# A Genetically Detoxified Derivative of Heat-labile *Escherichia coli* Enterotoxin Induces Neutralizing Antibodies against the A Subunit

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## Summary

*Escherichia coli* enterotoxin (LT) and the homologous cholera toxin (CT) are A-B toxins that cause travelers' diarrhea and cholera, respectively. So far, experimental live and killed vaccines against these diseases have been developed using only the nontoxic B portion of these toxins. The enzymatically active A subunit has not been used because it is responsible for the toxicity and it is reported to induce a negligible titer of toxin neutralizing antibodies. We used site-directed mutagenesis to inactivate the ADP-ribosyltransferase activity of the A subunit and obtained nontoxic derivatives of LT that elicited a good titer of neutralizing antibodies recognizing the A subunit. These LT mutants and equivalent mutants of CT may be used to improve live and killed vaccines against cholera and enterotoxinogenic *E. coli*.

Heat-labile enterotoxin (LT)[1] and cholera toxin (CT) are homologous proteins that cause intestinal fluid accumulation (1–3). CT is produced by *Vibrio cholerae* and is responsible for cholera, an epidemic diarrheal disease causing over 150,000 deaths each year (4, 5). LT is produced by enterotoxigenic *Escherichia coli* (ETEC), which cause most cases of travelers' diarrhea, and, in developing countries, are responsible for approximately two episodes of diarrhea in each child per year (4).

Both toxins are encoded by an homologous operon containing two overlapping genes coding for the A and B subunits, which contain the toxic and receptor binding domains, respectively (6–8). After synthesis the two subunits are exported to the periplasm where they are assembled into the final structure which contains five copies of the B subunit assembled in a compact ring-like structure (B oligomer), and one copy of the A subunit (9). As in the case of other bacterial toxins such as diphtheria, pseudomonas, and pertussis toxins, toxicity is mediated by the ADP-ribosyltransferase activity of the A subunit, which modifies essential GTP-binding proteins of eukaryotic cells (3, 10).

The immune response to CTs and LTs has been widely studied by using chemically inactivated toxoids and whole toxins, usually in the presence of Freund's adjuvant. These studies have shown that whereas immunization induces both anti-A and anti-B antibodies, toxin-neutralizing antibodies are elicited almost entirely by the B subunit (11–16). Based on these observations, vaccine development has been focused on live-attenuated (17, 18) or killed vaccines (19–21) containing only the nontoxic but immunogenic B subunit, and inducing mostly antibacterial protective immunity. The occurrence in Bangladesh of an outbreak caused by a strain with a different serotype (22, 23) against which the developed vaccines are ineffective, showed the weakness of this approach. Moreover, this emphasized the need to improve the current vaccines, possibly by inducing protective immunity against antigens such as CT, that are common to all cholera strains. In this work we produced a derivative of LT that is nontoxic and induces significant neutralizing antibodies against the A subunit. Such a molecule and equivalent derivatives of CT may be useful to improve vaccines against cholera and ETEC.

## Materials and Methods

*Plasmid Construction and Mutagenesis.* The 2-kb SmaI–HindIII fragment from plasmid pEWD299, containing the LT genes and the LT promoter region (24, 25), was subcloned into the Blue-Script KS vector (Stratagene, San Diego, CA) and was used in all subsequent studies. Site-directed mutagenesis was performed on single-stranded DNA, using the method of Zoller and Smith (26). LT-K63 was obtained using the following oligonucleotide for mutagenesis: 5′-GTTTCCACTAAGCTTAGTTTG-3′. LT-K7 was obtained using the oligonucleotide 5′-GATTATACAAGGCTGACT-3′. DNA manipulations were performed by standard procedures (27).

*Protein Purification.* LT and LT mutant proteins were purified

[1] *Abbreviations used in this paper:* CT, cholera toxin; ETEC, enterotoxinogenic *E. coli*; LT, *E. coli* heat-labile enterotoxin.

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from the periplasm of recombinant *E. coli* strains grown in a 20-liter fermentor, using controlled pore glass and A5M Sepharose columns as described by Pronk et al. (24). Gel filtration on a Sephadex 75 column (Pharmacia LKB, Uppsala, Sweden) was then used to separate the holotoxin from the free pentamer. This was done both on large-scale in order to obtain molecules virtually free of any B pentamer, and at the analytical level to determine the relative abundance of fully assembled molecules and free LTB in the material purified from periplasmic space. CT was purified from culture supernatant of a wild-type cholera strain, as described by Mekalanos (28).

