Functional Diversity of Heat-labile Toxins (LT) Produced by Enterotoxigenic Escherichia coli

DIFFERENTIAL ENZYMATIC AND IMMUNOLOGICAL ACTIVITIES OF LT1 (hLT) AND LT4 (pLT) 2

Juliana F. Rodrigues 1, Camila Mathias-Santos 1, Maria Elisabete Sbrogio-Almeida 5, Joaquim Cabrera-Crespo 2, Andrea Balan 6, and Luis C. S. Ferreira 4 2

From the 4 Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP 05508-000, the 4 Division of Technological Development, Butantan Institute, São Paulo, SP 05503-900, and the 4 National Laboratory of Biosciences, Research Centre of Energy and Material, São Paulo, SP 13084-971, Brazil

Heat-labile toxins (LTs) have ADP-ribosylation activity and induce the secretory diarrhea caused by enterotoxigenic Escherichia coli (ETEC) strains in different mammalian hosts. LTs also act as adjuvants following delivery via mucosal, parenteral, or transcutaneous routes. Previously we have shown that LT produced by human-derived ETEC strains encompass a group of 16 polymorphic variants, including the reference toxin (LT1 or hLT) produced by the H10407 strain and one group of 16 polymorphic variants, including the reference LT produced by human-derived ETEC strains encompass a also act as adjuvants following delivery via mucosal, parenteral, or transcutaneous routes. Previously we have shown that LT produced by human-derived ETEC strains encompass a group of 16 polymorphic variants, including the reference toxin (LT1 or hLT) produced by the H10407 strain and one variant that is found mainly among bacterial strains isolated from pigs (LT4 or pLT). Herein, we show that LT4 (with six polymorphic sites in the A (K4R, K213E, and N238D) and B (S4T, A46E, and E102K) subunits) displays differential in vitro toxicity and in vivo adjuvant activities compared with LT1. One in vitro generated LT mutant (LTK4R), in which the lysine at position 4 of the A subunit was replaced by arginine, showed most of the LT4 features with an ~10-fold reduction of the cytotoxic effects, ADP-ribosylation activity, and accumulation of intracellular cAMP in Y1 cells. Molecular dynamic studies of the A subunit showed that the K4R replacement reduces the N-terminal region flexibility and decreases the catalytic site crevice. Noticeably, LT4 showed a stronger Th1-biased adjuvant activity with regard to LT1, particularly concerning activation of cytotoxic CD8+ T lymphocytes when delivered via the intranasal route. Our results further emphasize the relevance of LT polymorphism among human-derived ETEC strains that may impact both the pathogenicity of the bacterial strain and the use of these toxins as potential vaccine adjuvants.

Enterotoxigenic Escherichia coli (ETEC) 3 is a major etiological agent of diarrhea, afflicting both young children and travelers in developing countries, with high morbidity and mortality rates. ETEC also causes diarrheal disease in livestock, especially in piglets, representing an economically relevant problem (1, 2). ETEC pathogenicity is directly linked to the production of fimbrial or afimbrial colonization factor antigens and heat-stable and/or heat-labile toxin (LT) (1). LT belongs to the family of AB5 toxins consisting of an enzymatically active A subunit, proteolytically processed into the larger and enzymatic active A1 domain and the shorter A2 domain and five B subunits that mediate binding to glycolipid and glycoprotein receptors of host cells. The B monomer (11.5 kDa) pentamer interacts with the A subunit (28 kDa) via non-covalent binding to the A2 domain. The A1 domain is responsible for ADP-ribosylation of stimulatory G protein, leading to uncontrolled elevation of the intracellular cAMP concentration. Consequently, ion permeases open, and chloride anions and water molecules are released, a hallmark of the watery diarrhea caused by ETEC infection (3).

The structure of LT is closely related to the biological functions of the toxin. The A subunit has an overall globular structure and is linked to the compact cylinder-like structure formed by the five B monomers, which expose the residues involved with binding to the host cell receptors. The enzymatically active A1 domain is linked to the long B helix of the A2 domain by means of a disulfide bridge between the A1-Cys187 and A2-Cys199 residues. The C-terminal portion alone of the A2 domain remains linked to the central cavity of the B oligomer, permitting the transfer of the A1 domain into the host cell cytoplasm following binding of the holotoxin to the cell membrane (4, 5). The toxin is activated following cleavage of a trypsin-sensitive loop and reduction of the disulfide bridge, leaving the free A1 domain. The A1 domain carries the catalytic site, which is delimited by the ADP-binding crevice that binds to NAD and subsequently transfers the ADP-ribose moiety to the target protein (6). Several site-specific mutants contributed to the understanding of LT structural/functional relationships, such as LTK63, in which the serine at position 63 of the A1 domain active site was replaced with lysine (7, 8). This LT mutant shows a complete knock-out of enzymatic activity but preserves the structural features of the parental toxin. Similarly, LTR72 (substitution of alanine to arginine at position 72 of the A1 domain) reduced ~100-fold the enzymatic activity of the toxin as a consequence of the charged
Functional Diversity of LT Produced by Natural ETEC Isolates

