses in both the systemic and mucosal compartments. However, even low doses of LT (and CT) induce side effects, such as diarrhea, loose stools, nausea, and vomiting, when given orally to human volunteers (1, 20, 26). In an attempt to diminish toxicity but retain adjuvanticity, amino acid substitutions have been introduced into LT to generate adjuvant-active but nontoxic mutants. One such example is LT(R192G), or mLT, in which a glycine was substituted for arginine in a proteolytically sensitive site in the A-subunit that separates A1 and A2, preventing cleavage by trypsin and related enzymes (8). In both in vitro assays and animal studies, mLTLT shows reduced toxicity (3, 8) but maintains adjuvanticity equivalent to LT, inducing a balanced Th1/Th2 cytokine and antibody subclass profile similar to native LT (2, 10, 15, 16, 18, 22–24, 30, 32, 34, 39, 40). In a randomized, placebo-controlled, escalating-dose study in adults, 2 to 50 μg of mLTLT given orally was well tolerated; however, with administration of 100 μg of mLTLT, 16.7% (2 of 12) of volunteers reported mild to moderate diarrhea (28). In contrast, as little as 5 μg of LT or CT causes diarrhea in adult volunteers (1, 20). Although 25 μg of mLTLT was safe and well tolerated when given alone, one-third of adults receiving an oral inactivated Helicobacter pylori whole-cell vaccine that included 25 μg of mLTLT experienced mild diarrhea from this formulation (17). We hypothesized that the synergistic activity of mLTLT with endotoxin from the whole-cell Helicobacter vaccine induced enough secretion to overcome the natural resorptive capacity of the intestine, resulting in the observed self-limited, mild diarrhea.

In an attempt to further detoxify mLTLT while preserving its adjuvanticity, an additional mutation was created in a putative pepsin-sensitive proteolytic site in the A2 domain, changing leucine 211 to alanine (L211A). This double mutant, LT(R192G/L211A), or dmLT, demonstrated adjuvanticity in mice at levels comparable to mLTLT in an oral H. pylori vaccine study (34) and in a sublingual Streptococcus pneumoniae vaccine study (23). In the present study we characterized dmLT...
for proteolytic sensitivity, enzymatic activity, toxicity, and adjuvanticity compared to native LT and mL. The potential contribution of dmLT to the field of vaccinology and oral adjuvants is discussed.

MATERIALS AND METHODS

Toxin purification. LT, mL, and dmLT were prepared by galactose-affinity chromatography in our laboratory as described previously (6, 8, 34). Briefly, toxins were purified from cultures grown overnight in a 10-liter fermenter. Cells were harvested by centrifugation and lysed in a microfluidizer (model M-110L; Microfluidics, Newton, MA). The cell lysates were dialyzed overnight in TEAN (0.05 M Tris, 0.001 M EDTA, 0.003 M Na2HPO4, 0.2 M NaCl; pH 7.5), clarified by centrifugation, and subjected to chromatography on separate immobilized β-galactose columns (Pierce, Rockford, IL). Toxins were eluted with 0.3 M galactose in TEAN and passed through an endotoxin removal column (Pierce, Rockford, IL). The composition and purity of each protein were confirmed by SDS-PAGE and a Limulus amebocyte lysate assay (Lonza Inc., Walkersville, MD). The endotoxin content of the final products was <1 endotoxin units/mg.

Visualization of proteins by SDS-PAGE. Toxins were heated for 5 min with a reducing agent at 95°C and run on a NuPAGE 10% bis-Tris gel (Invitrogen, Carlsbad, CA). Protein bands were visualized after staining with 0.25% Coomassie blue. For trypsinization, 10 µg of each toxin was subjected to 1 h or 18 h of digestion with 10 ng trypsin (Sigma, St. Louis, MO) at 37°C prior to electrophoresis.