*Immunoassays.* Western blotting was used to test the ability of the antibodies to recognize the A subunit, the B monomer, or the B pentamer. 3 μg of LT were run in each lane of a 15% polyacrylamide gel. Depending on whether the sample was boiled or not before SDS-PAGE, the B monomer or the B pentamer, respectively, were obtained. Sera were usually tested at a dilution of 1/500. Purified antibody fractions were usually tested diluted 1/70. Radioimmunoassay was used to test the specificity of the antibody fractions and the ability of LT-K63 or LTB to compete for antibody binding with wild-type toxin. The test was performed essentially as described (29). Microtiter plates (Microvil PVC microtiter plates; Dynatech Laboratories, Alexandria, VA) were coated with 10 μg/ml of purified γ-globulins, overnight at 4°C, and then saturated with BSA 1% for 2 h at 37°C. The assay was performed adding to each well $^{125}$I-labeled LT ($10^5$ cpm/well of chloramine-T labeled LT) mixed with 10-fold dilutions of competitor protein (10–10,000 ng/ml). After washing, the wells were counted in a gamma counter. Assays were performed in duplicate.

*Toxin Activity on Y1 Cells.* The morphological change caused by LT and CT on Y1 adrenal cells (30) was used to detect the toxic activity of LT and LT mutants and to measure the toxin-neutralizing titer of the antibodies. The assay was performed in microtiter plates using 50,000 cells/well. The results were read after 48 h of incubation. The assay detected 5 pg/well of wild-type toxin. In toxin neutralization experiments, 80 pg of LT or CT and serial dilutions of antibodies were preincubated at 37°C for 3 h before they were added to the cells.

*Animal Immunization.* Mice were immunized subcutaneously at days 0 and 15, and intraperitoneally at days 30 and 45, using either 3 or 25 μg of purified protein. The antigen was given in saline in all immunizations or mixed with Freund's adjuvant. The immunizations performed with Freund's adjuvant used complete adjuvant at day 0, incomplete adjuvant at days 15 and 30, and no adjuvant at day 45. Blood was taken 7 d (day 52) after the last immunization. Rabbits were immunized intradermally at day 0, subcutaneously at day 14, and intraperitoneally at days 28 and 56. The antigen was either given in saline in all immunizations or mixed with complete Freund's adjuvant at day 0 and incomplete adjuvant at days 14, 28, and 56. Blood was taken 15 d after the last immunization.

*Affinity Purification of Anti-A and Anti-B Antibodies.* The LTB subunit was purified (24) from the periplasm of an *E. coli* strain transformed with a plasmid encoding only the B subunit of LT. The affinity matrix was prepared by coupling 5 mg of LTB/ml of CNBr-activated Sepharose 4B (Pharmacia LKB), following the instructions of the manufacturer. 2 ml of serum were diluted to 6 ml with phosphate-buffered saline and applied to a column containing 0.5 ml of resin. The flow through was collected and the bound fraction of antibodies were eluted by 0.1 M glycine buffer, pH 2.7, containing 300 mM NaCl. The eluted fractions were neutralized immediately using a 1 M Tris-HCl buffer, pH 9. Fractions with an $OD_{280nm}$ above 0.05 were collected. To ensure that all anti-B antibodies were removed from the flow through, this was loaded again on the regenerated B column until the $OD_{280nm}$ of the eluted fraction was equal to, or below, 0.05. To use less serum, in some experiments the volumes were scaled down fourfold.

## Results

*Construction of Nontoxic Mutants of LT.* The crystallographic structure of ADP-ribosylating toxins (9, 31, 32) shows that all these enzymes have a cavity harboring the NAD-binding site that is delimited by a β-strand and an α-helix (Fig. 1, *bold line*). The catalytic residues (Arg7, Glu112, and His44 in the case of LT) surround this cavity on both sides (Fig. 1). To study the role of the amino acids involved in the structure and function of the NAD-binding and catalytic site, we designed and constructed by site-directed mutagenesis a number of LT mutants and tested them for the ability to form the AB structure and for toxicity on Y1 cells. The details of this work are described in separate manuscripts (33, 34). After an initial screening, we selected for further studies LT-K63, a mutant that was nontoxic, well-assembled, and among the most resistant to proteolytic attack. In this mutant, a small, polar residue of the β-strand (Ser 63), that is conserved also in CT and pertussis toxin, was changed by site directed mutagenesis to Lys 63. We reasoned that the large, charged Lys residue would fill the NAD-binding cavity, and possibly stabilize the structure through salt bridges with one of the glutamic acids in positions 110 and 112 which face the cavity.

