

Bacterial Protein Toxins: Current and Potential Clinical Use

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Abstract: Natural toxins are the product of a long-term evolution, and act on essential mechanisms in the most crucial and vital processes of living organisms. They can attack components of the protein synthesis machinery, actin polymerization, signal transduction pathways, intracellular trafficking of vesicles as well as immune and inflammatory responses. For this reason, toxins have increasingly being used as valuable tools for analysis of cellular physiology, and in the recent years, some of them are used medicinally for the treatment of human diseases.

This review is devoted to protein toxins of bacterial origin, specifically those toxins that are currently used in therapy or those under study for their potential clinical applications. Bacterial protein toxins are all characterized by a specific mechanism of action that involves the central molecular pathways in the eukaryotic cell. Knowledge of their properties could be used for medical purposes.

Keywords: Bacterial protein toxins, immunotoxins, therapeutic agents, vaccine adjuvant, drug delivery agent.

BACTERIAL PROTEIN TOXINS USED IN THERAPY

Bacterial protein toxins are the principal virulence factors of pathogenic bacteria and their production represents one of the main bacterial strategies to interact with mammalian cells. Bacterial toxins manipulate by diverse and sophisticated mechanisms the host cell functions in a way that can favor the survival and spreading of the microbes. Understanding how a bacterial toxin acts at a molecular level, is certainly of paramount importance in using the toxin as an instrument in controlling certain cellular pathways, thus converting a detrimental toxin effect to a favorable one.

In this section, we report a list of bacterial protein toxins used in therapy, briefly highlighting their main characteristics. The use of selected bacterial toxins as components of engineered molecules, referred to as immunotoxins, is also presented (Table 1).

Table 1. Bacterial Protein Toxins with a Potential Use in Therapy

TOXIN	Therapeutic Use:
Botulinum neurotoxin (BoNT) from <i>C. botulinum</i>	Dystonia Muscle tone disorders Autonomic nervous system disorders Cosmetic use
Lethal toxin (LF) from <i>B. anthracis</i>	Potential treatment of cancer
Pertussis toxin (PXT) from <i>B. pertussis</i>	Potential use in control of HIV replication
Cytotoxic necrotizing factor 1 (CNF1) from <i>E. coli</i>	Potential use in learning and memory enhancement
IMMUNOTOXINS	Cancer therapy

Botulinum Toxin from *Clostridium botulinum*

C. botulinum are gram-positive, anaerobic and spore-forming bacteria widely distributed in soil and water. Strains of *C. botulinum* produce seven different neurotoxins designated BoNT/A to G. Although differing in their biosynthesis, size, structure, and specific site of action, all botulinic neurotoxins bind and enter inside peripheral cholinergic terminals and cause a sustained block of acetylcholine release, with ensuing flaccid paralysis [1-3].

Botulinum neurotoxins are synthesized as a single polypeptide protoxin chain with a molecular mass of about 150 kDa [4]. After bacterial death and lysis, the protoxin undergoes a process of activation by bacterial proteases to yield the 50 kDa light chain and the 100 kDa heavy chain, held together by a disulfide bond. One exception is BoNT/E, which is activated by splitting into light and heavy chains only by gut proteases [3, 5, 6]. The heavy chain of botulinum neurotoxins is responsible for binding to specific surface receptors, whereas the light chain is a zinc-dependent metalloprotease that enzymatically cleaves specific Soluble N-ethylmaleimide-sensitive factor-Attachment protein Receptor (SNARE) proteins, namely vesicle-associated membrane protein (VAMP), synaptosome-associated protein of 25 kDa (SNAP-25) and Syntaxin, before they assemble into the complex responsible for vesicle docking (Fig. (1)) [1, 2]. To exert its action, active neurotoxin undergoes a 3-step process consisting of i) binding to a surface receptor; ii) internalization within an endosome with subsequent translocation of the light-chain portion of the toxin into the cytosol; iii) proteolysis of target proteins and the subsequent effect on neurotransmission [5, 7, 8]. However, the 7 mentioned toxins (BoNT/ A to G) act in a slightly different way on the targets [for more details see reference 9].

The enzymatic cleavage of SNARE proteins ultimately results in the observed clinical effects of muscle paralysis. The clinical effects include disfunction of the autonomic nervous system and in particular limb paralysis, dysarthria, facial muscle weakness, dyspnea, constipation, urinary retention, and ophthalmoplegia. Most patients survive botulism, but complete recovery is slow and may require mechanical ventilation [10].

Therapeutical Applications

Over the last 50 years, botulinum neurotoxin has been transformed from a cause of life-threatening disease to a medical therapy. In 1817 Justinus Kerner, who provided the earliest account of food borne botulism, recognized the potential for the therapeutic use for botulinum toxin [11].

After having been regarded for several decades as a potential bioterrorism weapon [12], the botulinum toxin was first used by Scott [13] as a muscle-weakening agent for the correction of experimental strabismus in monkeys. Since then, different commercial preparations were made available and the number of publications dedicated to the clinical uses of botulinum toxins has increased significantly [14]. Botulinum neurotoxin is employed in a range of neurological human pathologies characterized by hyperfunction of all types of peripheral cholinergic terminals but it also has become very popular as a cosmetic agent to reduce facial wrinkles [15].

Application in Neurological Disorders

Botulinum toxin (Botox) (Allergan, Inc, Irvine, CA) first gained clinical acceptance in 1989 by the Food and Drug Administration

