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The Cholera Toxin as a Biotechnological Tool

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1. Introduction

It was as early as 1886 when Robert Koch proposed that the symptoms caused by *Vibrio cholerae* were initiated by a "poison" produced by the pathogen. However, it was not until 1959 that this postulate could be demonstrated by reproducing the disease in an animal model [De, 1959]. Today, cholera toxin (CT) is known to exhibit toxic effects in human cells and produces dehydrating diarrhea in humans. It is produced almost exclusively by few serogroups of *V. cholera*, however, sometimes may be naturally produced by other organisms, as the opportunistic pathogen *V. mimicus* [Nishibuchi and Seidler, 1983; Spira and Fedorka-Cray, 1984].

CT has important immunological properties and for that reason it has been extensively used as a systemic and mucosal adjuvant because it enhances the immunogenicity of most antigens fused or co-administered with the toxin [Sanchez and Holmgren, 2008].

The aim of this chapter will be to describe the biotechnological utilities of CT, with special attention to its adjuvant effect as well as its application in the treatment of autoimmune diseases through its ability to generate oral tolerance.

2. Structure

CT belongs to the family of AB_5-type toxins, since it is composed of two subunits in a 1:5 ratio. The A subunit (CTA), of 28 kDa, is a heterodimer associated non-covalently to a homopentamer formed by the subunits B (CTB) of 56 kDa [Merritt et al., 1994; Vanden Broeck et al., 2007]. CTA is responsible for the biological activity and CTB binds to the cell membrane receptor [Holmgren et al., 1973; Lonnroth and Holmgren, 1973] (Fig. 1.).

CTA comprises 240 amino acids, and the 11.6 kDa B subunit monomers each have 103 amino acids. CTA is synthesized as a single polypeptide chain and is post-translationally modified through the action of a *V. cholerae* protease at position R192 [Mekalanos et al., 1979]. The cleavage of this amino acid, found in an exposed loop that extends from C187 to C199 residues, generates two fragments named CTA1 and CTA2, which remain linked by a disulfide bridge [Lencer and Tsai, 2003; Tsai et al., 2001]. The toxic activity (enzymatic ADP-ribosylating) activity of CTA resides in CTA1, whereas CTA2 serves to insert CTA into the CTB pentamer [Sanchez and Holmgren, 2011]. The C-terminal hydrophobic region including residues 162-192 of CTA1, plays a key role in toxicity. It triggers the ER-associated degradation (ERAD) mechanism (see section 3) and facilitates interaction with

the cytosolic ADP-ribosylation factors (ARFs) that serve as allosteric activators of CTA1 [Teter et al., 2006].

The remarkable stability of pentameric CTB is attributed to non-covalent interactions including 130 hydrogen bonds, 20 salt bridges, as well as tight packing of subunits via hydrophobic and pentamer-pentamer interactions. Consequently, the CTB pentamer is held together and remains as a complex unless boiled or monomerized by acidification at pH below 3 [Sanchez and Holmgren, 2008].

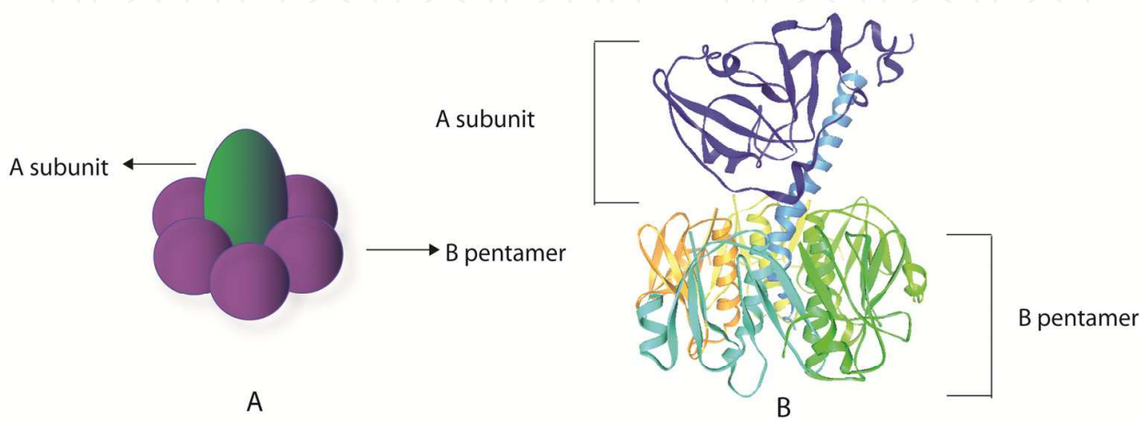

Fig. 1. Cholera toxin structure. A) Schematic model of cholera toxin. A subunit contains the toxic activity while B subunits bind to cells. B) Model based on X-ray crystallography analysis. Each subunit is represented by a different color. Adapted from Zhang et al 2005.

