Bacterial toxins as tools for mucosal vaccination

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Several studies have demonstrated that the biological properties of secreted bacterial toxins could be harnessed for the induction of mucosal and systemic immunity following application at epithelial surfaces. Although the properties and potential application of several of these toxins will be discussed in this review, special focus will be placed on *Pseudomonas aeruginosa* exotoxin A (PE). A non-toxic form of PE (ntPE) into which antigenic epitopes can be integrated appears to be a particularly promising vaccination tool, which is able to cross the polarized epithelia of the gastrointestinal, respiratory and reproductive tracts and selectively target macrophages and dendritic cells.

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▼ Carefully regulated relationships exist at all mucosal surfaces of the body that involve interactions between epithelial cells and bacteria that are either probiotic (commensal) organisms or pathogenic in nature. These interactions are strictly monitored by the immune system, which keeps this balance in favor of probiotic organisms that reside on the surface of these epithelial barriers. For pathogenic bacteria to obtain a dominant position in these arenas, they must shift this natural balance. Protein toxins, frequently secreted by these pathogenic bacteria following intimate bacterial–epithelial cell association, can provide such a shift. Some secreted toxins act at the plasma membrane of host cells where they interfere with local signaling pathways. This group includes the enterotoxin heat-stable Shiga toxin (ST) produced by *Escherichia coli* that targets guanylate cyclase of intestinal epithelial cells. Other toxins produce pores in host cell membranes. These toxins include toxin α from *Staphylococci* and *E. coli* hemolysin. Another group is composed of toxins that act within the host cell to alter (in most cases) an intracellular process through enzymatic activity. Toxins in this last group are the focus of this review.

Once secreted, some bacterial toxins enter into host cells through a receptor-mediated uptake mechanism [1]. To accomplish the multiple steps involved in uptake and trafficking, toxins are commonly composed of several subunits (single subunit toxins usually have multiple domains) with specific functions of receptor interaction, translocation and toxicity. For example, the diphtheria toxin of *Corynebacterium diphtheriae* (DT) and the anthrax toxin (AT) of *Bacillus anthracis* translocate into the host cell cytoplasm from acidified endosomes after endocytosis. Cholera toxin (CT) produced by *Vibrio cholerae*, the heat-labile enterotoxin of *E. coli* (LT), pertussis toxin (PT) secreted by *Bordetella pertussis*, *Pseudomonas aeruginosa* exotoxin A (PE) and *E. coli*-encoded shiga-like toxin 1 (SLT-1) all appear to enter the cytoplasm after trafficking through the endoplasmic reticulum (ER) of the host cell [2]. Shiga toxin produced by *Shigella dysenteriae* probably uses a similar retrograde route, being transported along a route through early endosomes to the *trans*-Golgi network (TGN) [3]. Once at the ER, unfolded toxins appear to enter the cytoplasm through the protein translocation complex of the ER, where they refold and express their toxic function [4].

Teleologically, secreted toxins are ultimately thought to facilitate the survival of a bacterium in its primary environments: water and soil. As opportunities arise for bacteria to use animals as either a food source or as a transmission vector, these secreted toxins found a role in establishing and/or maintaining infection. This point has been emphasized in several infection or survival models with pathogenic bacteria

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that have been modified to no longer express a particular toxin [5]. For example, CT induces an intestinal secretory event, causing the expulsion of *V. cholerae* to perpetuate its life cycle. Similarly, AT has been suggested to rapidly cause host death to provide a decomposing food source for *Bacillus anthracis*. PE is part of a complex of toxins secreted by *P. aeruginosa* that is involved in the accession of nutrients. Specifically, PE has been shown to stimulate fluid resorption in the lung, which might act to stabilize the bacteria in a preferred environment of infection [6]. A common feature of these toxins is that they frequently target cells that deal with immediate and/or long-term immune response events associated with aninfection. Thus, it is common to see ablation of neutrophils, as well as macrophage and dendritic cell populations, so-called antigen presentation cells (APCs), either at the site of infection or even systemically.

There are several general features associated with the actions of bacterial toxins that should be highlighted. First, these secreted proteins are not toxic to the bacteria that produce them. This is important because if they are ultimately to be used as a tool for immunization, it is important that methods are available to obtain them in sufficient quantities. Several methods have also now been identified for the controlled or regulated production of these proteins. Second, as part of their toxic actions, these proteins have the ability to enter into and frequently cross epithelial barriers. Many toxins use transport pathway(s) present in the host cell [7], whereas others disrupt the barrier properties of the epithelia by cleaving proteins that stabilize the intercellular barriers present between adjacent epithelial cells [8]. Alternatively, some exotoxins can directly damage epithelial cells to breech the epithelial barrier, but these would not be desirable for vaccine development. This property of transport across epithelial barriers provides an intriguing tool for introducing antigen to incite a mucosal immune response. Third, several toxins can selectively target APCs. Such toxins show particular promise for immunization because they might be used to activate and possibly deliver antigens directly to crucial cells involved in the establishment of a potent and durable immune response. Finally, some toxins can progress across epithelial barriers to gain access to the systemic circulation. In this way, their use in the delivery of antigens should lead not only to a mucosal immune response but also a systemic immune response that would provide an important complement of protection from infection.