Intracellular LT detection by Western blotting. Caco-2 cells (HTB-37) from ATCC (Manassas, VA) were propagated in minimal essential medium–20% fetal bovine serum (FBS) INT407 cells (CCL-6) from ATCC were propagated in Basal medium (Earle) in Eagle's BSS supplemented with 10% FBS. Confluent cell monolayers in 12-well plates were treated with 10 µg toxin, which in some cases was pretitransfused as described above. After 1, 3, 5, or 7 days, cells were extensively washed with cold phosphate-buffered saline PBS and lysed with 100 µl radiomunoprecipitation assay buffer (Cell Signaling, Beverly, MA) containing phenylmethylsulfonyl fluoride protease inhibitor. Cell lysates were run on a NuPAGE 10% bis-Tris gel and transferred to a nitrocellulose membrane by using the iBlot system (Invitrogen). Membranes were blocked with skim milk and then stained for 1 h with anti-LT A-subunit or anti-LT B-subunit polyclonal rabbit sera prepared in our laboratory. After three 5-min washes, membranes were incubated with donkey anti-rabbit antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Membranes were washed again and developed with ECL Western blotting substrate (Pierce) and photographed on a LAS4000 imager (FujiFilm, Tokyo, Japan). Band density pictures were created using Multi Gauge V3.1 software (FujiFilm).

Detection of enzymatic activity in human colonic epithelial cells by cAMP assay. Caco-2 cells were treated as previously described (3). Confluent monolayers were washed and pretreated with 20 µM Zardaverine (Sigma) in Eagle's minimal essential medium–1% FBS for 20 min before the addition of trypsinized toxins. After 3 h, cells were washed twice with cold PBS, and intracellular cAMP levels were assessed using a cAMP assay kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Patent mouse assay for in vivo enterotoxicity. The patent (nonocluded gut) mouse assay was performed as previously described (3). Female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in filter-top cages under sterile conditions. All animal studies were approved by the Tulane University Institutional Animal Care and Use Committee. Animals were fasted overnight but had water available ad libitum prior to the assay. Groups of five mice were administered oral doses of LT (5, 25, or 125 µg), mL (25, 125, or 250 µg), or dmLT (25, 125, or 250 µg) in 0.5 ml saline. The toxin preparations were administered intragastrically with a blunt-tip feeding tube (Popper & Sons, Inc., New Hyde Park, NY). After 3 h, animals were sacrificed by CO2 inhalation, and the entire intestine from duodenum to anus was carefully excised, excluding any residual mesenteric tissue. Tissue and carcasses were separately weighed, and individual gut/carcass (G/C) ratios were calculated. A G/C ratio of >0.88 was considered positive.

Oral immunizations with tetanus toxoid. For oral immunizations studies, adjuvanticity was based upon the ability of dmLT to enhance immune responses against coadministered antigens, and antigenicity is based upon the ability of dmLT to elicit anti-LT-specific antibodies. Tetanus toxoid (TT; Statens Serum Institute, Denmark) was used as the coadministered antigen. Groups consisting of 8 to 10 BALB/c mice each were immunized orally, once a week for 3 weeks. Oral immunizations consisted of 0.5 ml of the test preparation containing 100 µg of TT or 100 µg of TT admixed with 25 µg of mL or dmLT in saline delivered intragastrically with a blunt-tip feeding needle (Popper & Sons, Inc.). One week after the third immunization, animals were sacrificed. Blood was collected from each mouse by cardiac puncture, and serum anti-TT IgG and serum anti-LT IgG levels were determined by enzyme-linked immunosorbent assay (ELISA). For the anti-TT ELISA, serum samples were serially diluted in PBS–0.05% Tween 20 and added to microplates precoated with 0.1 µg of TT per well. For the anti-LT ELISA, serum samples were serially diluted in PBS–0.05% Tween 20 and added to microplates precoated with 10 µg of GM1 per well followed by 0.1 µg of LT per well. Anti-TT and anti-LT IgG levels were determined with anti-mouse IgG whole molecule conjugated to alkaline phosphatase (A1902; Sigma). Reactions were stopped with 2 N NaOH, and the absorbance at 405 nm was determined spectrophotometrically. The titer was defined as the serum dilution that produced an optical density at 405 nm reading of 0.2.

