

Genetically derived toxoids for use as vaccines and adjuvants

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Abstract

Until very recently, development of vaccines has been based on an empirical approach. For example, bacterial toxins have been detoxified using empirical chemical treatment. Progress in biotechnology and molecular biology has allowed the fine knowledge of the structure-function relationship of several bacterial toxins. Thanks to this, the genetic attenuation of bacterial toxins has been made possible. Following this approach, a genetically detoxified pertussis toxin has been produced. This molecule is now the component of an acellular pertussis vaccine, which has been shown to be highly immunogenic and efficacious in infants. The same strategy of molecular detoxification of bacterial toxins has been applied to cholera toxin and to the *Escherichia coli* heat-labile enterotoxin. Toxin mutants devoid of any toxic activity have been produced and shown in animals to be highly immunogenic and to exhibit strong adjuvanticity when administered at mucosal sites in conjunction with several antigens. These successful results show that rational design of stronger and safer vaccines is feasible. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

During co-evolution with their hosts, microorganisms have developed sophisticated strategies permitting their survival and spreading, while favoring modifications in the physiology of their hosts. The events which underlie the bacteria-host interactions are multiple and complex, and foresee the involvement of bacterial factors enabling invasion, growth, and survival of bacteria [1], as well as of human factors (such as those linked to the immune response) which counterbalance the first in the attempt to limit microbial invasion and growth, and eventually eliminate the invaders. Among the different strategies developed by bacteria to colonize their hosts there are the toxins. Bacterial toxins are proteins composed of two functional units: one has enzymatic activity once the toxin is translocated intracellularly following endocytic path-

ways after the other unit has attached to specialized structures on the cell membrane. The structure of bacterial toxins can vary, as well as their toxic activity. In any case, the final outcome of the intoxication is the cell death.

A well known family of bacterial toxins has NAD-dependent ADP-ribosyltransferase activity, which is exerted on different cellular substrates. Some of these toxins, such as pertussis toxin (PT), cholera toxin (CT), and the heat-labile enterotoxin (LT) of *Escherichia coli*, ADP-ribosylate defined amino acid residues of the α subunit of particular G proteins, a family of GTP-binding proteins involved in signal transduction [2]. The outcome will be a constitutive activation of adenylate cyclase, accumulation of the second messenger cAMP, and finally cell death. Other toxins, such as diphtheria toxin (DT) and *Pseudomonas aeruginosa* exotoxin A, specifically ADP-ribosylate the elongation factor 2, which can not participate any longer in protein synthesis, thus causing cell death. All ADP-ribosylating toxins share a high degree of similarity in their structure. They consist of a monomeric A subunit, containing the catalytic moiety

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of the enzyme, and a cell-membrane binding B subunit, which can be monomeric (e.g., DT) or polymeric, made of homomers (e.g., CT and LT) or heteromers (e.g., PT).

The genes coding for these toxins have been cloned and the regulation of expression of the genes is now well understood. Likewise, the three-dimensional structure of most of these bacterial toxins has been elucidated and, in some cases, crystals have also been obtained. All this work has allowed a fine molecular definition of the structure-function relationship and has made possible the total or partial knock-out of their enzymatic and toxic activity, thus rendering them suitable for the development of vaccines. The feasibility of this genetic approach in the development of new vaccines is best elucidated by the genetically detoxified PT, which has been shown to be efficacious in preventing pertussis in infants, and is now being used as an acellular pertussis vaccine, and by the genetically detoxified mutants of LT and CT, that have been shown to behave as strong mucosal adjuvants.

2. An 'ouverture': the cross-reacting materials of diphtheria toxin

DT is among the strongest toxins known: as little as 100 ng/kg of body weight is lethal in sensitive species, such as humans, monkeys, rabbits, guinea pigs [3]. This toxin is a 58 kDa protein, produced by lysogenic *Corynebacterium diphtheriae* strains as a single polypeptide that, after reduction of disulphide bonds, consists of two fragments, A and B. The crystal structure of the molecule [4] has shown a C (catalytic) domain, corresponding to fragment A, a T (transmembrane or translocation) domain, consisting of nine α helices, and a C-terminal R (receptor-binding) domain, corresponding to fragment B. This fragment binds to its receptor, the heparin-binding EGF-like growth factor precursor [5], which is present on most animal cells. After binding and internalization via receptor-mediated endocytosis, fragment B undergoes conformational changes in the acidic endosomal compartment; this allows hydrophobic interactions of the T domain with the endosomal membranes, with final translocation of fragment A into the cytoplasm [6], where it becomes enzymatically active and catalyzes the transfer of the ADP-ribosyl group of NAD to a histidine residue of the cytoplasmic elongation factor 2 (EF-2). The ribosylated EF-2 becomes inactive, and this causes inhibition of the protein synthesis and death of the eukaryotic cell [7].