In the present study, we show that LT1 and LT4 differ in functional, structural, and immunological features following mucosal administration to mice. Endowed with lower toxic effects, LT4 showed similar mucosal and systemic adjuvant effects for either antibody production or T cell-based immune responses but in contrast to LT1 showed Th1-biased immune responses in mice and stronger CD8+ T cell activation than LT1. Generation of a site-specific single mutant (LTK4R) allowed us to demonstrate that most features of LT4 can be attributed to the replacement of lysine with arginine at position 4 of the A1 domain, which affects the stability of the toxin and reduces the size of the ADP-binding pocket.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Plasmids and LT Purification—The genes encoding the LT1 and LT4 toxins from the H10407 and 1372-1 strains, respectively, were cloned into the PstI and KpnI sites of the pBSPKS(−) vector (24) under the control of their native promoters as described previously (22, 23). pML19 and pKS1372 plasmids, encoding LT1 and LT4, respectively, were used to generate a new LT derivative containing the sequence of LT1 but carrying arginine instead of lysine at position 4 (LTK4R). Both plasmids were first digested with NdeI and PstI enzymes, obtaining two linear DNA fragments (4,513 and 791 kb). The 4.5-kb fragment from the pKS1372 digestion was ligated to the 0.79-kb pML19 fragment comprising the elt operon sequence from position +383 of the eltA gene to the end of the eltB gene, resulting in the pK4R plasmid. Construction of the elt operons encoding the LTK63 and LTR72 toxins (7–9) was performed using the 1.34 kb KpnI-PstI fragment from pML19 as the template for polymerase chain reaction for splicing by overlapping extension, according to previously reported conditions (25). The external oligonucleotides used for PCR-mediated mutagenesis were FwLTpKnl 5′-CACGTTTACCCCTTTTCTTTTATCG-3′ and RvLTpKnl 5′-GTTCTCTGACAGAGACATG-3′. The mutations at positions 63 and 72, corresponding to Ser → Lys and Ala → Arg substitutions, respectively, were introduced using the following internal oligonucleotides: FwLT63 (5′-GTTCAGACTAAACTTATGTTTG-3′) and RvLT63 (5′-CAACCATTTGTGAATCTTTGACG-3′) for S63K replacement and FwLTR72 (5′-AGACTGCTCCTTTAAGAGGACG-3′) and RvLTR72 (5′-AGACTGCTCCTTTAAGAGGACG-3′) for A72R substitution. All LT toxins generated under in vitro conditions were submitted to automatic sequencing to confirm the complete elt operon sequence. The specific amino acid replacements of each tested LT are indicated in Table 1. The mutant operons were cloned into the pBSPKS(−) vector as described above, resulting in the plasmids pK63 and pR72.

Biochemical Evaluation of the Purified LTs—Following purification, LTs were activated by trypsin cleavage. Briefly, each toxin (5 μg) was treated with 150 ng of trypsin (Cultilab) in a final volume of 10 μl of TEAN (50 mM Tris-Cl, 1 mM EDTA, 50 mM NaCl) at 37°C for 15 min. Trypsinated LTs were then incubated for 30 min at 37°C in the presence of sera from BALB/c mice for the LT1a and LT1b strains and of BALB/c mice for the ETBs11 strain. The mixed contents were then centrifuged for 10 min at 10,000 × g, and the supernatants were used for the determination of LT activity.

Several other amino acid residues of the A1 domain also play a direct role on the enzymatic activity of the toxin, such as glutamic acid residues at positions 110 and 112 or arginine at position 7 (7, 10).

In addition to its role in ETEC pathogenesis, LT also has a long record of immunological uses based on the strong adjuvant effects exerted in mice following injection via parenteral, mucosal, and transcutaneous routes. Co-administration of LT with antigens results in increased production of antigen-specific mucosal (IgA) and systemic (IgG) antibodies as well as activation of T lymphocytes (11, 12). To avoid the mucosal toxicity exhibited by the native toxin, different LT mutants with reduced toxic effect but partially preserved adjuvanticity were tested in the murine model (13, 14). Among the several LT mutants generated under laboratory conditions, LTK63 and LTR72, with no or drastically reduced enzymatic activity, and LTR192G, lacking the trypsin cleavage site, have been intensively investigated as potential mucosal adjuvants in the mouse model (8, 9, 15).

Previous studies demonstrate that LT produced by ETEC strains isolated from humans and animals show significant natural polymorphisms. Two major LT groups have been defined based on distinct amino acid composition and biological activities. Type I LTs include the reference LT produced by the ETEC H10407 strain and cholera toxin (CT) produced by Vibrio cholerae, whereas type II LTs (LT-IlA and LT-IlB), produced mainly by ETEC strains isolated from nonhuman hosts, differ drastically in the B subunit amino acid sequence, showing distinct receptor affinity and immunological features (16, 17). Among the LT-I toxins, a major distinction has been established between toxins produced by ETEC strains isolated from humans (hLT) or pigs (pLT). pLT differs from hLT at six amino acid positions including three polymorphic sites at the A subunit (K4R, K213E, and N238D) and three polymorphic sites at the B subunit (S4T, A46E, and E102K) (18, 19). pLT and hLT also differ in biochemical (electrophoretic mobility) and antigenic features (20, 21).

Recently, we described the occurrence of 16 natural LT-I variants (LT1–LT16) among ETEC strains, isolated from diarrheal and asymptomatic human subjects (22). LT1, the most frequently found LT type, includes the reference LT produced by the H10407 strain (which in addition carries a unique polymorphic site, N238D, at the A subunit) as well as LT expressed by bacterial strains belonging to the serotypes most frequently associated with diarrhea. LT2 was the second most frequently expressed LT type by diarrheogenic ETEC strains and shares similar toxic and immunological features with LT1 (23). One LT variant, named LT4, showed the same amino acid composition as the previously reported pLT, and in vitro and in vivo results indicated that this LT type has reduced toxicity with regard to the most ubiquitous LT1 and LT2 types (22). Of note, pLT has been used for definition of the LT tertiary structure and as a template for the generation of nontoxic or attenuated LT derivatives used as vaccine adjuvants (4, 5, 7–9). In fact, during the last three decades, pLT and hLT were considered similar both with regard to their toxic effects and immunomodulatory activities in murine hosts, but no direct comparison of the two toxins has been reported so far.
3 mM NaN₃, and 0.2 mM NaCl, pH 7.4) at 37 °C. After 30 min, the samples were boiled for 5 min for characterization by SDS-PAGE or treated with 60 ng of trypsin inhibitor (Sigma) for the ADP-ribosylation assays. Determination of pH stability was carried out with diluted LT samples (5 ng/μl) in a final volume of 100 μl of 0.1 M Na₂HPO₄, and sodium citrate buffer was adjusted to pH levels ranging from 1 to 9. Following 2 h at room temperature, 10 μl samples were diluted in 90 μl of PBS, pH 7.0, and applied to wells of microtiter plates that were previously coated with GM1 followed by blocking with PBS (0.1% BSA). The GM1-ELISA was performed according to Lasaro et al. (22), using polyclonal antibody or monoclonal antibody LT39:13:1 (LT39) (supplied by Dr. J. Holmgren, University of Gothenburg, Institute for Vaccine Research, Gothenburg, Sweden), which recognize conformational epitopes of the A and B subunits, respectively.