RESULTS

Mutations in dmLT prevent proteolytic cleavage of LT-A into A1 and A2 but enhance A-subunit degradation. dmLT has two amino acid substitutions in the A-subunit, arginine 192 to glycine and leucine 211 to alanine (Fig. 1). Cleavage of LT-A by trypsin into A1 (21 kDa) and A2 (7 kDa) is required for activation of the molecule and is a key factor that distinguishes native LT from mL (8). To verify the retention of trypsin sensitivity by dmLT, we performed 1-h trypsin digestions and evaluated LT subunits by SDS-PAGE. Similar to mL, dmLT was insensitive to trypsin digestion of the A-subunit into A1 and A2 (Fig. 2A). However, we observed a decrease in the density of the dmLT A-subunit upon trypsin digestion and the appearance of faint 3- to 6-kDa bands, suggestive of alternative proteolytic processing. Significantly, the A-subunit of dmLT was not separated into A1 and A2 by trypsin treatment, which would indicate proteolytic activation of the toxin, but instead appeared to be completely degraded into a group of peptides smaller than 6 kDa. Long-term incubation of LT or either of the mutant toxins with trypsin completely degrades the A-subunit (data not shown), but dmLT A-subunit degradation occurs more rapidly (i.e., by 18 h) than either LT or mL (Fig. 2B). It should be noted that some degradation of native LT also occurs upon long-term incubation with trypsin, in addition to the cleavage into A1 and A2, but not as quickly or to the same extent as is seen with mL. The degradation of dmLT without
detectable creation of an A1 band was confirmed by a highly sensitive anti-LTA Western blot assay (Fig. 2C).

To verify these results in vivo, Caco-2 cells and INT407 cells, human colonic or embryonic epithelial cell lines, respectively, were treated with 10 μg of trypsinized or untrypsinized toxins for 24 h, and the A-subunits were detected by Western blotting. There was a significant, repeatable reduction in dmLT A-subunit bands in cell lysates that received trypsin-treated or untreated dmLT compared to LT- or mLT-treated cells, and this was more clearly visible in Caco-2 cells (Fig. 3A and B). As seen with the in vitro studies, the A-subunit of dmLT was not separated into A1 and A2 by trypsin treatment but was completely degraded. Further analysis of Caco-2 cells incubated with toxin for 3 to 7 days revealed a decreasing presence of all of the toxin A-subunits over time, but not the B-subunits. Moreover, in contrast to native LT, there was never an indication of A1 in either the mLT- or dmLT-treated epithelial cells. Collectively, these results suggest that dmLT is not separated into A1 and A2 by proteolytic cleavage but is more rapidly degraded than either LT or mLT in vivo as well as in vitro.

dmLT exhibits reduced enzymatic activity and no detectable toxicity in in vitro or in vivo assays. LT, mLT, and dmLT were evaluated for the ability to increase intracellular levels of cAMP in Caco-2 cells. As seen in Fig. 4A, 0.01 μg of native LT induced production of 390 pmol of cAMP in 3 h. In contrast, 1 μg of mLT or dmLT induced production of approximately 100 pmol of cAMP in 3 h, equivalent to that induced by 0.001 μg of native LT but still more than the negative control. We also assessed the ability of these toxins to induce intestinal fluid secretion after intragastric feeding in an in vivo patent mouse assay. Intragastric feeding of LT resulted in a dose-dependent increase in intestinal fluid secretion with higher gut-to-carcass ratios than in saline-fed mice (Fig. 4B). As previously reported (3), mLT induced significantly less fluid secretion than native LT at any dose up to 250 μg. Interestingly, mice receiving ≥125 μg of mLT had significant intestinal fluid secretion without an apparent dose-dependent effect once an initial threshold for secretion was achieved. This may reflect the residual activity of mLT that synergizes with lipopolysaccharide (LPS), as observed in volunteers when mLT was administered with Gram-negative whole-cell vaccines (17, 28). However, no fluid accumulation was observed in mice inoculated with dmLT in doses up to 250 μg. While there was a significant reduction in enterotoxicity as a result of the single mutation in the A1-subunit in mLT, our results indicate that the addition of a second amino acid substitution within the A2 piece of dmLT results in a lack of detectable enterotoxicity in vivo.

