Mucosal Adjuvanticity and Immunogenicity of LTR72, a Novel Mutant of *Escherichia coli* Heat-labile Enterotoxin with Partial Knockout of ADP-ribosyltransferase Activity

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Summary

Heat-labile *Escherichia coli* enterotoxin (LT) has the innate property of being a strong mucosal immunogen and adjuvant. In the attempt to reduce toxicity and maintain the useful immunological properties, several LT mutants have been produced. Some of these are promising mucosal adjuvants. However, so far, only those that were still toxic maintained full adjuvanticity. In this paper we describe a novel LT mutant with greatly reduced toxicity that maintains most of the adjuvanticity. The new mutant (LTR72), that contains a substitution Ala → Arg in position 72 of the A subunit, showed only 0.6% of the LT enzymatic activity, was 100,000-fold less toxic than wild-type LT in Y1 cells in vitro, and was at least 20 times less effective than wild-type LT in the rabbit ileal loop assay in vivo. At a dose of 1 μg, LTR72 exhibited a mucosal adjuvanticity, similar to that observed with wild-type LT, better than that induced by the nontoxic, enzymatically inactive LTK63 mutant, and much greater than that of the recombinant B subunit. This trend was consistent for both the amounts and kinetics of the antibody induced, and priming of antigen-specific T lymphocytes. The data suggest that the innate high adjuvanticity of LT derives from the independent contribution of the nontoxic AB complex and the enzymatic activity. LTR72 optimizes the use of both properties: the enzymatic activity for which traces are enough, and the nontoxic AB complex, the effect of which is dose dependent. In fact, in dose–response experiments in mice, 20 μg of LTR72 were a stronger mucosal adjuvant than wild-type LT. This suggests that LTR72 may be an excellent candidate to be tested in clinical trials.

Heat-labile enterotoxin (LT)[1] produced by enterotoxigenic *Escherichia coli* strains (1) and cholera toxin (CT) produced by *Vibrio cholerae* strains (2) are the causative agents of traveler's diarrhea and cholera, respectively. They show 80% homology in the primary structure (3, 4) and a similar 3-D structure (5). Both toxins are composed of two functionally distinct domains: the enzymatically active A subunit with ADP-ribosylating activity (6–8), and the pentameric B subunit that contains the monosialoganglioside (GM1) receptor–binding site (9, 10). The A subunit intoxicates eukaryotic cells by activating the protein Gs, a GTP-binding protein that regulates the levels of the second messenger cAMP (11, 12). In vivo, enhancement in cAMP levels alter ion transport, inducing secretion of water and chloride ions in the intestine (13).

Both CT and LT have the unique property of being very immunogenic by the oral and other mucosal routes, where most antigens are unable to induce an immune response. Even more interesting is the fact that they act as potent mucosal adjuvants and induce an immune response against coadministered antigens (14, 15). The adjuvanticity and the immunogenicity of CT and LT have been extensively studied in animal models with the aim of understanding the basis for these unique features and in order to develop mucosally delivered vaccines (16–18). However, their toxicity has precluded their use in humans (19). To overcome the problem of toxicity and understand the mechanism of action, two different approaches have been followed, one based on the use of the nontoxic B subunit (20, 21), and the other based on the generation of genetically detoxified derivatives of LT (22, 23) and CT (24, 25) by site-directed mutagenesis. These studies have shown that the most im-

[1] *Abbreviations used in this paper:* CT, cholera toxin; LT, heat-labile enterotoxin; rLTB, recombinant B subunit of LT.

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portant factor for immunogenicity is the ability to bind the receptor on eukaryotic cells. In fact, a nonbinding mutant of the B subunit of LT, containing the mutation Gly 33 → Asp, was found to be nonimmunogenic (26). On the other hand, the ADP-ribosylating activity was found unnecessary for immunogenicity since we and others showed that nontoxic derivatives of LT obtained by site-directed mutagenesis of the A subunit retained the immunological properties of the wild-type LT (23, 27, 28).

In the case of adjuvanticity, the results are less clear. Initially, the B subunit of LT (LTB) and that of CT (CTB) were reported to have an adjuvant effect. However, subsequent studies showed that those results had been compromised by the use of preparations contaminated by the active toxin (29). The use of recombinant LTB and CTB, free of contaminating enzymatic activity, confirmed that the B subunits are very poor mucosal adjuvants (30–32). This suggested that either the nontoxic A subunit per se or the enzymatic activity, or both, are necessary for adjuvanticity. The attempt to define the role of ADP-ribosylating activity in LT adjuvanticity has generated conflicting results. Lycke et al. (30) described a nontoxic derivative of LT (LTE112K) that, when coadministered with KLH by the oral route in mice, lacked the adjuvant properties of the wild-type LT, thus suggesting that the adjuvant activity of LT is linked to its ADP-ribosylating activity. We showed that LT derivatives (e.g., LTK7 and LTK63; references 32–34) devoid of any enzymatic activity and toxicity were still able to elicit an antibody response against the coadministered antigen in intranasally immunized mice. One of the mutants tested, LTK63, after intranasal immunization with a synthetic peptide, induced measles virus-specific CTL response (35), and strongly enhanced protection against *Helicobacter pylori* after intragastric immunization with *H. pylori* antigens (36, 37). However, the antibodies induced by the nontoxic LT mutants were somehow lower in titer and appeared later than when the wild-type toxin was used (32–34).

To address the above questions, we have produced a new LT mutant (LTR72) with greatly reduced enzymatic activity and toxicity, and compared its adjuvanticity and immunogenicity with those of the toxic wild-type LT, of a nontoxic AB molecule (LTK63), and of the nontoxic B subunit. For the first time, the results give a consistent picture of the relative role of the B subunit, the AB complex, and the enzymatic activity on the immunological properties of LT, and they show that a mutant such as LTR72 may be an optimal mucosal adjuvant. In fact, although it retains the adjuvanticity of wild-type LT, the greatly reduced enzymatic activity may allow for safe use in humans.