Rationale for toxin-based antigen delivery for mucosal immunization

Most pathogens that enter the body do so following an initial infection at a mucosal surface. The natural sequence of events for such infections that are resolved through corrective actions by the immune system involves the induction of a potent and durable immune response. Two types of immunity are invoked through these events. One is a mucosal immune response that is stimulated through the actions of APCs that pick up pathogens and ferry them back to local draining lymph nodes associated with mucosae where T- and B-cell lymphocyte populations are 'taught' to recognize and respond to selected pathogen components. Some lymphocytes and APCs associated with the initial site of infection circulate to other mucosal surfaces to distribute this immune information to other potential sites of infection. A significant population of APCs and lymphocytes is also retained at the mucosal site of infection. This retention at the initial site of infection as well as the dissemination of cells to other sites is a crucial function of the 'common mucosal immune system' [9]. In addition, once significant numbers of a pathogen cross an epithelial barrier, the systemic immune system leaps into action in a manner that is similar to that of the mucosal immune system. In this way, the mucosal immune response to a pathogen can be coordinated to the response achieved by the systemic immune system. With both systems working in concert, a rapid and protective immune response can be stimulated that frequently protects from re-infection [10].

This brief outline of events associated with mucosal and systemic immune responses provides a rationale for the induction of an optimal immune response. Antigens of a pathogen must get across an epithelial barrier, reach APCs and then be carried to sites of immune instruction in local lymph nodes (for mucosal immunity) and in tissue sites involved in systemic imune responses (such as the spleen). One additional and rather crucial event associated with a successful immune response is the recognition by the immune system of the severity of the infection. To this end, the immune system looks for cues, such as a structure that might be associated with a virus or bacteria as either a monomer or polymer. These agents are referred to as adjuvants, because they act to augment or modulate intercellular immune signaling as part of the immune response [11]. Although there is no clear understanding of the cellular mechanism(s) to explain how secreted bacterial toxins function as adjuvants, it is clear that the immune system is intensely activated by their presence [12].

It is difficult to directly compare adjuvant activities because of differences in the antigens being used, and differences in the specific or desired immune outcomes being assessed. Despite this, several inappropriate comparisons have been made; comparing immune responses following subcutaneous or intramuscular immunization with a mucosal immunization using an adjuvant that is clearly

optimized for one route or the other. It is even difficult to compare different mucosal adjuvants, because presentation by each can differ and require different forms of the antigen for an optimum response. One area in which direct comparisons should be made is in the evaluation of various forms of the same toxin. For example, Komase *et al.* have evaluated differences between various LT mutants (discussed more fully later) [13]. Another excellent example of such a study is where an LT mutant that lacks ADP-ribosylating activity was compared with another that still retains partial enzyme activity [14]. The data for such comparisons, however, are typically obtained in mouse models and might not reflect events in humans. In addition, the toxicity of these proteins can depend upon dose and route, as in the case of LT applied topically to skin versus the intestinal mucosa [15].

Modification of toxins for use in mucosal vaccination

Cholera toxin and heat-labile enterotoxin

It is clear that secreted bacterial toxins can incite a durable and protective immunity involving both the mucosal and systemic immune systems. The biggest problem with their use for the induction of a desired immune response is that these molecules can be incredibly toxic; repeated use of even small quantities of a potent toxin would not be acceptable in humans. Initial efforts to reduce the toxicity of these toxins through chemical modification resulted in significant loss in their adjuvant activity when applied at a mucosal surface. It is likely that this loss was caused by a disruption of the toxin's ability to enter into and progress within host cells as it normally does without this chemical modification. In particular, the transcytosis capacity of many of these toxins is lost after even mild chemical alteration. In an effort to better understand how to detoxify these secreted bacterial toxins yet retain their adjuvant activity, several studies have focused on determining the specific requirements of various toxin subunits or domains associated with cell binding, intracellular trafficking and toxicity for the adjuvant functions of these proteins.

CT and LT have been the most widely used toxins for mucosal vaccination [16,17]. Both CT and LT are composed of a pentamer of B subunits, which bind to a cell-membrane ganglioside (GM1), and one A subunit which targets the α-subunit of host cell GTP-binding proteins to activate adenylate cyclase and increase intracellular cAMP levels [16]. Mixing CT with purified virus-like particles of human papilloma virus type 16, a virus strongly connected to the onset of cervical cancer, allowed for potent mucosal antigen-specific immunizations following either oral or nasal inoculations [18]. It is important to note that these immunizations also resulted in systemic immune responses, which could be more important for protection from infection by this virus [19]. Intranasal and intragastric immunizations with a chimeric protein composed of the serine-rich *Entamoeba histolytica* protein antigen and subunits of CT induced persisting IgA antibodies and circulating antigen-specific IgG antibodies [20]. In general, the B subunits of CT alone are not as potent as the holotoxin for the induction of immunization [21]. This could be because of the suggestion that the ADP-ribosylating activity of the A subunit but not the GM1 binding actions of the B subunits is essential for the adjuvant function(s) of CT [22,23].

Recent studies have used genetic mutants of the CT holotoxin and assessment of these mutants have led to a similar correlation – the greater the reduction in toxicity, the less potent its actions as an adjuvant [16]. Like CT, LT has been shown to be a potential mucosal immunogen and adjuvant toward co-administered antigens. Neither GM1 binding nor the ADP ribosylating activity of LT are required for its adjuvant activity [24,25]. LT has been shown to be safe and efficacious as an adjuvant in humans as a component of an intranasal flu vaccine [26]. Administration of LT with recombinant *Helicobacter pylori* urease resulted in protection in non-human primates against *H. pylori* infection and this same approach appears to be safe and immunogenic in humans [27,28]. Intranasal administration of a fusion construct containing the 15 amino-terminal amino acids of type 5 streptococcal M protein and non-toxic B subunit of LT (LTB) resulted in the significant protection of mice from death after intraperitoneal challenge with type 5 streptococci [29]. Intranasal immunization with LTB alone and a truncated glycoprotein D of herpes simplex virus (HSV) type 1 produced both mucosal IgA and serum IgG antibody response [30].