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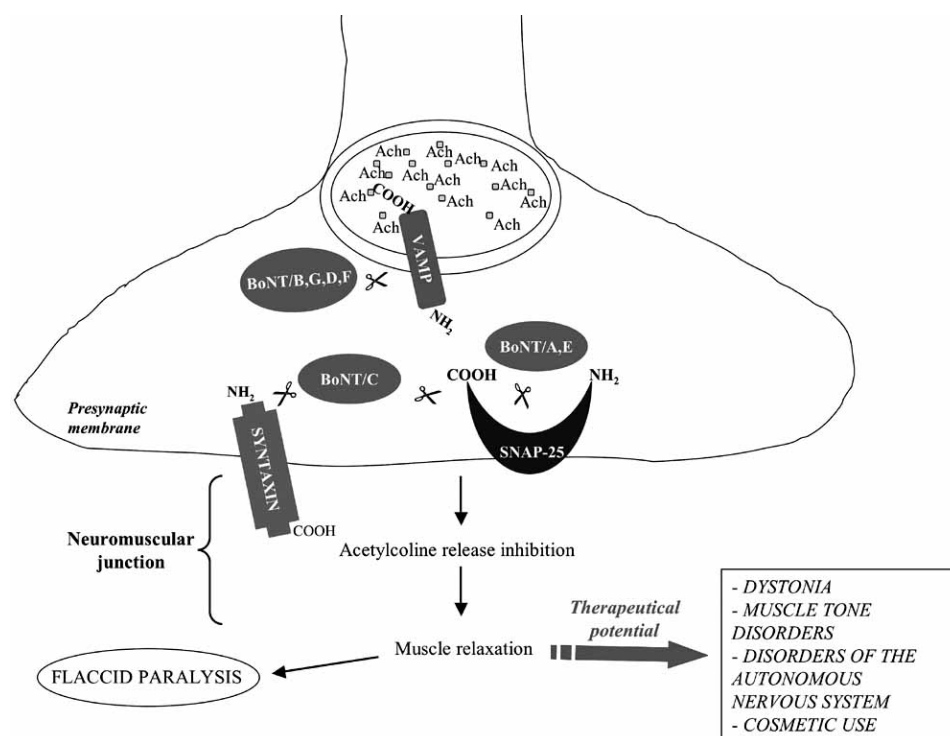


Fig. (1). *C. botulinum* neurotoxins.

Seven serotypes (A to G) of botulinic neurotoxins (BoNT) exist, and all bind and enter inside peripheral cholinergic terminals causing a sustained block of acetylcholine release and ensuing muscle relaxation and flaccid paralysis. This occurs because the toxins cleave and inactivate SNARE proteins required for the fusion of acetylcholine vesicles with the presynaptic membrane. In particular, BoNT B, G, D, F cleave VAMP, a protein localized on the vesicle membrane, BoNT C acts on syntaxin and SNAP-25, two proteins of the pre-synaptic membrane, while BoNT A and E act on SNAP-25.

The ability of BoNTs to induce muscle relaxation makes them potential therapeutical tools for treatment of different neurological disorders.

VAMP, vesicle-associated membrane protein; BoNT, botulinum toxin; SNAP-25, synaptosome associated protein of 25 kDa; Ach, acetylcholine.

(FDA) that approved it as a result of its marked benefits produced in patients with dystonia, a neurological disorder dominated by repetitive and patterned contractions of muscles producing abnormal movements and postures [16]. In particular, a marked improvement has been reported in a large percentage of patients with blepharospasm [17], oromandibular dystonia, manifested by involuntary jaw closure and bruxism [18], hemifacial spasm [17], cervical dystonia, also known as torticollis [19] and laryngeal dystonia manifested by spasmodic dysphonia [20]. Botulinum toxin injections have been also found to be useful in patients with Parkinson's disease and other neurodegenerative disorders or stroke related hemiplegia [21].

In addition, botulinum toxin has been used to treat disorders of muscle tone like spasticity associated with cerebral palsy, stroke, brain trauma, multiple sclerosis [22], but also painful muscle spasms and other painful disorders including headaches [23].

It is known that botulinum toxin interferes with transmission not only at the neuromuscular junction but also in the cholinergic autonomic parasympathetic and postganglionic sympathetic nervous system. It is therefore increasingly useful in the treatment of various disorders of the autonomic nervous system. In this context, an intradermal injection of botulinum toxin has provided a highly effective treatment for focal hyperhidrosis [24] (defined as excessive sweating of the palms, feet or axillae), or sialorrhoea, the last one associated with amyotrophic lateral sclerosis and other neurodegenerative disorders [25].

The array of disorders treated with this toxin include also various gastrointestinal, genitourinary and sphincter disorders. The following are specifically included, dysphagia caused by spasm of the cricopharyngeal component of the inferior constrictor of the

pharynx; achalasia and other oesophageal spasm; anismus associated with intractable constipation caused by rectal sphincter spasms or chronic anal fissure; overactive and neurogenic bladder; non-bacterial prostatitis and benign prostatic hyperplasia and vaginismus [26-28].

Recently, botulinum toxins have been used in cancer therapy to open tumor vessels allowing a more effective destruction of cancer cells by radiotherapy and chemotherapy [29]. Furthermore, in experimental epilepsy models, botulinum toxin delivery to the hippocampus is antiepileptogenic [30].

Cosmetic Applications

There is growing interest in the use of botulinum toxin in cosmetics. The treatment of wrinkles is one of the most common uses for the botulinum toxin. The excellent results in the treatment of furrows and frown lines on the forehead have been confirmed by several open and controlled studies [31]. However, inappropriate placement or dose of the toxin may result in loss of facial expression and disfiguring complications.

Many of the reported diseases treated with botulinum toxin were previously refractory to most pharmacological and surgical treatments. The remarkable therapeutic use of botulinum toxin lies in its ability to inhibit specifically and potentially involuntary muscle activity for an extended period.

Anthrax Toxin from *Bacillus anthracis*

Bacillus anthracis, the etiological agent of anthrax, is a gram-positive bacterium mainly present in the environment in the form of highly resistant spores [32]. The disease takes one of several forms: cutaneous, gastrointestinal or inhalational, depending on the route