Materials and Methods

Computer Modeling and Site-directed Mutagenesis of the LT Active Site

Mutations in position 72 were designed on the basis of the 3-D structure of LT (5, 22, 38). Computer analysis was performed on an INDIGO ELAN workstation (Silicon Graphics, Inc., Mountain View, CA), using the program INSIGHTII (Biosym Technologies, Inc., San Diego, CA). Energy calculations were performed using the program DISCOVER (Biosym Technologies, Inc.).

The 2Kb SmaI–HindIII fragment from plasmid pEWD299 containing the LT genes and the LT promoter region (3) was cloned in Blue-Script KS vector (Stratagene, San Diego, CA) and used as DNA template for site-directed mutagenesis (39). The mutations in position 72 were introduced using the following oligonucleotides for mutagenesis: 5′-GCTCACTTACGTGGACAGTCT-3′ for Ala → Arg substitution, 5′-GCTCACTTACATGGACAGTCT-3′ for Ala → His, 5′-GCTCACTTAGAGGGACAGTCT-3′ for Ala → Asp substitution. The mutation in position 63, corresponding to the Ser → Lys substitution, has been previously described (22, 27). DNA manipulations were performed using standard procedures (40).

Biochemical Characterization of the LTR72 Mutant

Purification. Wild-type LT, LTK63 (Ser → Lys), and LTR72 (Ala → Arg) mutants, and recombinant B subunit of LT (rLTB) were purified from the periplasm of the recombinant *E. coli* strain using three chromatographic steps on CPG 350 (Controlled Pore Glass; Serva, Heidelberg, Germany), A5m agarose (Bio-Rad Laboratories, Richmond, CA), and Sephacryl S-200 (Pharmacia Biotech AB, Uppsala, Sweden) resins as described (28, 41). The purified LTR72 protein was stored at 4°C and every 2 mo 100-μl samples were analyzed by HPLC using a Superdex 200 HR (Pharmacia Biotech AB) gel filtration column.

Trypsin Treatment. LT, LTR72, or LTK63 mutant proteins (45 μg) were treated with 9 μg of trypsin (Sigma Chemical Co., St. Louis, MO) in a final volume of 150 μl of 10 mM Tris, pH 7.5, at 37°C. 30-μl samples were collected after 5 and 30 min of incubation and the reaction was stopped by the addition of 3.6 μg of trypsin inhibitor (Sigma Chemical Co.). 10 μl of 4× concentrated electrophoresis sample buffer was added to each sample and the mixture was treated for 10 min at 95°C. Proteins were loaded on 15% SDS minigels and stained with Coomassie brilliant blue R-250 (Bio-Rad), or transferred onto a nitrocellulose membrane. In this case the membrane was incubated with a rabbit anti-LT polyclonal sera at a dilution of 1:300.

Functional Characterization of the LTR72 Mutant

ADP-ribosylation Assay. Different amounts of purified wild-type LT and LT mutants were analyzed for their ability to ADP-ribosylate polyarginine as described by Lai et al. (42).

In Vitro and In Vivo Toxicity. Toxicity of the mutants was tested in vitro using Y1 cells (43) and in vivo using the rabbit ileal loop assay as previously described (44). For the Y1 cells assay, serial twofold dilutions of LT, LTR72, LTK63, or rLTB were added to 5×10^4 cells/well on 96-well plates. The cells were then observed for morphological changes after 48 h. For the rabbit ileal loop assay, two New Zealand adult rabbits, ~2.5 Kg each, were used for each assay. 1-ml samples with various amounts of LT or LT mutants were injected into each loop. After 18-20 h, the liquid accumulated in each loop was collected and measured with a syringe. The experiment was performed four to six times in duplicate and the results are expressed as milliliters per centimeter.

Immunization of Mice

Mucosal immunogenicity and adjuvanticity of wild-type LT, LTR72 and LTK63 mutants, and rLTB were tested by immunizing groups of 10 BALB/c mice (4–6-wk-old females; Charles

River, Calco, Italy) intranasally with 1 μg of toxin and 10 μg of antigen, or with antigen alone. In the dose–response experiment, five mice were immunized with a constant amount of OVA (10 μg) and varying doses of wild-type LT, LTR72 (2.5 ng, 50 ng, 1 μg, and 20 μg), or LTK63 (50 ng, 1 μg, and 20 μg).

The animals were lightly anesthetized and immunized on days 0, 21, and 35 with a 15-μl volume per nostril. Immune responses were followed in the serum samples taken on days 0, 20, 34, and 52. The animals were killed and nasal lavages were performed by repeated flushing and aspiration of 1 ml of PBS containing 0.1% BSA (Sigma Chemical Co.).

Quantitation of Anti-OVA and Anti-LT Antibodies by ELISA

LT-specific antibodies were measured using a GM1 capture ELISA. Each well on 96-well plates (Greiner GmbH, Kremsmunster, Austria) was first coated with 150 ng of GM1 ganglioside (Sigma Chemical Co.) by overnight incubation at 4°C. Wells were then washed three times with PBS containing 0.05% Tween 20, and 50 ng of toxin were added to each well. Plates were then incubated for 2 h at 37°C. OVA-specific antibodies were assessed by coating each well with 60 μg/ml of OVA and incubated overnight at 4°C. The plates were washed and the wells were saturated with 1% BSA in PBS for 1 h at 37°C. Sera from individual mice were tested starting from a dilution of 1:50 in PBS and nasal lavages were tested starting from undiluted solution. Plates were incubated for 2 h at 37°C. Specific Ig were measured using a horseradish peroxidase–conjugated rabbit anti–mouse Ig (Dako, Glostrup, Denmark) (2 h at 37°C). Antibodies were then revealed by adding *o*-phenylenediamine (OPD) as substrate (Sigma Chemical Co.). After 10 min the reaction was blocked by the addition of 12.5% H_2SO_4 and the absorbance was read at 490 nm. ELISA titers were expressed as the reciprocal of the last dilution which gave on $OD_{490} \geqslant 0.3$ above the preimmune sera. Titers of specific IgA in the sera and in the mucosal lavages were measured using the biotin-conjugated goat anti–mouse IgA (α chain–specific; Sigma Chemical Co.) followed by horseradish peroxidase–conjugated streptavidin. Bound antibodies were revealed using OPD as substrate. ELISA titers were expressed as the reciprocal of the last dilution which gave on $OD_{490} \geqslant 0.2$ above the preimmune sera. The values were normalized using positive control sera in each plate.