The LT-K63 mutant produced in *E. coli* was exported to the periplasmic space and assembled into the A-B structure, at least as efficiently as the wild-type LT (Table 1), so that it could be purified to homogeneity by conventional methods (24). Typically, we obtained 80 mg of pure protein from a 10-liter fermentor. The purified mutant toxin was indistinguishable from wild-type LT in SDS-PAGE (Fig. 2 *a*), enzymatically inactive in the ADP-ribosylation assay, and nontoxic on Y1 cells (30), even when 18 μg of protein were used (Table 1). Since in this assay the wild-type LT is toxic at 5 pg, LT-K63 is at least $3.6 \times 10^6$ times less toxic than wild-type toxin. As expected, LT-K63 maintained the ability to bind the receptor ganglioside GM1, an activity that resides in the B pentamer. Furthermore, it was recognized both in competitive radioimmunoassay and Western blotting as efficiently as LT by a number of anti-LT polyclonal and monoclonal antibodies available in the laboratory (data not shown). In conclusion, LT-K63 is nontoxic, but otherwise indistinguishable from wild-type LT. In some of the studies, a second nontoxic mutant of LT was used for comparison (LT-K7). This mutant, containing a substitution of one of the residues important for catalysis shown in Fig. 1 (Arg7 → Lys), formed the AB structure less efficiently than LT, and had an A subunit that was more susceptible to proteolytic attack (Table 1).

*Freund's Adjuvant Influences the Specificity of the Immune Response to LT-K63.* The understanding of the immunogenicity of cholera and LT toxins has been complicated by both the toxic and the adjuvant activities of these molecules (11, 13–16). The availability of a nontoxic derivative of LT provided a unique opportunity to study the immune response to the molecule itself. First, mice were immunized with different doses