### Determination of Cytotoxic Activity of Purified Toxins

Mouse Y1 adrenal tumor cells were seeded in 96-well microtiter plates at a concentration of 5 × 10⁴ cells/well at 37 °C in a 5% CO₂ atmosphere. Following 16–18 h of incubation, the cells were used for evaluation of the cytotoxic effects induced by the tested toxins by means of cell morphology changes and determination of the intracellular cAMP concentration. In the first experiment, 100 μl of serial 2-fold dilutions of the purified toxins were inoculated into plates containing the Y1 cells, as reported previously (26). The plates were incubated at 37 °C for ~8 h and then analyzed for morphological changes (cell rounding effect) induced by the tested toxins. The LT concentrations causing morphological changes in 50% of the treated cells were determined for each tested toxin. In the second assay, 1 μg of each toxin was treated with 12 ng of trypsin in TEAN at 37 °C for 30 min and transferred to wells of microtiter plates with the cells. After incubation for different periods, the Y1 cells were subjected to complete lysis, and the intracellular cAMP concentrations were determined with the Amersham Biosciences cAMP enzyme immunoassay system. The concentrations of cAMP (pmol/ml) were calculated according to the manufacturer’s instructions.

### LT Cell Binding Assay

Mouse Y1 adrenal tumor cells (1 × 10⁶), diluted in 100 μl of DMEM with 10% FBS, were treated with different amounts of LT1 or LT4 ranging from 1 to 1,000 ng for 30 min. After incubation, the cells were fixed with 4% paraformaldehyde for 10 min and incubated with anti-LT serum (titer of 1 × 10⁵) diluted (1/500) in PBS containing 1% FBS (PBS-FBS) for 30 min. The cells were incubated with FITC-conjugated anti-mouse IgG (Invitrogen) at a dilution of 1/100 for 30 min and were suspended in 100 μl of PBS-FBS. The cells were washed three times in PBS-FBS at 4 °C after each procedure mentioned above. The stained cells were detected by FACS (Becton Dickinson, Mountain View, CA), and the data were analyzed using the FlowJo program.

### ADP-ribosylation Assay

The ADP-ribosylation activity of the purified LTs was measured by determination of 32P-NAD incorporation into TCA-precipitated material using synthetic poly-L-arginine as substrate. The reactions were performed at 25 °C for 2 h in 10 μl of 25 mM sodium phosphate buffer, pH 7.0, containing 20 mM dithioerythritol, 2 μM 32P-NAD (9.25 MBq, 29.6 TBq/mmol), 10 μg of poly-L-arginine (Sigma) (27), and 1–10 μg of toxin activated with trypsin as described above. The precipitation reaction with TCA was carried out as previously reported by Chung and Collier (28), and incorporated radioactivity was determined in a liquid scintillator.

### NAD⁺ and NADH Degradation Activity

The NAD⁺ and NADH degradation assays were based on previously described procedures (29–31). Briefly, the activated LTs (10 μg) were added to sodium phosphate buffer, pH 7.5, containing β-NAD⁺ or NADH (Sigma-Aldrich) at final concentration of 100 and 140 μM, respectively. Following different incubation times at 25 °C, NADH concentration was determined by reading the absorbance at 340 nm, and calculation of the remaining substrate concentration was determined after establishment of a standard curve. For the NAD⁺ hydrolysis assay, the reactions were performed at 37 °C and interrupted by KCN addition at different time points. NAD⁺ concentrations were determined after measurement of the absorbance at 340 nm based on a previously determined standard curve.

### Structural Homology Modeling of LT1 and LTK4R

Three-dimensional models of the A1 subunits of LT1 and LTK4R were generated using the Modeler 9v2 program (32) based on the atomic coordinates of the E. coli heat-labile enterotoxin (Protein Data Bank code 1LTS) (5). The three first residues of the N-terminal, which were missing in 1LTS model, were added to the models. Ten different models were built for each protein, and the simulated annealing procedure was applied to every model. The best one, according to the MODELLER objective function, PROCHECK (33), and Ramachandran plot statistics, was chosen for subsequent optimization. The overall stereochemical quality of each model was also evaluated with the residue corrections using COOT (34) and secondary structure matching superposition of the models according to Krissinel and Henrick (35). The structural model figures were prepared with Pymol (36).