OVA-driven Proliferative Response

14–20 d after two intranasal immunizations with LT or LT mutants in combination with OVA, two or three mice per group were killed and spleens were removed. Spleen cell suspensions were obtained and resuspended in complete DMEM containing 10% FCS, 2 mM L-glutamine, 15 mM Hepes, 100 U of penicillin/streptomycin, and 50 mM 2-ME. 2×10^5 spleen cells were seeded per well in U-bottomed 96-well plates and cultured in the presence of different concentration of OVA for 5 d. [^{3}H]thimidine was added (1 μCi/well) 16 h before the end of culture. Cells were then harvested with a cell harvester, and [^{3}H]thimidine incorporation was evaluated by liquid scintillation counting.

In Vivo Challenge with Wild-type LT

To determine the LD_{50} for wild-type LT by intraperitoneal route, groups of 10 BALB/c mice (9-wk-old females) were inoculated intraperitoneally with 12.5, 25, 50, and 100 μg of LT. The control group was inoculated with PBS. The mice were observed for death for 7 d. LD_{50} was determined as 20.4 μg. Then, 4-wk-old BALB/c mice were immunized intranasally with 1 μg each of LT, LTR72, LTK63, and LTB at days 0 and 21, and challenged with $2 \times LD_{50}$ of wild-type LT at day 35. The mice were observed for deaths for 7 d. Sera were collected at day 35 and anti-LT titers were analyzed by ELISA as described above.

Results

Biochemical and Biological Characterization of the LTR72 Mutant. After site directed mutagenesis of the A subunit of LT, we described, among other mutants, three mutants of the Alanine in position 72, an amino acid located in the internal face of the α helix that forms the cavity containing the nicotinamide adenine dinucleotide (NAD) binding site (22, 45). When tested in crude periplasmic extracts, two of the mutants showed some reduced toxicity in Y1 cells, suggesting that they could be potentially useful when LT mutants with some residual toxicity were needed (22). Subsequent analysis of purified preparations of the three mutants showed that although LTH72 (Ala → His) and LTE72 (Ala → Glu) had a toxicity indistinguishable from that of wild-type toxin (data not shown), the mutant LTR72 (Ala → Arg) showed greatly reduced toxicity. We therefore decided to further study this mutant and compare it to wild-type LT and to other nontoxic mutants previously obtained and characterized. Fig. 1 *A* shows the elution profile on Superdex 200 HR column of LTR72, LTK63, and wild-type LT. The three molecules had an identical elution profile, suggesting that the mutation Ala72 → Arg does not alter the structure of the A subunit, and that this is correctly assembled into the AB_5 structure. This elution profile remained unchanged up to 1 yr after storage of the LTR72 mutant at 4°C, suggesting that the molecule is also very stable (data not shown). To check whether minor conformational changes were present, we tested the sensitivity to proteases. Purified wild-type LT, LTK63, and LTR72 mutants were treated with trypsin at 37°C, and samples were collected at different times and analyzed by Western blot. The results (Fig. 1 *B*), showed that after 5 min of incubation, in all three molecules the trypsin caused an almost immediate nicking of the A subunit into the A1 and A2 domains, and that the nicking was complete after 30 min of incubation. No differences were detected in the sensitivity to proteases between LTR72, wild-type LT, or LTK63. Then we tested various functional activities. In vitro, the LTR72 mutant exhibited, as compared to wild-type LT, an ADP-ribosylation activity that was reduced by at least two orders of magnitude: the minimal amount to obtain a detectable activity was 0.5 μg for LT and 85 μg for LTR72. As expected, the LTK63 mutant was totally devoid of enzymatic activity (Fig. 1 *C*). In agreement with the reduced catalytic activity, the LTR72 was 100,000-fold less toxic than wild-type LT on Y1 cells (Fig. 1 *D*). In the rabbit ileal loop assay, at least a 20-fold higher amount of LTR72 was needed to start to induce a fluid accumulation. However, this mutant was never able to induce more than 1.5 ml/cm of fluid at any of the amounts tested (up to 200 μg), whereas LT induced >3.0 ml/cm already at very low amounts (50 ng; Fig. 1 *E*).