A large number of LT mutants have been evaluated for efficacy and safety [13]. An attenuated form of the LT holotoxin, having a mutation at amino acid residue 192 from arginine to glycine, has been shown to significantly increase the antibody response to a rotavirus immunogen when it was included in an intranasal delivery [31]. This same toxin adjuvant, termed LT(R192G), greatly enhanced the delayed-type hypersensitivity reaction to heat-killed *Candida albicans* [32]. LT(R192G) has also been shown to induce significant T-cell responses following oral administration [33]. Intranasal immunization with influenza hemagglutinin or *Salmonella dublin* and a non-toxic LT mutant (R72) induced potent immune responses [34,35]. Another non-toxic mutant, LT(S63K), has been investigated by X-ray crystallography and was found to retain a similar structure as native LT while modifying the active site [36]. LT(S63K) has now been shown to generate strong systemic proliferative and cytotoxic T-cell lymphocyte

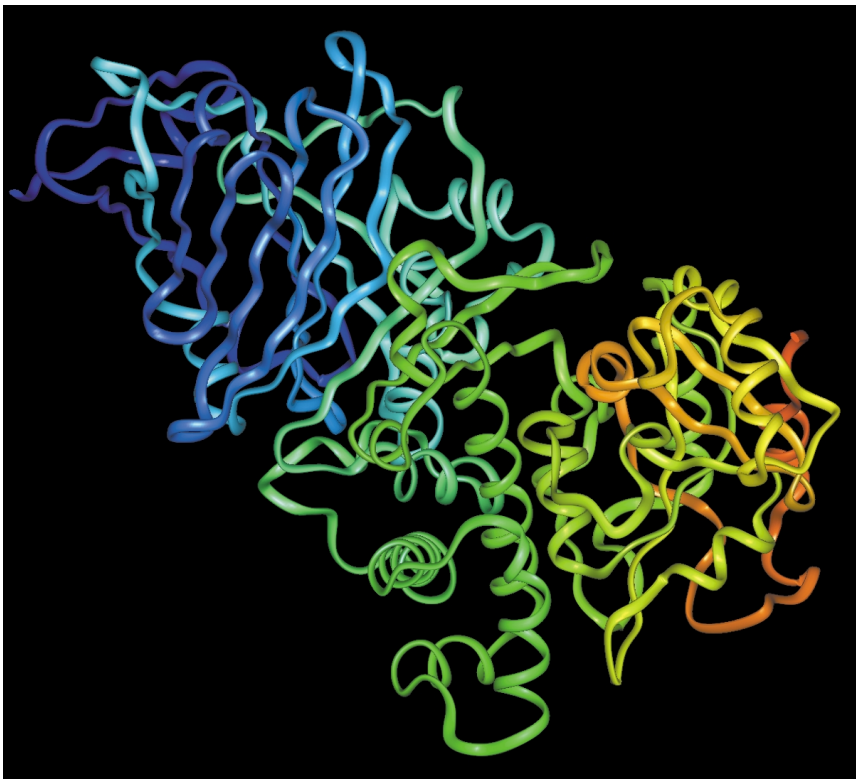

Figure 1. Schematic diagram showing the postulated structure of *Pseudomonas aeruginosa* exotoxin A. Structure based upon X-ray diffraction data [79]. This figure was generated using the RasMol program to demonstrate the various domains of this single chain protein of ~68 kDa. The protein has been colored (with amino acid residues) along a dark blue (1–102), blue (103–191), aqua (192–252), light green (253–301), green (302–401), olive green (402–452), yellow (453–502), golden (503–552), orange (553–602) to red (603–606) gradient depicting N- to C-terminal domains. The C-terminus is truncated because of its flexibility in the structure. Receptor binding interactions occur primarily within the dark blue region. The translocation domain is dominated by the green-colored regions and the kill domain is primarily yellow to orange.

Table 1. *In vitro* transcytosis of *Pseudomonas aeruginosa* exotoxin A (PE) constructs across Caco-2 monolayers

PE contruct	Chimera component	Percent (%) of 20 µg transported after 4 h	
		Apical to basolateral	Basolateral to apical
Toxic PE (68 kDa)	–	7	0.2
Non-toxic PE (ntPE)	–	6.5	0.1
NtPE–V3MN	26 aa loop	4	–
NtPE–V3Thai	26 aa loop	8	–
K57E ntPE (rbPE)	–	0.01	–
Inulin (4 kDa)	–	1.5	1.5

Lys57 to Glu conversion of ntPE (K57E) results in a 100-fold reduced binding (rbPE) affinity to CD91. The amount of transported protein was determined by enzyme-linked immunosorbent assay (ELISA).

(CTL) responses when co-administered with synthetic peptides [37].

Heat-stable Shiga toxin, Shiga-like toxin and pertussis toxin

Several other exotoxins have been scrutinized for their potential to safely induce potent mucosal immune responses. ST is composed of five B subunits that form a pore-like structure in association with a single A subunit. The receptor-binding non-toxic B-fragment of ST (STB) has been used to target dendritic cells and B cells [38]. SLT-1 from *E. coli* strain 0157:H7 is an analogue of ST. Its 32 kDa enzymatic A subunit is non-covalently associated with five 7.6 kDa B subunits that bind strongly to the cell membrane lipid globotriaosylceramide (Gb3) of host cell plasma membranes. A variant of SLT, (E167Q), has been shown to be non-toxic and still able to induce antibodies [39]. PT, like many of the other toxin adjuvants described in this review, has an AB_5 structure and causes toxicity by its ADP-ribosylating activity. A mutant form of PT that lacks ADP-ribosylation activity PT(9K/129G) retains its potent mucosal adjuvant activity following intranasal delivery [40].