In 1971, Uchida and coworkers showed that, following mutagenesis of corynebacteriophage β tox⁺ with nitrosoguanidine, several phages could be isolated that

encoded non-toxic proteins [8]. These proteins were called cross-reacting material (CRM), since they were immunologically related to DT [9], and represented the first tool to dissect the functional domains of DT and identify the amino acids responsible for the enzymatic activity of fragment A and for the binding and translocation properties of fragment B. These observations were subsequently confirmed and extended when mutants of DT obtained by site-directed mutagenesis became available.

Of the different mutants generated by random chemical mutagenesis, CRM197 has showed the most promising results. In fact, CRM197, which bears the single Glu52 → Glu mutation in the A subunit, is totally devoid of enzymatic activity [10]. Although less stable and less immunogenic than the toxin [11], both stability and immunogenicity of CRM197 were significantly increased when the molecule was subjected to a very mild treatment with formaldehyde [12], i.e., at concentrations which are unsuitable for detoxification, but which have clearly been shown to potentiate the stability and immunogenicity of this and of several other bacterial toxins present in vaccine formulations [13]. Adult volunteers immunized with a diphtheria-tetanus vaccine containing CRM197 instead of diphtheria toxoid showed a significant boosting of the pre-existing anti-diphtheria toxin antibody response [14]. However, if CRM197 is not employed yet as a vaccine against diphtheria, it is being, currently, widely used as a carrier molecule for the production of conjugated vaccines containing capsular polysaccharides from encapsulated bacteria. CRM197-based conjugate vaccines against *Haemophilus influenzae* have been largely shown to be highly immunogenic and efficacious in infants and have now been licensed [15]. Together with other conjugated vaccines, their use has contributed to the reduction of the circulation of this bacterium in countries where the vaccines have been introduced. Furthermore, CRM197-based conjugate vaccines against group A and group C *Neisseria meningitidis* [16–19], and against several serotypes of *Streptococcus pneumoniae* [20] are at a very advanced stage of development.

3. Vaccine development 'à la carte': the genetic attenuation of the pertussis toxin

PT represents one of the major virulence factors of *Bordetella pertussis*, and it has been proposed that whooping cough can be a toxin-mediated disease [21]. Indeed, PT is produced by *B. pertussis*, but not by *Bordetella bronchiseptica* or *Bordetella parapertussis* [22], which do not cause the classical clinical symptoms characteristic of whooping cough. Furthermore, it is well known that the bacterial product, later identified

as PT, had several toxic effects in mice at very low concentrations. For example, 0.5 and 10 ng of PT render mice susceptible to death after histamine challenge, enhance IgE antibody production, potentiate anaphylactic phenomena, enhance insulin secretion, and induce lymphocytosis. Finally, doses as low as 500 ng are lethal to mice [23].

The PT is an ADP-ribosyltransferase of 105 kDa with a classical AB₅ heteromeric structure [24]. The A domain (S1 subunit), monomeric, contains the enzymatic activity and intoxicates eukaryotic cells by binding NAD and transferring the ADP-ribose to a cysteine residue present in the XCGLX motif in the C-terminus of the α subunit of many GTP-binding proteins, such as G_i, G_o, G_t, and G_{gust} involved in signal transduction [25]. The non-toxic B domain is a heteropentamer consisting of one S2, one S3, two S4, and one S5 subunit, noncovalently linked. Through the S2 and S3 subunits [26], it binds to cell membrane glycoproteins with a branched mannose core and an *N*-acetyl glucosamine attached [27]. The S1 subunit is thus translocated inside the mammalian cells where it interacts with the G proteins and blocks their activities. The PT subunits are coded by an operon, contain a signal peptide, and are cotranslationally transported into the periplasm where the holotoxin is assembled [28]. Secretion of the toxin takes place after assembly, and is more efficient in the presence of the A (S1) subunit [29].