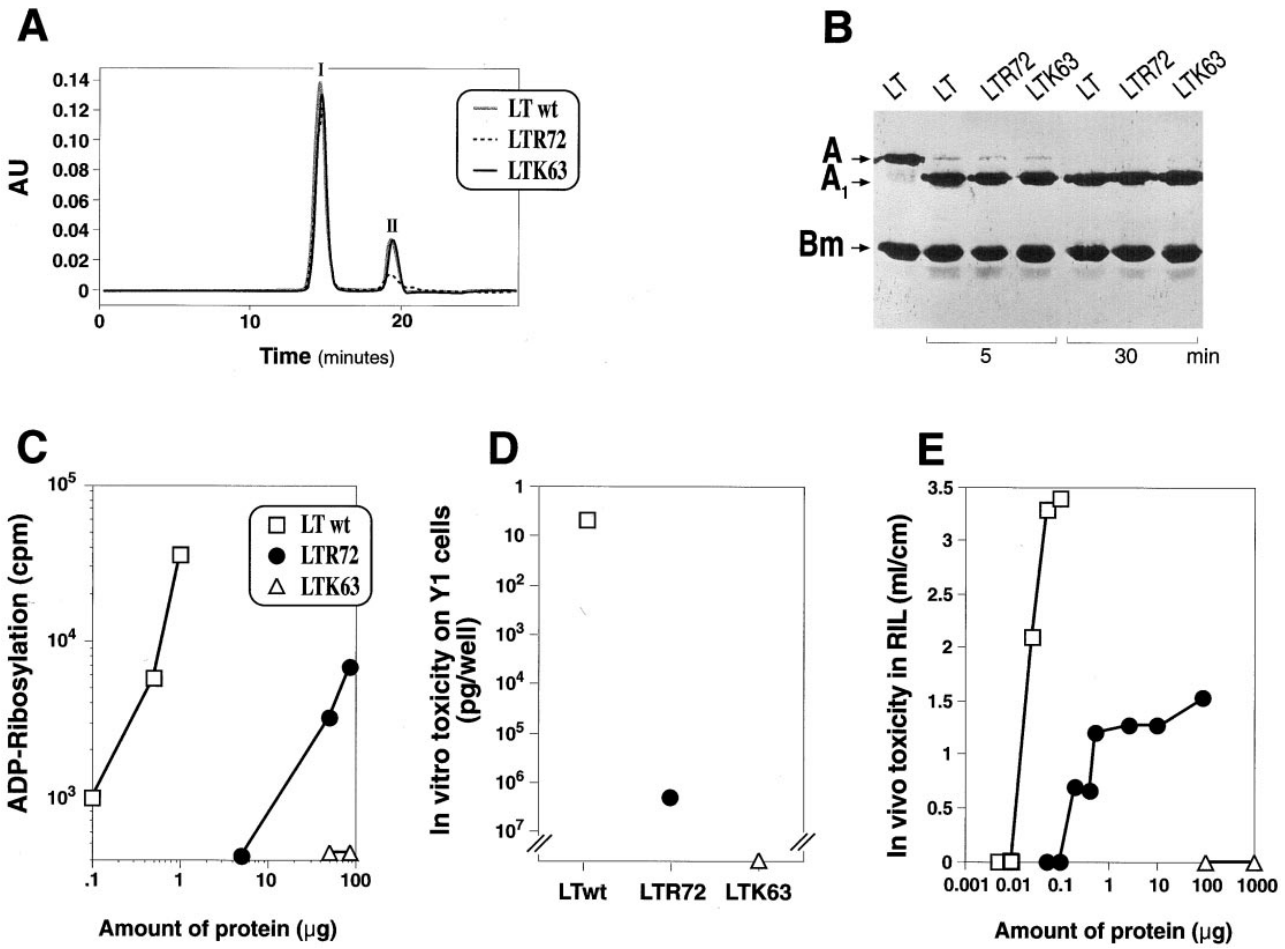

Figure 1. Comparison of biochemical and biological properties of LTR72, LTK63, and wild-type LT. (*A*) Chromatographic profiles of LTR72, LTK63 and wild-type LT on Superdex column (peak I corresponds to the holotoxin, peak II to the EDTA contained in the buffer). (*B*) Western blot of the untreated wild-type LT and of the wild-type LT, LTR72, and LTK63 after 5 and 30 min of incubation with trypsin. (*C*) In vitro ADP-ribosylation using polyarginine as substrate. (*D*) In vitro toxicity on Y1 cells at 48 h. (*E*) In vivo toxicity in the rabbit ileal loop assay.

As previously reported, LTK63 was completely nontoxic both in Y1 cells and in the rabbit ileal loop assay (46).

Mucosal Adjuvanticity of LTR72. Groups of mice were immunized intranasally three times with 10 μg of OVA alone or with 10 μg of OVA and 1 μg of wild-type LT, LTR72, LTK63, or rLTB. Serum anti-OVA specific antibody titers were determined after each immunization. As shown in Fig. 2, wild-type LT and the mutant LTR72 induced the highest anti-OVA antibody response. LTK63 induced an intermediate level; the B subunit gave quite a low response. The antigen-specific antibody response was detectable already after a single immunization with LT and LTR72, whereas, as previously described (33, 34), the nontoxic LTK63 mutant required at least two immunizations to induce anti-OVA antibodies in serum.

OVA-specific IgG isotypes were measured in pools of sera of the last bleeding (Fig. 3). High titers of anti-OVA IgG1, IgG2a, and IgG2b antibodies were induced in the groups of mice receiving the wild-type LT or the LTR72 mutant as an adjuvant. IgG2b antibodies were almost undetectable in the mice receiving the nontoxic LTK63 mutant. OVA-specific IgG3 antibodies were never detectable. It is interesting to note that, in the presence of wild-type LT, the predominant antigen-specific IgG subclass was the IgG2a, whereas in the presence of the mutant LTR72 and LTK63, higher titers of serum anti-OVA IgG1 were detected. Finally, in the group of mice receiving OVA in the presence of LTB, only very low titers of anti-OVA IgG1 antibodies were found.