3. Binding and mechanism of action

CT is secreted through the outer membrane of *V. cholerae* and its toxic action begins when its B subunit binds to the high-affinity monoganglioside GM1 receptor. GM1 is a glycolipid commonly found in caveolae, organized membrane structures enriched in glycolipids, cholesterol and caveolin, involved in endocytosis and transcytosis, cellular transport and signal transduction [Shin and Abraham, 2001]. These membrane structures are present in various cell types, including immune cells [Thomas et al., 2004]. Each B subunit monomer has a binding site for GM1, however, the CTB pentamer has a much higher binding affinity for the receptor due to the important role played by a single amino acid from an adjacent B subunit that enhances this action [Merritt et al., 1994]. After binding to the receptor, CT enters human intestinal cells through endocytosis and is transported from early endosomes to the Golgi. Endocytosis of CT may follow one of three pathways: (i) lipid raft/caveolae mediated endocytic pathway, (ii) clathrin mediated endocytic pathway, or (iii) noncaveolar clathrin-independent pathway [Chinnapen et al., 2007]. GM1 is the vehicle for retrograde transport of the CT holotoxin from the plasma membrane to the ER [Fujinaga et al., 2003]. In the ER, the disulfide bond that links CTA1 and CTA2 to CTB is reduced and a protein disulfide isomerase mediates the dissociation of CTA1 from CTA2/CTB. CTA1 moves from the ER to the cytosol by the ERAD dislocation mechanism, wich recognizes misfolded proteins in the ER and exports them to the cytosol for degradation by the 26S proteasome [Massey et al., 2009]. Once inside the host cells, CTA1 catalyzes the transfer of an ADP-

ribose unit from NAD^+ oxidizing agent to an arginine residue of Gs protein. This covalent modification leads to the loss of GTPase activity of the Gs protein, which remains attached to GTP, keeping the adenylate cyclase (AC) enzyme active that will produce increasing amounts of cAMP. Over 100 times the normal concentration of cAMP, the intestinal mucosa cells open a Cl^- channels in the cytoplasmic membrane, resulting in an influx of ions and water to the gut lumen that causes the characteristic acute diarrhea of cholera [Spangler, 1992]. As little as 5 μg of purified CT administered orally is sufficient to induce significant diarrhea in human volunteers while ingestion of 25 μg of CT elicits a full 20 litres cholera purge [Levine et al., 1983].

4. Immune properties

Adjuvants are substances that have the ability to enhance the immune response when co-administered with poor immunogenic molecules. CT is a bacterial immunogen with a great function as an adjuvant to a variety of antigens when given by systemic and mucosal route whether these are linked to or simply mixed with the toxin, generating a long-term immune response (Elson 1989; Vajdy and Lycke 1992).

These properties may be explained by three main characteristics of the molecule. First, CT is remarkably stable to proteases, bile salts and other compounds in the intestine. Secondly, its high affinity to GM1 ganglioside receptor, which is present on most mammalian cells including the M cells covering the Peyers patches, as well as all antigen-presenting cells (APC), facilitates the uptake and presentation of the toxin to the gut mucosal immune system. Finally, CT has strong inherent adjuvant and immunomodulating activities that depend both on its cell binding capability and its enzymatic ADP-ribosylating function (Sanchez and Holmgren 2008).

Pioneer studies carried out in 1972 showed that CT delivered by the intravenous route with a foreign antigen behaved as an adjuvant [Northrup and Fauci, 1972], a fact confirmed later by several groups using a number of unrelated antigens of little immunogenicity [Bianchi et al., 1990; Elson and Ealding, 1984]. Additional studies revealed that upon co-administration of CT and antigen through parenteral, mucosal, and transcutaneous routes resulted in substantial enhancement of mucosal immunoglobulin A (IgA) and serum IgG responses to the co-administered antigen [Chen and Strober, 1990; Drew et al., 1992; Reuman et al., 1991]. In addition to enhancing humoral immune responses, CT also augmented cellular immune responses to co-administered antigens enhancing induction of $CD4^+$ T helper (Th) and class I-restricted cytolitic T lymphocyte responses [Nurkkala et al.; Simmons et al., 1999]. In most cases, CT induced a Th2 bias response [Lavelle et al., 2004; Okahashi et al., 1996]. However, other studies have reported Th1 [Sasaki et al., 2003; Taniguchi et al., 2008] or mixed Th1/Th2 responses following oral, sublingual and intranasal immunization with antigens in the presence of CT [Cuburu et al., 2007; Fecek et al., 2010]. More importantly, subsequent studies showed that CT elicited a long-term memory response and thus was detectable long after the initial immune response [Soenawan et al., 2004; Vajdy and Lycke, 1992].

