Non-GM1 Ganglioside-Targeting Bacterial Toxins and Derivatives with Mucosal Adjuvant Activity

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Abstract

Studies using the enterotoxin cholera toxin and the related heat-labile toxin I of *Escherichia coli* as adjuvants have established that effective immunity can be provided in mucosal tissues and the general blood stream by vaccines administered by the oral or nasal route. Despite this knowledge and other recognized advantages, needleless mucosal vaccines rarely appear in the health care market and classic injected vaccines remain the norm. Safety issues related to the adjuvants needed for oral and nasal vaccines are the chief factor that hampers their generalized use in humans. Initial strategies to overcome the toxicity of enterotoxin adjuvants consisted of removing the enzymatic activity of these molecules. The binding of these toxins to their ganglioside receptors was later found to be a major source of safety concern regarding nasal vaccines. Non-ganglioside-binding bacterial toxins and their derivatives are being evaluated as alternatives for the development of effective and safe mucosal vaccines. The CTA1-DD adjuvant, which acts by B-cell targeting of the enzymatically active cholera toxin A subunit, represents one of the strategies to induce immunity by nasal immunization without targeting central nervous system tissues. More recently, we found that the edema toxin of *Bacillus anthracis*, which binds to target cells via the anthrax toxin receptors and delivers its adenylate cyclase subunit, is an effective adjuvant for nasal vaccines. Interestingly, the unique receptors of anthrax toxin derivatives appear to prevent their accumulation in the central nervous system.

Keywords :

adjuvant, anthrax, mucosal, vaccine

Mucosal Vaccines and Mucosal Immunity

The intestinal mucosa can selectively take up foreign antigens into highly specialized sites such as the Peyer's patches or gut-associated lymphoreticular tissues in the small intestine for the initiation of immune response. Similarly, antigens entering the organism via the respiratory tract are taken up into inductive sites of the nasopharyngeal-associated lymphoreticular tissues. These tissues contain a full repertoire of antigen-presenting cells as well as B and T lymphocytes for subsequent immune responses. When appropriate mucosal antigen stimu-

lation occurs, interactions between antigen-presenting cells and effector cells lead to induction of mucosal and serum antibody responses and T cell-mediated immunity. The induction of mucosal and systemic immunity is made possible by the unique homing characteristics of effector cells triggered in mucosal inductive sites. In fact, after stimulation in mucosal inductive sites, effector B and T cells selectively migrate to distant mucosal effector sites, where they and their progeny carry out immune functions.

Stimulation of mucosal inductive sites for the

provision of mucosal and systemic immunity can only be achieved with appropriate mucosal adjuvants or delivery systems. Furthermore, adjuvants for vaccines can be described in terms of the T cell-mediated immunity or humoral (antibody)-mediated immunity, which they principally induce. Cell-mediated immunity responses are clearly supported by CD4⁺ Th1 cells that preferentially produce interferon- γ , interleukin-2, and lymphotoxin- α (IFN- γ , IL-2 and LT- α). The Th1 cells and derived cytokines activate macrophages for more effective immunity against intracellular bacteria and help B cells in IgG subclass antibody responses that are effective in opsonophagocytosis after complement activation. Other adjuvants more effectively induce Th2-type cells, which selectively produce interleukins IL-4, IL-5, IL-6, IL-10, and IL-13 and help B cells produce noncomplement-activating IgG subclasses, IgE, and IgA.

Ganglioside-Binding Bacterial Enterotoxins as Mucosal Adjuvants

The major enterotoxins produced by *Vibrio cholerae* and *Escherichia coli*, termed native cholera toxin (nCT) and the heat-labile toxin I of *E. coli* (nLT-I) respectively, represent the most studied mucosal adjuvants for oral and nasal vaccines. These macromolecules are composed of two structurally, functionally, and immunologically separate A and B subunits (1-3). These two AB₅ enterotoxins, toxins that consist of five identical 11.6-kDa peptides, differ from each other in that the B subunit of nCT (CT-B) only binds to GM1 ganglioside (4), whereas the B subunit of nLT-I (LT-B) is less selective and binds to GM1 as well as asialo GM1 and GM2 (5-7). After the binding of the B subunit to epithelial cell GM1 or GM2 receptors, the A subunit reaches the cytosol and after activation there, binds to nicotinamide adenine dinucleotide (NAD) and catalyzes ADP-ribosylation of Gs α (8). Mucosal exposure of nCT and nLT-I stimulates the production of S-IgA and serum antibodies, which are almost entirely restricted to CT-B or LT-B (9). Both toxins are also potent mucosal adjuvants for unrelated proteins administered in the

