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# LTK63 and LTR72, two mucosal adjuvants ready for clinical trials

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## Introduction

The most powerful mucosal immunogens recognised to date are cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin (LT), the molecules that cause the debilitating watery secretions typical of cholera and traveller's diarrhoea, respectively (Mekalanos et al., 1983; Spangler, 1992). The anti-toxin response induced is so potent that a strong immune response is also activated against foreign bystander molecules that are present simultaneously at the mucosal surface (Elson, 1989). Nevertheless, the use of CT and LT as mucosal immunogens and adjuvants in humans has been limited by their toxicity (Levine et al., 1983). The three-dimensional structure of CT and LT has been solved (Sixma et al., 1991) and these results have prompted attempts to dissect, using genetic techniques, the mucosal immunogenicity and adjuvant activity of CT and LT from their toxicity. Such studies have allowed to design non toxic but immunogenic molecules and to define the roles of the receptor-binding B domain, the A subunit, and the enzymatic activity of LT and CT in mucosal immunogenicity and adjuvanticity.

## LT and CT structure-function relationship

CT and LT have high homology (80 % identity) in their primary structure (Dallas and Falkow, 1980; Spicer et al., 1981) and super-imposable tertiary structures (Sixma et al., 1991). Both toxins are composed of an enzymatically active A subunit that is responsible for the toxicity and a pentameric B oligomer that binds the receptor(s) located on the surface of the eukaryotic cells (Rappuoli and Pizza, 1991) (Fig. 1). CT binds mostly to the GM1 ganglioside (Holmgren et al., 1973), while LT binds not only GM1 but also to other glycosphingolipids and glycoprotein receptors (Sugii, 1989).

The A subunit is divided into the globular (enzymatically active) $A_1$ and the carboxy-terminal $A_2$ domains that remain linked by a disulphide bridge between the $A_1$-Cys187 and the $A_2$-Cys199. Proteolytic cleavage of the loop and reduction of the disulphide bridge are both necessary in order to generate the enzymatic activity (Gill and Rappaport, 1979). The A subunitis an enzyme with ADP-ribosylating activity that binds NAD and transfers the ADP-ribose group to the stimulatory α subunit of Gs, a GTP-binding protein which regulates the activity of adenylate cyclase.

The ADP-ribosylation of Gs leads to a permanent activation of adenylate cyclase with an intracellular accumulation of cAMP which causes an increase in secretion of water and chloride ions in the intestine (Field et al., 1989). The NAD-binding and catalytic site is a cavity, formed by a β-strand (the floor of the cavity) and an α-helix (the ceiling of the cavity) that contains the amino acids essential for catalysis: Arg7 and Glu110-Glu112 (Domenighini and Rappuoli, 1996). A peculiar feature of CT and LT is that the basal ADP-ribosyltransferase activity is enhanced by interaction with the 20-kDa

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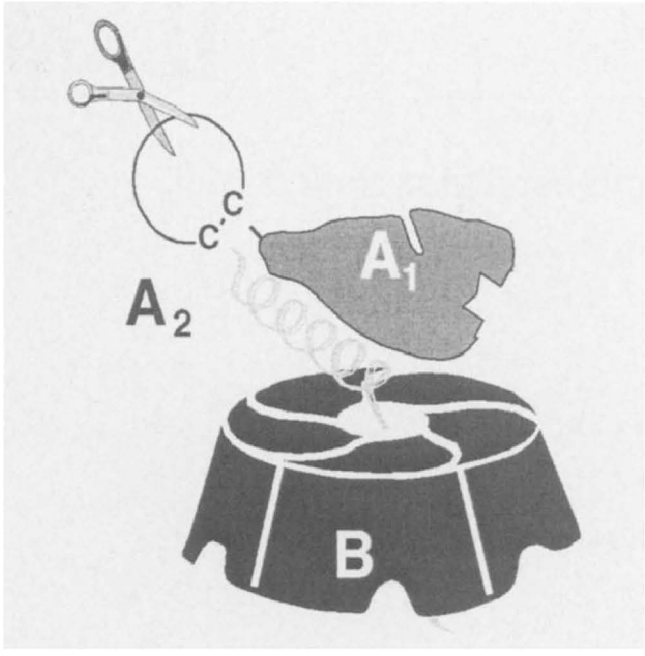


**Fig. 1.** Schematic representation of LT and CT, showing the A and B subunits and the site of proteolytic cleavage of the loop between the A1 and A2 domains.