CT also acts as mucosal adjuvant against a variety of pathogens. Examples include, tetanus toxoid [Jackson et al., 1993], *Helicobacter felis* [Jiang et al., 2003], *Schistosoma japonicum* [Kohama et al., 2010], *Helicobacter pylori* [Raghavan et al., 2002], and *Sendai virus* [Liang et al., 1988]. There are many other examples where it was shown that CT has significant potential

for use as adjuvant for mucosally administered antigens [Clapp et al., 2010; Jhon Carlos Castaño Osorio, 2002].

5. Mechanism of adjuvant activity

The mechanism of adjuvanticity of CT is still unclear but is has been related to: (i) the induction of increased permeability of the intestinal epithelium leading to enhanced uptake of co-administered antigens; (ii) the induction of enhanced antigen presentation by various APC; (iii) the promotion of isotype differentiation in B cells leading to increased IgA formation; and (iv) exhibition of complex stimulatory as well as inhibitory effects on T cell proliferation and cytokine production. Among these many effects, those leading to enhanced antigen presentation by various APC are probably of the greatest importance [Sanchez and Holmgren, 2011].

As mentioned before, the polarity of the immune response generated by CT is a matter of debate. Some studies indicate that CT primes naïve T cells *in vitro* and drives them towards a Th2 phenotype, with production of interleukins IL-4 (a cytokine needed for B cell differentiation), IL-5, IL-6 and IL-10, but little IFN-γ (a cytokine needed to evoke Th1 responses) and suppression of IL-12 production by dendritic cells (DC) [Braun et al., 1999; Klimpel et al., 1995; Wilson et al., 1991]. Moreover, after immunization of animals with CT co-administered antigens, IL-4 levels were significantly elevated in gut-associated tissues and in spleen, while the levels of IFN-γ either decreased or remained static [Akhiani et al., 1997; Marinaro et al., 1995]. These results are supported by evidence of increased secretory IgA, serum IgA and IgE levels [Adel-Patient et al., 2005; Bourguin et al., 1991], and higher titers of IgG1 than IgG2a [Glenn et al., 1998; Lycke et al., 1990].

In contrast, others have reported that CT induces a mixed Th1/Th2 type of immune response with the production of IFN-γ and IL-4 [Fromantin et al., 2001; Imaoka et al., 1998]. In addition, it has been shown that CT induces strong Th17-type responses after intranasal delivery [Datta et al.; Lee et al., 2009].

Furthermore, CT markedly increased antigen-presentation by DC, macrophages, and B cells [Bromander et al., 1991; George-Chandy et al., 2001]. Also, CT upregulates the expression of MHC/HLA-DR molecules, CD80/B7.1 and CD86/B7.2 co-stimulatory molecules, as well as chemokine receptors CCR7 and CXCR4, on both murine and human DC, among other APC [Cong et al., 1997; Gagliardi et al., 2000]. Importantly, CT also induced the secretion of IL-1β from both DC and macrophages. IL-1β not only induces the maturation of DC, but also acts as an efficient mucosal adjuvant when co-administered with protein antigens and might mediate a significant part of the adjuvant activity of CT [Staats and Ennis, 1999]. Treatment with CT has been demonstrated to induce maturation and mobilization of DC [Lavelle et al., 2003]. Also, CT interferes with the differentiation of monocytes into DC, giving rise to a distinct population (Ma-DC), which displays an activated macrophage-like phenotype, induces a strong allogeneic and antigen specific response, and promotes the polarization of naïve $CD4^+$ T lymphocytes toward a Th2 profile [Raghavan et al., 2010]. In additon, CT enhanced IL-6 secretion by peritoneal mast cell [Leal-Berumen et al., 1996] and production of IL-1β, IL-6, and IL-10 together with inhibition of IL-12, TNF-α, and nitric oxid in macrophages [Cong et al., 2001], depleted the $CD8^+$ intraepithelial lymphocyte population [Flach et al., 2005], and induced isotype differentiation of B cells acting synergistically with IL-4 [Salmond et al., 2002]. Recent studies show that CT enhances STAT3 gene expression