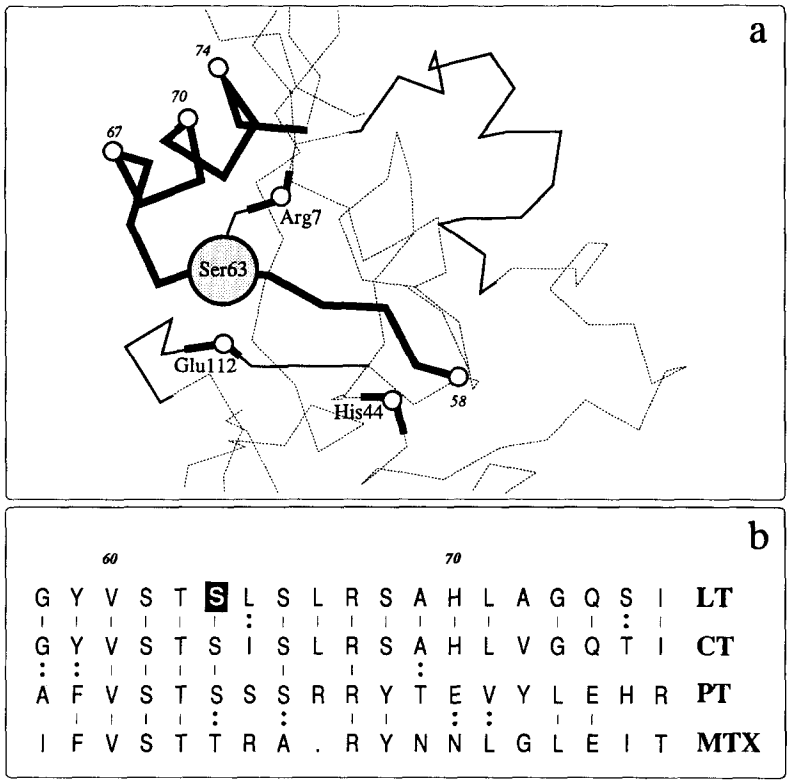


**Figure 1.** (*a*) α-Carbon structure of the active site of LT. The *bold trace* indicates the amino acids 58–76 folded in a β-strand followed by an α-helix, which form the NAD-binding site. The catalytic amino acids Arg7, Glu112, and His44 are also indicated by a *bold trace*. The *intermediate trace* indicates the other sequences that are important for NAD-binding and catalysis. The position of the amino acid modified in LT-K63 and LT-K7 is indicated. (*b*) Amino acid homology in the region of Ser63 between LT, CT (8), pertussis toxin (PT) (46), and Mosquitocidal toxin (MTX) (47).

of LT-K63, with or without Freund's adjuvant, and the sera obtained were analyzed for antibody response to LT by Western blotting. As shown in Fig. 2 *b* (lanes *1* and *2*), mice immunized with 3 μg of LT-K63 with adjuvant recognized only the B subunit, whereas mice immunized without adjuvant recognized only the A subunit. When 27 μg of LT-K63 were used for immunization (Fig. 2 *b*, lanes *3* and *4*), the antibody response changed quantitatively, but a similar trend was observed: sera from mice immunized with adjuvant recognized mostly the B subunit and those from mice immunized without adjuvant recognized mostly the A subunit. Immunization of rabbits gave results similar to those reported in Fig. 2 *b*. These results suggested that immunization in the presence of Freund's adjuvant may direct the immune response towards the B subunit of LT.

*Fractionation of Anti-K-63 Antibodies.* To study further the properties of the antibody response to LT, the anti-A and anti-B fractions of the rabbit sera were separated by affinity chromatography using a column containing immobilized recombinant B pentamer as shown schematically in Fig. 3 *a*.

**Table 1.** *Comparison of the Properties of LT, LT-K7, and LT-K63*

| | LT | LT-K7 | LT-K63 |
|---|---|---|---|
| Amino acid change | / | Arg7 → Lys | Ser63 → Lys |
| Codon change | / | CGT → AAG | TCT → AAG |
| ADP-ribosylation | Positive at 0.5 μg | Negative at 3.0 μg | Negative at 3.0 μg |
| Percent unassembled B5 in purified protein* | 7% | 25% | 2% |
| Resistance to Trypsin‡ | + | − | + |
| Binding to GM1 | + | + | + |
| Toxicity on Y1 cells | Toxic at 5 pg/ml | Nontoxic at 18 μg/ml | Nontoxic at 18 μg/ml |

* The concentration of the free oligomer in the proteins purified from periplasm reflects the ability of the mutant A subunit to form the holotoxin (33).
‡ Incubation for 5 min of 0.35 mg/ml of LT, LT-K7, and LT-K63 in the presence of 0.07 mg/ml of trypsin resulted in complete degradation of the A subunit of LT-K7. The A subunits of LT and LT-K63 and the B subunits of all mutants were unaffected by the treatment.

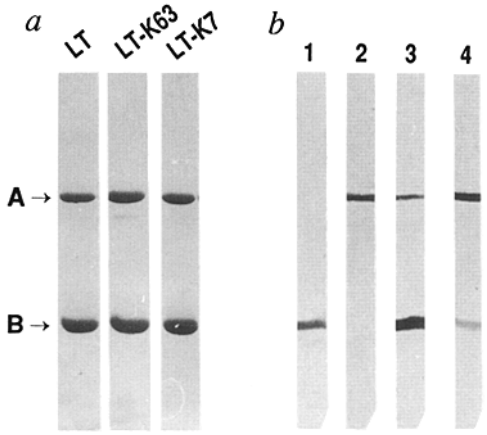


**Figure 2.** (*a*) SDS-polyacrylamide gel showing the purified LT, LT-K63, and LT-K7. (*b*) Western blotting to detect the anti-A and anti-B monomer antibodies. Mice in lanes *1* and *2* were immunized with 3 μg of LT-K63; mice in lanes *3* and *4* received 27 μg of antigen. Freund's adjuvant was used for the immunizations shown in lanes *1* and *3*. The data reported were obtained with a pool of six sera. Individual mice were also tested and found to behave as shown in the figure.