of entry of infectious spores. The cutaneous is the most common form, manageable with antibiotics, whereas the gastrointestinal anthrax is a much more serious systemic disease. However, the most deadly anthrax is the inhalational form, which is extremely rare and occurs when spores are taken up by alveolar macrophages and transported to lymph nodes, where germination occurs. The bacteria then produce anthrax toxins, which inhibit the innate and adaptive immune response. This enables the bacterium to escape the lymph-node defense barrier to reach the blood system causing bacteremia and toxemia, which rapidly kills the host [32, 33]. *B. anthracis* produces toxins that are crucial for the establishment of infection and pathogenesis and the anthrax toxins consist of three distinct proteins: the protective antigen (PA), the edema factor (EF) and the lethal factor (LF). Binary combination of these secreted proteins comprise the two anthrax toxins: PA combined with LF is called lethal toxin (LeTx) and PA combined with EF is known as edema toxin (EdTx). LF and EF act enzymatically on intracellular substrates: LF is a zinc-dependent metallo-protease that cleaves and inactivates most isoforms of mitogen-activated protein kinase (MAPK) kinases (MEKs) whereas EF is a calcium- and calmodulin-dependent adenylate cyclase that causes a dramatic increase in cytoplasmic cAMP, leading to an imbalance of water homeostasis [34]. PA escorts LF and EF to the interior of the host cell by binding to at least two different cell surface receptors [35] and then being cleaved by membrane endoproteases of the furin family. Cleaved PA molecules assemble into heptamers, which then associate with EF and LF. PA heptamers enter lipid rafts and are quickly endocytosed into acidic compartments [36] where the low pH triggers a conformational change of the complex, allowing translocation of LF and EF into the cytosol where they exert their enzymatic activity (Fig. (2)). By acting on their cytosolic targets, the two toxins functionally impair cells of both the innate and adaptive immune systems. In particular, they i) block neutrophil chemotaxis and ROS production; ii) induce cell death in macrophages; iii) impair dendritic cells; iv) have effects on T and B cells, decreasing

production of growth-promoting cytokines and blocking proliferation [for a review see 34]. In this way, both toxins contribute to establishment of infection by promoting bacterial dissemination and causing disease.

A better understanding of the toxins will help in the design of much-needed anti-toxin drugs and the development of new toxin-based medical applications.

Therapeutical Applications

Treatment of Cancer

As stated previously, anthrax LF selectively inactivates MEKs. Inappropriate activation of these kinases has been implicated in tumorigenesis, tumor metastasis and tumor angiogenesis [for a review see 37]. This raised the possibility that anthrax LF might be used to counteract increased levels of MEKs activity in tumors, LF representing a novel and potentially useful chemotherapeutic agent. Analysis of different human tumor cell lines derived from melanomas, breast tumors and colon tumors showed that their growth was inhibited by LeTx treatment. In addition, when (V12) H-ras transformed NIH 3T3 cells were treated with LeTx *in vitro*, their transformed phenotype was reverted. These results show that LeTx is capable of inhibiting tumor-like properties of transformed cells *in vitro*. Initial observations indicate that LeTx effectively blocks tumor growth *in vivo*. Preclinical studies with anthrax LeTx show the sensitivity of malignant melanoma cell lines in tissue culture and anti-tumor efficacy in melanoma xenograft models [38]. Additional studies to define the maximal tolerated doses and dose-limiting toxicity of anthrax lethal toxin in rodent and primate models should pave the way for Phase I studies testing the efficacy of the anthrax lethal toxin in patients with metastatic melanoma.

The use of anthrax toxin will also be described in the section devoted to immunotoxins.

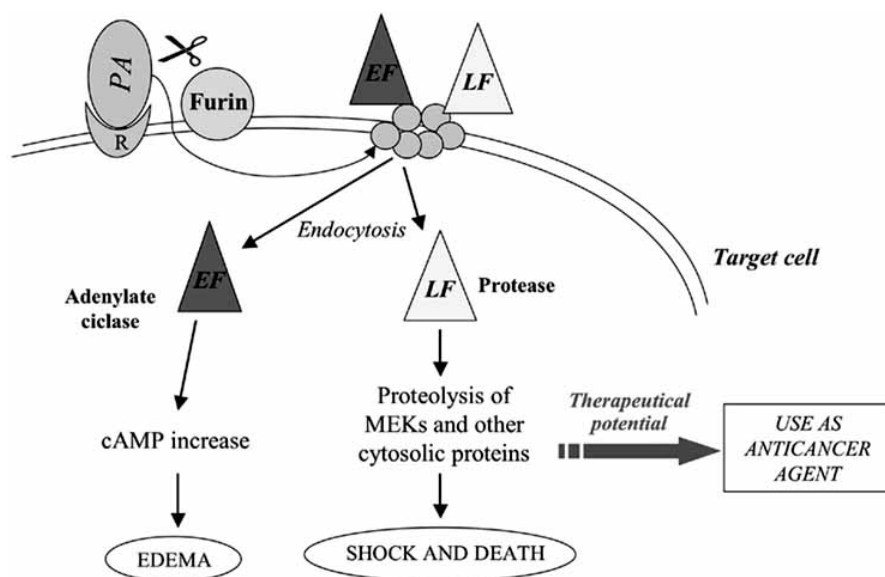


Fig. (2). *B. anthracis* lethal factor.

The anthrax toxin consists of three distinct proteins: the protective antigen (PA), the edema factor (EF) and the lethal factor (LF). After binding to a host cell surface receptor (R), PA is cleaved by membrane endoproteases of the furin family. Cleaved PA molecules assemble into heptamers, which then associate with EF and LF. PA heptamers are endocytosed into acidic compartments, where a conformational change of the complex allows translocation of LF and EF into the cytosol. LF and EF act enzymatically on intracellular substrates: LF is a zinc-dependent metallo-protease that cleaves and inactivates most isoforms of MEKs, whereas EF is a calcium- and calmodulin-dependent adenylate cyclase that causes a dramatic increase in cytoplasmic cAMP, leading to an imbalance of water homeostasis.

Anthrax LF might be used to counteract increased levels of MEKs activity in tumors, thus representing a novel and potentially useful chemotherapeutic agent.

Mitogen-Activated Protein Kinase (MAPK) kinases (MEKs).

Pertussis Toxin from *Bordetella pertussis*

B. pertussis, the aetiological agent of whooping cough, produces numerous toxins including the pertussis toxin (PTX). PTX is composed of different subunits organized in a typical A-B type structure. The B oligomer (PTX-B) is responsible for binding PTX to target cells, whereas the A moiety exhibits ADP-ribosyltransferase activity. After binding to the cell receptors, the toxin most likely follows the retrograde transport to the endoplasmic reticulum. Probably from there, the A moiety translocates into the cytosol where it expresses the ADP-ribosyltransferase activity using NAD as donor and trimeric G-proteins as acceptors [39]. ADP-ribosylated G-proteins lose their ability to transduce signals and this constitutes the molecular basis of most PTX actions. Recent evidence demonstrates that a p38 MAPK activity is possibly linked to the ADP-ribosylation of G-proteins, leading to alteration in lung permeability [40]. These studies provide mechanistic information on the pathogenesis of PTX-induced lung toxicity.