### Molecular Dynamics

Possible movements and secondary structure changes of the LT1 and LTK4R models were determined in molecular dynamic simulations with the GROMACS 4.0 package (37). Simulations of 10 ns were performed using the solvated proteins. Each model was immersed into a cubic box filled with water molecules. To relax the binding geometries, the energy potential of the system was minimized using the steepest descent method until convergence. Simulations were performed using the GROMOS43a1 force field and the SPC solvent model. LINCS (38) and Particle Mesh Ewald algorithms were used for constraining of the bond lengths and to calculate the electrostatic interactions, respectively. The

### Table 1

| Toxin    | A1 domain | A2 domain | B subunit |
|----------|-----------|-----------|-----------|
| LT1      | Lys Ser   | Ala Lys   | Asn Ser   | Ala Glu   |
| LT4      | Arg Ser   | Ala Lys   | Asp Thr   | Glu Lys   |
| LTK4R    | Arg Ser   | Ala Lys   | Asn Ser   | Ala Glu   |
| LTR72    | Lys Ser   | Arg Lys   | Asn Ser   | Ala Glu   |
| LTK63    | Lys Lys   | Ala Lys   | Asn Ser   | Ala Glu   |

*The positions correspond to the amino acid sequence of the mature protein (without signal sequence).*
temperature was kept constant using a Berendsen thermostat. After energy minimization, the system was subjected to thermalization using temperatures from 50 K up to 250 K. Trajectories were analyzed using GROMACS (37) and VMD (39).

**Mouse Immunization Experiments**—Female C57BL/6 mice aged 8–12 weeks were supplied by the Isogenic Mouse Breeding Facility of the Department of Parasitology of the Institute of Biomedical Sciences at São Paulo University. The animals were subjected to light anesthesia (75 mg/kg ketamine (10%) plus 10 mg/kg xylazine (2%)) and inoculated via the intranasal route with PBS (10 μl) containing 30 μg of ovalbumin (OVA) (Sigma) alone or combined with 10 μg of toxin (LT1, LT4, LTK4R, LTR72, or LTK63). The immunization regimen consisted of three doses given at 2-week intervals. Bleeds from the retro-orbital plexus were performed 1 day before each inoculation and 2 weeks after the last dose. Two weeks after the last dose, the animals were euthanized, and lung washes and spleens were collected individually. For lung washes, PBS (1 ml) was injected into the lungs and then aspirated through an incision in the trachea. The serum samples and lung washes were stored at −20°C until the detection of antibodies by ELISA, whereas the spleens were processed immediately to obtain spleen cells.

**Quantitation of Antibodies by ELISA**—LT-specific serum IgG and mucosal IgA titers were determined by GM1-ELISA (40), whereas anti-OVA antibody levels were evaluated by conventional ELISA (41). Serum anti-LT and anti-OVA IgG (total IgG and IgG1/IgG2 subclasses) titers were measured with a horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Sigma-Aldrich). The assays were carried out in duplicate samples and repeated at least three times. The results are expressed as titers, defined as the highest sample dilution with an A_492 nm ≥ 0.2 above preimmune sera or lung washes.

**Cytokine Measurements**—For evaluation of cytokine production, the animals were euthanized 2 weeks after the immunization protocol, and spleen cells were harvested according to a previously reported protocol (42). Spleen cells (1 × 10^7 viable cells/ml) were cultured in a CO_2-containing atmosphere at 37°C in the presence or absence of OVA (5 μg/ml), a class I (K^b^-restricted CD8^+ T cell peptide epitope of OVA (257–264), named OVA_{257–264}, or a class II (I^-A^b^-restricted CD4^+ T cell peptide epitope of OVA (265–273), named OVA_{265–273}, respectively. Finally, the color reaction was developed for 5–10 min using diamobenzidine as the substrate (Sigma-Aldrich). The slides were counterstained with Mayer’s hematoxylin and examined by optical microscopy. Representative fields were photographed with a digital camera (ColibriX4500; Nikon).

**Statistical Evaluation**—Statistical analyses were performed using the Student’s t test for comparisons of means. The p values <0.05 were considered statistically significant.

**Ethics Statement**—All of the handling procedures and experiments involving mice were approved by the committee on ethical use of laboratory animals from the Institute of Biomedical Sciences of University of São Paulo in accordance with the recommendations in the guidelines for the care and use of laboratory animals of the National Committee on the Ethics of Research.
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RESULTS

In a previous study, we reported that one LT type (LT4) produced by an ETEC strain isolated from one asymptomatic child in Brazil has the same sequence as pLT, originally found among ETEC strains isolated from swine hosts (22). Our findings also indicated that LT4 showed decreased toxicity, both under in vitro and in vivo conditions, when compared with the reference LT (LT1 or hLT) expressed by human-derived ETEC strains, particularly by the reference ETEC H10407 strain (22). Indeed, LT4 showed an ~10-fold reduction in cytotoxic activity in Y1 cells when compared with LT1 (16.7 ng/ml of LT4 and 1.9 ng/ml of LT1 caused the cell rounding effect in 50% of the cells) (Fig. 1). The same result was observed with whole bacterial cell lysates in which the amount of LT4 has been determined by GM1 ELISA (data not shown). Two previously reported nontoxic LT-derivatives, LTK63 and LTR72 (generated by site-specific mutagenesis using the LT1 sequence as template) (8, 9), were also tested under the same conditions and showed no or much reduced toxicity to Y1 cells, respectively (Fig. 1). LT1 and LT4 differ in three polymorphic amino acids in the A subunit: K4R, K213E and N238D. The K213E and N238D are shared by other LT types with toxicity similar to LT1. Furthermore the asparagine at position 238 is present only in the LT encoded by the H10407 strain. Thus, we generated a LT1 derivative with the amino acid replacement at position 4 of the A1 domain (lysine replaced by arginine) (LTK4R). As shown in Fig. 1, LTK4R has similar cytotoxic effects when compared with LT4 (Fig. 1).