The specificity of the fractions obtained was confirmed by Western blotting analysis (Fig. 3 *b*); unfractionated sera recognized the B pentamer, the A subunit, and the B monomer (Fig. 3 *b*, lanes *1* and *2*). After passage through the B column, the unbound fraction contained antibodies that recognized only the A subunit (Fig. 3 *b*, lanes *3* and *4*), while the fraction eluted from the column recognized only the B pentamer and the B monomer (Fig. 3 *b*, lanes *5* and *6*, respectively).

Radioimmunoassay was then used to detect conformational epitopes that could be lost in Western blotting. The binding of $^{125}$I-labeled LT to purified antibodies immobilized onto microtiter wells was completed with cold LT or recombinant B oligomer. As shown in Fig. 3 *c* (*top*), the antibodies that did not bind the B affinity column and recognized only the A subunit in Western blotting, also bound LT efficiently in this assay, but did not bind the B oligomer. These data indicate that the antibodies present in the unbound fraction of the column recognized only LT epitopes that are present on the A subunit, or that are induced by the presence of the A subunit. To simplify, these will be indicated as anti-A antibodies. As expected, the antibodies retained by the B affinity column recognized equally well LT and the B pentamer (Fig. 3 *c*, *bottom*), confirming that they were indeed specific only for the B subunit.

*Toxin Neutralizing Antibodies.* Since a protective immune response to LT should induce toxin-neutralizing antibodies, we investigated the ability of the antibodies induced by LT-K63 to neutralize the activity of the wild-type toxin on Y1 cells. Table 2 shows that unfractionated sera from rabbits immunized with LT-K63 and Freund's adjuvant had a toxin neutralizing titer of 1/20,480, which was approximately fourfold higher than that present in sera from rabbits immunized without adjuvant, which was 1/5,120. After passage through the affinity column containing immobilized B pentamer, the anti-A antibodies had a neutralizing titer of 1/1,280 in sera obtained without adjuvant and 1/40 in sera obtained with

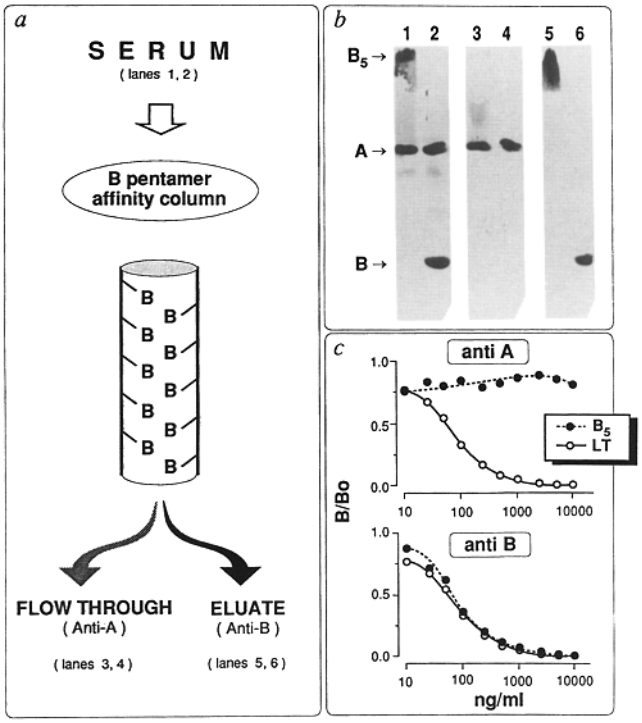


**Figure 3.** (*a*) Schematic representation of the method used to separate the anti-A antibodies from the sera. (*b*) Western blotting analysis showing the specificity of the sera fractionated with the method shown in *a*. Each fraction of the serum was tested against LT that had not been boiled before electrophoresis in order to maintain intact the B pentamer (lanes *1*, *3*, and *5*), and against LT that had been boiled before electrophoresis in order to dissociate the B pentamer in monomeric B subunits (lanes *2*, *4*, and *6*). The B pentamer is indicated as *B5*, while the monomeric B subunit is indicated as *B*. Lanes *1* and *2* show the reaction of the serum before passage through the B affinity column. Lanes *3* and *4* show the specificity of the serum not bound to the B column (anti-A). Lanes *5* and *6* show the specificity of the anti-B fraction eluted from the column. (*c*) Radioimmunoassay showing the ability of LT and the B pentamer to compete with $^{125}$I-labeled LT for binding to anti-A and anti-B antibodies.