However, it is now clear that PTX-B alone directs cellular responses [41]. In particular, it is a rapid and robust stimulus for p42/p44 MAP kinase (ERK) activity in endothelium [40]. Other signaling pathways are stimulated by PTX-B and they involve PKC, Ca^{2+} and tyrosine kinases as well [42-44]. These phenomena lead to multiple effects including DNA synthesis in B cells, proliferation of T lymphocytes, potentiation of Th1-dependent immune responses via up-regulation of IFN and IL-2, and inhibition of IL-1-induced IL-2 expression and prostaglandin production (Fig. (3)) [41, 45].

Therapeutical Applications

Control of Viral Infection

The whole PTX as well as a non-toxic derivative of this molecule, has been reported to down-regulate the replication of the virus SIVmac251 in infected macaques by inducing a transient lymphocytosis [46]. However, the vast majority of reports highlighting a therapeutic potential of PTX against HIV-1, deal with PTX-B, the binding portion capable of transducing a variety of cellular signals. In particular, PTX-B can block the infectious process of HIV-1 *in vitro* at multiple levels [45, 47, 48]. It affects the entry of R5 but not X4 strains into primary T lymphocytes by cross-deactivation of one of the major HIV-1 co-receptor, CCR5, thus affecting infection by the viruses that use this receptor [45]. PTX-B also blocks the post-entry step of HIV-1 replication, using a mechanism that affects the transcription and stability of HIV-1 mRNA [47]. In addition, PTX-B potentially inhibited cytokine induced virus expression in chronically infected promonocytic U1 cells, thus suggesting the potential value of such toxin as an agent that can prevent reactivation of latent HIV provirus [48, 49]. Finally, experiments with a surrogate model of HIV-1 infection, that is, SCID mice reconstituted with human peripheral blood leukocytes (hu-PBL-SCID) infected with a CCR5-dependent (R5) HIV-1 strain, demonstrated that PTX-B potentially inhibited virus infection and replication [50].

No matter which molecular pathway is exploited by PTX-B, its described activities make it an attractive candidate for anti-HIV therapy as it can inhibit virus production both after re-infection and after reactivation of the latent virus. The anti-viral potential of PTX is not restricted to HIV-1. In fact, PTX also delays lymphocytic

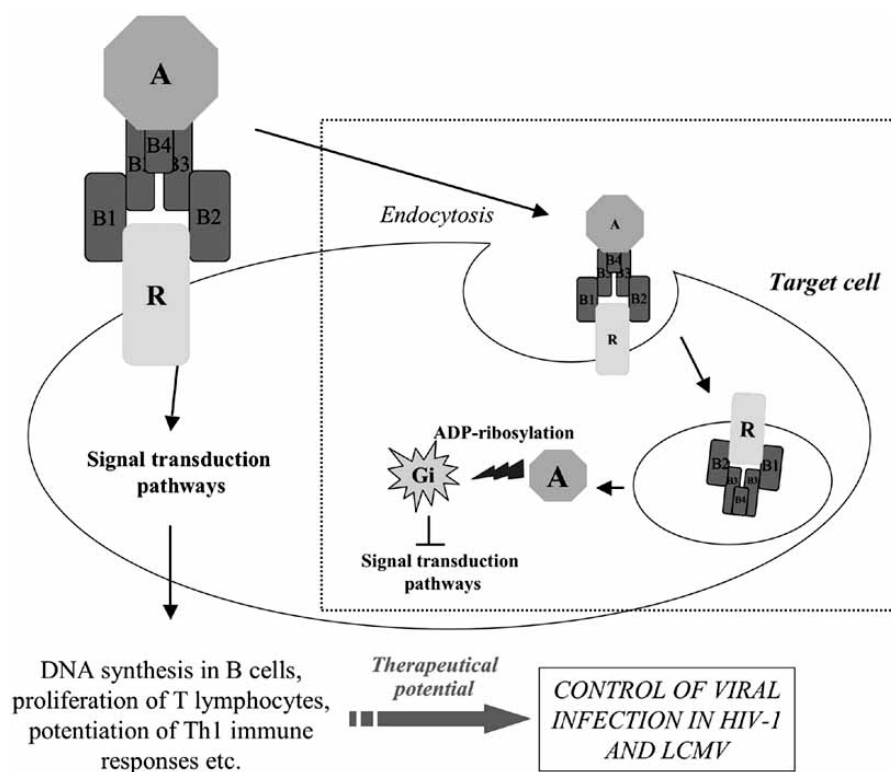


Fig. (3). *B. pertussis* toxin.

Pertussis toxin (PTX) is composed of a B oligomer (B), responsible for binding PTX to target cells, and a moiety that exhibits ADP-ribosyltransferase activity (A). After binding to the cell receptors (R), the toxin is endocytosed and the A moiety translocates into the cytosol where it expresses the ADP-ribosyltransferase activity on heterotrimeric G proteins. ADP-ribosylated G-proteins lose their ability to transduce signals. Furthermore, the binding of the B oligomer to the cell receptor is able to trigger different signal transduction pathways which, in turn, can lead to DNA synthesis in B cells, proliferation in T lymphocytes, and others.

This last mechanism of action of PTX has been exploited to develop potential therapeutic tools to control viral infection of HIV-1 and LCMV1viruses.

choriomeningitis virus (LCMV) dissemination after intragastric inoculation in mice.

PTX has no direct effect on LCMV replication, but it modulates integrin expression in part by blocking chemokine signals, thus inhibiting the G protein-coupled chemotactic response of macrophages infected during the initial exposure to the virus [51].

Cytotoxic Necrotizing Factor 1 (CNF1) from *Escherichia coli*

E. coli is a normal inhabitant of the human intestine that becomes highly pathogenic following the acquisition of virulence factors, including a protein toxin named CNF1. CNF1-producing *E. coli* strains are the most common pathogens in extraintestinal infections [52, 53] and, occasionally, they are detected in isolates from feces of children with diarrhea [54-56].