dmLT is a potent oral adjuvant for coadministered antigen. The cAMP and patent mouse assays demonstrated that dmLT has reduced enzymatic activity compared to native LT and no detectable enterotoxicity in the patent mouse assay. It was also important to demonstrate that dmLT retains two important
functions: the ability to serve as an oral adjuvant for coadministered antigens and the ability to induce anti-LT antibodies. For these studies, groups of mice were immunized orally with TT alone or admixed with mLT or dmLT, and anti-TT or anti-LT antibodies were determined by ELISA. As seen in Fig. 5A, animals immunized with TT in conjunction with either mLT or dmLT had a significant increase in serum anti-TT IgG compared to animals immunized orally with TT alone.

In addition to its ability to act as an oral adjuvant for coadministered antigens, LT may also be a protective antigen in vaccines against enterotoxigenic E. coli (33), and some studies have suggested that antitoxin immunity alone may be sufficient to protect against ETEC diarrhea. As seen in Fig. 5B, animals immunized with TT in conjunction with either mLT or dmLT also had a significant increase in serum anti-LT IgG. Because of the structural similarities between LT and CT, anti-LT antibodies exhibit immunologic cross-reactivity with cholera toxin (4, 5). Therefore, our results suggest that dmLT could be an effective component of an oral anti-ETEC or cholera vaccine in addition to functioning as an adjuvant for coadministered antigens.

**DISCUSSION**

Despite the fact that the adjuvant properties of LT and CT have been known for more than 20 years (7, 11), there are no available oral vaccines containing these molecules as adjuvants, primarily because they are both very potent enterotoxins. A number of attempts, with various degrees of success, have been made to reduce or eliminate the enterotoxicity of LT and CT so that they can safely be used as oral adjuvants or immunogens (8, 9, 29, 34). Most of those attempts have focused on modulating the active site of the A-subunit, the site of NAD binding, hydrolysis, and initiation of ADP-ribosyl transferase activity. Our initial attempts here focused instead on the proteolytically sensitive bond that joins A1 and A2; this followed findings from our earlier studies (6), which demonstrated that LT and CT, like many other bacterial toxins (e.g., diphtheria toxin, Pseudomonas exotoxin A), require proteolytic activation to be fully enzymatically active/toxic. Our previous studies had also indicated that some degree of residual enzymatic activity is required for LT to function as an oral adjuvant (3), and so we specifically avoided altering the active site of the molecule.

Our first initiative was to create a mutation that made LT insensitive to trypsin cleavage by substituting a glycine for an arginine at position 192 (R192G, or mLT). We have extensively studied this molecule, and there are more than 75 published articles on the adjuvant activity of mLT in a variety of animal models by different routes and with different bacterial, viral, fungal, and parasitic antigens. We have also completed a number of human trials with mLT alone or in combination with killed Campylobacter (37) or killed Helicobacter (17) whole-cell oral vaccines. We were surprised to find that formulations that consisted of whole killed Gram-negative cells and a previously identified safe but effective dose of mLT
adjuvant caused mild diarrhea in approximately 30% of vaccine recipients in both sets of trials. Since neither bacterial nor adjuvant component alone caused diarrhea, we concluded that residual activity of mLIT acted synergistically with LPS from the Gram-negative killed whole cells to induce sufficient fluid movement to overwhelm the resorptive capability of the bowel (i.e., secretion). We therefore looked for an alternative, additional attenuating mutation outside the active site of the molecule. We identified a putative pepsin site within the A2 piece of the A-subunit and constructed a double mutant containing the original R192G mutation and subsequently a second mutation, L211A.