adjuvant. Similar results were obtained in mice (Table 2). Therefore, although immunization with LT-K63 and Freund's adjuvant induced higher neutralizing titers than immunization without adjuvant, in the presence of the adjuvant the anti-A–neutralizing antibody titers were lower than those obtained immunizing without adjuvant. From the quantitative point of view, the anti-A–neutralizing antibodies represented 0.2–3.2 and 25% of the total antibodies, depending on whether immunization was performed in the presence or in the absence of the adjuvant, respectively. These results indicate that when immunization is performed in the presence of Freund's adjuvant, the ability to induce toxin-neutralizing antibodies resides almost entirely in the B subunit, whereas in the absence of the adjuvant, the A subunit also induces a significant fraction of toxin-neutralizing antibodies. To verify whether the ability to induce high titer of anti-A antibodies is a property unique to LT-K63, rabbits were immunized with and without adjuvant with wild-type LT and with LT-K7. As in the case of LT-K63, immunization without adjuvant induced anti-A–neutralizing antibodies; however, these were never superior to 12.5% of total neutralizing antibodies. In

**Table 2.** *LT-neutralizing Titer of Anti-LT-K63 Antibodies*

| | Rabbits | | Mice | |
|---|---|---|---|---|
| | − Adjuvant | + Adjuvant | − Adjuvant | + Adjuvant |
| Serum LT-K63 | 1/5,120 | 1/20,480 | 1/5,120 | 1/20,480 |
| Anti-A LT-K63 | 1/1,280 | 1/40 | 1/1,280 | 1/640 |
| (% Anti-A) | (25%) | (0.2%) | (25%) | (3.2%) |

Unfractionated mouse and rabbit sera and of their anti-A fractions are shown.

the presence of adjuvant the anti-A fraction represented only 0.2–1.5% of total neutralizing antibodies. We therefore conclude that immunization with LT, KT-K63, and LT-K7 in the absence of adjuvant induces a significant titer of anti-A–neutralizing antibodies, and that LT-K63 appears to be the best molecule in doing so. In the presence of Freund's adjuvant, however, the anti-A–neutralizing antibodies are almost absent.

*Anti-LT-A Antibodies Neutralize CT More Efficiently Than anti-LT-B Antibodies.* Although highly homologous in amino acid sequence, LT and CT are known to induce antibodies with low cross-neutralization activity. Since the enzymatically active moiety is the portion of the molecule that is most conserved between LT and CT, we expected that the anti-A antibodies had an increased ability to neutralize CT. To verify this, we compared the neutralizing activity on LT and CT of an anti-LT-K63 serum obtained by immunizing with Freund's adjuvant which recognized almost exclusively the B subunit, with that of the affinity purified anti-A antibodies. The concentration of the antibody preparations was adjusted so that both of them neutralized 80 pg of LT at a dilution of 1/512. When tested for neutralization of 80 pg of CT, the anti-B antibodies had a titer of 1/32, whereas the anti-A antibodies had a titer of 1/128. This finding confirmed our expectation that anti-LTA antibodies are more efficient than anti-LTB in neutralizing CT.