CNF1 is 113 kDa monomeric protein toxin that in eukaryotic cells binds to its receptor, reported to be the receptor of laminin [57], and is endocytosed and released into the cytoplasm by an acidic-dependent mechanism [58]. Once in the cytoplasm, CNF1 exerts its enzymatic activity that is represented by deamidation of a pivotal glutamine residue of the GTP-binding proteins Rho, Rac, and Cdc42 (glutamine 63 of Rho or glutamine 61 of Rac and Cdc42) giving rise to a glutamic acid [59-61]. The GTPases modified by CNF1 belong to the Rho family, a class of highly conserved regulatory proteins [62]. The glutamine residue modified by CNF1 lies in the *switch 2* domain of Rho proteins, which is involved in GTP hydrolysis; thus, the modification exerted by CNF1 blocks the Rho GTPases in their GTP-bound activated state.

Activated Rho GTPases are recognized by cells that degrade them by means of the ubiquitin-proteasome pathway, thus lowering

their amount and activity in cells (Fig. (4)) [63]. By activating the Rho GTPases, CNF1 is able to manipulate several cellular functions, such as cell differentiation [64], DNA transcription [65], cell cycle progression [66] as well as the organization of the actin cytoskeleton [67]. Furthermore, it has been reported that CNF1 is indeed not cytotoxic, but rather confers to cells a resistance to apoptotic stimuli [68]. In particular, activity on the Rho GTPases that are powerful cellular regulators [62] renders CNF1 an attractive putative "drug" in different therapeutic purposes.

Therapeutic Applications

Learning and Memory

In this context, we have recently reported that constitutive activation of Rho family GTPases in mice, induced by a single intracerebroventricular injection of CNF1, results in rearrangements of actin in cortical neural cells, enhanced synaptic transmission and significantly improved learning and memory [69]. These effects are persistent for months and are not seen in animals injected with an enzymatically inactive version of the toxin (CNF1 C866S), in which the enzymatic activity was abolished by substituting serine to cysteine at position 866. It is known that learning and memory depend on the morphology and dynamics of dendritic spines, their development and structural plasticity being controlled by Rho GTPases and actin dynamics. In primary neuronal cells from the mouse cortex, we have demonstrated that CNF1 induced actin changes and dendritic spine formation (69). These findings raise the possibility that pharmacological agents could be used to enhance the changes in neuronal connectivity associated with memory formation, and the cytoskeletal components are identified as a possible targets for such agents. CNF1 can thus be regarded as endowed

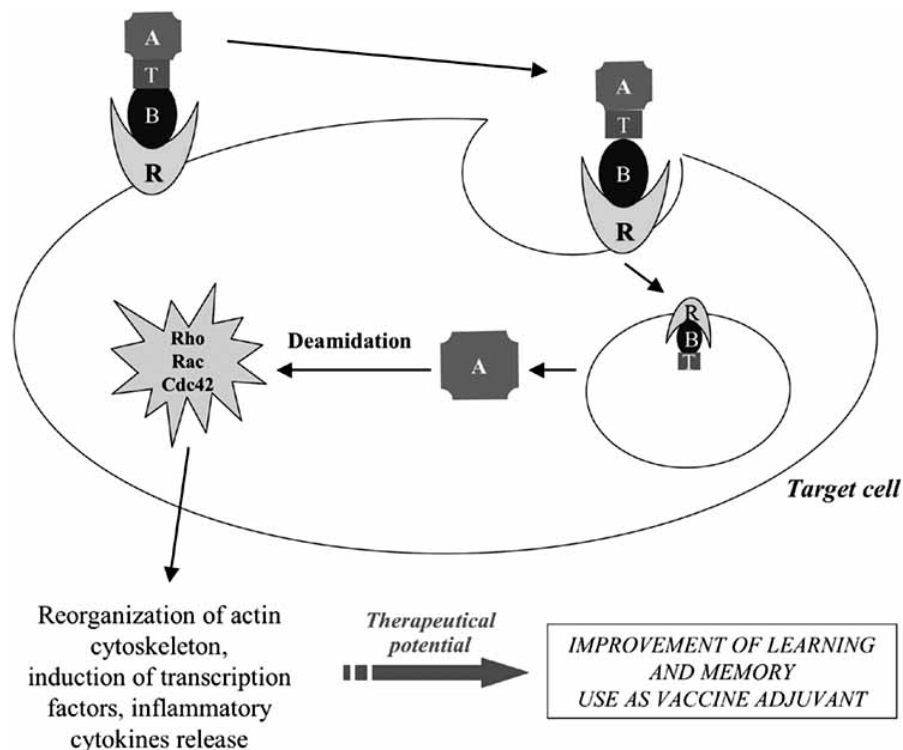


Fig. (4). *E. coli* CNF1.

CNF1 is a monomeric A-B toxin composed of a receptor-binding (B) domain and an enzymatically active (A) one, separated by a hydrophobic domain (T), which allows the translocation of the A moiety across the endosome membrane. The enzymatic activity of CNF1 is represented by deamidation of a specific aminoacidic residue of the small GTP-binding proteins Rho, Rac, and Cdc42. This modification blocks the Rho GTPases in their activated state, thus inducing different cell responses, among which the reorganization of actin cytoskeleton, and the induction of transcription factors and inflammatory cytokines release.

The ability of CNF1 to act on the actin network and to modulate the inflammatory response has been exploited to improve learning and memory and to design new vaccine adjuvants.

with long-lasting cognition enhancing properties. Overall, the results suggest that learning ability can be improved through pharmacological manipulation of neural connectivity.

Furthermore, as well as improving cognition in healthy patients, these drugs could, in theory, be used to alleviate the cognitive impairments exhibited by patients with conditions such as Alzheimer's Disease.

Immunotoxins

Immunotoxins are hybrid molecules created by coupling an antibody or antigen with part or all of a toxin. The hybrid molecule combines the specificity of the antibody or antigen with the toxicity of the toxin. An immunotoxin can kill a cell through the binding of specific cell surface receptors, internalization and the delivery of the toxin moiety to the cell cytoplasm. First generation of immunotoxins were created by chemically conjugating an antibody to a protein toxin. Currently, immunotoxins are produced by using the recombinant DNA techniques and the principle of protein engineering, and the recombinant chimeric proteins are produced in *Escherichia coli* [70].