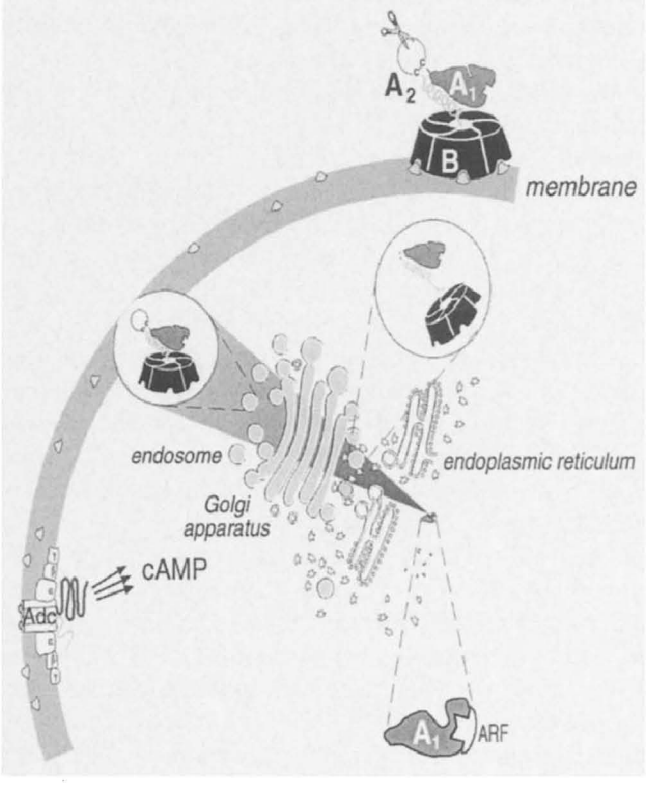


**Fig. 2.** Sequence of events that take place during cell intoxication: the toxin binds the receptor located on the plasma membrane of eukaryotic cells, it is internalized into vesicles. The vesicles are transported to the Golgi where the holotoxin is disassembled. The A subunit is transported from the Golgi to the ER, the A or the A1 subunit is translocated from the ER to the cytosol, where it could interact with the soluble ARF. The activated A1 migrates to the plasma membrane where the substrate Gs is located.

guanine-nucleotide-binding proteins, known as ADP-ribosylation factors or ARFs (Moss and Vaughan, 1995) which play also a crucial role in vesicular membrane trafficking. Whether ARF activates CT and LT within eukaryotic cells remains still unknown.

The sequence of events that takes place during cell intoxication is schematically shown in Figure 2 and can be summarised as follows (Bastieaens et al., 1996; Majoul et al., 1996): the toxin binds the receptor located on the surface of eukaryotic cells, it is internalised into vesicles, transported to the Golgi and dissociated. The A subunit is transported from the Golgi to the endoplasmic reticulum (ER) compartment, while the B subunit persists in the Golgi and is later degraded. The A subunit (or the A1 domain) is translocated, by an unknown mechanism, from the ER to the cytosol where, according to in vitro data, it can interact with the soluble ARF leading to activation. Then the active A1 subunit ADP-ribosylates the α subunit of Gs located at the plasma membrane.