Pseudomonas *exotoxin A*

PE [41] is made by virtually all clinical isolates of *P. aeruginosa* [42]. This 67 kDa single-chain protein has three domains [43]: domain I binds to the α_2 macroglobulin receptor (CD91); domain II is responsible for translocation and domain III contains an enzymatic activity that ADP-ribosylates elongation factor 2 (Fig. 1). Between domains II and III is a short loop of unknown function known as the Ib loop. Proteolytic processing of PE results in the generation of a 37 kDa C-terminal fragment containing domains II, Ib and III. This fragment traffics to the ER and ultimately gains access to the cytoplasm of the host cell to incite apoptosis through the inhibition of protein synthesis [44]. Early studies using

PE used its ability to access the cytoplasm of target cells to achieve the type of peptide processing required for major histocompatibility complex (MHC) class I pathway presentation [45]. Our studies have shown that PE can rapidly and efficiently move across intact epithelia (Table 1) to target local APCs [46]. These observations highlight several important features related to the biology of PE (Fig. 2). PE is capable of moving across polarized epithelia using a receptor-mediated endocytosis process that restricts it from significant intracellular processing. The result is that the entire molecule is released from the basolateral membrane of epithelial cells, allowing for the targeting of PE to both mucosal and systemic APCs [46].

The application of PE, as a mucosal adjuvant, became plausible when methods to make it less toxic were identified. Initially, chemical de-toxification of PE was used to circumvent this problem [47]. Through genetic mutations, deletion of a glutamic acid at position 553 (ΔE553) was found to produce a completely non-toxic form of PE (ntPE) that retained its conformational integrity [48]. Additional studies have demonstrated that ntPE could be used as a potent mucosal immunogen that also induces a strong systemic immune response [49]. This outcome could partially have been because of the ability of PE (and ntPE) to efficiently move across intact epithelial barriers, which has been shown *in vitro* (Table 1) and *in vivo* (Fig. 3). Others have deleted the terminal portions (ΔE576-613) to obtain another form of PE that lacks ADP-ribosylating activity [50], although it is currently unclear whether this mutant traffics and behaves in a similar manner to native PE. Box 1 outlines the rationale for developing ntPE as a mucosal vaccine delivery system.

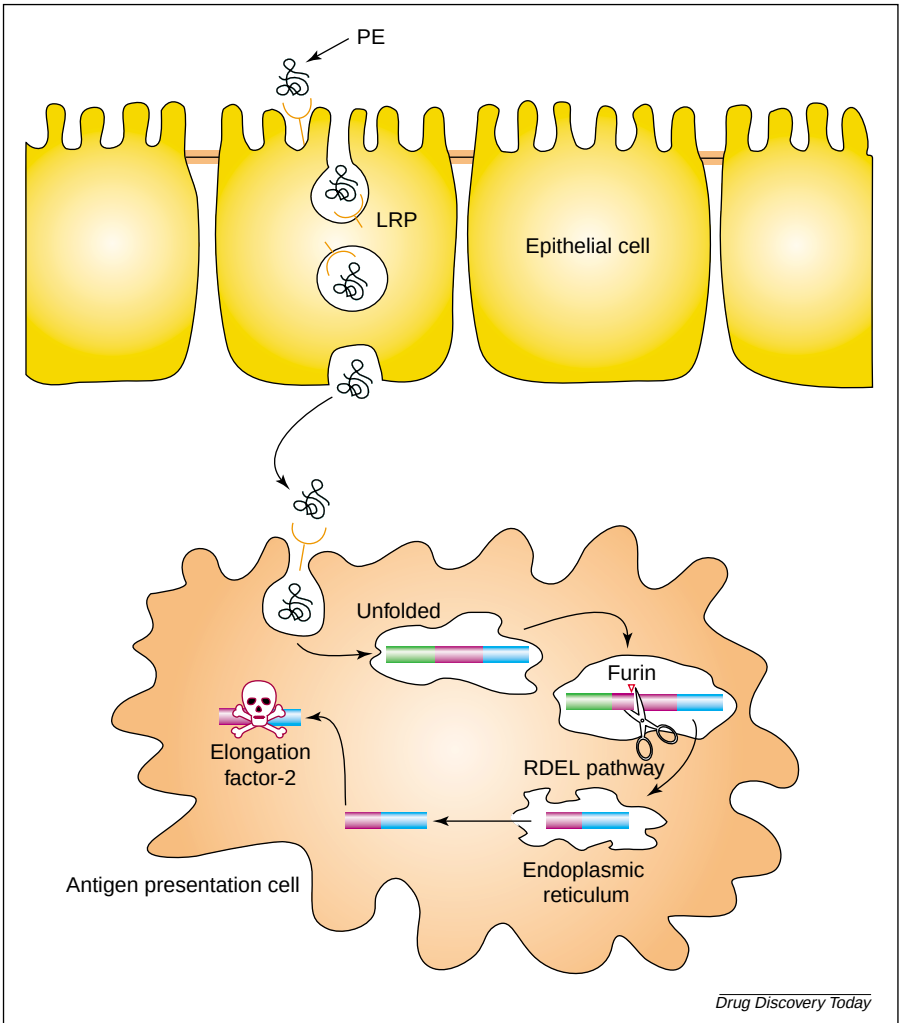

Figure 2. Cartoon of *Pseudomonas aeruginosa* exotoxin A (PE) trafficking. PE enters cells via CD91, also known as the low-density lipoprotein-receptor-like protein (LRP), expressed ubiquitously by epithelial cells and antigen presentation cells (APCs). PE traverses polarized epithelial cells, without killing them, to target CD91 expressed on APCs. After entering, the C-terminal half of PE (see Fig. 1) accesses the cytoplasm where it kills the APC cell by ADP-ribosylation of elongation factor 2 to induce apoptosis. Once PE enters cells through CD91 binding and endocytosis, PE traffics differently in polarized and non-polarized epithelial cells, allowing for the selective destruction of APCs. Once PE enters an APC, it kills these cells through ADP-ribosylation of elongation factor 2. This can occur because PE traffics differently in APCs versus polarized epithelial cells. In APCs, PE is cleaved by the protease furin. One half of PE (containing the toxin activity) then traffics through the actions of C-terminal REDEL amino-acid sequence that acts as an ER retention signal. Once in the ER, the toxic domain probably enters the cytoplasm via movement through the protein translocation complex of the ER.