The cell binding ligand may be a growth factor, a cytokine, an antibody or a fragment of an antibody, and the toxin is usually the catalytic portion of either exotoxin A from *Pseudomonas aeruginosa* (PE) or diphtheria toxin (DT) produced by *Corynebacterium diphtheriae*. Although being different in their sequence and structure, both PE and DT ADP-ribosylate the Elongation Factor 2 (EF-2), thus blocking proteins synthesis and inducing cell death (Fig. (5)) [71-73]. Recently, the anthrax toxin has been also used for the production of immunotoxins (see above the anthrax toxin section) to specifically target tumor cells.

Therapeutical Applications

The goal of immunotoxin therapy is to target a cytotoxic agent to cell surface molecules. The subsequent internalization of the cytotoxic agent will cause cell death. Immunotoxin, produced with the bacterial protein toxins DT and PE, kills the targeted cells following the blockade of protein synthesis [70].

Developing this type of therapy has aroused great interest in the past two decades, especially in the field of cancer therapy.

Immunotoxin treatment greatly differs from that of chemotherapy, in its action and toxicity profile, and it has the potential to improve the systemic treatment of incurable tumors with existing modalities. They rely on tumor cells that often express surface receptors or other molecules, which distinguish them from their normal surrounding tissues. Thus, immunotoxins are constructed by using ligands that recognize those surface receptors over-expressed on tumor cells.

Immunotoxins are optimal for treating hematological malignancies, since malignant cells are often intravascular and accessible to intravenously administered drugs. Furthermore, patients often lack sufficient immunity to make antibodies against the toxin. On the other hand, targeting solid tumors with immunotoxins is much more difficult because not only are the cellular junctions tighter and the tumor cells more packed, but also patients are less immunosuppressed and thus likely to make neutralizing antibody to the toxin. Different tumors are successfully treated with the use of immunotoxins and an increasing amount of ligands are currently exploited to construct these efficient immunotoxins. In particular, hematologic malignancies like hairy cell leukemia (HCL), Hodgkin's lymphoma, T-cell lymphoma and chronic lymphocytic leukemia (CLL), are treated by means of immunotoxin. Currently, a

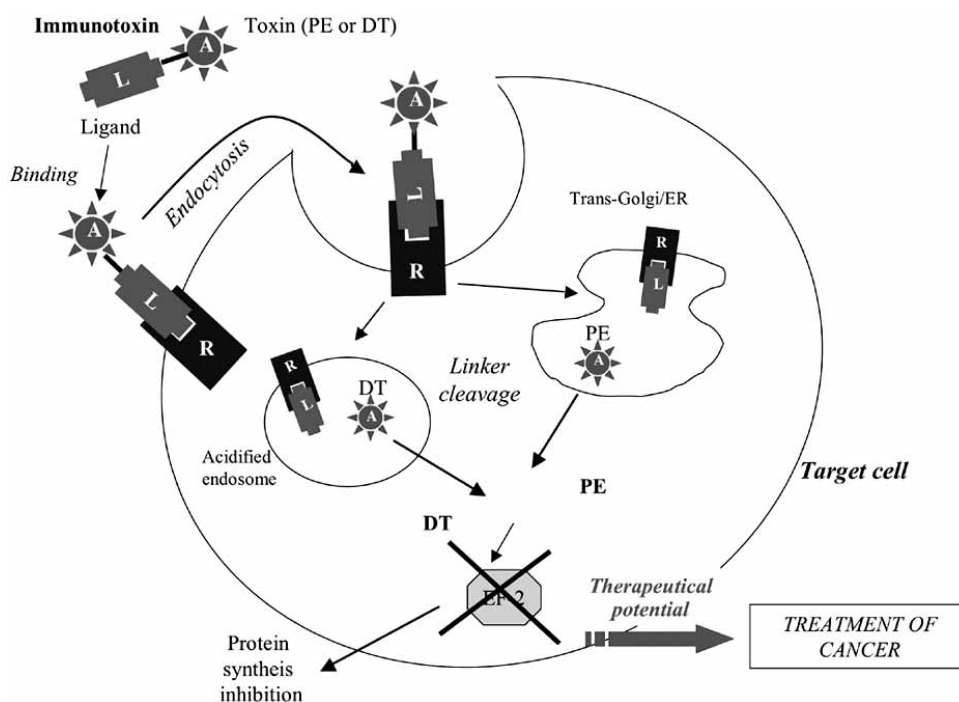


Fig. (5). Mechanism of action of immunotoxins.

Immunotoxins are chimeric proteins composed of a ligand subunit (L), which recognizes surface receptors (R) overexpressed on target cells, fused to the catalytic portion of a toxin (A), which ADP-ribosylates the Elongation Factor-2 (EF-2), thus blocking proteins synthesis and inducing cell death. The ligand subunit may be a growth factor, a cytokine, a monoclonal antibody or a fragment of an antibody, while the toxin moiety is generally the enzymatic domain of the exotoxin A from *P. aeruginosa* (PE) or diphtheria toxin (DT) produced by *C. diphtheriae*. After the ligand subunit binds to the surface of the target cell, the immunotoxin is internalized by endocytosis and the enzymatic fragment of the toxin translocate into the cytosol. Once into the cytosol, the toxin molecule blocks protein synthesis thus killing the target cell.

Immunotoxins that recognize antigens over-expressed on cancer cells can be used as anticancer therapies.

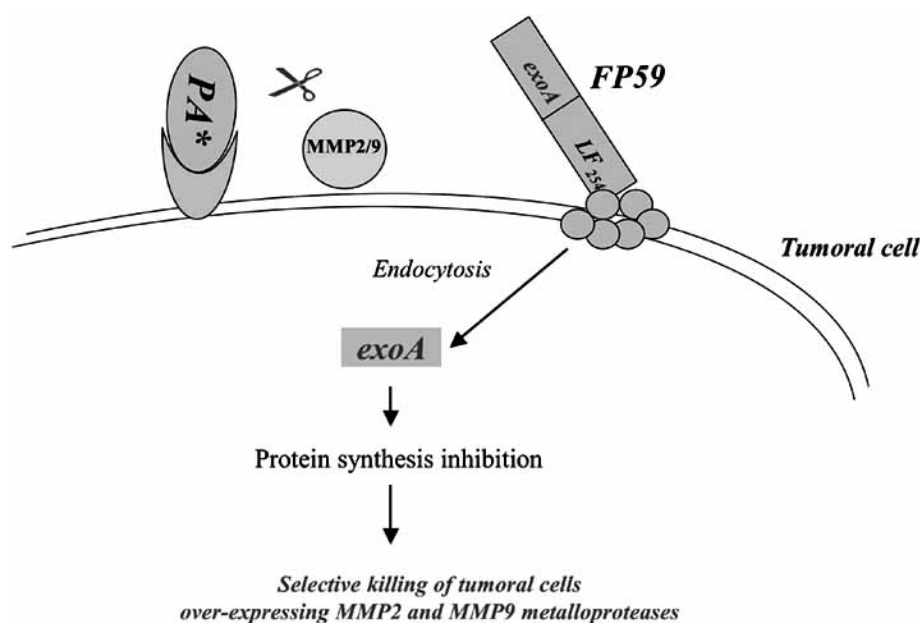


Fig. (6). Engineered anthrax toxin.