## Genetically detoxified LT and CT derivatives

### Mutants in the LT and CT catalytic sites

In order to study the structure-function relationship of CT and LT and to find molecules that are non-toxic but still active as mucosal adjuvants and immunogens, more than fifty different molecules have been produced by site-directed mutagenesis and analysed for their biochemical and immunological properties. These studies have shown that most site-directed mutations in the A subunit either do not influence enzymatic activity or reduce it by only two- to ten-fold. Other mutants do not assemble, assemble poorly into the AB structure or are unstable during storage. However, some mutants are efficiently assembled, are stable during storage and exhibit no detectable or very low ADP-ribosylating activity (Pizza et al., 1994; Fontana et al., 1995; Magagnoli et al., 1996). This class of mutants includes LTK63 and CTK63 (containing a serine-to-lysine substitution in position 63 of the A subunit). These holotoxoids have no detectable enzymatic activity and no toxicity in vitro or in vivo, even when huge amounts of them are used (Figs. 3 and 4) (Fontana et al., 1995; Giuliani et al., 1998).

Therefore, we can consider them to be complete knock-outs of enzymatic activity. The crystal structure of LTK63 has been solved and has shown a complete identity to the wild-type LT across the entire molecule, with the exception of the active site where, the bulky side chain of the lysine 63 fills the catalytic cavity, thus making it unsuitable for the enzymatic activity (Van den

Akker et al., 1997). Furthermore, many other biological properties of LTK63 are maintained intact, including receptor and ARF binding (Stevens et al., 1999).

A further class of mutant molecules comprises LTR72 (with an alanine-to-arginine substitution in position 72 of the A subunit) and CTS106 (with a proline-to-serine substitution in position 106 of the A subunit). These mutants, have approximately 1% of the wild-type ADP ribosylating activity, a reduced ($10^4$–$10^5$-fold) toxicity in vitro against Y1 cells, and 1% toxicity in vivo in the rabbit ileal loop assay (Figs. 3 and 4) (Fontana et al., 1995; Giuliani et al., 1998).

## Mutants in the protease-sensitive loop

Mutants in this region have been constructed with the aim to make the loop insensitive to proteases and, thus, render the toxin not susceptible to the activation necessary for enzymatic activity and toxicity. Among the many mutants constructed, LTG192 is the best characterised. In this LT mutant, the arginine in position 192 is replaced by a glycine (Giannelli et al., 1997; Cieplak et al., 1995; Dickinson and Clements, 1995). In vitro, the mutant is completely resistant to trypsin treatment; in vivo, proteases other than trypsin may partially cleave the loop and activate the toxin, since toxicity is detectable. The toxicity observed in Y1 cells is approximately $10^3$-fold lower than that of wild-type toxin during the first 8 hours of incubation and becomes only 5–10 times lower than wild type following longer incubation (Giannelli et al., 1997). In practice, this molecule takes longer to be activated but delivers approximately the same total enzymatic activity as the wild type. The difference is that the delivery of the active toxin is diluted over a longer period of time. In vivo, in the rabbit ileal loop, only very little difference in toxicity is observed between LTG192 and wild-type LT (Giannelli et al., 1997) (Figure 3).

## Immunological properties of CT derivatives

CT mutants have been evaluated for their ability to act as mucosal adjuvants for coadministered bystander antigens (Douce et al., 1997). The results of this study, summarised in Figure 5, show that there is a direct correlation between adjuvant activity of the CT mutants and their ADP-ribosylation activity. In fact, the wild-type CT induces the highest antigen-specific immune response. The CTS106 mutant, which retains some enzymatic activity is a less effective mucosal adjuvant compared to wild-type CT but more effective than the CTK63, the mutant devoid of enzymatic activity. The CTK63 induces a weak adjuvant response which is comparable to that induced by the recombinant CTB.