During the seventies, it was shown in Japan that the development of acellular pertussis vaccines was feasible. These vaccines were prepared by concentration of *B. pertussis* Tomaha I strain supernatant, extraction and fractionation of antigen, and final inactivation with formaldehyde. These preparations were known to contain different proportions of PT, filamentous hemagglutinin (FHA), and agglutinogens (pertactin and fimbriae), they were less reactogenic than conventional DTP vaccine, and exhibited efficacy similar to that obtained by DTP [30]. These results prompted several manufacturers in Europe, USA, and Japan to formulate acellular pertussis vaccines containing defined doses of purified *B. pertussis* antigens, which were chemically inactivated. All acellular vaccines produced so far contain PT, some contain PT and FHA, the majority of them contain PT, FHA, and pertactin, and some contain PT, FHA, pertactin, and fimbriae-2 and -3 [31]. The general approach for inactivation of the bacterial antigens present in these vaccine formulations has consisted of different chemical treatments, such as with formaldehyde, glutaraldehyde, formaldehyde plus glutaraldehyde, hydrogen peroxide, or tetranitromethane [31]. It is, however, recognized that chemical treatment of PT with formaldehyde and glutaraldehyde is associated with significant reversion rates [32]. For example, the early vaccines produced in

Japan contained active PT at levels comparable to those found in whole-cell vaccines [30].

The production of mutant PT molecules by random mutagenesis using nitrosoguanidine treatment, as in the case of *C. diphtheriae*, was attempted but not pursued for vaccine development [33,34]. On the other hand, the expression of the PT operon in *E. coli* and the use of the purified recombinant toxin as an immunogen failed, because the assembly of the subunits composing the B domain of the toxin did not take place correctly when the proteins were expressed in *E. coli* [35], and because the conformational epitopes of the S1 subunit recognized by neutralizing antibodies were not formed in the protein expressed in *E. coli* [36].

In order to develop a PT molecule suitable for inclusion in acellular vaccines, i.e., a well assembled holotoxin molecule, devoid of any toxic (i.e., enzymatic) activity, several new S1 mutants were produced in *E. coli*, then by homologous recombination the chromosomal PT operon of *B. pertussis* was replaced with an operon containing the functional promoter and the PT coding genes with the amino acid substitutions [37]. The mutant *B. pertussis* strains produced a well assembled PT which, after purification, was undistinguishable from the wild-type toxin. Some mutants retained some residual enzymatic activity and in vitro toxicity on CHO cells. One, bearing substitutions at residues 9 (Arg → Lys) and 129 (Glu → Gly) in the S1 subunit, was totally devoid of any enzymatic activity and of any in vitro toxicity. Finally, the PT mutants retaining the neutralizing B-cell epitope contained in the S1 subunit were well recognized by human T-cell clones specific for S1 subunit epitopes, and conferred protection upon immunization in a mouse model of intracerebral challenge with wild-type *B. pertussis* [37,38].

As compared to the PT wild-type, the mutant carrying the substitutions at positions 9 and 129 did not exhibit any toxic effects: it did not induce leukocytosis, even at doses as high as 50 μ g/mouse; it did not cause insulin secretion, nor did it cause death after sensitization with histamine [38]. Interestingly, very low doses were able to induce high neutralizing titers of antibodies in mice and guinea pigs, which were not affected by mild treatment with formaldehyde [38,39]. Other studies have demonstrated that the genetically inactivated PT was also devoid of other toxic properties of the wild type PT. For example, as compared to wild type PT, the mutant had greatly reduced the ability to induce IgE in vitro [40] and in vivo [41,42], to induce long-lasting enhancement of nerve-mediated intestinal permeabilization of antigen uptake [41], to inhibit IL-1-mediated IL-2 release in EL4 6.1 cells [43], and to inhibit neutrophil migration [44].

On the contrary, the non-toxic properties of PT

were still conserved in the mutant PT, such as T-cell mitogenicity, hemagglutination [39], platelet activation [45], and mucosal adjuvanticity [42]. Finally, using a large panel of PT subunit-specific monoclonal antibodies, it has been shown that most of the monoclonal antibodies failed to recognize chemically detoxified PT molecules; on the contrary, their binding to the genetically detoxified PT was fully conserved, and in some cases even enhanced [46]. While confirming previous findings on mutant PT molecules [37–39], these data clearly demonstrate that chemical detoxification of PT destroys most of the epitopes important for neutralizing activity of antibodies, whereas such epitopes are fully conserved in the PT mutant obtained by genetic engineering.