same vaccine when given by oral, nasal, or even parenteral routes (10, 11). Native cholera toxin has been shown to elicit adjuvant responses by inducing antigen-specific CD4⁺ Th2-type cells, which in turn produce high levels of IL-4, IL-5, IL-6, and IL-10. These Th2 cells support the subsequent development of systemic IgG1 and IgG2b subclasses, IgE, and mucosal S-IgA antibody responses (12). On the other hand, oral immunization with nLT results in plasma IgM, IgG2a, IgG1, IgG2b, and mucosal S-IgA antibody responses, which are associated with CD4⁺ Th1-independent and IL-4-independent Th2-type responses with IFN- γ , IL-5, IL-6, and IL-10 production (13). More recent studies with chimeras of nCT and nLT have shown that the binding B subunits of these enterotoxins played a major role in determining the Th1-type or Th2-type nature of the immune responses induced by molecules as adjuvants (14).

A number of studies have shown that ADP-ribosyl transferase activity is not required for nasal adjuvanticity. Thus, two mutants of LT-I with single amino acid substitutions, called R7K (15) and R192G (16), were shown to be nontoxic but retain adjuvant properties when administered along with protein antigens by the nasal or oral routes, respectively (15, 16). On the other hand, two mutants of cholera toxin (mCTs), designated S61F and E112K, which harbor single amino acid substitutions in the ADP-ribosyltransferase active center complex, lack ADP-ribosyltransferase activity and diarrheagenicity (17). Both mCTs are effective adjuvants and are comparable with nCT when given parenterally or nasally (17). In addition, a *brevibacillus*-derived chimera CTA-E112K/LT-B that lacks ADP-ribosyltransferase activity was recently developed and found to act as a mucosal adjuvant for nasal immunization and induce LT-I-like responses (18).

Ganglioside-Binding and Safety of Oral and Nasal Mucosal Adjuvants

After the B subunit of the enterotoxins binds to the epithelial cell GM1 or GM2 receptors, the A subunit reaches the cytosol and after activation there, binds to NAD and catalyzes ADP-ribosylation of Gs α (2).

This GTP-binding protein activates adenylyl cyclase and elevates cyclic adenosine monophosphate (cAMP); in epithelial cells, this results in the secretion of water and chloride ions into the small intestine for a subsequent watery type of diarrhea (19). While diarrhea is the primary limiting factor of the use of oral enterotoxin in humans, major safety concerns with mucosal adjuvants and delivery systems such as their potential to trigger unwanted inflammatory responses and to target central nervous system (CNS) tissues are limiting factors for nasal vaccination. Studies with enterotoxin adjuvants (20) suggest that these adverse effects are in large part mediated by the ADP-ribosyl transferase activity and the nature of the cellular receptors targeted. Both nCT and nLT-I bind to GM1 on epithelial cells and require endocytosis followed by transport across the epithelial cell to reach the basolateral membrane. The GM1 gangliosides are also abundantly expressed by cells of the CNS, and their concentration in neuronal and microglial cells varies during the development of different cell types in different regions of the brain. Previous studies have shown that when given nasally to mice, nCT or CT-B entered the olfactory regions (specifically, the olfactory nerve epithelium and olfactory bulb) by mechanisms that appeared to be selectively dependent on GM1 (20). These studies have also shown that cholera toxin as an adjuvant promotes the uptake of unrelated protein antigens, administered nasally in the same vaccine, into the olfactory nerve epithelium (20). The targeting of CNS tissues by nasally administered bacterial enterotoxins is strongly believed to be the chief risk-inducing factor for cases of Bell's palsy (facial paresis) in Switzerland among individuals who received the Berna nasal influenza vaccine (21). Thus, case-series analysis identified an increase in incidence of Bell's palsy in individuals who received the nasal, but not the parenteral, inactivated influenza vaccine (21). The relative risk of Bell's palsy following nasal vaccination was estimated to be 19 times higher than in the controls and the vaccine was withdrawn from the market. The fact that the peak of incidence occurred between 31

and 60 days after vaccination suggests that an induced response, rather than some direct toxic effects, led to the palsy (21). No side effects were reported following nasal application of CT-B to twelve patients with IgA nephropathy (22), suggesting that some mutants of cholera toxin or LT-I that lack enzymatic activity may not be harmful when used in appropriate vaccine formulations.