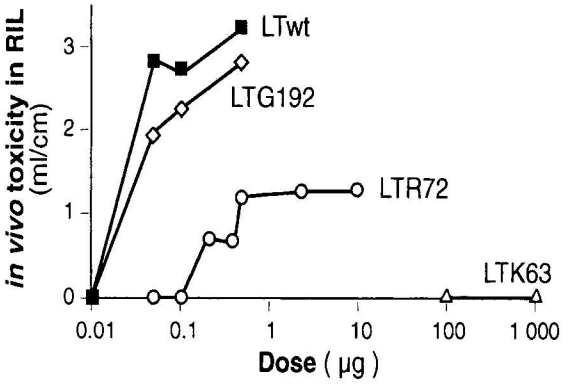


**Fig. 3.** In vivo toxicity in the rabbit ileal loop assay of LT derivatives. Toxicity is expressed as the ratio of the amount of fluid collected in each loop to the length of the loop.

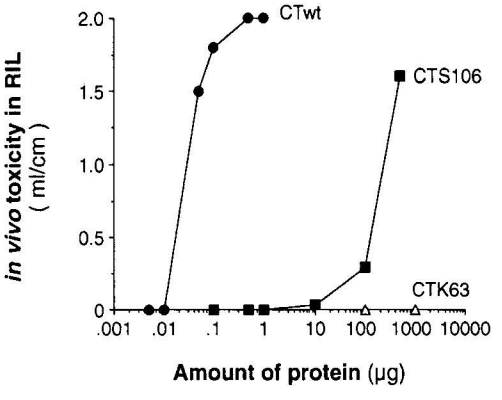


**Fig. 4.** In vivo toxicity in the rabbit ileal loop assay of CT derivatives. Toxicity is expressed as the ratio of the amount of fluid collected in each loop to the length of the loop.

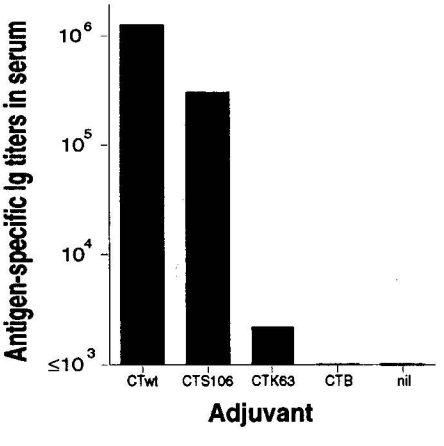


**Fig. 5.** Ig immune response to bystander antigens in sera of mice immunised intranasally with wild-type CT and its genetically detoxified derivatives as adjuvants.

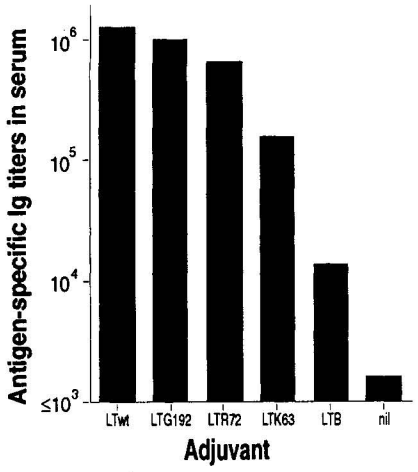


**Fig. 6.** Ig immune response to bystander antigens in sera of mice immunised intranasally with wild-type LT and its genetically detoxified derivatives as adjuvants.

Other CT mutants devoid of enzymatic activity and toxicity but still active as mucosal immunogens and adjuvants have been described in the References. However, for the interpretation of those data is important to point out that in those experiments, the amount of CT mutants used were ten times higher than that of wild-type CT (Yamamoto et al., 1997).

## Immunological properties of LT derivatives

The ability of the LT mutants to act as mucosal immunogens and adjuvants in the animal model has been extensively characterised. The immunological properties of the LTG192, LTR72 and LTK63 mutants have been compared to those of the wild-type LT and of the recombinant LTB. The results of this study are summarised in Figure 6. As expected, no differences were observed in the adjuvant activity of the LTG192 mutant and the wild-type LT. LTR72, the mutant retaining residual levels of enzymatic activity exhibited a mucosal adjuvanticity similar to that induced by the wild-type LT and higher than that induced by the LTK63, the mutant devoid of any enzymatic activity and toxicity.