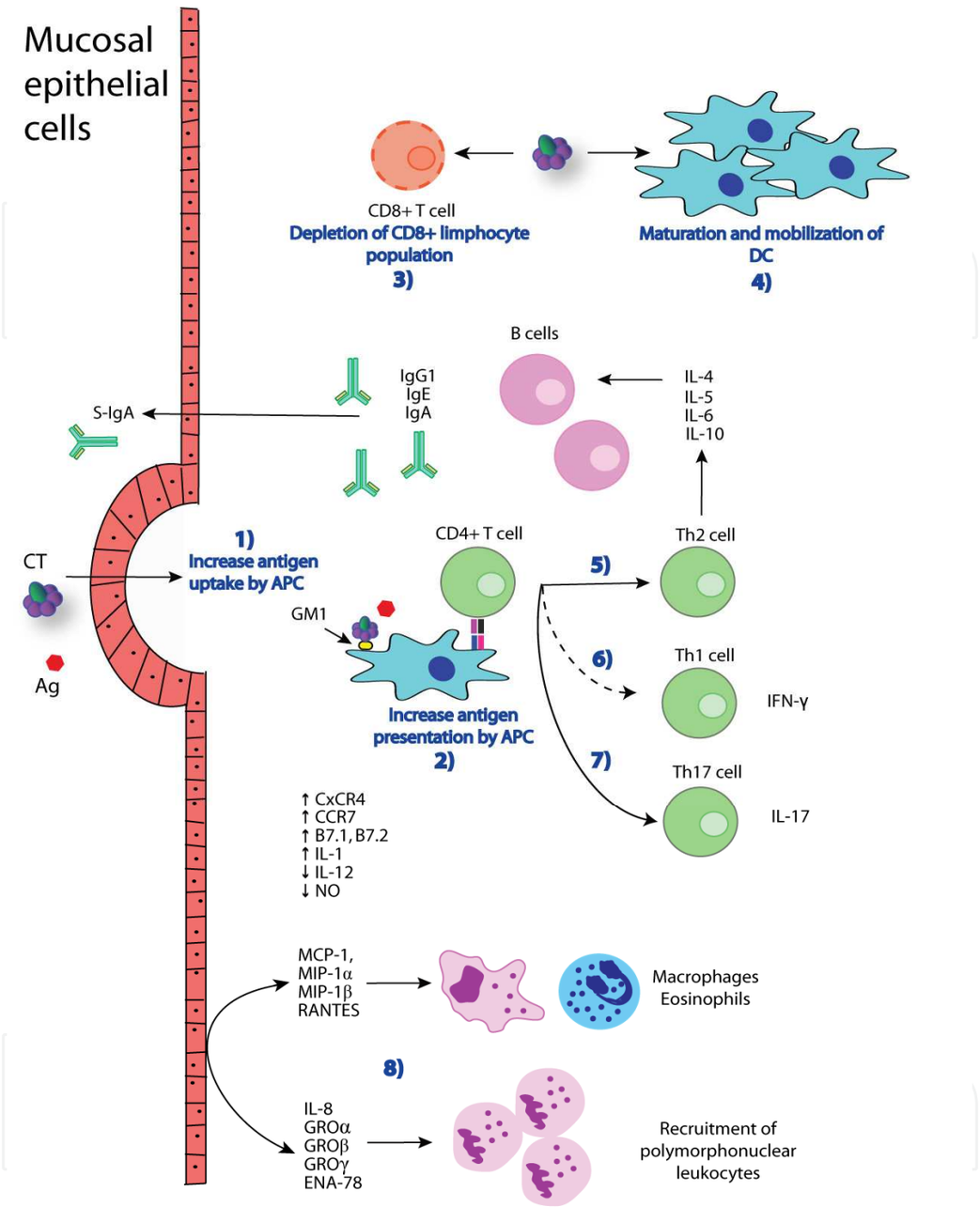

Fig. 2. Proposed mechanism of action by CT as a mucosal adjuvant. CT induces increased permeability of the intestinal epithelium leading to 1) enhanced uptake of co-administered antigens and 2) enhanced antigen-presentation by various APC. 3) It causes the depletion of $CD8^+$ lymphocyte population that may produce inhibitory cytokines, and 4) induces maturation and mobilization of DC. In addition, 5) CT promotes a strong Th2 dominant response to bystander antigens, and can either 6) induce or inhibit a Th1 response. Moreover, 7) CT induces strong Th17-type responses. Furthermore, 8) mucosal epithelial cells contribute to the adjuvant activity of CT by secreting a number of chemokines and acting on polymorphonuclear leukocytes, macrophages, eosinophils and T cells.

in murine B cells, and may critically modulate immune responses in both a pro-inflammatory and anti-inflammatory direction, depending on the circumstances and the types of cells involved Sjoblom-Hallen et al., (2010).

It has been suggested that mucosal epithelial cells may also play a role in adjuvanticity. Human epithelial cells express and secrete high levels of the chemoattractant cytokines IL-8, GROα, GROβ, GROγ, and ENA-78 in response to stimulation with TNF-α, IL-1β, or infection with enteroinvasive microorganisms. These chemokines attract and activate polymorphonuclear leukocytes. Activated epithelial cells also secret MCP-1, MIP-1β, MIP-1α, and RANTES, which variably act on monocytes/macrophages, eosinophils, and subpopulations of T-cells [Freytag and Clements, 2005]. One possibility is that CT interacts with epithelial cells triggering expression of one or more immunomodulatory factors that recruite APC and immune effector cells or activate those cells, or both [Lopes et al., 2000; Soriani et al., 2002].