In all experiments, OVA-specific IgA antibodies were never detectable in sera of mice after one or two intranasal immunizations (data not shown). After a third immunization, a serum IgA response was detected when wild-type LT or the mutants LTR72 and LTK63 were used as adjuvants (Fig. 4 *A*). Mice immunized with the rLTB plus OVA did not show any detectable IgA response, whereas only one mouse of the control group (i.e., those receiving OVA without any adjuvant) showed detectable titers of anti-OVA IgA. A similar pattern of IgA response was observed at the mucosal level. After three immunizations,

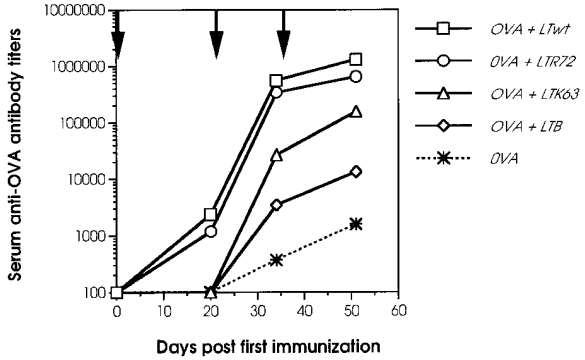

Figure 2. Anti-OVA serum Ig antibody response. Immune responses to OVA in sera of mice immunized intranasally with OVA alone, or OVA in combination with wild-type LT, LTR72, LTK63, and LTB, at time 0 after one, two and three immunizations. Results are expressed as mean titers of OVA-specific antibodies in the sera of mice on days 0, 20, 34, and 52.

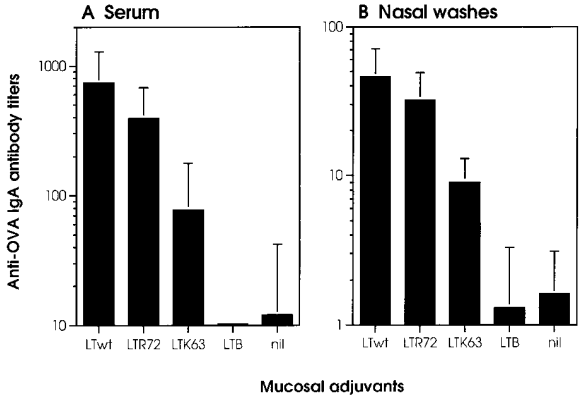

Figure 4. Anti-OVA serum and mucosal IgA antibody responses. IgA immune responses to OVA in sera (*A*) and in nasal washes (*B*) of mice immunized intranasally three times with OVA alone or OVA in combination with wild-type LT, LTR72, LTK63, and rLTB. Results are shown as mean titers and error bars indicate the standard deviation from the mean titer.

OVA-specific IgA antibodies were found in nasal washes of mice receiving the antigen along with wild-type LT, LTR72, or LTK63, but not in the absence of adjuvants or in the presence of rLTB (Fig. 4 *B*).

OVA-specific T Cell Priming. Spleen cells from mice immunized intranasally twice with OVA alone or combined with a mucosal adjuvant were restimulated in vitro in the presence of different concentrations of OVA. Fig. 5 shows the results of a representative experiment. It is clear that intranasal coadministration of the antigen with wild-type LT or with the LTR72 and LTK63 mutants induced a priming of OVA-specific T cells in vivo, which was much stronger than that detectable after immunization with OVA alone or with OVA given in the presence of rLTB. Interestingly, the OVA proliferative response induced by LTR72 was lower than that observed with cells from mice immunized with OVA plus wild-type LT, but higher than that observed with cells from mice immunized with OVA plus LTK63.

Comparative Adjuvanticity of LT, LTR72, and LTK63. The comparative adjuvanticity of LT, LTR72, and LTK63 versus OVA was tested in a dose–response experiment. Mice were immunized using a constant dose of OVA (10 μg) and doses of mucosal adjuvant ranging from 2.5 ng to 20 μg. As shown in Fig. 6 *A*, wild-type LT induced high titers of anti-OVA antibodies already at 2.5 ng, which increased at 50 ng and reached a plateau at 1 μg. No further increase was observed raising the amount of wild-type LT to 20 μg. On the contrary, LTR72 and LTK63 showed a dose response at all amounts tested. The adjuvanticity of

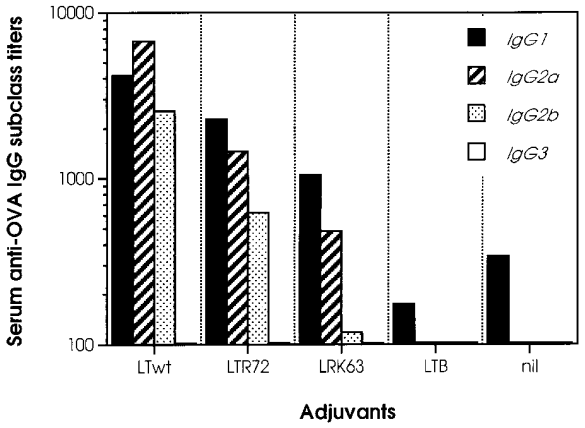

Figure 3. Serum anti-OVA IgG subclasses response. IgG suclasses response in sera of mice immunized intranasally with OVA alone, or OVA in combination with wild-type LT, LTR72, LTK63, and rLTB after three immunizations. Titers were calculated by using dilutions of pooled sera.

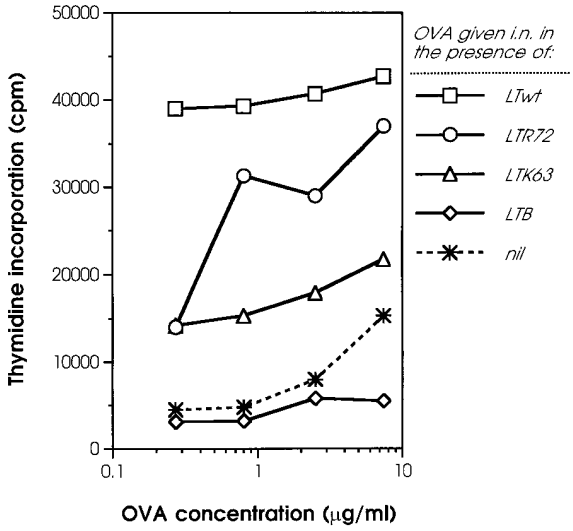

Figure 5. T cell proliferative response to OVA. T cell proliferative response to OVA in spleen cells from mice immunized intranasally for two times with OVA alone or OVA in combination with wild-type LT, LTR72, LTK63, and rLTB.