To further investigate the biological properties of LT4 and LTK4R, we measured the amount of intracellular cAMP that accumulated in Y1 cells treated with different concentrations

FIGURE 1. Reduced in vitro cytotoxic effects of LT4 and LTK4R in cultured Y1 cells. A, Different amounts of purified toxins were added to microplate wells seeded with Y1 cells (5 × 10⁴ cells/well). The cells were incubated for 8 h at 37 °C and inspected for altered morphology using a reverse microscope. The samples were: NC (Y1 cells without addition of toxin as negative control), LT1 (1.9 ng/ml), LT4 (16.7 ng/ml), LTK4R (15 ng/ml), LTR72 (310 ng/ml), and LTK63 (1,000 ng/ml). The toxin concentrations shown in the insets represent the amounts of LT causing cytotoxic effects in 50% of the Y1 cells following an incubation period of 8 h.

FIGURE 2. LT4 and LTK4R show reduced enzymatic activity but preserved receptor binding affinity and stability at different pH levels when compared with the reference LT produced by the ETEC H10407 strain. A, cAMP production of different tested LTs. Except for the LT sample in lane 1, all of the LT aliquots were treated with trypsin (6 ng/μl for each 1 μg of toxin) for 30 min at 37 °C. All of the samples were separated on 17% polyacrylamide gels and stained with Coomassie Brilliant Blue. Lanes 1 and 2, LT1; lane 3, LT4; lane 4, LTK4R; lane 5, LTR72; lane 6, LTK63. MW, PageRuler unstained protein ladder (Fermentas). B, cAMP (pmol/ml) levels measured in Y1 cell lysates following incubation with the tested LTs. cAMP concentrations were measured in Y1 cells following exposure to 1 μg of the tested LT for 1 h. The values are represented as the means ± S.D. of three independent experiments. The results obtained with cells exposed to LT4 and LTK4R were significantly different from those detected with cells exposed to LT1 (*, p < 0.01). C, LT4 and LTK4R show reduced ADP-ribosylation activity with regard to LT1. The assays were carried out with poly-L-arginine as the substrate and 32P-NAD as the co-factor. LT1, LT4, and LTK4R were tested at concentrations ranging from 1 to 10 μg/reaction. The values are expressed as the means ± S.D. of three independent experiments. The samples were: LT1 (■), LT4 (▲), and LTK4R (●). The results obtained with LT1 were significantly different from those measured with LT4 and LTK4R (**, p < 0.002; ***, p < 0.04). D, binding of LT1 and LT4 to Y1 cells. The cells were incubated with the tested LT (1 μg) for 1 h and labeled with mouse anti-LT and FITC-conjugated anti-mouse IgG antibodies before analysis of emitted fluorescence intensity in a flow cytometer. The samples were: LT1 (■), LT4 (▲), and LTK4R (●). E, binding of LT4 and LTK4R to Y1 cells. The samples were treated as described in E, except for the use of a B subunit-specific monoclonal antibody in the GM1-ELISA. The samples were: LT1 (■), LT4 (▲), and LTK4R (●).
of trypsin-activated toxins (Fig. 2, A and B). Under the tested experimental conditions, LT4 and LTK4R did not show any differential sensitivity to trypsin activation, but only one-fourth of the LT1 effect was observed (5.37 nmol of cAMP/ml for LT1 and 1.44 and 1.41 nmol of cAMP/ml for LT4 and LTK4R, respectively) (Fig. 2, A and B). Under the same conditions, LTR72 showed a stronger attenuation (161 pmol of cAMP/ml), whereas LTK63 showed no significant activity (Fig. 2B). Further evidence of the reduced toxicity of LT4 was obtained in ADP-ribosylation experiments. Using 

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{^{32}P-NAD}^\text{+}
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as the co-factor and poly-l-arginine as the substrate, LT4 and LTK4R showed a similar 10-fold decrease in ADP-ribosylation activity when compared with LT1 (Fig. 2C). As expected, LTK63 and LTR72 showed no or much reduced activity, respectively, compared with LT1. Because LT4 displays three additional polymorphic sites in the B subunit (S4T, A46E, and E102K), we would like to know whether the reduced toxicity could reflect, at least in part, reduced binding to surface-exposed cell receptors. LT4 and LT1 displayed the same binding affinity to Y1 cells, as demonstrated following incubation of cells with the purified proteins and fluorescence detection in a flow cytometer (Fig. 2D). Finally, we measured the stability of the A and B subunits of the toxins incubated at different pH levels. As indicated in Fig. 2, no significant difference could be observed in the stability of the A and B subunits of LT1, LT4, and LTK4R upon incubation at pH values ranging from 1 to 9.