A proposed mechanism of action of CT as adjuvant is shown in Fig. 2.

6. Genetic modifications of CT

The inherent enterotoxicity of CT has limited its widespread use as a vaccine component and adjuvant. In dogs, protection due to CT occurred only with doses that caused transient, sometimes severe, diarrhea [Pierce et al., 1982]. Moreover, murine models demonstrated that intranasal sensitization with CT as adjuvant led to increased lung inflammation with a massive recruitment of macrophages as well as accumulation in the olfactory nerves, epithelium and the olfactory bulbs of mice after binding to GM1 gangliosides [Fischer et al., 2005]. These limitations have led to mucosal strategies involving nontoxic mutants and purified B subunits.

Although early reports showed that mutants without the ADP-ribosyltransferase activity lack their adjuvant properties [Lycke et al., 1992], later studies showed that non-toxic mutants retained their adjuvant and immunogenic properties [Douce et al., 1997; Yamamoto et al., 1997] without central nervous system (CNS) toxicity [Hagiwara et al., 2006]. This suggests that the ADP-ribosyltransferase activity is not essential for its immunogenic properties, though it contributes to the adjuvant effect.

In a different approach, the CTA1 fragment linked to a synthetic analogue of *Staphylococcus aureus* protein A, the D fragment with affinity for APC, [Agren et al., 1997], proved to be non-toxic [Eriksson et al., 2004]. The fusion protein CTA1-DD binds specifically to immunoglobulins on the surface of antigen-presenting B cells through the DD polypeptide, and induces the ADP ribosylation by CTA1. Although this produces a good immune response when administered intranasally, it has been shown not to work as well after oral administration. This limitation was overcome by fusing CTA1-DD with immunostimulating complexes, such as ISCOMs (lipophilic immune stimulating complexes), producing both Th1/Th2 responses at systemic and mucosal levels [Andersen et al., 2007]. A recent report showed that CTA1 potently enhances a GeneGun-delivered DNA prime for human and simian immunodeficiency viruses antigens boost in macaques and mice [Bagley et al., 2011].

7. Immunological and adjuvant properties of CTB

Several studies using different conditions and routes of administration have described that CTB has several immunomodulatory properties opening many perspectives for future therapeutic and biotechnological applications. In this regard, intranasal immunization of women with CTB resulted in the production of long-lasting IgG and IgA anti-CTB in serum, nasal and vaginal secretions in a dose-dependent manner [Bergquist et al., 1997].

However, its capacity as mucosal adjuvant has proven to be much less than that of the toxin when given together with non-coupled antigens by the oral route [Sanchez and Holmgren, 2008]. Recombinant CTB has been successfully used as a mucosal adjuvant in vaccines for human use such as the cholera vaccine itself [Quiding et al., 1991], and the vaccine against enterotoxigenic *E. coli* that causes diarrhea [Peltola et al., 1991; Qadri et al., 2000]. Analogously, CTB proved to be good adjuvant for a *Streptococcus pneumoniae* cellular vaccine [Malley et al., 2004] and a severe acute respiratory syndrome-associated coronavirus vaccine [Qu et al., 2005] when administered intranasally in mice.

Given the potential of CTB as a regulator of the immune response, this subunit has been produced in various biological systems such as *Vibrio cholerae* [Sanchez and Holmgren, 1989], *Escherichia coli* [Arimitsu et al., 2009], *Bacillus brevis* [Goto et al., 2000], *Lactobacillus paracasei* and *plantarum* [Slos et al., 1998], in the yeasts *Hansenula polymorpha* [Song et al., 2004] and *Saccharomyces cerevisiae* [Mohsen and Rezae, 2005], and in silkworm [Gong et al., 2005]. In addition, CTB has been expressed successfully in tomato [Jani et al., 2002], lettuce [Young-Sook Kim, 2006], rice [Oszvald et al., 2008], tobacco [Hein et al., 1996], carrots [Kim et al., 2009], banana [Renuga et al., 2010] and potato transgenic plants, [Arakawa et al., 1997] where ubiquitin fusion enhances CTB expression [Mishra et al., 2006]. CTB may induce systemic immune responses in mice after gavage of the animals with the transgenic vegetal [Jiang et al., 2007]. The advantage of this approach is that plants present a low-cost agricultural-based effective production system. Different formulations, such as encapsulation in liposomes or microspheres with antigens [Seo et al., 2002] or combined with vesicles or liposomes containing antigens [Harokopakis et al., 1998; Lian et al., 1999] were also successfully tested.