We have a working hypothesis of how the original R192G mutation reduces the enzymatic activity of LT. After internalization, CT and LT are transported by retrograde transport through the Golgi cisternae to the endoplasmic reticulum (ER) (19). Once in the ER, the A1 chain must be transported across the membrane into the cytosol. Evidence suggests that A1 may be transported through the Sec61p channel (31). Tsai et al. (32) demonstrated that protein disulfide isomerase (PDI) in the lumen of the ER functions to disassemble and unfold CT (and presumably LT) once its A-chain has been proteolytically cleaved at Arg192 (38). The unfolding of A1 by this redox-driven chaperone could facilitate the transport of A1 into the cytosol from the ER back into the cytosol, where it interacts with its two substrates, Gsα and NAD. Tsai et al. (38) also demonstrated that proteolytic processing is required for CT to be unfolded by PDI, and this is prevented by the R192G substitution. The failure to unfold LT and CT in the ER could either retard or reduce the transport of the A-subunit to the cytosol or, if the entire A-subunit were transported to the cytosol, interfere with the binding of ADP-ribosylating factors (ARFs) to A1 (36). ARF binding to A1 greatly increases the enzymatic activity of LT-A, and when A2 is present it interferes/alters this interaction (41). Alternatively, there could be some residual but minor cleavage of A1 and A2 intracellularly. Studies are under way in our laboratory to determine which, if any, of these processes actually occur in vivo.

The attenuation of LT that results from the L211A mutation clearly affects a different process. An L211A single mutant has approximately the same safety profile in cAMP and patent adjuvant activity as the single R192G mutant (data not shown). While we originally identified the leucine at position 211 as a putative pepsin site, there is no evidence that it is cleaved by pepsin either in vitro or in vivo (data not shown). We have, however, determined that the double mutant is more sensitive to trypsin or intracellular proteolytic degradation than either native LT or mLIT; there was no proteolytic activation produced by separation of the A-subunit into A1 and A2 but there was complete degradation of the molecule. Since CT and LT hijack the ER-associated protein degradation (ERAD) pathway to reach the cytosol but avoid proteolytic degradation once there (13, 35), it is tempting to speculate that the reduced enterotoxicity of dmLT compared to mLIT is a consequence of its more rapid breakdown in the cytosol.

dmLT was determined to induce significantly less cAMP accumulation in cultured cells than native LT (dmLT is approximately equivalent to mLIT in this 3-h assay) and to completely lack enterotoxicity in the patent mouse assay. The non-dose-responsive secretion seen with 125 μg and 250 μg of mLIT is completely eliminated with the second attenuating mutation at L211. Nonetheless, dmLT has oral adjuvancy for a coadministered antigen equivalent to mLIT and retains the ability to induce anti-LT antibodies. The latter property may be important for inclusion of an LT adjuvant/toxoid in an oral ETEC vaccine. To date, our published studies have shown that dmLT functions as an oral adjuvant in a Helicobacter vaccine (34) and as a sublingual or buccal adjuvant in a whole-cell pneumococcal vaccine in mice (23).

LT and CT have also been examined as adjuvants for coadministered antigens by other mucosal (intranasal, sublingual, and rectal), topical (transcutaneous), and parenteral (intradermal and subcutaneous) routes. Intranasal use of these molecules or their mutants in humans has serious safety risks and has been associated with Bell’s palsy (21, 27). By routes other than intranasal, these molecules must retain some enzymatic activity to maintain their adjuvant function. Although our primary focus at the moment is oral vaccines, dmLT is expected to be effective by other routes, similar to LT and mLIT. There have not been significant safety concerns associated with other immunization routes, but these need to be investigated thoroughly. The oral route of delivery has tremendous promise for both ease of administration and for fewer short- and long-term safety risks, as millions of individuals naturally ingest organisms that produce LT every year and the only specific adverse effect seems to be diarrhea. The key to LT and CT applicability in human vaccines is to be able to reduce enterotoxicity while preserving adjuvanticity. In this study, we have demonstrated that dmLT exhibits both of these properties.

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