The safety and immunogenicity of genetically inactivated PT has been tested in clinical trials, both in adult volunteers and in infants and children, as a monovalent mutant PT alone [47,48], in association with FHA and pertactin [49,50], and also with FHA and pertactin in association with diphtheria and tetanus toxoids (DTaP) [51]. These trials showed that the different acellular vaccine formulations containing the non-toxic PT mutant were extremely safe, and much safer than whole-cell pertussis vaccines. Furthermore, all formulations induced high titers of anti-PT neutralizing titers and very strong antigen-specific T-cell proliferative responses. Interestingly enough, the immune response induced by these vaccines in adult volunteers lasted for a long time, since both antigen-specific antibody and CD4⁺ T-cell responses were still detectable at significant levels four years after vaccination [52].

In a comparative phase II trial conducted at the National Institutes of Health, USA, safety and immunogenicity of thirteen acellular pertussis vaccines were investigated in infants [31]. The final formulation of the pertussis vaccine with the genetically inactivated PT contained only 5 µg mutant PT, 2.5 µg FHA, and 2.5 µg pertactin, whereas the chemically inactivated vaccines contained 5 to 10 times more antigens per dose. A striking finding of this trial was that, despite its very low dose, mutant PT was more immunogenic than chemically detoxified PT, both in terms of ELISA antibody titers and of neutralizing antibody titers. The difference between the genetic versus chemical PT preparations was even more evident when the results were normalized by µg of antigen per dose, with the mutant PT being 10 to 20 times more immunogenic than the chemically detoxified PT [53]. Administration of a fourth dose of vaccines to the same infant population confirmed the high degree of safety of acellular vaccines, and the superior immunogenicity of the genetically detoxified vaccine over the chemically detoxified vaccines [54].

Similarly, in a double-blind placebo-controlled phase III efficacy trial carried out in Italy and involving

about 16,000 infants, despite the lower content of pertussis antigens (5, 2.5, and 2.5 µg of mutant PT, FHA, and pertactin, respectively), infants who received the recombinant DTaP had significant higher titers of anti-PT and neutralizing antibodies as compared to infants receiving conventional whole-cell DTP, and to infants receiving DTaP containing the chemically detoxified PT (composed of 25, 25, and 8 µg of PT, FHA, and pertactin, respectively) [55]. In this trial, the DTaP containing the genetically detoxified PT exhibited high levels of efficacy against whooping cough (84%) [55]. This level of efficacy has been recently confirmed in a very large efficacy trial carried out in Sweden and involving more than 80,000 infants [56,57]. During a 33-month follow up in the Italian efficacy trial, the infants experienced fewer pertussis cases than those receiving an efficacious DTaP vaccine containing a higher dose of PT prepared by conventional chemical inactivation [58]. It is interesting to note that if the two DTaP vaccines were equally efficacious against disease in the stage going from 1 month to 17 months after the third immunization, the DTaP vaccine containing the genetically detoxified PT showed a superior efficacy in the early stage (from the first immunization to 1 month after the third), when protection is believed to be partial, and at later stages (from 17 to 30 months after complete immunization), when vaccine-induced antibody response has declined. Since the two vaccines differed in the quality of the PT antigen contained, it is very likely that the superior protective efficacy of the vaccine containing the genetically inactivated PT was indeed due to the PT component, which induced a qualitatively better response, i.e., able to confer protection at stages when the quantity of the antibodies is not per se sufficient to protect against disease.

These clinical studies have shown that a rational molecular approach to the development of safe and strong vaccines is feasible. This approach is undeniably superior to the conventional empirical approach of chemical detoxification of antigens, since it allows to reduce the immunizing dose of antigens while keeping a strong and long-lasting immunity in vaccinees and, at the same time, reducing the risk of unwanted side effects. Furthermore, these successful results clearly showed that a similar molecular approach could be applied to other bacterial toxins.

4. Non-toxic LT and CT mutants as strong mucosal adjuvants

CT is known to be a powerful immunogen when given orally in its native form to animals [59], and a strong adjuvant for antigens co-administered at the same mucosal surface; similar characteristics of muco-

sal immunogenicity and adjuvanticity are shared by LT [60–62]. Their use in humans is, however, limited by the fact that CT and LT are extremely toxic, and very low doses are sufficient to induce severe diarrhoea in human volunteers [63]. The knowledge of the molecular structure of CT and LT has allowed a rational approach towards the genetic removal of the toxic activities of these molecules, while retaining their powerful immunogenicity and adjuvanticity for mucosal immunization.