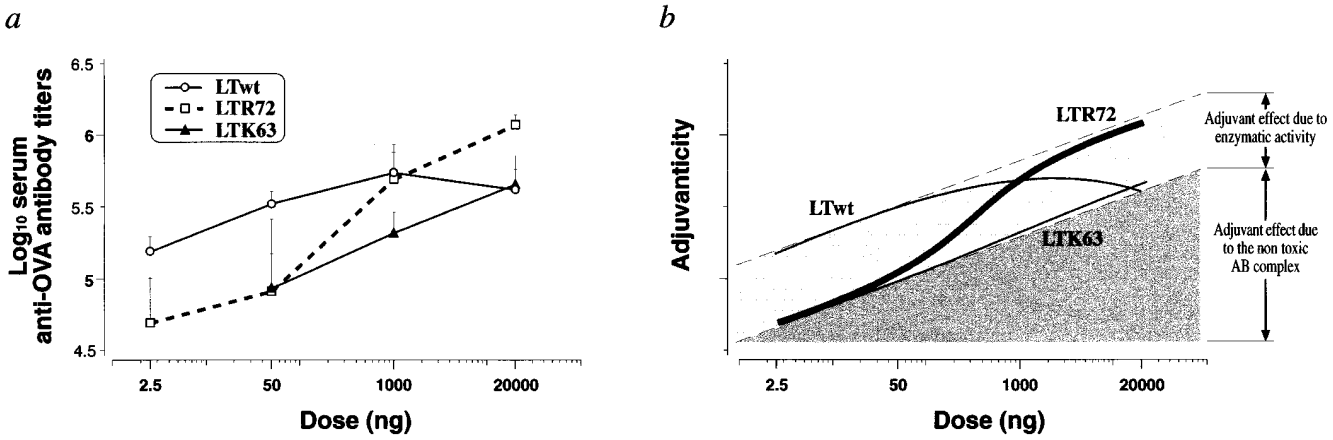

Figure 6. Serum Ig anti-OVA responses in a dose–response experiment. (*a*) Ig immune response in sera of five mice after three intranasal immunizations with 10 μg of Ova and 2.5 ng, 50 ng, 1 μg, and 20 μg of wild-type LT or LTR72 and 50 ng, 1 μg, and 20 μg of LTK63. Results are shown as mean titers and error bars indicate the standard deviation from the mean titer. (*b*) Schematic representation showing the dose-dependent adjuvanticity of LTK63, LTR72, and wild-type LT derived from *a*. The figure shows that the adjuvanticity of the nontoxic AB complex (*darker area*) is dose dependent, while the adjuvanticity of the enzymatic activity (*lighter area*) is dose independent.

LTR72 was lower than that of wild-type LT at 2.5 and 50 ng, but at 1 μg it was comparable to that induced at plateau value by wild-type LT and, remarkably, at 20 μg it was even higher. The adjuvanticity of LTK63 was similar to that of LTR72 at 50 ng and increased at 1 μg and 20 μg. However, the dose–response curve of LTK63 was such that at 1 μg it reached a value lower than that of wild-type LT and LTR72, whereas at 20 μg it was similar to that of wild-type LT and lower than that of LTR72. These data show that although the adjuvanticity of wild-type LT is saturated after a given dose, possibly because of the detrimental effects deriving from its toxic properties, the adjuvant effect of LTR72 and LTK63 increases at higher doses. Furthermore, at high doses LTR72 can be a stronger mucosal adjuvant than wild-type LT.

Immunogenicity after Mucosal Delivery and Protection against a Challenge With Wild-type LT. We next addressed the mucosal immunogenicity and whether the observed immune response protected mice against an intraperitoneal challenge with the toxic wild-type LT. First, we determined the LD_{50} of LT by intraperitoneally inoculating groups of mice with increasing doses of LT and recording the mortality rate after 7 d. The determined LD_{50} was 20.4 μg (data not shown). Then, groups of mice were immunized intranasally at days 0 and 21 with 1 μg of LT, LTK63, LTR72, or rLTB. The control group received diluent only (PBS). On day 34 mice were challenged intraperitoneally with $2 \times LD_{50}$ of wild-type LT and observed for mortality for the following 7 d. The results showed that all mice immunized with wild-type LT, LTR72, and LTK63 survived to the challenge, whereas only 30% of mice immunized with the rLTB survived. As expected, all mice in the control group died (Fig. 7 *A*). The serum anti-LT antibody titers in mice were determined after two imunizations and just before the challenge; the results are shown in Fig. 7 *B*. Sera of mice immunized with LT, LTK63, and LTR72 contained very high and comparable levels of anti-LT antibodies. In mice intranasally immunized with rLTB, anti-LT antibody titers were 10–20 times lower than those found in mice immunized with the wild-type LT, or with the LTR72 or LTK63 mutants. Interestingly, Fig. 7 *B* also shows that the three mice immunized with the rLTB that survived to the intraperitoneal challenge with wild-type LT had serum anti-LT antibody titers significantly higher than those found in the sera of immunized mice that did not survive. These data suggest that serum anti-LT antibody response participates in the effector mechanisms mediating protection against lethal challenge with toxic LT.