Immunization outcomes using toxin-based vaccines

General aspects

Antigens taken up by APCs are typically either bound to MHC class II molecules and returned to the cell surface for CD4+ helper cell stimulation, or presented by MHC class I complexes as peptides to stimulate the production of CD8+ CTLs [51]. APCs, such as macrophages and dendritic cells, also have an alternative MHC class I pathway that presents peptides of extracellular origin or non-native structure, which is important in responses to infections by intracellular pathogens [52]. Once an antigen is processed and presented by an APC through MHC class I or II, antigen

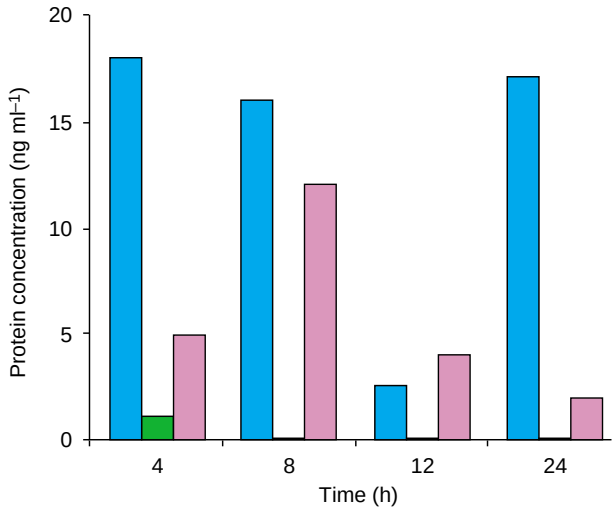

Figure 3. Systemic uptake of *Pseudomonas aeruginosa* exotoxin A (PE; blue) and the non-toxic form (ntPE; purple) following intra-tracheal application. Ten microliters of phosphate buffered saline containing 1 mg ml^{-1} of PE or ntPE (10 μg total protein) was applied on the tracheal surface of mice (N = 4 per group). Serum samples, collected over 24 h following application, were assayed for the presence of PE using an L929 cell-killing assay calibrated against authentic PE. The addition of the blocking antibody, M-40-1, to these PE serum samples was used to verify assay specificity (green). Using authentic PE, an enzyme-linked immunosorbent assay (ELISA) was validated to determine the amount of ntPE present in serum samples. The increased levels of PE at early and late times might reflect events associated with secondary uptake pathways because of the toxic actions of the protein, whereas ntPE levels present a profile anticipated for receptor-mediated uptake.

Box 1. Overall rationale for ntPE as a mucosal vaccine delivery system

- A genetically-derived non-toxic bacterial toxin
- Antigen epitopes readily introduced as loops or linear sequences
- Easily prepared in large quantities (using recombinant technology)
- Readily purified using standard chromatographic methods
- Introduced epitopes retain at least near-native conformation
- Method of verifying proper folding of chimera (use of fully toxic form)
- Stable lyophilized formulations
- Easily applied to mucosal epithelial surfaces
- Delivers antigens efficiently across intact epithelial barriers
- Delivers antigens to the cytoplasm of antigen presentation cells (APCs)
- Induces a mixed Th1/Th2 response following mucosal immunization
- Produces potent and durable antigen-specific systemic IgG response
- Produces potent and durable antigen-specific systemic sIgA response
- Does not produce an IgE response.

These statements can be made for a few, or in some cases many, of the bacterial toxins currently being examined for use in mucosal vaccination. The primary reason that ntPE appears as a promising carrier for mucosal immunization is that these statements are all true for chimeras generated using this molecule.

information is passed from the APC to T cells and B cells [53]. B cells, once activated, transform into plasma cells and begin making large amounts of an antibody that recognizes the presented antigen. Activated T cells can be induced to take on a variety of characteristics. They can become memory cells to provide long-term protection, effector helper cells to support and sustain the events of an immune response or suppressor cells that protect against autoimmunity. Following stimulation, naive $CD4^+$ T cells (Th0) can be transformed into either a T-helper type 1 (Th1) or a T-helper type 2 (Th2) population [51]. Th1 cells are primarily responsible for cell-mediated immunity and Th2 cells for humoral or antibody immunity.

Toxin-driven immune responses

Mucosal immunization with CTB has been used to stimulate both serum IgG and mucosal IgA responses [54] and can incite a Th1 response [55]. Other researchers have shown CT to induce a mixed Th1/Th2 response following mucosal immunization [56]. A non-toxic CT mutant, in which a negatively charged glutamic acid at residue 112 is replaced by a positively charged lysine (E112K), has been shown to preferentially inhibit Th1-type $CD4^+$ cell responses [57]. Not only does the adjuvant activity of CT appear to be interleukin-12 (IL-12)-independent [58], CT inhibits the production of IL-12 and its receptor [59]. Because IL-12 is a cytokine that has a major stimulatory activity for Th1 and counter-regulatory activity for Th2 functions, it is unclear how CT has been shown repeatedly to incite potent Th1 responses. Indeed, a non-toxic form of CT (S61F) induces mucosal and systemic immune antibody responses that are mediated via $CD4^+$ Th2-type cells [60]. CT and LT were used in studies that implicated the production of cAMP as a modulator of Th1 or Th2 cytokines [61], although others have suggested that neither GM1 binding nor ADP-ribosylating activity are required for the adjuvant activities of LT [24].