Adjuvant Activity of Non-GM1-Binding Type II Heat-Labile Enterotoxins LT-IIa and LT-IIb

Two serogroups of heat-labile enterotoxins have been distinguished on the basis of distinct immunoreactivity. Serogroup I consist of CT and the LT-I of *E. coli*, which includes two antigenic variants isolated from humans and pigs, designated LTh-I and LTp-I, respectively (23, 24). The serogroup II enterotoxins include the LT-II of *E. coli* (25). Based on immunoreactivity and amino acid sequence homology, two antigenic variants of LT-II, designated LT-IIa and LT-IIb, have been isolated (25-27). These antigenic variants are structurally related but antigenically distinct; they share the inability to bind to GM1 gangliosides, but they have different binding specificities for ganglioside receptors (7). For example, the monosialoganglioside GM1 is now recognized to be the high-affinity receptor for CT and LT-I, while the B subunit of LT-IIa binds with high affinity to GD1b and less strongly to GM1, GT1b, GQ1b, GD2, GD1a, and GM2 (7). Unlike CT and LT-IIa, the LT-IIb lacks affinity for GM1 but has been shown to bind with high affinity to the di-sialoganglioside GD1a (7).

Nasal administration of LT-IIa or LT-IIb enhanced systemic antibody responses to the concurrently administered vaccine antigen (28). The adjuvant activity of nasal LT-IIa and LT-IIb also led to increased antigen-specific antibody responses in saliva and vaginal secretions, indicating that both molecules are mucosal adjuvants. Analysis of T helper cytokine responses showed that the adjuvant activity of both LT-IIa and LT-IIb involves mixed Th1 and Th2 responses with both antigen-specific IL-4-producing and IFN- γ -producing cells (28). The

mixed pattern of CD4⁺ Th cell cytokines responses induced by the LT-II molecules was further supported by *in vitro* studies in which CT, but not the LT-II molecules, reduced the expression of CD40 ligand on CD4⁺ T cells and the subsequent alteration of TNF- α and IL-12 p70 production by both autologous monocytes and monocyte-derived dendritic cells (29). Other studies showed that, unlike GM-1-binding enterotoxins, LT-II enterotoxins were inactive in stimulating IL-1 and IL-8 secretion by antigen-presenting cells (30). In sharp contrast to the holotoxins, the B pentamers of LT-IIa and LT-IIb stimulated cytokine production, suggesting a link between the absence of the A subunit and increased proinflammatory properties (30).

Single-point substitution mutants of LT-IIa and LT-IIb that have no detectable binding activity for their major ganglioside receptors [e.g., LT-IIa (T34I) and LT-IIb (T13I)] were developed to address whether the binding of LT-IIa and LT-IIb to their specific ganglioside receptors is essential for adjuvant activities (31). While these mutant enterotoxins exhibited only a low capacity for inducing the production of cAMP in a macrophage cell line, they were able to enhance the systemic immunity from a concurrently administered vaccine antigen (31). However, only LT-IIb (T13I), which retained some binding activity for lymphoid cells, had the capacity to strongly potentiate both mucosal and systemic antibody responses (31). These studies suggest that the major ganglioside receptors for LT-IIa and LT-IIb are not essential for their adjuvant activities.

Non-Ganglioside-Binding Subunits or Derivatives of Cholera Toxin and Heat-Labile Toxin I of *E. coli* as Mucosal Adjuvants

The studies summarized above suggest that neither GM1 nor the other ganglioside receptors for bacterial enterotoxins are required for these molecules to act as adjuvants when delivered by the nasal route. In context, the role of the binding B subunit of these toxins has been questioned. Furthermore, because the adverse effects that have hampered the use of bacterial enterotoxin in human oral or nasal

vaccines are primarily due to the affinity of these molecules for GM1 (and probably other gangliosides), new cellular targets for mucosal adjuvants have been investigated.

Could CT-A and LT-A be effective nasal adjuvants?