Discussion

Safety Profile of LTR72. The unique property of LT to act as a potent mucosal adjuvant and immunogen has stimulated many studies aimed at understanding the mechanism of action and generating nontoxic derivatives suitable for use in human vaccines. Thus far, the best reagents and candidate adjuvants for vaccines have been the nontoxic, recombinant LTB subunit (47), the nontoxic, enzymatically inactive LTK63 mutant (33–37), and LTG192, a mutant containing the Arg → Gly substitution in position 192, in the trypsin-sensitive loop of the A subunit. This mutant has an intact catalytic site but cannot be activated in vitro because of the resistance of the loop to trypsin digestion. In vivo, proteases other than trypsin are likely to activate in part the LTG192. This is because LTG192 on Y1 cells showed a toxicity 1,000-fold lower than wild-type after an 8-h incubation, whereas it was only 5-fold lower after 24 h of incubation (46). In the ileal loop, the toxicity of LTG192 was barely distinguishable from that of wild-type LT (46, 48, 49). Here we have described a new mutant in the catalytic site (LTR72) having ~100-fold reduced enzymatic activity, 100,000-fold reduced toxicity in the Y1 cells, and at least 20-fold reduced toxicity in the rabbit ileal loop assay.

The question is whether LTR72 mutant can be safely

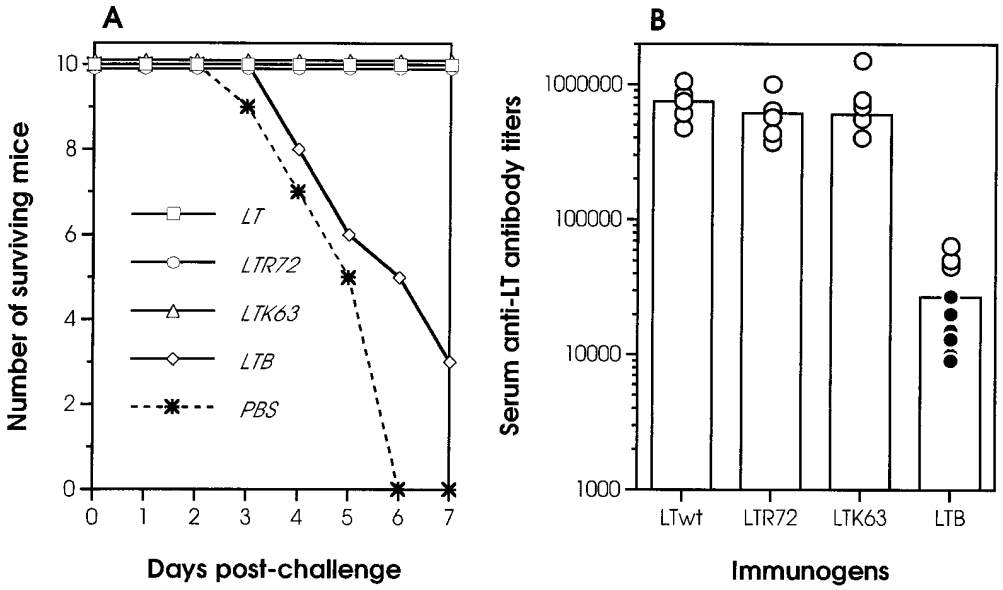

Figure 7. Protection of intranasal immunization with wild-type LT, LTR72, and LTK63 against systemic challenge with wild-type LT. (*A*) Mice survival to the challenge with 2 × LD_{50} of wild-type LT after two intranasal immunizations with 1 μg of wild-type LT, LTR72, LTK63, and LTB. Mice were observed for 7 d after challenge. (*B*) Ig anti-LT immune response in sera of mice before challenge. Antibody titers of individual mice survived to the challenge are reported as white dots; antibody titers of mice which did not survive to the challenge are reported as black dots.

used in humans. So far the experience in humans is limited: CTB has been safely used at a 1-mg oral dose (20) and up to a 100-μg intranasal dose (50). In the case of LT, the only published information refers to the use of LTG192 (51). This was safe orally at 5-, 25-, and 50-μg doses, whereas at a 100-μg dose it induced mild diarrhea in some of the volunteers. Based on the above data, it is likely that LTR72, which in the rabbit ileal loop is at least 20-fold less toxic than LTG192, can be used safely in humans at high doses. Since the immunogenic and adjuvant dose is predicted to be in the range of 25–200 μg, it is likely that LTR72 has the appropriate safety window to be safely used in the open population, including adults and children.

Adjuvanticity Derives from both the Nontoxic AB Complex and the Enzymatic Activity. rLTB, LTK63, LTR72, and wild-type LT have been compared in this study to determine which is the optimal mucosal adjuvant and to understand the role played in adjuvanticity of the following factors: the B subunit, the nontoxic AB complex, and the enzymatic activity.

Adjuvanticity is a complex feature because two properties of LT (the nontoxic AB complex and the enzymatic activity) seem to contribute independently to the adjuvant effect. Therefore, the final adjuvanticity of LT results from both properties. Here we have confirmed that the B subunit is a very poor mucosal adjuvant, whereas LTK63 acts as a good mucosal adjuvant, showing that the nontoxic AB complex has an adjuvant effect despite the absence of any ADP-ribosylating activity. However, the antibody titers to the coadministered antigens induced by LTK63 were lower than those obtained using the wild-type toxin and were detected only after two immunizations. In marked contrast, the mutant LTR72 generated anti-OVA responses after one intranasal immunization, in a manner equivalent to that induced by wild-type LT. These data show that the presence of enzymatic activity enhances the adjuvanticity of LT mutants. However, the full enzymatic activity of wild-type LT is not necessary and mutants with only 0.6% enzymatic activity, such as LTR72, retain most of the adjuvant properties of wild-type LT. The above conclusions based on antibody levels were fully confirmed by the ability of the mutants tested of inducing antigen-specific T cell proliferative responses: LT and LT mutants, but not rLTB, were able to enhance priming of OVA-specific $CD4^+$ T lymphocytes. It is reasonable to speculate that this enhanced antigen-specific T cell priming was responsible for an enhanced antigen-specific B cell help, which was at the basis of the higher and faster OVA-specific IgG and IgA antibody response in mice receiving LT or LT mutants as mucosal adjuvants.