Nasal vaccination with a mutant of LT (R192G) induced a dominant Th1 response, whereas LT itself produces a mixed Th1/Th2-type response [62]. Intranasal immunization with the R72 mutant of LT provides a Th1 response

[34,35] and the R72 mutant appears to induce a more powerful Th1 response than the K63 mutant [63]. Others have also shown that although oral immunization with the LT(K63) mutant induces local and systemic antibody responses, this adjuvant fails to induce a strong cellular response [33]. The non-toxic LT mutant S63Y produced a mixed Th1/Th2 response, whereas the non-toxic LT derivative Δ110/112 produced primarily a Th2 response in mice following intranasal immunization [64]. LTB can also induce lymphocytes isolated from Peyer's patches of the small intestine to express early Th1-type cytokines (IFN-γ and IL-2) and late Th2 cytokines (IL-4, IL-5 and IL-6). The late-stage cytokine profile might dominate over time because intragastric administration of LTB produces primarily a Th2-type response [65]. PT has been used to facilitate the delivery of antigens to the cytoplasm to enhance MHC class I presentation [66] and induce CTL responses for anti-tumor vaccination [67]. A non-toxic PE mutant (9K/129G) drives primarily a Th2 response following nasal vaccination [40].

Table 2. Th1 and Th2 induction following mucosal immunization with an ntPE chimera containing the V3 loop of MN gp120 in mice

Antigen-specific immunoglobulin isotype	T-cell helper subtype	Fraction (%) of total immunoglobulin G
IgG1	Th2	55
IgG2a	Th1	20
IgG2b	Th2	20
IgG3	Th1	5
IgE	–	None detected

Serum samples were analyzed for the presence of IgG isotypes and IgE specific for the V3 loop of gp120 following three mucosal inoculations (either oral, rectal or vaginal) at two-week intervals as described [49].

Using the Δ553 version of PE (ntPE), it was found that a mixed Th1/Th2 response is achieved (Table 2), and potent and durable induction of both secretory IgA and systemic IgG responses [49]. A rationale for the exceptional potency of ntPE has been suggested. Complexes of heat shock proteins (HSPs) and antigenic peptides are taken up by macrophages and dendritic cells and efficiently presented by MHC class I-restricted molecules. The uptake of these HSP–antigen complexes occurs through the CD91 cell-surface receptor [68]. PE (and ntPE) enter cells through receptor-mediated endocytosis using the CD91 receptor [69]. Studies assessing the cytokine response of mice following mucosal application of PE and ntPE have demonstrated increases in the systemic levels of IL-12 (Fig. 4), a potent cytokine central to the induction of durable immunity [70]. Although these results appear most promising, it must be pointed out that the majority of studies performed, to date, evaluating the immune responses to ntPE, as with all of the bacterial toxin-based adjuvants discussed above, have been performed in rodents. Thus it is currently unclear as to which of these outcomes will translate to humans.

Antigen incorporation into toxin-based vaccines

Overall strategy

Mucosal immune responses have been obtained using soluble antigens by simply delivering them in a non-covalent mixture with a bacterial toxin adjuvant. In essence, the presence of the toxin activates the immune system sufficiently so that, in its heightened state of alert, it efficiently organizes an immune response against other non-self components identified in the same local region. This approach, however, causes concern on several fronts.

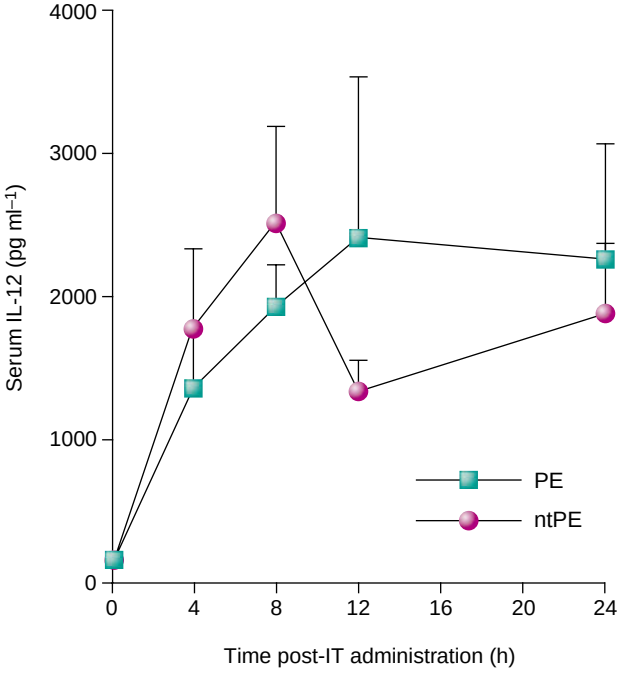

Figure 4. Interleukin-12 (IL-12) levels in serum following intratracheal (IT) application of either *Pseudomonas aeruginosa* exotoxin A (PE) or non-toxic PE (ntPE). Mice were dosed, while anesthetized, with 10 μg of PE or ntPE in 10 μl of phosphate buffered saline. Serum samples (N = 4 animals for each time point) were analyzed for the presence of interleukin-12 (IL-12) using a commercially available kit (R&D systems, Minneapolis, MN, USA).

Optimal immune responses require that the antigen, like the bacterial toxin adjuvant, has the capacity to deliver itself across intact epithelial barriers. Such antigens are commonly whole, attenuated pathogens. In an effort to vaccinate with only selected antigens and reduce the potential unwanted autoimmune responses that might occur following immunization with whole pathogens [71], subunit antigens have become more in demand. Subunit antigens typically do not have the capacity to transport themselves across epithelial barriers and thus high doses of antigen are required to achieve a reasonable immune outcome [40]. In addition, bacterial toxin adjuvants typically gain access to the cytoplasm of APCs with great efficiency. This is an optimal outcome for the maximal delivery of antigen for presentation to the immune system. Thus, a strong case can be made for the covalent coupling of subunit antigens with these bacterial toxin adjuvants to maximize the immune response and minimize the dose when administering a mucosal vaccine.