CT is an AB₅ toxin which consists of a monomeric A subunit of 27 kDa, with ADP-ribosyltransferase activity, and of a B subunit composed of five identical monomers (of 11.5 kDa, each) which bind to the cellular receptor, the GM1 ganglioside [64], present on most epithelial cells. The A subunit contains two domains, linked together by a loop formed by a disulfide bridge: the A1 is enzymatically active; the A2 domain is a long α helix inserted in the central hole formed after the assembly of the five B monomers [65]. Following internalization, the A subunit is activated by proteolytic cleavage of the loop linking together A1 and A2 domains. After trafficking through the Golgi and the endoplasmic reticulum compartments, the A subunit is translocated into the cytoplasm, where it binds NAD and transfers an ADP-ribose group to the arginine present in the LRXR~~V~~X~~T~~ motif of the α subunit of the G proteins G_s, G_t, and G_{olf} [66]. The outcome of this intoxication will be accumulation of cAMP, increase in the chloride and water secretion from the intestinal cells, and diarrhoea [67].

LT shares about 80% homology with CT [68,69] and their structures both in the A and B subunits are very similar [65]. Both toxins share numerous biological activities, although some differences exist between the two toxins, which may account for the differences in their biological and immunological properties.

Two different approaches have been followed in order to develop non-toxic CT and LT derivatives with strong mucosal adjuvanticity. The first one has been based on the observation that the non-toxic pentameric B subunit of CT and LT (CTB and LTb, respectively) also exerted mucosal adjuvanticity [70]. CTB is present in the formulation of a whole-cell killed cholera vaccine, which has been extensively studied in endemic areas in Asia and South America [71]. Further studies, however, showed that the adjuvant effect of these preparations was due to the presence of contaminating traces of wild-type toxins [72]. Moreover, when recombinant CTB and LTb became available, it was clear that B subunits were very poor mucosal adjuvants [73–75].

The second approach is based on the generation of genetically detoxified mutants of LT [76,77] and CT [74,78–80]. This approach has been made feasible, thanks to the knowledge of the three-dimensional

structures of these proteins. The NAD-binding cavity of their A subunits consists of a β -strand bent over an α -helix, forming the floor and the ceiling of the cavity, respectively. This has allowed the identification of amino acid residues which are critical for the enzymatic activity, thus as potential targets for site-directed mutagenesis [81]. Other LT mutants have been generated by substituting amino acid residues in the loop of the A subunit, with the aim of rendering the toxin insensitive to proteases, thus not susceptible to the activation which leads to the enzymatic activity and, in turn, to toxicity [82,83].

Among the several LT and CT mutants generated, those referred to as LTK7 (Arg7 $\rightarrow$ Lys) [75], LTK63 (Ser63 $\rightarrow$ Lys) [84,85], LTR72 (Ala72 $\rightarrow$ Arg) [85], LTR192G (Arg192 $\rightarrow$ Gly) [82,83], CTK63 (Ser63 $\rightarrow$ Lys), CTS106 (Pro106 $\rightarrow$ Ser) [79,86] and CTS61F (Ser61 $\rightarrow$ Phe) [80] have been characterized in detail for the induction of systemic and local immune response to co-administered antigens.

LTK7 and LTK63 are fully devoid of enzymatic and toxic activity, whereas LTR72 retains some residual enzymatic activity. All these mutants behaved as strong mucosal adjuvants for all the antigens tested when given intranasally, orally, or vaginally, inducing very strong systemic and mucosal antigen-specific antibody responses. The mutant retaining a residual enzymatic activity, LTR72, was the strongest adjuvant, as compared to the fully non-toxic LTK63 mutant. In fact, it induced antigen-specific antibodies at titers similar to those induced by wild-type LT, and the induction of these antibodies required only one intranasal administration of antigen plus adjuvant. Furthermore, mice immunized with antigen along with LTR72 exhibited the strongest proliferative responses in vitro of antigen-specific CD4⁺ T lymphocytes, very similar to that observed in mice receiving wild type LT as an adjuvant [85]. In addition, the use of these mutants (e.g., LTK63) as mucosal adjuvants significantly enhanced the level of protection achieved in mice following oral immunization with *Helicobacter pylori* antigens and challenge with live bacteria, both in the prophylactic [87] and therapeutic approaches of vaccination [88]. These data are in full agreement with those previously obtained in mice immunized intranasally with the tetanus toxin fragment C along with the LTK7 mutant and then challenged systemically with tetanus toxin [75]. Thus, not only the mutants behave as a strong mucosal adjuvants, but they are also able to favor protective immunity in appropriate animal models of challenge.