Mutated anthrax toxin protective antigen (PA*) consists of PA proteins in which the furin protease cleavage site is replaced by sequences selectively cleaved by metalloproteases (MMP) MMP-2 and MMP-9. These MMP-targeted PA proteins are activated rapidly and selectively on the surface of MMP-overexpressing tumor cells. The activated PA proteins permit internalization of a recombinant cytotoxin FP59, consisting of anthrax toxin lethal factor residues 1–254 fused to the ADP-ribosylation domain of PE that induces selective killing of MMP-overexpressing human tumor cell lines.

number of clinical trials, including those targeting the less accessible solid tumors such as breast, colon, stomach, pancreas, cervix and lung cancer, are in progress [a detailed description is out of the scope of this paper, for more details see references 74–76].

Regarding the anthrax toxin, the unique requirement of PA to be activated on the surface of the target cell has provided an opportunity to re-engineer this protein to make its activation dependent on either Matrix Metalloproteinases (MMPs) [77] or urokinase Plasminogen Activator (uPA) [78]. Both molecules are overexpressed in a variety of tumor tissues and tumor cell lines, their expression correlating to tumor malignancy and metastasis.

Mutated anthrax toxin protective antigen (PA) proteins were constructed in which the furin protease cleavage site was replaced by sequences selectively cleaved by MMPs [77] or activated by uPA [78]. These PA proteins were activated rapidly and selectively on the surface of tumor cells overexpressing such molecules thus causing the internalization of the recombinant anthrax toxin that selectively kills tumor cells (Fig. (6)). Interestingly, antitumor efficacy of the urokinase activation-dependent anthrax toxin has been reported also in *in vivo* human tumor model [79, 80].

BACTERIAL PROTEIN TOXINS USED AS ADJUVANTS OR DRUG DELIVERY AGENTS

Some bacterial protein toxins have been described to act as adjuvant or drug delivery agent (Table 2). Adjuvants are vaccine additives that enhance the elicited levels of antibodies and T lymphocyte responses [81] in ways that presumably mimic natural infection, whereas drug delivery agents are compounds able to promote absorption of therapeutic agents thus increasing their effectiveness [82].

CT from *Vibrio cholerae* and LT from *E. coli*

CT and LT are closely related molecules that function as virulence factors in *V. cholerae* and *E. coli* infections, respectively. Both toxins are composed of an A and B subunit of which the B

subunit forms a homopentamer structure that binds to surface receptors of glycosphingolipid nature, virtually present in all cells, including enterocytes, dendritic cells, macrophages, B and T lymphocytes [83]. The A subunit is composed of a globular A1 domain and an A2 domain that interacts with the B subunit. A proteolytic cleavage of the loop between the two A domains and reduction of a disulfide bond facilitates the ADP-ribosyltransferase activity that is exerted once the A1 fragment enters the cytosol. The target for ADP-ribosylation is the Gs proteins of the adenylate cyclase complex and the result is an increased cAMP production [84].

Table 2. Bacterial Protein Toxins Used as Potentiation or Delivery Agents

TOXIN	Therapeutic Use:
Cholera toxin (CT) from <i>V. cholerae</i>	Mucosal adjuvant
Zonula occludens toxin (ZOT) from <i>V. cholerae</i>	Mucosal adjuvant Drug delivery agent
Cytotoxic necrotizing factor 1 (CNF1) from <i>E. coli</i>	Mucosal adjuvant
Pertussis toxin (PXT) from <i>B. pertussis</i>	Mucosal adjuvant
Adenylate Cyclase (AC) from <i>B. pertussis</i>	Mucosal adjuvant

Vaccine Adjuvant

Beside being potent immunogens [85, 86], CT as well as LT can act as adjuvants for the enhancement of mucosal and serum antibody responses to a mucosal co-administered antigen, resulting in long-term memory to antigens [87, 88]. 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 diarrhoea in human volunteers.

An immune response occurs against these toxins when used as adjuvant. However, high levels of toxin specific IgA at the inductive site, reduce but do not completely inhibit the mucosal adjuvanticity [89] so that CT and LT remain the most potent mucosal adjuvants known to date. CT and LT have immunomodulatory effects on different cell types that may facilitate adjuvanticity [90-92]. However, the interaction of these toxins with dendritic cells is thought to be of major importance for mediating their adjuvant effects [93, 94].

Two different approaches have been followed in order to develop non-toxic CT and LT derivatives with strong mucosal adjuvanticity. The first has been based on the use of the non-toxic B subunits alone. The results obtained demonstrated B-subunits were very poor mucosal adjuvant [95, 96]. The second approach has been based on the generation of genetically detoxified mutants of CT and LT where mutations in the enzymatically active A subunit were introduced. Several mutants were generated and all behaved as strong mucosal adjuvants [97-100] for all antigens tested when given intranasally, orally or vaginally, inducing very strong systemic and mucosal antigen-specific antibody responses [101]. Interestingly, one of these mutants, LTK63, is being used in Phase I clinical studies where influenza vaccine was given intranasally with LTK63 as an adjuvant. Results are promising since LTK63 enhanced mucosal IgA responses to the influenza virus [102, 103]. The fine molecular mechanisms of mucosal adjuvanticity of CT and LT mutants are still not fully understood, but it is reasonable to hypothesize that the enzymatic activity of the A subunit is not necessary in the adjuvanticity of mutants. Moreover, the holotoxin structure exerts an important role in adjuvanticity since mutants completely lacking the A subunit are weak adjuvants [101].