To correlate the functional differences observed between LT1 and LT4 with specific structural alterations, we modeled both the LT1 and LTK4R A1 domains based on the LT crystal structure available from the Protein Data Bank (Protein Data Bank code 1LTS) (5). Two structural models for the LT1 and LTK4R A1 domains were generated with molecular modeling tools, and the Ramachandran plots showed that more than 95% of the residues were positioned in the most favored regions, and none were located in disallowed regions (data not shown). Superposition of the two models showed that LTK4R and LT1 exhibited the same three-dimensional structure (root mean square deviation of 0.1 Å), but the pattern of hydrogen bonds at the N-terminal end was changed (Fig. 3). Essentially, two new bonds are present in the LTK4R model: Arg4/NH2 with the hydroxyl group of the Tyr145 and Asn1/N with the oxygen of Arg151, suggesting that the N-terminal domain of this protein is less flexible than the same region in LT1 (supplemental Table S1). Indeed, additional interactions of the LTK4R B1 strand residues (which include Arg3 and the loop covering the NAD-binding pocket) further suggest that this region shows structural differences compared with LT1 (supplemental Table S1). To investigate how such altered bond patterns at the A1 N-terminal end would affect the structure of overall A1 domain and particularly the NAD-binding pocket, molecular dynamic simulations were performed with LT1 and LTK4R. First, each model was minimized to 30 ps and then subjected to the dynamic simulation for 10 ns. The results were recorded to permit evaluation of: (i) structural changes to the A1 domain, (ii) flexibility of the N-terminal region (amino acids 1–6), and (iii) movement of the β-sheet strand (B1) facing the active site crevice (supplemental Fig. S1). After the simulation, both proteins acquired different conformations, as demonstrated by the root mean square deviation values determined from the initial to the final models (2.0 and 2.8 Å, respectively, for LTK4R and LT1 initial models, and 3.2 Å between the final models). The dynamic simulation data demonstrated that LTK4R took a shorter time period than LT1 to achieve a minimum energy state, confirming the reduced flexibility of the LTK4R A1 domain (supplemental Fig. S1, A and B). In addition, the dynamic simulation showed that the N-terminal residue interactions present in the LTK4R A1 domain (including those formed by Asn1, Gly2, and Arg3) helped to stabilize the protein when compared with LT1 (Fig. 4B). Such interactions in the LTK4R N-terminal end further accentuated the displacement of the β1 strand, affecting interactions and the size of the NAD-binding pocket (Fig. 4). Displacement of the β1 strand in LTK4R places the Arg7 residue into a position that favors its coordination through five interactions with four residues (Phe52, Val53, Arg54, and Ser56) (Fig. 4C and supplemental Table S1), leading to a contraction of the loop encompassing residues 52–55 and reducing the NAD-binding crevice. In contrast, in LT1 this loop is displaced away, and Arg7 makes just one contact with Val53 (Fig. 4D and supplemental Table S1). The calculated contact area of residues at the NAD-binding pocket was reduced from 1,145 Å² for LT1 to 1,108 Å² for LTK4R. The positioning of the important residues Ser61 and Ser63 is also changed in both proteins (Fig. 4, C and D).

To determine whether the predicted structural alteration of the NAD-binding pocket would cause a significant impact in the enzymatic activity of LT4 and LTK4R, we measured the

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\text{in vitro NAD}^+\text{ hydrolyzing activity of the toxins. As expected}
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from previous observations (31), the LT1 NAD$^+$ hydrolyzing activity showed low kinetics, and only 14% of added substrate was degraded following 18 h of incubation. Interestingly, under the same experimental conditions, LT4 and LTK4R showed an approximately 50% reduction of the enzymatic activity of LT1, and 7% of added NAD$^+$ was degraded at the same time point (supplemental Table S2). We also have compared the reactivity of LT1, LT4, and LTK4R using NADH as substrate, and again, LT1 showed a higher \textit{in vitro} decrease in the NADH concentration when compared with LT4 and LTK4R submitted to the same experimental conditions (supplemental Table S2). Thus, the experimental data support the conclusions drawn from the molecular dynamic simulations and indicated that the differential toxicities of LT4 and LTK4R reflect a small but biologically significant alteration of the NAD-binding pocket of the toxins with regard to the reference LT1.

We also evaluated the different immunological properties of LT1 and LT4, including their mucosal immunogenicity and adjuvant effects. C57BL/6 mice were immunized intranasally with three doses of OVA co-administered with one of the tested LTs, and the anti-LT and anti-OVA serum (IgG) and lung wash (IgA) antibody titers were measured. As indicated in Fig. 5, both LT4 and LTK4R retain their immunogenicity, as demonstrated by the anti-LT serum IgG and lung wash IgA titers. The anti-LT IgG responses detected in mice immunized with LTK63 and LTR72 were approximately 1 order of magnitude lower than the titers observed in mice immunized with LT1, LT4, or LTK4R (Fig. 5A). Mice immunized with LT1, LT4, or LTK4R elicited similar anti-OVA serum antibody responses, whereas lower quantities of anti-OVA IgA antibodies were detected in lung washes of mice immunized with OVA and LT4 (Fig. 5). The anti-OVA serum and mucosal antibody titers in mice immunized with OVA mixed with LTK63 or LTR72 were significantly lower. These results indicated that LT4, and particularly LTK4R, have preserved immunogenicity and adjuvant effects overall with respect to LT1 and are more potent than LTK63 and LTR72 with regard to the generation of serum and mucosal antibodies.