## Discussion

We have shown that nontoxic mutants of LT may be used to induce good titers of toxin neutralizing antibodies against the A subunit in mice and rabbits. This finding was unexpected, since the A subunit has been generally considered unable to induce neutralizing antibodies. Indeed, cholera vaccines have been developed assuming that the toxin neutralizing immunity resides entirely on the B subunit, and that the in vivo protection is mediated mostly by the antibacterial antibodies. The availability of nontoxic mutants of LT and of equivalent mutants of the homologous CT may provide an opportunity to improve vaccines against cholera and ETEC. The two prototypes of cholera vaccines are the CVD 103-HgR (17, 18, 35), a live cholera strain containing a deletion of the gene coding for the A subunit and the killed BS-WC vaccine, a vaccine containing 1 mg of purified B subunit and $10^{11}$ killed bacteria (19–21, 36). Both vaccines showed good clinical efficacy against cholera induced by 01 strains, and were expected to solve the cholera problem in a definitive way. However, the recent epidemic of the Bengal strain 0139 against which both vaccines are ineffective (23, 37), made several years of vaccine development obsolete (22, 38). To generate vaccines that will cover also this strain, a great effort is presently dedicated to develop live and dead vaccines that include a mixture of 01 and 0139 cells. Nevertheless, such vaccines would not protect against serotypes other than 01 and 0139 that may arise in the future, as occurred recently for the Bengal strain. An alternative way to generate vaccines covering the 0139 strain and possibly also new strains that may arise in the future, would be to improve the protection conferred by the toxin, a molecule that is present in all pathogenic cholera strains and also present in ETEC. A role of the immunity against the B subunit of CT in protection against disease has been demonstrated in several instances. Anti-CTB antibodies are known to be synergistic with anti-lipopolysaccharide antibodies in inducing protection against challenge with live *V. cholerae* (39). In the field trial in Bangladesh, during the first 6 mo, protection was much higher (85%) in the group that received the BS-WC vaccine containing the B subunit and bacterial cells, than in the group that received the bacterial cells alone (58%) (20, 40). In the same clinical trial, the BS-WC vaccine conferred a good protection against diarrhea caused by LT producing ETEC, which are known to share only the toxin antigen with *V. cholerae* (41). In a subsequent study in travelers to Morocco, the antitoxin immunity induced by the BS-WC vaccine was confirmed to protect from *E. coli*–induced diarrhea (42).

In conclusion, the evidence that immunity against the B subunit can confer protection against disease encourages the development of nontoxic LT and CT molecules that may increase the protection mediated by the antitoxin immunity. The use of a fully assembled molecule such as LT-K63, instead of the B subunit alone would increase the ability of the immunity obtained to crossneutralize cholera or other serological variants of LT. In addition, the nontoxic mutants would provide a target for the immune system larger than the B subunit alone. For instance, the A subunit, which is a polypeptide of 239 aminoacids, should help to overcome the genetic restriction that is expected with a polypeptide of only 105 aminoacids, such as the B subunit, and that has been described in the mouse model (43).

We have observed that LT-K63 induces a higher proportion of anti-A–neutralizing antibodies than wild-type toxin

and LT-K7. While the data available are not enough to conclude whether this is statistically significant, it is not unlikely that the ability to induce anti-A-neutralizing antibodies may be different in different mutants. In fact, the neutralizing epitopes of the A subunit are likely to be conformational and the ability to induce an immune response against them may depend on the stability of the 3-dimensional structure. Several observations suggest that the Ser63 → Lys mutation may increase the stability of the structure of the A subunit, and possibly the ability of this molecule to induce toxin neutralizing antibodies. For instance, LT-K63 is more stable than LT-K7 in the presence of trypsin and more efficient than LT-K7 and wild-type LT in assembling the holotoxin in the periplasm, suggesting that the A subunit of LT-K63 is conformationally more competent than the wild-type to form a stable AB5 structure (Table 1). The crystal structure of these molecules, which is progress, should help to answer some of these questions. Preliminary data on the structure of LT-K7 indicate that in this mutant, one of the loops that face the active site has an increased motility, which may justify the increased susceptibility to proteases of LT-K7.

*Role of Freund's Adjuvant in the Immune Response to LT-K63.* Freund's adjuvant has been generally used to enhance immune responses in animal immunization. The possibility that the use of the adjuvant could bias the specificity of the immune response has not been a great concern. Here we have shown that when a nontoxic mutant of LT is used to immunize mice or rabbits, the quality of the immune response is changed if Freund's adjuvant is used. In particular, the presence of Freund's adjuvant decreases dramatically the antibody response towards the A subunit both in Western blotting and in toxin-neutralizing activity. This finding may explain the very low immunogenicity of the A subunit reported in the literature. The effect of Freund's adjuvant could be explained by its hydrophobic nature that could result in the preferential denaturation of some epitopes. In the case of the A subunit, that is known to be loosely associated to the B pentamer and to be able to interact with hydrophobic membranes (44), Freund's adjuvant may induce a change in conformation and possibly the dissociation from the B pentamer.

In general, the finding reported here for LT-K63 and recently on recombinant pertussis and diphtheria antigens (45) should induce a critical reevaluation of the immunogenicity data reported using Freund's adjuvant and a careful selection of the adjuvants to be used for immunization.

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