Zonula Occludens Toxin (ZOT) from *Vibrio cholerae*

Pathogenic strains of *V. cholerae* produce several endotoxins, including the Zonula Occludens toxin (ZOT) [104]. ZOT is of bacteriophage origin and is composed, at the origin, by a single polypeptide chain of 45 kDa localized in the *V. cholerae* outer membrane. Following cleavage, a carboxyl terminal fragment of 12 kDa is excreted that is probably responsible for the biological effect of the toxin [105]. The 12 kDa fragment of ZOT binds to a specific receptor, whose surface expression differs on various cells, and activates a complex intracellular cascade of events that involve a dose and time-dependent protein kinase C α - related polymerization of actin filaments, strategically localized to regulate the paracellular pathway [106]. The result is the modification of the cytoskeletal organization and the opening of the intercellular tight junctions (TJ) thus leading to a transient increase of the permeability of the paracellular route [104].

Drug Delivery Agent

The main route of absorption of hydrophilic drugs (proteins, peptides and others) is the paracellular route that is governed by TJ. *In vitro* experiments in rabbit ileum demonstrate that ZOT reversibly increased intestinal permeability of insulin by 72% and immunoglobulin by 52% in a time-dependent manner [107]. ZOT was also tested in an *in vivo* primate model of diabetes mellitus where insulin was intragastrically administered in combination with ZOT. Measurements of blood insulin levels revealed that insulin bioavailability increased from 5.4% in controls to 18% when ZOT was used as enhancer of drug delivery [108]. Recently, the ability to enhance drug delivery has been demonstrated to reside in a small part of the ZOT molecule. In fact, a novel synthetic hexapeptide derived from ZOT (AT1002) enhanced the absorption of both, cyclosporine A [109] and heparin [110]. Interestingly, ZOT as well as

its 12 kDa active fragment were introduced as potential absorption enhancers for delivery of the therapeutic agents also to the central nervous system [111, 112].

Vaccine Adjuvant

The ability of ZOT to reversibly alter intestinal epithelial tight junctions, was exploited to deliver soluble antigens through the nasal mucosa for the induction of antigen-specific systemic and mucosal immune responses. Intranasal immunization of mice with ovalbumin (Ova) and recombinant ZOT, induced anti-Ova serum immunoglobulin G (IgG) titers that were approximately 40-fold higher than those induced by immunization with antigen alone [113]. ZOT interferes also with tolerance induced by both nasal and oral administration of soluble protein antigens. Rossi and coworkers [114], using a model of systemic tolerance in which a reduced production of interferon-gamma (IFN-gamma) and interleukin-2 (IL-2) was induced upon administration of gliadin alone, demonstrated that this immune suppression was reverted in mice receiving gliadin together with ZOT. It is known that down-regulation of such cytokines is associated to a suppression of the T-cell proliferation, and ZOT co-administration with gliadin completely reverted this phenomenon. Recombinant fragments of ZOT are being tested for their mucosal adjuvant activity that can provide promising tools for the development of mucosal vaccine [115].

CNF1 from *E. coli*

We have already described the *E. coli* toxin CNF1 in the "Bacterial protein toxins used in therapy" section. Now we report its use as a vaccine adjuvant, a characteristic that was first described a few years ago [116].

Vaccine Adjuvant

Previous studies have demonstrated that CNF1 induces secretion of pro-inflammatory and immunomodulatory cytokines via the activation of Rho proteins [117, 118]. The subsequent investigation of the toxin immunomodulatory effects in a mouse model of oral administration showed that, similarly to cholera toxin, CNF1 elicits adjuvanticity anti-OVA responses, both systemic and mucosal [115]. Animals orally immunized with OVA, a prototype soluble antigen, co-administered with CNF1 displayed serum IgG anti-OVA antibody responses comparable to those elicited by cholera toxin. Interestingly, immunization with CNF1 C866S, a catalytic inactive CNF1 mutant, failed to enhance serum anti-OVA responses in animals co-fed OVA, demonstrating that the enzymatic activity of CNF1 is necessary for the adjuvanticity to occur. In the same vein, the *Bordetella* dermonecrotic toxin (DNT), a closely related Rho activating toxin, also showed adjuvant properties. These findings strongly suggest that the host response to CNF1, in addition to promoting innate immune effectors and regulators, may also stimulate the adaptive immune system. Manipulation of Rho proteins thus provides a possible new approach for the development of effective mucosal immunoadjuvants.

AC from *Bordetella pertussis*

B. pertussis produces a second important protein toxin of 1706 aminoacids known as Adenylate Cyclase (AC) toxin [119]. It expresses haemolytic and calmodulin-dependent adenylate cyclase activities, both important at the early stages of infection. The catalytic and calmodulin binding domain is located in 400 amino-terminal residues whereas haemolysis resides in the carboxy-terminal domain. This domain also mediates binding and internalization of the toxin into target cells. The translocation of the catalytic domain appears to occur directly at the plasma membrane and does not require endocytosis and vesicular trafficking.

Vaccine Adjuvant

AC toxin was shown to be highly antigenic either in mice or in humans [120-121]. Some studies report that AC acts as mucosal adjuvant for other antigens when co-administered by the intra-nasal route in mice. For this purpose, two forms of AC have been used, the enzymatically active form and the toxin lacking the enzymatic adenylate cyclase activity. In all cases the adjuvanticity effect was greater with the enzymatically inactive form [120, 122].

Also a genetically modified form of PTX (already described in the "Bacterial protein toxins used in therapy" section), PT-9K/129G, devoid of the A-protomer-specific enzymatic activity but retaining a functional PTX-B subunit, has been successfully used as a mucosal adjuvant for a tetanus vaccine in mice [123].

CONCLUSIONS

As an overview, although detrimental to the susceptible host during an infection, the activities of several bacterial toxins can be exploited for medical applications. As reported above, derivatives of some toxins have been incorporated into human vaccines because of their adjuvant properties while other toxins can be "engineered" as cell-killing components of immunotoxins. Importantly, some of these "magic bullets" are now approved for the treatment of some types of cancer. Furthermore, research on toxins has led to its use in cosmetics for the reduction of wrinkles. In concluding, scientific research has permitted the conversion of these harmful bacterial molecules into other powerful and beneficial drugs, used for the treatment of human diseases.

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