Not all bacterial toxins are amenable to the covalent association with antigens. For example, a molecule referred to as the zona occludens toxin (Zot) of *V. cholerae* acts as an adjuvant by facilitating the uptake of antigens through a proteolytic function that does not involve trafficking to the cytoplasm of APCs [8]. Further, covalent coupling of antigens is not always straightforward because many of these are highly complex molecules and it is unclear what aspects of these molecules are crucial for an optimal immune response. As stated earlier, disruption of bacterial toxin function can also destroy its adjuvanticity. Therefore, appropriate chemical coupling strategies are not always obvious. Highly complicated coupling methods have sometimes been used to retain the adjuvant activity of a bacterial toxin [72]. Despite these concerns, several successful immunizations have been achieved using antigens in which antigens are coupled to toxins using fairly straightforward chemical approaches. A more elegant approach involves the genetic introduction of antigens into bacterial toxin adjuvants. In both cases, however, there are many ways for a coupling or integration to fail (coupling at the receptor binding domain of the toxin or disruption of normal protein folding because of a bulky integrated sequence), and only a few ways for them to succeed (the few that are published). Once coupled or integrated, an additional concern relates to the stability of the toxin–antigen complex. Toxins, by themselves are usually stable even in the gastrointestinal tract. However, modifications to incorporate antigens can decrease the stability of the resulting chimera. In this case, other delivery platforms (e.g. microparticles and lipid structures) might be required to stabilize the vaccine at some mucosal surfaces.

Immune outcomes to antigens incorporated into a toxin carrier

A genetic fusion between the amino-terminal 254 amino acids of the lethal factor component of AT with the gp120 portion of the HIV-1 envelope protein was found to deliver antigen to isolated cells for MHC class I presentation [73]. A similar approach has also been taken to deliver HIV-1 antigens following intramuscular injection [74]. Fusion of a model tumor antigen, Mage 1, to the B subunit of ST resulted in the MHC class I-restricted presentation consistent with CTL induction [38]. Fusion of SLT1 with a peptide derived from the influenza virus matrix protein leads to the intracellular liberation of the peptide and the induction of a CTL response [2]. The optimal application of these fusion proteins has been achieved following injection rather than mucosal application. Thus, a price was paid for covalently joining the bacterial toxin to the antigen – a loss in the capacity of the toxin to efficiently transport across epithelial barriers, thus precluding mucosal immunization.

Some approaches, however, have spared this important delivery function provided by bacterial toxins. A fusion construct containing the 15 amino-terminal amino acids of type 5 streptococcal M protein and LTB resulted in significant protection of intranasally immunized mice from death after intraperitoneal challenge with type 5 streptococci [29]. Novel modifications to the C-terminus of LTB that facilitate the site-directed chemical coupling of antigens and the development of LTB as a carrier for mucosal vaccines have been described [75]. These LTB fusion proteins had modified versions of a short (14 amino acid) spacer epitope, Pk tag, attached at their C termini. Following site-directed chemical coupling of antigens to the cysteine residue within the Pk tag (LTB–Pk.cys), the LTB antigen conjugates retained their ability to bind GM1 on the surface of eukaryotic cells. Intranasal immunization of mice with an experimental antigen (HRP) chemically linked to LTB–Pk.cys induced high levels of anti-HRP antibodies that could be detected in the serum, saliva and nasal and lung washes.

Immune response to antigens incorporated into non-toxin *Pseudomonas* exotoxin A

The non-toxic form of PE (ntPE) provides a unique opportunity for antigen incorporation into a safe bacterial toxin that can be used to deliver antigens at mucosal surfaces. The eight amino acid Ib loop of ntPE can be replaced with much larger loops of amino acids without any apparent effect on transcytosis across epithelial barriers or on normal cellular trafficking [76,77]. Cell killing studies can be performed using the fully toxic form of these chimeras (containing E553) to verify that any modifications made to

the toxin by antigen introduction have not altered CD91 binding, cell entry or normal intracellular trafficking. This normal trafficking by ntPE chimeras appears to include antigen delivery into the cytoplasm of APCs [46]. Loops of amino acids that are used to replace the Ib loop are constrained by a disulfide bond and retain a conformation that is recognized by monoclonal antibodies generated to the same loop structure in the parent protein [76,77]. In addition, conserved amino acid changes in the terminal domain of ntPE can be used to insert helper T-cell epitopes (Fig. 5). The resulting nontoxic chimera then has the ability to efficiently transport across epithelial barriers, target APCs and deliver these incorporated antigens into the cytoplasm of these APCs. Also, because the ntPE molecule, like other toxins, is recognized as a potent adjuvant, the immune response induced by these chimeras is potent and durable. Mucosal stimulation of serum levels of antigen-specific IgG over 500 μg ml^{-1} can be obtained in mice more than one year since the last mucosal immunization [49].