CTB is a useful carrier protein for induction of mucosal IgA antibodies against chemically coupled antigens. In this regard, mice immunized intraduodenally with the horseradish peroxidase (HRP) covalently coupled to CTB showed a 33–120 fold higher level of IgA anti-HRP in intestinal washes as well as increased levels of serum IgG anti-HRP [McKenzie and Halsey, 1984]. In addition, CTB chemically conjugated to the protein I/II of *Streptococcus mutans* when administered in mice by oral [Russell and Wu, 1991], intranasal [Wu and Russell, 1998], and intragastric routes [Wu and Russell, 1993] results in the production of antistreptococcal IgG and IgA in serum and mucosa, as well as the presence of large numbers of antibody-secreting cells in salivary glands, mesenteric lymph nodes, and spleens. Similar results were found with CTB conjugated to human gamma globulin (HGG) and the recombinant *Neisseria gonorrhoeae* transferrin binding proteins, TbpA and TbpB. Vaginal and intranasal immunizations with CTB-HGG resulted in high levels of anti-HGG antibodies [Johansson et al., 1998], while rCTB-TbpA and rCTB-TbpB administered intranasally induced antibody responses in the serum and genital tract [Price et al., 2005]. Moreover, CTB was chemically conjugated to type III capsular polysaccharide from

Streptococcus group B [Shen et al., 2000] or to protein-polysaccharide conjugates [Bergquist et al., 1995] and in both cases, after subcutaneous administration, high levels of specific antibodies were detected. In addition to generating humoral response, simian immunodeficiency virus (SIV) virus-like particles (VLP) chemically conjugated to CTB showed higher levels of cytokine IFN-γ-producing splenocytes and cytotoxic-T-lymphocyte activities of immune cells than VLPs plus CTB, indicating a generation of a Th1 response in mice by CTB-VLP [Kang et al., 2003]. Finally, CTB chemically conjugated to the *Plasmodium vivax* ookinete surface protein, Pvs25, proved to be a potent transmission-blocking antigen in both intranasal and subcutaneal routes in mice [Miyata et al., 2010], and to protect against pharyngeal colonization by group A *streptococcus* when conjugated to the widely shared C repeat region of M6 protein [Bessen and Fischetti, 1990].

	Antigen	Route	CTB administration	Reference
Proteins	Nucleoprotein of Influenza A virus	in	co-administered	[Guo et al., 2010]
	Hepatitis B virus surface antigen	in	co-administered	[Isaka et al., 2001]
	MSP4 5 malaria protein	Oral	co-administered	[Wang et al., 2003]
	OVA	im	co-administered	[Rolland-Turner et al., 2004]
	HIV-1 gp41	sl	chemically coupled	[Hervouet et al., 2010]
	Epitopes from *Schistosoma mansoni* glutathione-S-transferase	in	genetically fused	[Lebens et al., 2003]
Polysaccharide	Group B Streptococcus Type III Capsular Polysaccharide	in, oral, rectal, and vaginal	chemically coupled/co-administered	[Shen et al., 2000]
	Lipopolysaccharide from *V. cholerae* O1, serotype Inaba	sc	chemically coupled	[Gupta et al., 1998]
	Pseudomonas aeruginosa polysaccharide	Oral	co-administered	[Abraham and Robinson, 1991]
Micro-organisms	Measles virus	in, ig	co-administered	[Muller et al., 1995]
	Influenza virus	in	co-administered	[Yang et al.]
	Pneumocystis carinii	in	co-administered	[Pascale et al., 1999]

Table 1. Antigens towards which CT has adjuvant activity. in: intranasal, im: intramuscular, sl: sublingual, sc: subcutaneous, ig: intragastric.

Another way of using CTB as an adjuvant is in genetic constructions based on the toxin and heterologous antigens. In general, these hybrid molecules are composed of antigens fused to the amino [Laloi et al., 1996; Song et al., 2004] or carboxyl [Kim et al., 2004; Wang et al., 2010] terminus of CTB, being GM1-binding much more efficient in the latter case [Liljeqvist et al., 1997], but also protein epitopes have been introduced at internal positions in CTB

[Dertzbaugh and Elson, 1993]. Some examples of genetic incorporation of epitopes to CTB include triple glutamic acid decarboxylase [Gong et al., 2009], dodecapeptide repeat of the serine-rich *Entamoeba histolytica* protein [Zhang et al., 1995] and human insulin B-chain [Sadeghi et al., 2002]. There are many studies showing the induction of immune responses through immunization of mice with CTB fused to soluble antigens expressed both in bacteria [Larsson et al., 2004; Lee et al., 2003; Sun et al., 1999; Tsuji et al., 2003] and in transgenic plants [Jani et al., 2004; Matsumoto et al., 2009]. In all cases there was generation of IgG and IgA antigen-specific antibodies and, in some cases, protection. Some examples of the adjuvant action of CTB are shown in Table 1.