In addition to enhancing the amount of antibodies elicited in vaccinated mice, LTs modulate the pattern of immune responses by means of selective T helper cell activation. Under our experimental conditions, LT1, LTK4R, and LTR72 induced similar IgG1/IgG2c subclass profiles regarding serum anti-LT antibodies, whereas mice immunized with LT4 or LTK63 induced a more biased Th1 pattern with lower IgG1/IgG2c profile (Fig. 5E). Similar results were also observed for the anti-OVA IgG subclass responses, and again mice immunized with LT4 developed a more Th1-biased response than mice immunized with LT1 (Fig. 5F). To further demonstrate the different immunomodulatory properties of LT1 and LT4, we measured some cytokines secreted by spleen cells harvested from immunized mice usually employed as indicators of the induced Th-type response pattern. The total amount of
FIGURE 5. LT4 and LTK4R show preserved immunogenicity and adjuvanticity compared with the reference LT1. C57BL/6 female mice were immunized intranasally with three doses of OVA alone (30 μg) or OVA co-administered with one of the tested LT (LT1, LT4, LTK4R, LTR72, or LTK63) (10 μg). A–D, individual serum samples or pooled lung wash samples, collected 2 weeks after the last immunization dose, were tested for serum IgG (A and B), mucosal IgA (C and D), anti-LT (A and C), and anti-OVA (B and D) levels. The bars represent the mean antibody titers detected in sera or in lung washes of animals subjected to the different immunization regimens. The antibody titer values detected in mice immunized with OVA only were deducted from the results obtained with animals immunized with OVA in the presence of the tested LTs. *, p < 0.05 compared with LT1. Each ELISA determination was repeated at least twice, and each immunization group was carried out with at least six animals. E and F, serum IgG1 and IgG2c subclass titers were determined in mice immunized with OVA and one of the tested toxins, both for anti-LT (E) and for anti-OVA (F) antibodies. IgG1 (black columns) and IgG2c (white columns) subclass titers are representative of three assays performed independently and were measured in pooled serum samples harvested from mice subjected to the same immunization regimen. The error bars in E and F represent the means ± S.E. of the antibody titers measured in the serum pool harvested from each immunization group.
INF-γ detected in culture supernatants of cells from mice immunized with OVA plus LT1, LT4, or LTK4R were approximately 1 order of magnitude higher than the levels detected in mice immunized with LTK63 and LTR72 following in vitro stimulation with purified OVA. Moreover, supporting the data based on the serum IgG subclass responses, mice immunized with LT4 or LTK63 elicited more biased Th1 responses with INF-γ/IL5 ratios of 10.6 and 12.3, respectively, compared with mice immunized with LT1, LTK4R, or LTR72 with INF-γ/IL5 ratios ranging from 2.7 (LTR72) to 3.6 (LTK4R) (Fig. 6A). Similar results were observed when the levels of secreted IL-10 were measured, whereas no significant IL-4 levels could be detected in culture supernatants of spleen cells harvested from the different immunization groups (Fig. 6D and data not shown). These results further indicated that LT4 promotes a more Th1-biased immune response than LT1 and LTK4R.

To establish the T cell populations involved in INF-γ production by OVA-stimulated spleen cells following immunization with LT1 or LT4, the cells were stimulated with peptides corresponding to the dominant CD8+ T cell-restricted (OVA257–264) and CD4+ T cell-restricted (OVA265–280) epitopes specifically recognized by MHC-I or MHC-II molecules of C57BL/6 mice, respectively. The data show that CD8+ T cells are mainly involved in the secretion of INF-γ following stimulation with OVA, CD4+ T cells are the main IL-5 producers, and both CD8+ and CD4+ T cells are involved in the secretion of IL-10 detected in spleen cell culture supernatants of mice immunized with LT1, LT4, and LTK4R (Fig. 6).

Next, we determined the activation of antigen-specific CD8+ T cell responses in mice immunized with different LTs using two experimental approaches. First, we measured the total number of INF-γ-secreting CD8+ T lymphocytes by ELISPOT assay carried out with spleen cells stimulated with the OVA257–264 peptide (Fig. 7A). The results show that LT1, LT4, and LTK4R have similar adjuvant activity concerning activation of CD8+ T lymphocytes. Mice immunized with LTR72 or LTK63 elicited 5- or 10-fold lower CD8+ T cell activation, respectively, following stimulation with the same peptide. We also measured the cytotoxic activity of OVA-specific CD8+ T lymphocytes using an in vivo approach based on the lysis of CFSE-labeled cells previously exposed to the OVA257–264 peptide. As shown in Fig. 7B, mice immunized with LT1, LT4, and LTK4R developed higher CD8+ T cell cytotoxic responses when compared with mice immunized with LTK63 and LTR72. Mice immunized with LT4 developed the highest cytotoxic response (45% lysis), followed by mice immunized with LTK4R (39% lysis) and LT1 (35% of lysis). These results demonstrated that LT1, LT4, and LTK4R presented similar adjuvant effects required to activate a cytotoxic CD8+ T cell response in mice.

At present, one of the main concerns regarding the clinical use of LT or CT as mucosal adjuvants is the observed neurotoxicity following administration via the intranasal route. As shown in Fig. 8, greater amounts of toxin are detected in the olfactory bulbs of mice treated with LT1, whereas mice treated with LT4 and LTK4R have much lower toxin counts in the olfactory bulbs 24 h after admin-
These results indicated that LT1 and LT4 differ in nervous system tropism.

**DISCUSSION**

Type I heat-labile toxins produced by ETEC strains encompass a group of genetically and functionally conserved proteins presenting similar enzymatic and immunomodulatory activities. Previous evidence has shown that the genetic diversity of LT produced by ETEC strains derived from humans is rather high, encompassing 16 toxin types distinguished by the presence of at least one polymorphic amino acid residue in either the A or B subunits (22). In addition, our initial observations suggested that one LT variant (LT4), sharing the same amino acid sequence as the previously reported pLT, would be less toxic than the prevailing LT types (LT1 and LT2), encoded by bacterial strains isolated from subjects with diarrhea (22). In this study, we reported that purified LT4 shows a 10-fold reduction in ADP-ribosylation activity, leading to lower cAMP accumulation in cultured Y1 cells without any measurable alteration on either cell binding activity or overall stability. These findings indicate that the polymorphic sites located at the B subunit are not responsible for the reduction in LT4 toxic effect. In fact, the lower enzymatic activity of LT4 was specifically ascribed to the presence of an arginine residue at position 4 of the A1 domain (corresponding to lysine in the LT1 sequence) that conferred a less flexible N-terminal region and a more complex bond profile that results in a decrease in the NAD-binding pocket. Despite the reduced toxic activity, LT4 showed no significant reduction in adjuvant function. Indeed, LT4 had a stronger Th1-biased immunomodulatory effect, resulting in a more efficient activation of antigen-specific cytotoxic CD8\(^{+}\) T cells compared with LT1 and previously reported attenuated LT forms (LTK63 and LTKR72). Collectively, the present data demonstrate that LT produced by ETEC strains represent a group of genetically and functionally diverse proteins that may impact both the epidemiology of the disease and the potential biotechnological applications of the toxins as vaccine adjuvants.
region, changing the conformation of the active site and reducing the size of the NAD-binding crevice. These structural molecular data are supported by experimental observation that LT4 and LTK4R have lower NAD-hydrasising activity in comparison with LT1. In addition, the presence of arginine at position 4 conferred a decreased mobility of the A1 domain under dynamic simulation conditions, suggesting that although less toxic, LT4 achieves a more energetically stable condition when compared with LT1.