As expected, the LTR192G mutant was completely resistant to trypsin cleavage in vitro [83,89]. However, when tested in vitro on Y1 cells, the LTR192G mutant was 1000 fold less toxic than wild-type LT after 8 h incubation; but only 5 to 10 times less that LT following

longer incubations [89], suggesting that the A subunit of this mutant requires longer time to be activated. Furthermore, no difference was observed between LT and the LTR192G mutant *in vivo*, in the rabbit ileal loop test.

LT mutants (e.g., LTK7) have been shown to behave as strong adjuvants also after subcutaneous immunization. As expected, in this case the antigen-specific antibody response is detectable at the systemic, but not at the mucosal level [75]. Very recently, it has been shown that wild-type CT and LT act as adjuvants also when directly applied onto the skin of animal together with the antigen [90]. It may be predicted that the mutants described above may exert the same effect after transcutaneous delivery along the antigen. Furthermore, intranasal immunization of peptides from measles virus, reproducing CD8⁺ epitopes together with the LTK63 mutant, also induce systemic priming of peptide-specific cytotoxic CD8⁺ T lymphocytes [91], suggesting that the use of these mutants at the mucosal level may represent an easy and feasible procedure to induce protective cytotoxic responses. However, the role of the CD8⁺ cells induced in this manner in the effector mechanisms of the immune response remain to be determined in appropriate animal models of protection.

The fine molecular mechanisms of mucosal adjuvanticity of LT and CT mutants are still not fully understood, but the available data allow to draw some reasonable hypotheses. For example, non-binding mutants are unable to behave as immunogens nor as mucosal adjuvants [92]. These data support the concept that binding of these molecules to the mucosal surfaces represents a prerequisite for both immunogenicity and adjuvanticity.

In addition, the fact that non-toxic mutants of LT (e.g., LTK63) behave as strong mucosal adjuvants suggests that the enzymatic activity of the A subunit is not necessary *per se* in the adjuvanticity of the mutants, and that the holotoxin structure exerts an important role in adjuvanticity, since LTb, completely lacking the A subunit, is a weak adjuvant. The first hypothesis is, however, in disagreement with previous observations showing that a fully non-toxic LT mutant (Glu → Lys at position 112) did not exhibit any adjuvanticity [74]. This disagreement is probably due to the different biological properties of CT and LT mutants, to their stability, or to the route of immunization employed by different groups. In fact, the fully non-toxic LTK63 mutant is a strong mucosal adjuvant [84,85], whereas the homologous mutant of CT (CTLK63) is a very weak adjuvant [86].

Finally, LTR72 and CTS106, which retain a residual enzymatic activity and toxicity *in vitro* and *in vivo*, exert the strongest mucosal adjuvanticity, with properties approaching those of wild-type LT [85,86]. In

dose-response experiments of adjuvanticity, very low doses (ng) LT exerted significant mucosal adjuvanticity, whereas the mutants were very weak. By increasing the doses, the adjuvant effect of LTK63 and LTR72 increased linearly, with LTR72 behaving better than the non-toxic LTK63 and reaching the adjuvant effect of LT, which at the highest doses did not perform better than LTR72 [85]. It is then logical to hypothesize that the contribution of the enzymatic activity to the adjuvanticity is dose-dependent, with an evident toxic effect *in vivo* with the wild-type LT, but not with the LTR72 mutant. The adjuvant effect observed with the non-toxic LTK63 molecule may be explained by a correct trafficking through the vesicular compartments, e.g., the Golgi apparatus, and to the ability of the mutated A subunit to be directed to the endoplasmic reticulum, and translocated into the cytoplasm. It will be important to understand whether and how the interaction of the different mutants with the different cellular compartments contributes to the enhancement of the immune response to co-administered antigens.

It can be concluded that a rational genetic approach has led to the design of molecules, that in animal models behave as strong mucosal adjuvants. The final answer on the possible use of these mutants in mucosally delivered vaccines for human use will come from human trials. CTb has been shown to be safe at 1 mg/dose after oral delivery, and at 0.1 mg/dose after intranasal delivery [70]. The LTR192G mutant, which has toxic activity *in vitro* and *in vivo*, was safe at doses of 5, 25, and 50 µg, but induced diarrhoea in some volunteers receiving 100 µg/dose [93]. The LTR72 mutant, which is much less toxic than LTG192, and the LTK63 mutant, which is totally devoid of toxicity, are expected to exhibit a better safety profile.

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