One of the strategies for using CTB as an adjuvant genetically fused to antigens has been described by Arêas *et al*. and is based on the expression vector called pAEctxB (Fig. 3.). In the generation of the vector, the gene ctxB was modified to ensure that the codons were those most frequently used by *E. coli, L. casei* and *S. typhimurium* [Areas et al., 2002]. The genetically engineered ORF was then cloned into the expression vector pAE [Ramos et al., 2004] and includes two consecutive restriction sites Mlu*I* and Hind*III*. The resulting vector allows expression, under the control of a T7 promoter, of proteins fused to the C-terminus of CTB with 6 histidine residues at the N terminus, which facilitate protein purification by immobilized metal ion affinity chromatography.

The pAE-ctxB plasmid was used to clone the pneumococcal surface adhesin A (PspA) [Areas et al., 2004], the *Leptospira interrogans* protein LipL32 [Habarta et al., 2010], the fatty-acid binding protein from *Schistosoma mansoni* S14 [Henrique Roman Ramos, 2010], and the *Bordetella pertussis* type III secretion system effector protein Bsp22 (Olivera et al., unpublished results). Intradermal immunization with CTB-PspA induced high titers of anti-PspA IgG and partially protected mice after challenge with *S. pneumonia* [Areas et al., 2005]. Moreover, intranasal immunization with CTB-PsaA protected mice against colonization with *S. pneumoniae* without alteration of the natural oral or nasopharyngeal microbiota of mice [Pimenta et al., 2006]. CTB-Sm14 itself was not able to reduce *Schistosoma mansoni* worm burden on intranasally immunized BALB/c mice, but reduced the hepatic granulomas around trapped eggs. CTB-LipL32 generated higher specific titers in mice immunized without external adjuvant than co-administration of CTB with LipL32, supporting CTB-LipL32 as a promising antigen for use in the control and study of leptospirosis.

8. CTB for mucosal immunotherapy

Mucosal administration by the oral, sublingual or nasal routes of many antigens can induce peripheral tolerance. Mucosal-induced tolerance has been recognized for a long time as a promising approach to prevent or treat allergic or autoimmune disorders and is characterized by a decreased immune response to systemic immunization with the same antigen [Sun et al., 2009; Sun et al., 1994]. In this regard, promising results have been obtained with auto-antigen coupled to CTB in order to induce oral tolerance. Although not known the mechanism by which CTB conjugated to antigens has the ability to potentiate the induction of oral tolerance, it is believed that in addition to the processes already mentioned before for CT, it may result in selected DC subsets with increased ability to induce different types of TGF-β-expressing suppressor T cells including $CD4^+$ $CD25^+$ Tr cells [Holmgren et al., 2005] and a direct depletion of effector T cells since CTB induces CD4+ and CD8+ T cell apoptosis [Christelle Basset, 2010].