**Table 1.** Adjuvant properties of LTK63 and LTR72.

| Adjuvant LTK63 | LTR72 | Antigen | Route of immunization | Animal model | Reference |
|---|---|---|---|---|---|
| + | + | Ovalbumin | Intranasal | Mice | Giuliani et al., 1998; Douce et al., 1997 |
| + | | Fragment C of tetanus toxin | Intranasal | Mice | Douce et al., 1997 |
| + | | CTL epitope of measles virus | Intranasal | Mice | Partidos et al., 1966, 1999 |
| + | + | Diphtheria,Tetanus, Pertussis | Intranasal | Mice | K. Mills, University of Ireland* |
| + | + | FHA, 69K and PT9K/129G of *Bordetella pertussis* | Intranasal | Mice | Ryan et al., 1999 |
| + | | Subunit influenza vaccine | Intranasal | Mice | Barchfeld et al., 1999; Barackman et al., 1999 |
| + | | gD2 of Herpes Simplex Virus | Intranasal | Mice | O'Hagan et al., 1999; Ugozzoli et al., 1998 |
| + | | p24 and gp120 of HIV | Intranasal | Mice | D. O'Hagan, Chiron SpA, Emeryville (USA)* |
| + | | Meningococcus C conjugate | Intranasal | Mice | G. Del Giudice, IRIS, Siena (Italy)* |
| + | + | Pneumococcus polysaccharide | Intranasal | Mice | Jakobsen et al., 1999 |
| + | | Ovalbumin | Intravaginal | Mice | Di Tommaso et al., 1996 |
| + | | Subunit influenza vaccine | Intranasal | Rabbits and minipigs | D. O'Hagan, Chiron SpA, Emeryville (USA)* |
| + | | CagA, VacA and urease of *H. pylori* | Oral | Mice and beagle dogs | Ghiara et al., 1997; Marchetti et al., 1998; G. Del Giudice, IRIS, Siena (Italy)* |
| + | | KLH | Oral | Mice | Douce et al., 1999 |

* unpublished observations.

The LTK63 behaved as a strong mucosal adjuvant although the activity was reproducibly reduced in comparison to wild-type LT and to LTR72 but significantly higher than that of the recombinant LTB.

Both LTK63 and LTR72 mutants are able to elicit an antibody response against co-administered bacterial and viral antigens and synthetic peptides (see Table 1), to strongly enhance protection against challenge in appropriate animal models, and to favour priming of antigen-specific CTL. The adjuvant activity for the different antigens is detectable following intranasal, vaginal or oral immunization, not only in mice but also in other species (Table 1).

Interestingly, LTK63 is consistently a better immunogen than LTB (Pizza et al., 1994), suggesting an important role for the enzymatically inactive A subunit in the induction of an immune response, a property that may reflect not only the larger number of B and T cell epitopes provided by the A subunit but also its ability to influence intracellular events, such as antigen processing and presentation.

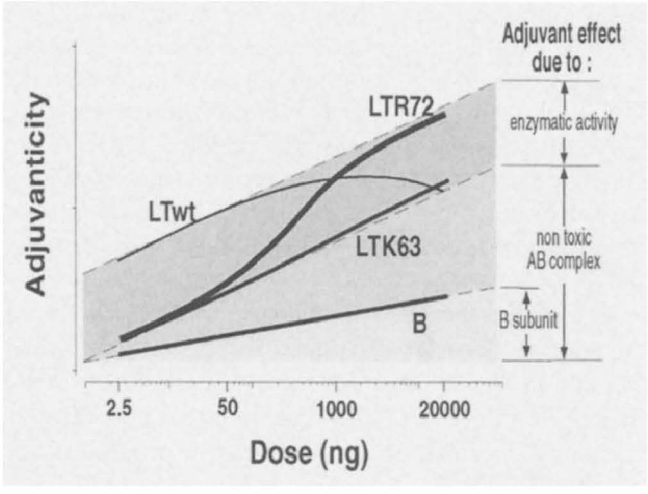


**Fig. 7.** Results of a dose-response experiment, showing the relative contribution of the B subunit, the non-toxic AB complex, and the enzymatic activity to adjuvanticity.