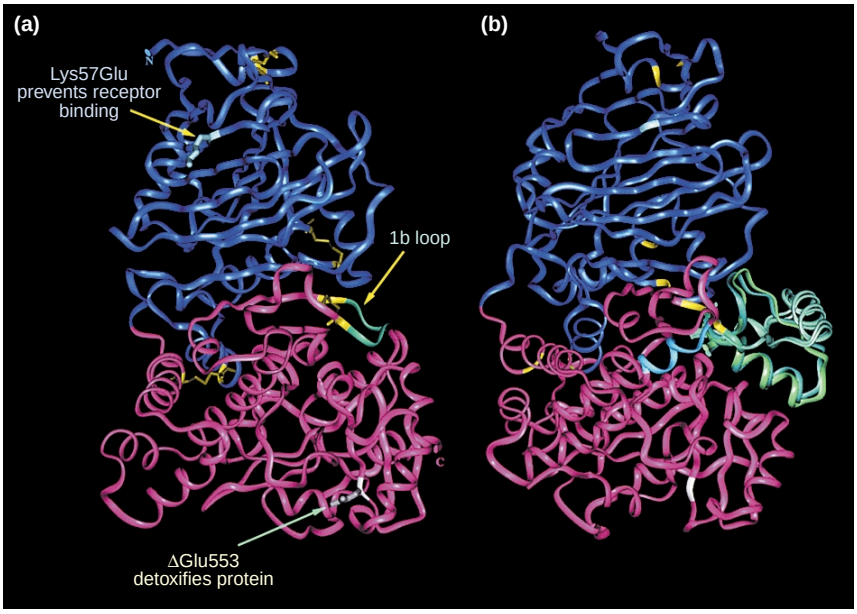

Figure 5. Schematic diagram of *Pseudomonas aeruginosa* exotoxin A (PE) showing structural features important for generating chimeric mucosal vaccines. Structures based upon X-ray diffraction data [79]. **(a)** Ribbon structure of PE. Normal processing of 67 kDa PE involves the furin-mediated cleavage of Arg279–Gly280 (depicted as the blue to magenta transition). Reduction of the disulfide bond (yellow) immediately below and to the right of the furin clip-site is required for the separation of the 28 kDa N-terminal (blue) and 37 kDa C-terminal (magenta) domains. Deletion of Glu553 (white; bottom) results in a non-toxic PE mutant (ntPE). Exchange of a glutamic acid residue (white; top) for Lys57 results in a reduced-binding toxin mutant (rbPE) with a 100-fold decreased interaction with CD91. **(b)** Two integrated peptide antigen epitopes are depicted. One shows the removal of the Ib loop (aqua green) and replacement with an amino acid sequence that includes flanking cysteine residues to recreate the base-constrained structure at this site. Note that the crystal structure suggests no obvious limitations for the size or conformation of the Ib replacement sequence. Alternatively, conserved amino acid exchanges in areas such as the helical domain from Glu346 to Gln353 (blue) can be used to introduce other antigenic peptide epitopes.

Repeated immunizations with ntPE chimeras provide striking immune responses with each inoculation [49]. This is somewhat surprising because one might assume that the immune system would learn to recognize not only the delivered antigen but also the toxin domains involved in the delivery of that antigen. It is possible that the immune-dominant components of bacterial toxins are not involved in crucial functions of the toxin. In fact, cystic fibrosis patients who are chronically infected with *P. aeruginosa* in their lungs frequently have serum antibodies that recognize PE but do not appear to block its toxic actions [42]. In this way, toxins can be repeatedly effective when secreted by the pathogen and, in the case of ntPE, can be used repeatedly to deliver antigen to a variety of antigens. Another genetically detoxified mucosal adjuvant, LT(K63) has also recently been shown to behave in a similar manner. In these studies, two different antigens were used sequentially to demonstrate that there was no adverse effect on immunization by the presence of preexisting immunity to the toxin [78].

Summary and prospective

Mucosal immunization is the most rational method to protect individuals from pathogens that infect through exposure at epithelial barriers. Traditionally, subcutaneous or intramuscular injections of attenuated pathogens mixed with an adjuvant have been used to raise a systemic immune response. In general, this approach primes the systemic immune system but does not necessarily result in a protective mucosal response. Thus, previous vaccine efforts have focused on helping the immune system resolve infections rather than impeding the initial infection. Secreted bacterial toxins, like those discussed in this review, are being examined to stimulate a protective mucosal immune response. The application of PE as a targeted mucosal delivery vehicle represents a new approach for the development of this protective immunity against pathogens that infect at mucosal surfaces. PE efficiently transports across intact epithelial

barriers and selectively targets professional APCs of the immune system. In this way, PE mimics many aspects of pathogens that cross mucosal surfaces and infect or cripple the function of APCs present just beneath epithelial barriers. We have examined ntPE chimeras containing antigens of bacterial and viral pathogens and found them to induce both mucosal and systemic immune responses following a simple application to an epithelial surface in mouse models. The true utility of ntPE chimeras as a mucosal vaccine tool, however, must await intensive safety and clinical efficacy studies in humans.

From a more generalized perspective, the knowledge that has recently been obtained concerning the uptake and intracellular trafficking of bacterial toxins has led to a better understanding of how such proteins can subvert and exploit endogenous pathways across intact epithelial barriers. Toxins bind to a select population of extracellular receptors that have well-defined uptake pathways. Upon entry into cells, toxins escape the normal degradation route that leads to lysosomal structures. Following escape from degradation pathways, toxins frequently traffic to the TGN, a sort of 'Grand Central' station of the cell. These toxins frequently use a strategy that mimics the retrieval of ER proteins through vesicular transport from the TGN back to the ER. Alternately, a toxin might use a different pathway to move across a polarized epithelial cell and enter into the systemic circulation. Some toxins use their association with particular lipids present in these vesicles, whereas others bind to protein receptors during these various trafficking events. Overall, the protein complexes that constitute secreted bacterial toxins have mastered the mechanisms of entry into cells and selective movement between the compartments present inside these cells. Extension of such knowledge, and derivatives of it, should provide new promise for future efforts in the transcellular and cell-specific targeting of protein and peptide therapeutics and anticancer agents. For centuries bacterial toxins have been the blight of civilization. The time could now be right to use our knowledge of the cellular actions of these same toxins to provide a variety of benefits to humankind.

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