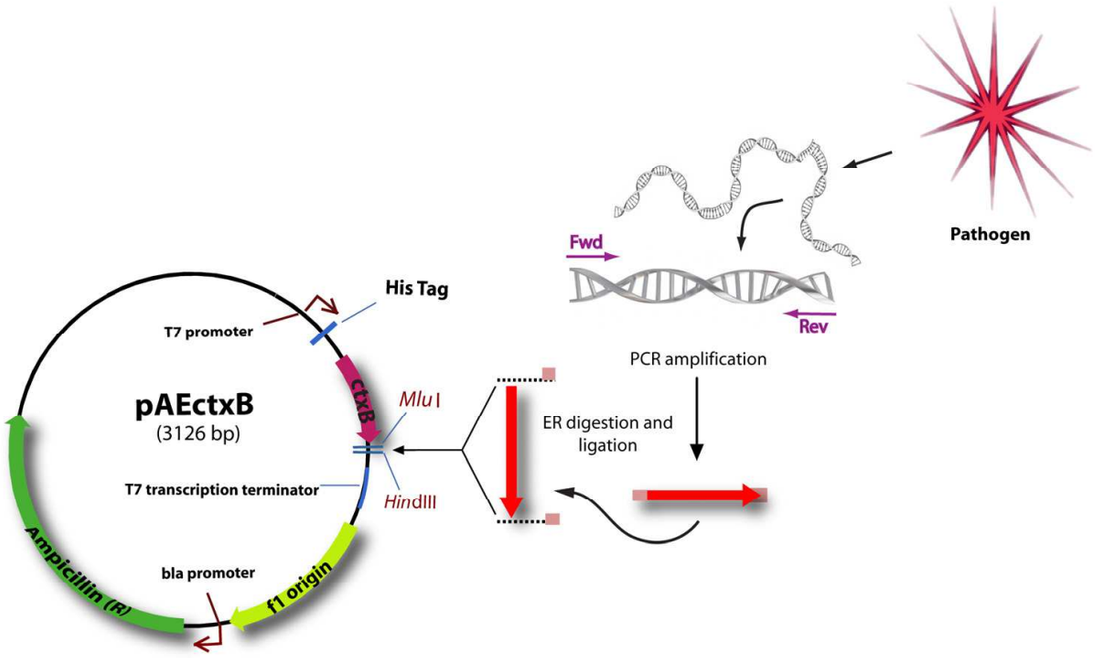

Fig. 3. Cloning strategy into pAEctxB plasmid

Oral delivery of CTB conjugated to myelin basic protein protected mice [Sun et al., 1996; Yuki et al., 2001] and rats [Sun et al., 2000b] against the development of experimental autoimmune encephalomyelitis. It was proposed that the inhibitory effect was a result of both the induction of TGF-β-producing Tr cells and down-regulation of IFNγ, IL-12, TNFα, MCP-1 and RANTES in the CNS [Wang et al., 2009].

Oral administration of a CTB-insulin conjugate prevented diabetes in non-obese diabetic (NOD) mice [Arakawa et al., 1998; Bergerot et al., 1997; Gong et al., 2007; Petersen et al., 2003; Ploix et al., 1999], which was associated with a reduction in IFNγ production and Tr cell migration into pancreatic islets [Aspord et al., 2002; Sobel et al., 1998]. On the other hand, oral administration of CTB-proinsulin fusion protein showed an increased expression of IL-4 and IL-10 in the pancreas of NOD-treated mice, suggesting that Th2 lymphocyte-mediated oral tolerance is a likely mechanism for the prevention of pancreatic insulitis [Ruhlman et al., 2007].

Oral delivery of CTB conjugated to a 60 kDa heat-shock protein derived peptide prevented mucosal induced uveitis in rats, an effect that was associated with enhanced IL-10 and TGF-β, and reduced IL-12 and IFN-γ production [Phipps et al., 2003]. Furthermore, a I/II phase clinical trial of the same peptide conjugated to CTB administered orally to 8 patients allowed the withdrawal of all immunosuppressive drugs in 5 of the 8 patients without a relapse of uveitis [Stanford et al., 2004].

In addition, oral administration of CTB in mice inhibits the induction of trinitrobenzene sulfonic acid-induced colitis and reverses such colitis after it has been established. This inhibition is associated with suppression of IL-12 and IFN-γ production [Boirivant et al., 2001; Coccia et al., 2005]. In a recent clinical trial, 40% of patients with active Crohn's disease responded to treatment with CTB [Stal et al., 2010].

CTB conjugates were also effective in the induction of tolerance to type II collagen, leading to a suppression of chondritis in a model of autoimmune ear disease [Kim et al., 2001]. Oral administration of allogeneic antigen linked to CTB induced immunological tolerance against allograft rejection [Sun et al., 2000a]. Finally, transconjunctival immunotherapy using CTB could suppress clinical effects for experimental allergic conjunctivitis in guinea pigs [Oikawa et al., 2011].

9. Conclusion

CT has been studied for over 40 years. Both CT and its non-toxic derivatives or its B subunit, have shown to be excellent mucosal adjuvants. The possibility to use them as biotechnological tools in the development of new vaccines is being intensively studied in the present. In recent years, the prospect to use CTB fused to different protein antigens became relevant because these proteins can be expressed in high levels in a soluble form and directly purified in their active form, requiring only one fermentation step. In addition, several reports have shown that CTB can generate oral tolerance to different conjugated antigens, opening ways for the treatment of autoimmune diseases. Hopefully, future studies will focus on the use of CTB in such important issues.

10. Acknowledgements

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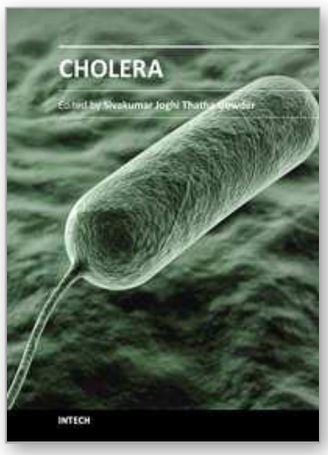

Cholera

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Cholera, a problem in Third World countries, is a complicated diarrheal disease caused by the bacterium Vibrio cholerae. The latest outbreak in Haiti and surrounding areas in 2010 illustrated that cholera remains a serious threat to public health and safety. With advancements in research, cholera can be prevented and effectively treated. Irrespective of "Military" or "Monetary" power, with one's "Own Power", we can defeat this disease. The book "Cholera" is a valuable resource of power (knowledge) not only for cholera researchers but for anyone interested in promoting the health of people. Experts from different parts of the world have contributed to this important work thereby generating this power. Key features include the history of cholera, geographical distribution of the disease, mode of transmission, Vibrio cholerae activities, characterization of cholera toxin, cholera antagonists and preventive measures.

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