### The non-toxic AB complex and the enzymatic activity contribute independently to the adjuvanticity

The results reported in Figure 6 show that although the enzymatic activity is not necessary for adjuvanticity, the presence of low levels of enzymatic activity may be useful to induce a faster and higher immune response to co-administered antigens.

The availability of molecularly defined mutants has allowed to clarify the role and the relative contribution of the B subunit, the non-toxic AB complex and the enzymatic activity to adjuvanticity. Adjuvant activity of LTK63, LTR72, wild-type LT and LTB were evaluated in a dose-response experiment (Giuliani et al., 1998) and the results are reported in Figure 7. LTK63 had no detectable adjuvant activity at 2.5 ng, and a low adjuvant activity at 50 ng that increased in a dose-dependent manner. The B subunit had a low adjuvant activity even at very high doses. Wild-type LT, in marked contrast, had an adjuvant activity already at 2.5 ng suggesting that at this dose the enzymatic activity provides the adjuvant effect. Increasing the dose of LT, the adjuvant activity increased in a dose-dependent manner, with a curve parallel to LTK63, suggesting that an increase in the enzymatic activity did not provide any further benefit over LTK63. Finally, when the dose of LT reached 20 μg, the curve reached a plateau, possibly because of the toxicity of the molecule at this dose. Interestingly, the LTR72 behaved in an intermediate way: like LTK63, it had no adjuvant activity at low doses, and a minimal adjuvant activity at 50 ng. Above this dose, it had an adjuvant activity similar to that of LT and at very high doses even better than LT, possibly because it is not affected by the toxicity induced by high doses of LT. The interpretation of these results is that enzymatic activity provides a dose-independent adjuvant effect. In order to have this effect a threshold of activity is needed. This threshold is reached already at 2.5 ng with LT and approximately at 1 μg with LTR72. The non-toxic AB complex, on the other hand, provides an adjuvant activity that is dose-dependent and independent from the enzymatic activity. One question is: what is present in the enzymatically inactive A subunit that confers to the non toxic AB complex an activity that is higher than that present in the B subunit alone? The A subunit, in addition to the enzymatic activity has other functions such as binding to ARF, that may provide a signal to the immuno-competent cells. In addition, the enzymatically inactive A subunit is still able to interact with the vesicular transport system, to be transported to the Golgi, to undergo retrograde transport from the Golgi to the endoplasmic reticulum (Majoul et al., 1998) and to be translocated to the cytosol where it could bind specifically the ARF factors. One of these activities or a combination of them may be responsible for the adjuvanticity of the non-toxic AB complex.

## Conclusions and relevance for human therapeutics

LT and CT are potent mucosal immunogens and adjuvants in model systems. These proteins are resistant to protease degradation in vivo and target immune cells and epithelial cells, factors that contribute to their mu-

cosal immunogenicity. Non-toxic mutants of LT and CT can have adjuvant activity, and holotoxoid molecules appear to be more immunogenic and potentially better adjuvants than the B subunits alone. However, enzymatic activity associated with the A subunit can contribute to overall adjuvant activity. The A subunit may also contribute to adjuvanticity by interacting with regulatory proteins inside target cells using mechanisms independent from the ADP-ribosylating activity.

The availability of non-toxic derivatives of LT with adjuvant activity opens up an opportunity for their evaluation in humans. To date we have no evidence that these molecules can act as mucosal adjuvants in humans. Clinical trials are expected to proceed in the near future.

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