The first suggestion that LT-A alone could act as a nasal adjuvant came from studies by de Haan and colleagues to dissect the respective roles of the LT-A and LT-B subunits in the adjuvanticity of LT-I. These authors reported that the adjuvanticity of LT-B required GM1-binding activity, whereas GM1-binding was not essential for the adjuvant activity of nLT-I (32). This observation led the authors to test LT-A alone and they found recombinant LT-A alone acts as a potent mucosal adjuvant for a concomitantly administered antigen (32). The same authors later reported that recombinant LT-A could stimulate systemic and mucosal antibody responses toward a concomitantly administered protein vaccine antigen (33). The LT-A used in these studies was expressed as fusion proteins with an N-terminal 10 histidine-tag to facilitate purification on nickel-chelate columns. It remains unclear whether the long histidine tail produced conformational changes in the LT-A molecule. Alternatively, the long histidine tail may allow interactions with CT-A that do not normally take place. In this regard, nCT was reported to exhibit affinity for nickel through three His residues present in CT-B (34). Regardless of the mechanisms involved, it is striking that the authors reported responses after nasal immunization with this LTA(His)10 that were comparable in both magnitude and serum immunoglobulin isotype and subtype distributions to those observed after concomitant immunization with LT or LT-E112K (33).

Another group of researchers addressed the possibility that CT-A could act as an adjuvant for a poorly antigenic protein (i.e., ovalbumin) concomitantly administered by the nasal route (35). Their studies showed that CT-A elicits both mucosal and systemic immunity to antigens. The mechanisms underlying the adjuvant activity of LT-A and CT-A remain to be elucidated. The more likely possibility

is that the histidine tail on these recombinant proteins leads to conformational changes that in turn facilitate the binding of LT-A and CT-A to cell receptors still to be identified.

The B cell-targeting CTA1-DD adjuvant

A model of adjuvant was developed by combining the ADP-ribosylating ability of CT with a dimer of the Ig-binding fragment D of *Staphylococcus aureus* protein A (36). This CTA1-DD molecule directly binds to B cells of all isotypes, including naive IgD⁺ cells, but not to macrophages or dendritic cells (37, 38). Immunizations of FcεpsilonR (common FcRγ-chain)-deficient and FcγRII-deficient mice demonstrated that FcγRII-expressing cells are not required for the adjuvant function of CTA1-DD (38). In contrast with CT, CTA1-DD was highly efficient at augmenting antibody responses in athymic mice, demonstrating that T cell-independent responses were also enhanced by this adjuvant. In normal mice, both CT and CTA1-DD, but not the enzymatically inactive CTA1-R7K-DD mutant, were efficient enhancers of T cell-dependent as well as T cell-independent responses, and both promoted germinal center formation following immunization. Although CT augmented apoptosis in antigen receptor-activated B cells, CTA1-DD strongly counteracted apoptosis by inducing Bcl-2 in a dose-dependent manner, with a mechanism that was independent of the CD19 co-receptor. However, in the presence of CD40 stimulation, apoptosis was low and unaffected by CT, suggesting that the adjuvant effect of CT is dependent on the presence of activated CD40 ligand-expressing T cells (38). Despite the lack of a mucosal binding element, the B cell-targeted CTA1-DD molecule was an equally strong adjuvant as CT. Moreover, the adjuvant effect was TLR4-independent and absent in the mutant CTA1-E112K-DD, which lacks enzymatic activity. The CTA1-DD adjuvant augmented germinal center formation and T cell priming in the draining lymph nodes, and contrary to CT, promoted a balanced Th1/Th2 response with little effect on IgE antibody production. The adjuvant CTA1-DD did not induce inflammatory

changes in the nasal mucosa, and most importantly, did not bind to or accumulate in the nervous tissues of the olfactory bulb (39).

Anthrax edema toxin as a new mucosal adjuvant

Bacillus anthracis expresses the protective antigen (PA), the lethal factor (LF), and the edema factor (EF), which combine to form two AB-type toxins (40-42). The combination of PA and EF forms edema toxin (EdTx), while the combination of PA and LF forms lethal toxin. The PA subunit targets cells via the anthrax toxin receptor 1 (ATR1), which resembles tumor endothelial marker 8 (TEM8) (43), and the related ATR2, which is similar to capillary morphogenesis gene 2 (CMG2) (44). The EF is an adenylate cyclase that increases intracellular cAMP levels (45-47) and induces edema (45). Intradermal concomitant immunization with a DNA plasmid encoding the N-terminal fragment of LF, which shares homology with the N-terminal fragment of EF, was reported to induce higher anti-PA antibody responses than immunization with a single plasmid encoding PA (48). Edema toxin delivers its adenylate cyclase EF subunit into target cells following binding of PA on its membrane receptors, the ATRs. It has been recently reported that ATR1/TEM8 is expressed by epithelial cells (49). However, previous studies have shown that PA binds more effectively to the basolateral membrane of polarized epithelial cells (50), suggesting that ATRs may not be expressed at the apical membrane of these cells.