A similar amino acid replacement in a nearby position, substitution of arginine with lysine at position 7, has a more dramatic impact on the enzymatic activity of the toxin (8, 43). Despite a direct interaction with NAD, the arginine at position 7 also contributes to the displacement of the loop (residues 47–56) that covers the NAD-binding crevice. Thus, our results indicate that both arginines are involved in the positioning of the 47–56 loop, but in contrast to Arg7, replacement of Arg4 with Lys enhances the enzymatic activity of the toxin by giving more mobility to the A1 domain, which favors opening of the active site crevice and interaction with a cofactor molecule. Further structural comparisons of the two toxins under inactive and activated conditions and in the presence of the NAD molecule might reveal further structural features that may correlate with the observed functional differences between LT1 and LT4.

The LT adjuvant effects reflect the combined contribution of both A and B subunits as well as the administration route. Via the mucosal route (intranasally), recombinant nontoxic LT derivatives induce strong antigen-specific humoral responses when co-delivered with soluble antigens (44). In contrast to GM1 binding-deficient LT mutants, loss of the ADP-ribosyltransferase activity does not impair the adjuvant effects, as demonstrated by the LTK63 (44–46), but reduces the immunomodulatory potency with regard to the native toxin. Our results indicated that a 10-fold reduction of the enzymatic activity did not reduce either the immunogenicity or the adjuvant activity of LT4 or LTK4R with regard to the LT1. More relevantly, the cytotoxic CD8+ T cell adjuvant effects of LT4 and LTK4R were similar or higher than that detected with the reference toxin, a feature that is significantly reduced both in LTK63 and LTR72 (47, 48). T cell adjuvants are key components for any vaccine formulation aiming induction of prophylactic and/or therapeutic protection to chronic or degenerative diseases. Although reduced residual toxicity or a lack of residual toxicity represents an essential feature of LT derivatives to be delivered via a mucosal routes, LT derivatives with enhanced T cell adjuvant effects might represent important tools for the development of vaccine formulations delivered via parenteral routes in which the inherent LT toxicity does not represent a significant concern.

There is a growing interest in the discovery of vaccine adjuvants capable of stimulating type 1 immune responses that favor activation of T cells required for the control of intracellular pathogens and tumors. LT derivatives are known to elicit balanced Th1/Th2 immune responses, whereas CT derivatives clearly favor Th2-biased responses (14). Our data show that mice immunized with LT4 favored a type 1-biased immune response when compared with mice immunized with LT1 and LTK4R, suggesting that amino acid residues at other positions, either at the A or B subunits, play a relevant role on the immunomodulatory properties of LT. A better understanding of the impact associated with specific residues involved in the immunomodulatory role of LT, as well as in inhibition of NF-κB production (49), will certainly help gain knowledge of the molecular mechanisms associated with the adjuvant effects of these molecules. Additionally, this will contribute to the more rational development of reagents, allowing for activation of tailored immune responses. In this aspect, the notion that natural LT derivatives may offer hints for the discovery of new immunological reagents emphasizes the importance of studies dealing with detection and functional characterization of virulence-associated factors of ETEC strains.

Previous experimental and clinical evidence demonstrated that LT derivatives administered via the nasal route show central nervous system tropism and tissue injuries that may be ascribed to the facial paralysis experienced by some individuals submitted to nasal instillations with nontoxic LT derivatives (50–52). One promising alternative to avoid such side effects might be the use of recombinant B subunit supplemented with a trace amount of the holotoxin (53) or the finding of LT-derivatives with lower nervous tissue tropism. In the present study, LT4 and LTK4R showed a greatly reduced neuronal tropism in mice inoculated via the intranasal route compared with LT1. So far, no specific structural or functional feature has been clearly associated with the tissue tropism of these toxins, but the finding that natural LT variants show decreased tropism to nervous cells should help to elucidate particular features of this interesting cellular interaction feature displayed by different LT forms.

One of the most relevant contributions of the present study was the observation that purified pLT (LT4) displays distinct biological and immunological characteristics in regard to hLT (LT1). Such a finding contrasts with the established role of pLT on the induction of secretory diarrhea in pigs and with recent evidence based on piglets inoculated with isogenic recombinant ETEC strains expressing either pLT or hLT (54, 55). Because previous studies regarding pLT effects were mainly based on live ETEC cells or whole cell lysates and considering the prevailing notion that both toxins were supposedly identical in their biological activities, it is not surprising that studies aiming the biochemical and immunological characterization of hLT and pLT using equimolar amounts of purified toxins were not reported previously. In addition, it may be expected that a small reduction in the pLT toxic activity with regard to hLT would have an undetectable in vivo impact in a susceptible host.

In the past, type I LTs produced by ETEC strains have been considered a group of toxins devoid of any relevant genetic diversity. The present observations, in conjunction with our previous evidence (22), show that LTs produced by wild ETEC strains isolated from human hosts represent a group of genetically distinct toxin types, and at least some of them express distinct functional characteristics. The present findings indicate that deeper analyses of LT natural genetic variants may offer interesting insights about the complex epidemio-
logical features of ETEC-associated diarrhea and the discovery of new biotechnological uses for these remarkable bacterial toxins.

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