A dose–response curve (Fig. 6 *A*) provided the rationale for the relative contribution to adjuvanticity of the nontoxic AB complex and of the enzymatic activity. The rationale is schematically represented in Fig. 6 *B*. At a low dose (2.5 ng), only wild-type LT showed an adjuvant effect, suggesting that this is entirely due to the enzymatic activity present in wild-type LT. At higher doses (between 50 ng and 1 μg), the adjuvant effect of LT and LTK63 showed a parallel growth, suggesting that the increase observed in this part of the curve is only due to the enzymatically inactive AB complex present both in LTK63 and LT, and that the difference is due to the adjuvant effect of the enzymatic activity present in LT only. At doses higher than 1 μg, the LT curve reaches a plateau possibly because of toxicity. The adjuvanticity of LTR72 follows the curve of LTK63 at very low doses (up to 50 ng), but joins the curve of LT at 1 μg likely because at this dose the enzymatic activity reaches the threshold level. Then the adjuvant effect of LTR72 continues to increase along the theoretical curve of wild-type LT beyond the LT because it is not affected by the toxicity induced by high doses of LT. In conclusion, the data confirm that the adjuvant effect derives from the inde-

pendent contribution of the enzymatic activity and the nontoxic AB complex. For the enzymatic activity in our experiments, 2.5 ng are enough and the increase above this level does not significantly enhance adjuvanticity. Conversely, the adjuvant effect of the nontoxic AB complex is dose dependent, so that the higher adjuvanticity corresponds to the higher dose. This makes LTR72 an ideal adjuvant since, without incurring in problems of toxicity, it can take advantage of the adjuvant effect induced by the combination of low levels of enzymatic activity and high doses of the nontoxic AB complex.

Mechanisms of Adjuvanticity. It is not clear yet through which mechanism(s) LT and LT molecules exert their mucosal adjuvanticity. It has been postulated that these molecules may activate macrophages (or possibly other antigen-presenting cells) by increasing the expression of costimulatory molecules such as B7-1 and/or B7-2 (52). However, it is not possible to rule out that LT may potentiate some intracellular events involved in uptake, processing, and presentation of antigen by antigen-presenting cells. In the case of CT, it has been reported that the adjuvant activity of CT could be mediated by its ability to enhance antigen presentation by macrophages, and this effect has been associated with increased production of IL-1 (53). Several studies have shown that CT could enhance production of IL-1 and IL-6 by macrophages or epithelial cells, and that the ADP-ribosylation activity of CT could contribute to these effects (54). In the case of LT, it has been reported that oral immunization with wild-type LT promotes B cell isotype switching and subclass responses to IgG1, IgG2a, IgG2b, and IgA; it induces a mixed $CD4^+$ Th1-Th2–type response, and in vitro stimulation of $CD4^+$ T cells resulted in synthesis of IFN-γ and IL-5, and low levels of IL-4 (55). After the results reported in this work, it would be interesting to use the reagents described here to define which of the above mechanisms is triggered by the enzymatic activity and which by the nontoxic AB complex.

It is possible that during internalization and retrograde transport through the Golgi, the A subunit could reach and interact with some unidentified proteins within the target cells, altering some signal transduction pathways.

Immunogenicity and In Vivo Protective Efficacy of LT Mutants. Here we have shown that both LTK63 and LTR72 are as immunogenic as wild-type LT and more immunogenic than rLTB. This indicates that the enzymatic activity is not needed and that the receptor binding is not enough for optimal immunogenicity. We have previously demonstrated that the nontoxic mutants of LT are able to induce in vitro neutralizing antibodies against the A subunit when used to immunize mice and rabbits by systemic route (27, 28), and that mucosal immunization with wild-type LT and LTK63 induces significant levels of anti-A antibodies (56). In the present study we have also shown that the levels of anti-LT antibodies induced in mice immunized intranasally with the holotoxin were higher than those induced in mice immunized with the rLTB. However, so far we have not addressed the question of whether the anti-A antibodies induced were able to neutralize the toxin in vivo. Here we have shown that the antibodies induced by intranasal immunization with the mutant holotoxins are more efficient in the neutralization of the toxic activity of LT in vivo compared to those induced by the rLTB. The in vivo mouse model used has been previously proposed for evaluation of antitoxic protective effect of cholera vaccines (57) and is based on the pathophysiologic effects induced by the systemic challenge of CT such as weight loss, atrophy of the spleen and thymus, and, when the dose is high enough, death (58). We have adopted the same model to LT, and have shown that all mice immunized intranasally with the mutant holotoxins were protected from the lethal challenge with wild-type LT, whereas, of those immunized with rLTB, only mice with highest antibody titers survived. A possible explanation is that the presence of an A subunit, even inactive, could increase the immunogenicity of the entire molecule, by providing a larger number of T and B cell epitopes, which may turn out to be those relevant for the induction of protective immune response. It has been shown that binding to target cells is required for immunogenicity (26) and therefore all the events associated to the induction of an immune response initiate after the binding of the toxin to its own receptor. It could be possible that, after binding and internalization in the antigen-presenting cells, the A subunit could influence events such as antigen processing and presentation.

Conclusions. We have shown that although ADP-ribosylation activity is not necessary for the adjuvanticity of LT, the presence of low levels of enzymatic activity may be useful to induce a faster and higher immune response to coadministered antigens. This makes LTR72 an ideal adjuvant since without incurring problems of toxicity, it can take advantage of the adjuvant effect of low levels of enzymatic activity and of high doses of nontoxic AB complex. Both LTK63 and LTR72 are excellent mucosal adjuvants and studies in humans are required to establish which is the best candidate. Perhaps the slightly different properties of the two molecules may be exploited for different applications in different vaccines. The mutants are also very good immunogens able to induce a protective immunity against LT and may therefore be useful as components of antidiarrheal vaccines.

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