We recently investigated whether an EdTx derivative could act as a mucosal adjuvant like the enterotoxins CT and LT-I, and promote S-IgA and systemic antibody responses to nasally concomitantly administered vaccine antigens (51). The presence of EdTx sharply increased antigen-specific plasma antibody responses to the nasally concomitantly administered protein vaccine and promoted mostly IgG1 and IgG2b antibody subclasses. EdTx as an adjuvant was able to stimulate antigen IgA and IgG antibodies in the saliva, indicating that this toxin is a mucosal adjuvant (51). We also found that the adjuvant activity of EdTx promotes antigen-specific

CD4⁺T cells secreting both Th1-type (IFN- γ) and Th2-type (IL-4) cytokines (51). Like most AB-type toxins, EdTx was immunogenic and induced high plasma and mucosal antibody responses against the binding subunit PA itself. This is interesting because current anthrax vaccines are PA-based and aim at promoting anti-PA antibodies. In this regard, EdTx as an adjuvant induced anti-PA antibodies in both the plasma and external mucosal secretions and provided an extra bonus for anthrax immunity (51).

The possibility of GM1-binding adjuvant targeting the olfactory nerve epithelium or other CNS tissues limited its use to experimental laboratory animals. Unlike the B subunit of CT and LT-I, anthrax PA failed to accumulate in the olfactory nerve epithelium, olfactory bulbs, or brains 24 hours following nasal delivery. Nasal PA concomitantly administered with EF did not increase PA accumulation in olfactory or brain tissues. Finally, nasal delivery of EdTx failed to induce inflammatory cytokine responses in the nasopharyngeal-associated lymphoreticular tissues and in nasal washes.

Conclusion

Despite their superior ability to promote potentially protective mucosal and systemic immunity to mucosally concomitantly administered vaccine

antigens, major safety issues prevent the use of CT and LT-1 in human oral or nasal vaccines (Table 1). However, the studies summarized here show that bacterial toxins or their components remain viable options for the future design of safe vaccines, provided that appropriate cell targeting systems are included. Such alternative targeting approaches include the GM1 receptors targeted by LT-IIa and LT-IIb, the B cells targeted by the synthetic analogue of protein A present on CTA1-DD, and the ATR targeted by anthrax PA. These strategies allowed the safe delivery of a functional ADP ribosyl transferase subunit of CT or LT-I, or the adenylate cyclase of anthrax EdTx. The fact that EdTx very efficiently promoted anti-PA antibody responses in both saliva and plasma could have important implications for improving the efficacy of current PA-based anthrax vaccines and for developing mucosal vaccines that could protect against multiple agents of infectious diseases.

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Table 1. Non-GMI-targeting bacterial toxins and derivatives tested as adjuvant for mucosal vaccines^a.

Bacterial Products or Derivatives	Molecular targets	Known target cells	Immune responses promoted	
			Systemic	Mucosal
LT-IIa	GD1b >> GT1b, CG1b, GD2, GD1a, GM2, GM1	Macrophages (M ϕ) Dendritic cells (DC), T cells	Yes	Yes
LT-IIb	GD1a	M ϕ , DC, T cells	Yes	Yes
CT-A	Unkown	Unkown	Yes/(?) ^b	Yes/(?) ^b
LT-A	Unkown	Unkown	Yes/(?) ^b	Yes/(?) ^b
CT-A1-DD	Surface immunoglobulins	B cells	Yes	Yes
EdTx	ATR1/TEM8, ATR2/CMG2	MO, DC, T cells	Yes+	Yes

^aMost studies examined the adjuvant activity for nasal vaccines.

^b(?) denote controversial findings due to lack of understanding of mechanisms involved and/or only partial information about potential contaminants.

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